Carbon isotopic fractionation in *Methanosarcina barkeri* and the study of anaerobic microbial communities of saline springs in West Central Manitoba.

By

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Abstract

Stable carbon isotope fractionation during methanogenesis is affected by the availability of substrates. The effects of different substrates on methanogen biomass, total lipid extract, biomarkers and methane under both abundant and limiting substrate conditions were studied. *Methanosarcina barkeri* was grown with methanol, acetate, trimethylamine (TMA) and H_2/CO_2, and carbon isotope fractionation in methane production was greatest with methanol, followed by H_2/CO_2, TMA and acetate. In contrast, biomass was isotopically lightest in *M.barkeri* grown on methanol, followed by TMA, H_2/CO_2 and acetate. Generally, fractionation was greater in cultures grown with abundant substrate availability as compared to those supplied with limiting substrate. During autotrophic growth, fractionation was greatest during slower growth for both methane and biomass production. The results of these fractionation studies under controlled laboratory conditions can be applied to the interpretation of isotopic signatures for methane and methanogen biomarkers, and ecological processes, in marine environments.

Several hypersaline springs off the western shore of Lake Winnipegosis, MB support unique microbial mat communities. These low temperature springs contain water with a mean salinity as high as 6.1%. Studies were undertaken to contrast the anaerobic microbial communities of these springs, specifically the methanogens and sulphate-reducing bacteria (SRB), and their contributions to biogeochemical cycling in these mats. Comparisons of lipid profiles revealed changes in the proportions of the dominant fatty acids related to the amount of mat growth. Cultures of SRB and methanogens were established with six different

substrates. Methanogenic cultures grew best on TMA and methanol, but could use formate, H_2/CO_2 and glycine betaine as well. In contrast, H_2/CO_2 was the preferred substrate of the SRB enrichment cultures, which were also able to use formate, but not TMA, the breakdown product of the compatible solute glycine betaine. Maximum methane production occurred at 5% salinity. The lipid composition of the mats, including methanogen biomarkers, and the results of the enrichments on different substrates and at different salinities, suggest that methanogenesis in these springs is supported by compatible solutes whereas sulphate reduction is linked to availability of hydrogen and formate.

Chapter 1
Literature Review.

1.1 Stable isotopes in microbial ecology

Stable isotopes of many elements such as hydrogen, carbon, nitrogen, oxygen and sulphur exist naturally in the environment. The most abundant naturally occurring form of these elements is always the lighter isotope, with the heavier isotopes present in quantities of 5% or less. These biologically important isotopes, with their low atomic masses, have a large relative mass difference between the heavy and light isotopes. Therefore, these stable isotopes are readily measurable despite the fact that they are not radioactive.

The ratio of stable isotopes in compounds can be measured via mass spectrometry. Observing their relative depletion and/or concentration in molecules is made easier by the rarity of heavier isotopes. The recent development of gas-chromatography, isotope ratio mass spectrometry (GC-IRMS) has enabled researchers to separate, as well as identify and measure, the isotopic ratio of a wide range of organic compounds.

Some enzymes preferentially use the lighter isotopes, which results in an isotopically light product relative to the reactant pool. This effect, known as fractionation, is caused by slight differences in bonding energy between the isotopes (Hoefs 1997). The fractionation, or difference in the isotopic composition between the reactants and products, is the most meaningful measure, rather than the absolute isotopic values. The isotopic composition of a compound (A) is often expressed as the "delta" (δ) value, which is defined as:

$\delta_A = \frac{(R_A - R_{std})}{(R_{std})} \times 10^3$ (‰) where R_A is the ratio of the heavy isotope to the lighter isotope in A and R_{std} is the same ratio in a standard of known isotopic composition. (Hoefs 1997).

There are many ways of expressing isotopic fractionation, some of the most common are the ε, α and Δ notations as defined below:

$\varepsilon_{A/B} = (\alpha_{A/B} - 1)10^3$ from Hayes 1993. ε is known as the fractionation effect.

$\alpha_{X\text{-}Y} = R_X/R_Y$ from Hayes 2001. α is known as the fractionation factor.

$\Delta_{y\text{-}x} = \delta_y - \delta_x \approx 10^3 \ln \alpha_{X\text{-}Y}$ from Hayes 2001.

Recently**,** the study of stable isotopes has been applied to microbial ecology. While the use of stable- or radioisotope- labelled substrates can be useful for many measurements, they are often difficult to introduce into the environment and may alter the abundance of natural substrates (Londry *et al.* 2004). Therefore studying naturally occurring stable isotopes ensures confidence that fractionations observed are unaffected by such additions and/or manipulations.

1.2 Carbon isotopic fractionation

The approach of using stable carbon isotopes has increased in microbial ecology because carbon is so ubiquitous in biomolecules. It is found in everything from nucleic acids to lipids. Most carbon on Earth is found in the form of ^{12}C (~ 98.89 %) while a minor portion is found as ^{13}C (~ 1.11 %). Carbon isotopic values are expressed as ratios of heavy to light isotopes in units of ‰ (a version of R from above) as:

$$\delta^{13}C = \frac{^{13}C/^{12}C \text{ of sample} - {}^{13}C/^{12}C \text{ of standard}}{^{13}C/^{12}C \text{ of standard}} \times 1000$$

The standard used for carbon isotopes is the marine carbonate, Peedee Belemnite (PDB), from the Cretaceous Peedee Formation in South Carolina with a $^{13}C/^{12}C$ value of 0.0112372 (Craig 1957).

Due to the larger relative mass differences between isotopes of smaller, lighter molecules, they will be fractionated to a greater extent than larger, heavier ones. Therefore, larger fractionations are typically seen with autotrophic growth, which relies on CO_2, as compared to heterotrophy (Hayes 2001). In this way, carbon fixation processes establish the isotopic relationship between an organism and its carbon source (Hayes 2001). This is also true of methanol and other carbon substrates used by methylotrophs. Another example is methane; as a small one carbon molecule, some of the largest fractionations are associated with methane (Krzycki *et al.* 1987). While much fractionation can occur during methane production, further carbon fractionation can also occur through the consumption of methane by methanotrophs. This in turn results in extremely light carbon that can be used in diagnosing the presence of methanotrophs. This is a useful tool when attempting to interpret the origin of certain biological products.

Largely due to the actions of the enzyme RUBISCO, photosynthetic carbon fixation via the Calvin cycle is associated with $\delta^{13}C$ values near –25‰ and molecules with negative $\delta^{13}C$ values, near or below this (ie. more negative than), are generally considered to be of biological origin. Other forms of autotrophy also result in discrimination against ^{13}C, including the reductive acetyl CoA pathway (ε = 2-52‰, depending on the enzymes involved) and the reverse TCA cycle (ε = 4-13‰), although the 3-hydroxypropionate cycle does not appear to involve much carbon

fractionation (Preuß *et al.*1989, Hayes 2001). Heterotrophy typically results in much less dramatic carbon fractionations and is well described by "you are what you eat" (Boschker and Middelburg 2002). Although isotopic fractionation later in the biosynthetic process will also affect cells, an organism's carbon isotopic make up is primarily determined by its carbon source and the way it assimilates carbon (Pancost and Damsté 2003).

Several other factors have been found to affect biological carbon isotopic fractionation including temperature, substrate availability, $\delta^{13}C$ of substrates, cellular carbon budget, the biosynthetic pathways used and carbon flow at metabolic branch points (Hayes 1993). When all else is equal, fractionation is believed to be greater at lower temperatures because molecules of light isotopes react more readily than those of heavier isotopes; and this effect (due to differences in bonding energies) is greater at lower temperatures (Hoefs 1997). For example, an experiment with methanogenic enrichment cultures grown with methanol found different fractionation factors dependent on temperature (Rosenfeld and Silverman 1959). The culture grown at 30˚C exhibited a fractionation factor (α) between substrate and methane of 1.083, while the culture grown at 23˚C showed a greater fractionation factor (α) of 1.094.

While enzymes will preferentially use lighter isotopes, they are capable of using the heavier ones as well. Therefore, if an enzyme has a limited substrate supply, such as in a "closed" system, and uses most of the substrate available, little fractionation will be seen. In a study of carbon fractionation in the methanogen *Methanobacterium thermoautotrophicum*, it was determined that initially, the

$\delta^{13}C_{CO2}$ = -41.9‰ and $\delta^{13}C_{CH4}$ = -65‰ (Games and Hayes 1978). When growth halted because the substrate CO_2 became limiting, the $\delta^{13}C_{CH4}$ was - 43‰. An earlier case was seen with a methanogenic enrichment culture taken from mud in the Pacific Ocean. In this case the substrate was methanol, and the $\delta^{13}C_{CH4}$ ranged from -83.8‰ initially to a final reading of +49.9‰ (Rosenfeld and Silverman 1959). This means that while laboratory studies in "open" systems with abundant substrate supplies can display maximal potential carbon fractionations, environmental conditions often result in natural fractionations that are much lower. Unlike most cultures grown in the lab, microbes in the environment usually face limited substrate availability.

The metabolic pathways involved in assimilation will also affect carbon fractionation. Not all enzymes fractionate carbon to the same degree; therefore, some pathways will result in isotopically lighter products than others. As mentioned above, not all autotrophic pathways involve equal carbon fractionations. In a study looking at this, the $\Delta\delta^{13}C$ values of bulk cellular carbon of pure bacterial cultures growing autotrophically were measured and it was determined that there were indeed differences between the fractionation among different pathways (Preuß *et. al* 1989). However, they found that there was also considerable variation in the carbon fractionation experienced between different microorganisms using the same pathway. Under similar temperature and pH growth conditions, *Desulfobacterium autotrophicum* and *Acetobacterium woodii,* which both use the reductive acetyl CoA pathway, were found to have very different $\Delta\delta^{13}C_{cell\text{-}CO2}$ values (-35.5‰ for the former and -20.7‰ for the latter) (Preuß *et. al* 1989).

The flow of carbon at pathway branching points is not always equal, and this also affects the $\delta^{13}C$ values of metabolic products. If the carbon flow to one product (A) is greater, the other product (B) will be isotopically affected (Hayes 2001). For simplicity, assimilated CO_2 in a methanogen growing autotrophically can flow either to biomass production or methane generation. If more carbon is directed to methane production (A), the isotopic value of biomass (B) will be affected. If the enzymes converting assimilated carbon to methane fractionate this carbon such that the methane is highly ^{13}C depleted, the assimilated carbon pool available to biomass production is relatively ^{13}C enriched. This will necessarily result in the $\delta^{13}C$ values of biomass to be isotopically heavier than that of CH_4 produced.

Another consideration to take into account is that not all cellular components within an organism will have the same $\delta^{13}C$ value. It has been found that carbohydrates are slightly isotopically heavier (by ~ 1‰) than proteins and nucleic acids, while lipids are ^{13}C depleted (Hayes 2001). This has been known for some time, Abelson and Hoering first reported that lipids are isotopically light in 1961. There are also isotopic differences within classes of cellular components; different amino acids in proteins and different fatty acids in lipids may have varying isotopic values.

Among the cellular components, a very useful group of compounds in microbial ecology are lipid biomarkers. Lipids are relatively simple to extract, and therefore make attractive targets for carbon isotopic fractionation studies among different organisms. Lipids have a wide variety of structural and functional diversity. Some lipids are very rare and are found in a limited group of microorganisms.

Biomarkers are biosynthetic products unique to a particular organism (or group of organisms), which can be used to signal their presence in environmental samples without the need for culturing. Several lipid biomarkers have been identified in microorganisms, such as hydroxy-archaeols in methanogens (Sprott 1992), hopanoids in aerobic bacteria (Ourisson *et al.* 1987) and methanotrophs (Summons *et al*. 1988), sterols in Eukarya (Volkman 2003) as well as unique fatty acids like i17:1 in sulphate reducing *Desulfovibrio* species (Vainshtein *et al.* 1992).

This approach can also be used to correlate isotopic signatures with a particular organism (or group of organisms). The study of natural microbial communities has been greatly assisted by combining knowledge of biomarker identifications with the development of stable isotope analytical methods. The result has been that many studies of a wide diversity of microorganisms have been done. Generally, bacteria exhibit a ^{13}C depletion of 2-4‰ between fatty acids and biomass under aerobic conditions (Pancost and Sinninghe Damsté 2003), although some bacteria have been reported to be capable of even greater fractionations (Abraham *et al.* 1998). Individual fatty acids of the cyanobacterium *Synechocystis*, which uses the pentose phosphate pathway, were found to be as much as 9.1‰ depleted in ^{13}C relative to biomass (Sakata *et al.* 1997). Lipids of the anaerobic, phototrophic green sulphur bacteria that use the TCA cycle, are approximately 4‰ enriched in ^{13}C relative to biomass (van der Meer *et al.* 1998). A study of two anaerobic iron reducing bacteria, *Geobacter metallireducens* which uses the TCA cycle and *Shewanella algae* which uses the serine pathway, found that the carbon isotopic fractionation effect (ε) between biomass and fatty acids was greater in *Shewanella*

(-10.9 to -15.5‰) than in *Geobacter* (-4.5 to -8.6‰) (Zhang *et al.* 2003). The fatty acids of sulphate reducing bacteria (SRB) are also usually depleted in ^{13}C relative to their biomass. Three different SRB were grown under both autotrophic and heterotrophic conditions and in each case, autotrophic growth resulted in more ^{13}C depletion than heterotrophic growth (Londry *et al.* 2004). In fact, this study reported that autotrophic growth of *Desulfotomaculum acetoxidans* resulted in isotopically depleted fatty acids, with an average $\Delta\delta^{13}C_{FA-CO2}$ of –37‰.

The highly ^{13}C depleted lipids of aerobic methanotrophs are very diagnostic indicators. Products of methanotroph biosynthesis can be extremely isotopically light because their substrate (methane) is already a very isotopically light carbon source, and they are capable of fractionating this carbon even further. Pure culture studies have indicated that fatty acids of aerobic methanotrophs can be ^{13}C depleted as much as 10 to 15‰ relative to biomass (Jahnke *et al.* 1999). In 1994, Summons *et al.* reported that *Methylococcus capsulatus* and *Methylomonas methanica* were capable of maximum ^{13}C fractionation into biomass of approximately 30‰. Several studies have found evidence of ancient aerobic methanotrophy through the discovery of greatly ^{13}C depleted hopanoids in a variety of environments such as the Green River Formation (Collister *et al.* 1992), the Messel Shale (Freeman *et al*. 1990) and in ancient marine settings (Yamada *et al.* 1997, Köster *et al.* 1998). Hopanoids are formed through diagenesis of bacteriohopanepolyols produced by aerobic bacteria, and remain stable over geologic time scales (Brocks and Summons 2001).

While these compounds are very useful in microbial ecology, some care must be taken when interpreting carbon isotopic fractionation in biomarker lipids and fatty

acids. Summons *et al.* (1994) found that a single organism can produce biomarker lipids with significantly different isotopic compositions if the isotopic composition of the substrate, isotopic fractionation during assimilation, or the relative abundances of lipids changes during the growth cycle. Pure culture investigations have shown that the fatty acids of organisms are not isotopically identical even within the same organism (Abraham *et al.* 1998). The $\delta^{13}C$ values of fatty acids in a species of SRB (*Desulfobacter hydrogenophilus*) were found to vary between +15‰ and -30‰ (Londry *et al.* 2004). Obviously, carbon isotopic fractionation is a complicated phenomenon, which varies not only between groups of microorganisms on a large scale, but also within individual microbes. This complicates interpretations of carbon isotopic signatures of these molecules.

Studies of ancient preserved biomolecules have also been aided by the knowledge of how environmental factors affect carbon isotopic fractionation by different organisms. As mentioned earlier, temperature has an important effect on fractionation, as does substrate availability. Ancient biomolecules were subject to the same fractionation effects, and this is reflected in their $\delta^{13}C$ values. Further study of microbial carbon fractionation is critical to understanding how modern and fossil sedimentary records should be interpreted.

Most ancient molecular fossils remaining today are remnants of biological lipids. These biomarker molecules contain information about ancient biodiversity, trophic relationships and environmental conditions (Brocks and Summons 2001). The portion of ancient sedimentary organic matter that is not soluble in solvents is known as kerogen, and is formed from lipids combining with degradation-resistant

macromolecules. The isotopic values of this material can be interpreted with the information available from both modern environment samples and pure culture work. For instance, the oil industry uses the carbon isotopic values of kerogen biomarkers to estimate the potential for petroleum production as well as the temperature history in particular sedimentary basins (MacKenzie 1984, Radke *et al.* 1997).

Though cyanobacteria and aerobic methanotrophs both produce hopanoids, they can often be differentiated through structural differences, and those produced by methanotrophs can have much lower $\delta^{13}C$ values. Through comparing the carbon isotopic content of geohopanoids and geoporphyrins (produced by cyanobacteria) from Messel shale, it has been determined that the very light geohopanoids (as light as -65‰) originated from methanotrophs and not from photosynthetic organisms (Hayes *et al.* 1987). Identifying the source of geohopanoids with intermediate carbon isotopic signatures was more difficult. From the $\delta^{13}C$ values of the porphyrins found, hopanoids produced by cyanobacteria would be expected to have $\delta^{13}C$ values of approximately -32‰ (Jahnke *et al.* 1999). An investigation into the carbon isotopic fractionation of hopanoids in methanotrophs under methane limiting conditions found that these compounds could be ^{13}C-enriched up to 10‰ relative to the substrate methane (Jahnke *et al*. 1999). Therefore, they found it was not possible to distinguish the hopanoids from cyanobacteria or heterotrophic bacteria from those produced by some methanotrophs under these conditions based on either chemical structure or isotopic signature. However, in some cases, structural differences among hopanoids can indicate their origin. For example, 3*β*-methylhopanoids are produced by methanotrophs and acetic acid bacteria (Summons and Jahnke 1992, Zundel and

Rohmer 1985 a,b,c), while 2*β*-methylhopanoids are produced mainly by cyanobacteria (Bisseret *et al.* 1985).

Large depletions of ^{13}C in Archaean age kerogens point to methane as an important part of the early carbon cycle. These kerogens are believed to have very low $\delta^{13}C$ contents through the consumption of biogenic methane in combination with fractionation exhibited by aerobic methanotrophs (Hayes 1983). This in turn offers some clues as to when the Earth changed from anoxic conditions to supporting aerobic life (Summons *et al.* 1994).

1.3 Carbon isotopic studies in methanogens

Today, of course, methane production is of great importance to global warming. Although present in lower amounts than carbon dioxide, methane is 21 times more potent as a greenhouse gas by volume. After years of rapid increase, atmospheric methane levels remained stable at 1751 ppbv (parts per billion by volume) between 1999 and 2002 (Dlugokencky *et al*. 2003). Studies of the isotopic composition of methane in the atmosphere estimate that 74% is from recent microbiological activity (Whitman *et al.* 1992). Approximately one third of current methane emissions come from wetland sediments (where methanogens are known to thrive). Therefore, there is much interest in methanogenic microorganisms today.

As a small, one carbon molecule, methane is subject to very large isotopic fractionations. It has the largest known variation of the $^{13}C/^{12}C$ ratio of any organic compound (Kryzycki *et. al* 1987). It has been found with $\delta^{13}C$ values as low as -110‰ (Botz *et al*. 1996), also making it the lightest hydrocarbon known. Since the first report of “light” (-83.8‰) methane production from a Pacific Ocean deep sea

mud sample grown on methanol (Rosenfeld and Silverman 1959), carbon isotopic fractionation in methanogens has been studied extensively. From these studies, it has been determined that biologically formed methane has an isotopic signature below (ie. more negative than) -55‰, while that of thermally produced methane (for example, from ancient carbon compounds including petroleum) is isotopically heavier than this value (Kryzycki *et. al* 1987). The oil and gas industry has exploited this knowledge to distinguish between methane formed biologically from that formed through thermal degradation of organic matter, which can indicate locations of petroleum reserves.

Methanogens produce methane with a wide range of fractionation effects, even with simple substrates like H_2. A study of the $^{13}C/^{12}C$ fractionation factors of three methanogens growing autotrophically in pure cultures on H_2/CO_2 revealed that although each microbe fractionated this carbon into isotopically light methane, the fractionation was not of the same magnitude (Games and Hayes 1978). *Methanobacterium thermoautrophicum* exhibited the least fractionation ($\alpha = 1.025$) while that of *Methanosarcina barkeri* was intermediate ($\alpha = 1.045$) and that of *Methanobacterium* strain M.o.H. was the greatest ($\alpha = 1.061$). Therefore, although these microorganisms use the same substrate and presumably the same pathway (only one autotrophic pathway has been documented in methanogens, but genome sequences suggest possible alternatives), they produce isotopically different methane.

Carbon isotopic fractionation studies of methanogens growing heterotrophically have also provided insight into the wide range of $\delta^{13}C$ values for CH_4. One study of *Methanosarcina barkeri* growing on acetate indicated that isotope

exchange occurs between the carbonyl group of acetyl-CoA and CO_2 (Fischer and Thauer 1990). Another study, looking at the carbon isotopic fractionation of acetate in a methanogenic marine sediment, found that the two carbon atoms of acetate vary isotopically (Blair and Carter 1992). This discovery, that not all carbon atoms within a compound are isotopically identical, was an important step in the understanding of fractionation during methanogenesis. Whether this is a common feature of all organic substrates for methanogens is still unknown.

The diversity of substrates and pathways available to some methanogens leads to even greater ranges of $\delta^{13}C_{CH4}$ possibilities. A study of carbon fractionation in *M. barkeri* under abundant substrate conditions has shown that fractionation ($\delta^{13}C_{CH4\text{-}substrate}$) is greatest when the substrate is methanol (-73.65 ‰), followed by CO_2 (-45.95 ‰) and finally acetate (-21.2 ‰) (Krzycki *et. al* 1987). The reduction of methyl coenzyme M to methane is the common step of methanogenesis from these three substrates (Figure 1.1). The varying carbon fractionations indicate that the enzyme catalyzing this reaction, methylreductase, is not responsible for the bulk of ^{13}C discrimination during methanogenesis. Another study of *M.barkeri* growing on trimethylamine (TMA) found a large carbon isotopic fractionation between substrate and methane, with a fractionation factor, $\varepsilon_{CH4\text{-}TMA}$, of 50.2‰ (Summons *et al.* 1998).

In addition to methane, methanogens produce biomass, which is also isotopically depleted. The first investigation into carbon isotopic fractionation of biomass during methanogensis studied *Methanobacterium thermoautotrophicum* growing under autotrophic conditions (Fuchs *et al.* 1979). When H_2/CO_2 was supplied at a low gassing rate of 5mL/min, the $\Delta\delta^{13}C_{cell\text{-}CO2}$ was +1.285 ‰. Fractionation

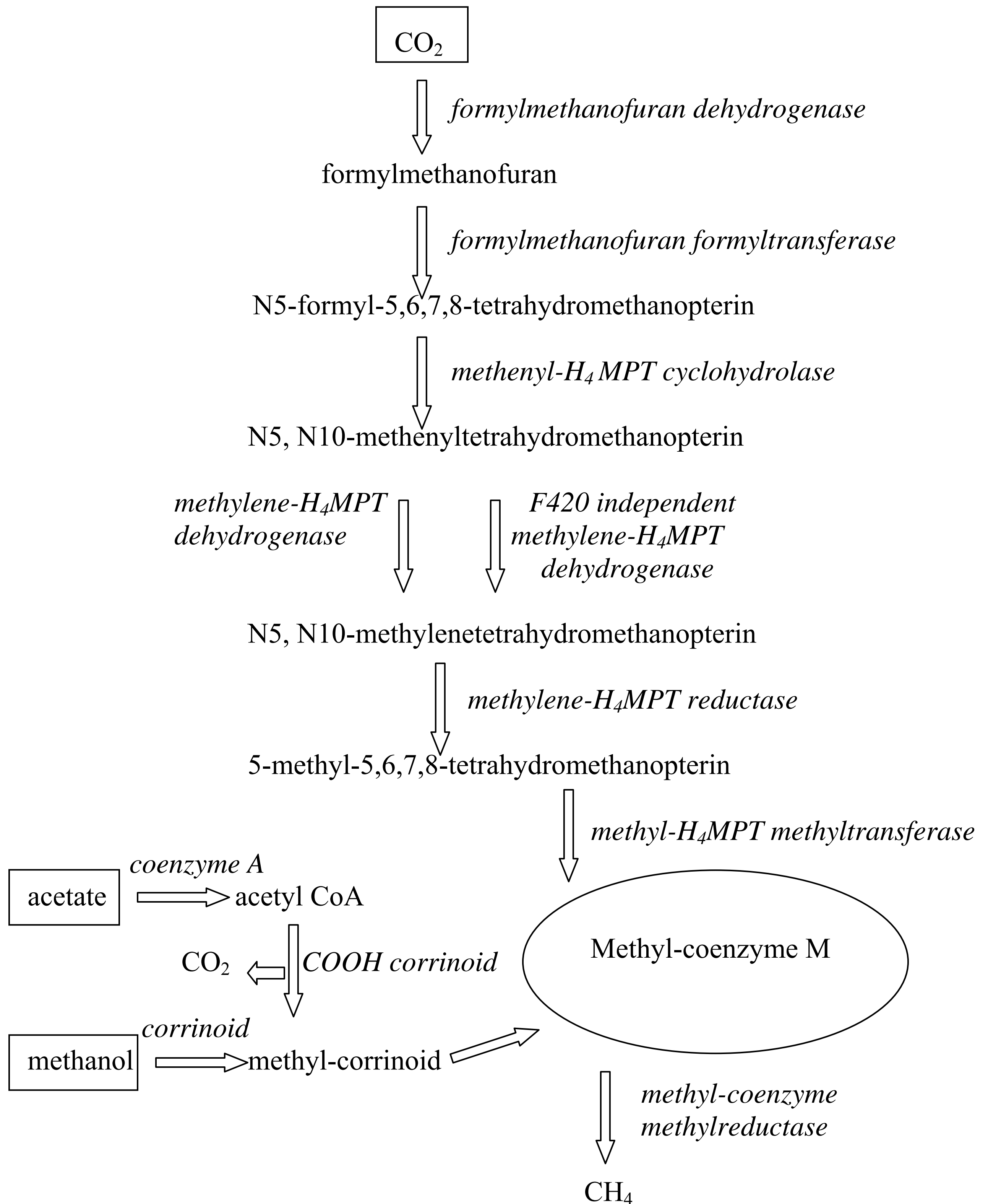

Figure 1.1 Methanogenesis from CO_2, acetate and methanol.
Enzymes are displayed in italics, substrates displayed in boxes.

increased when the gassing rate was increased to 300mL/min, and the $\Delta\delta^{13}C_{cell\text{-}CO2}$ was -1.907 ‰. Although the isotopic fractionation was not large in this case, it was measurable. During this experiment, *M. thermoautotrophicum* was grown at its temperature optimum of 65˚C. Therefore, it was suggested that mesophilic methanogens would be expected to produce isotopically lighter biomass.

A later study of *Methanobacterium formicicum* did indeed report a larger carbon fractionation in biomass. In this case, cultures were grown autotrophically at 34˚C and exhibited $\Delta\delta^{13}C_{cell\text{-}CO2}$ values as great as -7.1 ‰ (Balabane *et al.* 1987). A large investigation into the fractionation involved in different carbon fixation pathways found that *Methanococcus thermolithotrophicus* was capable of an isotope effect, $\varepsilon_{cell\text{-}CO2}$, of as much as 26.7 ‰ when grown on H_2/CO_2 (80%:20%) supplied at a pressure of 250 kPa and a temperature of 65˚C (House *et al.* 2003). The same study also reported that autotrophically grown cultures of *Methanococcus igneus* (grown at 85°C) and *Methanopyrus kandleri* (grown at 100°C) were capable of fractionating substrate carbon into biomass with isotope effects of approximately 20‰. These findings indicate that temperature may not be as important in carbon isotopic fractionation as originally believed.

The lipids in Archaea like methanogens are distinct structures, which contain ether linked phytanyl side chains made up of repeating five carbon isoprene units, in contrast to the ester-linked fatty acids used by Bacteria. These isoprenoid lipids are often used as archaeal biomarkers. While ^{13}C depletion of methanogenic biomarkers does occur, this is not always observed in natural environments depending on many variables (Summons *et al.* 1998). For instance, methanogen biomarkers from Ace

Lake (Antarctica) were enriched in ^{13}C up to 10‰ relative to photoautotrophic biomarkers (Schouten *et al.* 2001). On the other hand, samples from the Kazan mud volcano in the Eastern Mediterranean Sea contained the methanogen isoprenoid sn-3 hydroxyarchaeol, which was found to have a $\delta^{13}C$ of as low as -111.2 ‰ (Werne *et al.* 2002).

Anaerobic oxidation of methane (AOM) occurs in marine sediments. It is a globally important process, in that approximately 90% of the methane produced from the anaerobic ocean environment is recycled in these sediments (Reeburgh *et al.* 1993). This, of course, is very critical to the Earth's atmosphere as methane is a potent greenhouse gas. In addition, AOM also converts terminally reduced carbon into more accessible forms for many organisms in these sediments (Orphan *et al.* 2001). The discovery of very isotopically light archaeal lipids near methane seeps suggest that Archaea are involved in this process (Elvert *et al.* 1999, Hinrichs *et al.* 1999, Hinrichs *et al.* 2000, Thiel *et al.* 1999, Pancost *et al.* 2000).

One study compared rDNA surveys with lipid analysis of Archaea and Bacteria that were associated with methane seep sediments from different sites along the Californian continental margin (Orphan *et al.* 2001). Through this investigation, two groups distantly related to the *Methanosarcinales* were found and designated as ANME-1 and ANME-2. Bacterial fatty acids thought to belong to SRB were also found to be highly ^{13}C depleted, suggesting they too are involved in AOM. When the researchers combined 16S rDNA surveys with whole cell *in situ* hybridization, they found clusters of ANME-2 Archaea with *Desulfosarcina* and *Desulococcus* SRB species. It has been suggested that the ANME-2 archaea may be methanogens

operating in reverse, producing acetate or H_2, which could be readily used by the SRB (Orphan *et al.* 2001). The use of an ion probe to analyse these clusters provided a clear link between the molecular evidence for the methanogens and SRB and the extremely light carbon isotopic values of these clusters, indicating their role in AOM.

Researchers have proposed that the main substrates for methanogenesis in marine environments are methanol and/or methylamines rather than H_2/CO_2 or acetate, due to the presence of sulphate-reducing bacteria (SRB) competing for these substrates (Oremland and Polcin 1982). While methanogens and SRB often inhabit the same anaerobic environments, sulphate reducers are able to outcompete methanogens for substrates like H_2/CO_2 and acetate. Methanogens are able to survive in these conditions when "noncompetetitive" methylated substrates such as methanol or trimethylamine (TMA) are available.

TMA is commonly found in saline and hypersaline environments because it is a breakdown product of the osmoprotectant glycine betaine (Figure 1.2), which is used by a variety of organisms. TMA is readily used as a carbon source by a limited group of methanogens within the *Methanosarcinales*. Many microorganisms are able to degrade glycine betaine, and this can be done under both aerobic and anaerobic conditions (Oren 1990). Anaerobically, this degradation can result in the production of TMA through many reactions. The reductive breakdown of glycine betaine by the marine isolate *Sporomusa* results in the formation of TMA and acetate (Möller *et al.* 1984). Other anaerobes can use different compounds as electron donors for this cleavage to TMA and acetate. *Clostridium sporogenes* is able to use the amino acids alanine, valine, leucine and isoleucine as well as hydrogen (Naumann *et al.* 1983).

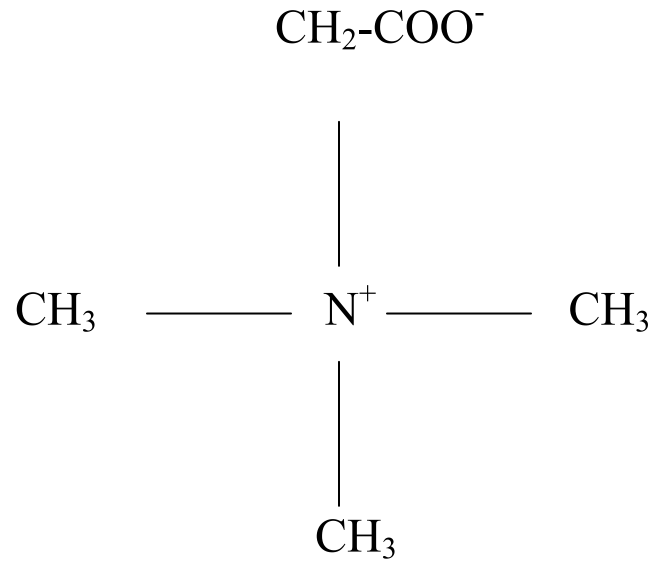

Figure 1.2 Structure of glycine betaine.

Eubacterium acidaminophilum can also use amino acids as well as hydrogen, formate or malate (Hormann and Andreesen 1988, Zindel *et al.* 1988). Glycine betaine can also be oxidized to CO_2 and TMA by many *Desulfuromonas* strains (Heijthuijsen and Hansen 1989). Other possible sources of TMA in saline environments include choline, which many bacteria are able to degrade, including sulphate reducers, as well as trimethylamine N-oxide, a common osmotic solute of marine fish species (Oren 1990).

Methylotrophic methanogenesis from TMA has been studied in *M. barkeri* and the Antarctic methanogen *Methanococcoides burtonii* (Summons *et. al* 1998). In *M. barkeri*, the isotope effect (ε) for the conversion of TMA to methane was 50.2‰, that of TMA to biomass was 20.2‰ and the phytanyl chains of the polar lipids were -18‰ compared to biomass (when grown at 35°C). The isotope effects in *M. burtonii* grown at 15°C were found to be even larger at 71‰ for TMA to methane, 49.6‰ for TMA to biomass and 79.9‰ for TMA to phytanyl lipid chains.

Of course, carbon isotopes are not the only ones fractionated by organisms. Hydrogen is also very useful in studying isotopic fractionation because it has the largest relative mass difference between the heavy and light isotopes of any element, making it subject to very large isotopic fractionations. Hydrogen can exist as ^{1}H or the heavier ^{2}H (also known as deuterium) and the isotopic ratio is represented as δD.

Interestingly, methanogenesis in marine and freshwater environments often results in isotopically distinct methane. This has led to the estimate that 70% of methane produced in freshwater environments is formed through acetate dissimilation and the remaining 30% through CO_2 reduction (Takai 1970, Smith and Mah 1966). In

contrast, CO_2 reduction is believed to dominate methanogenesis in marine environments. This results in distinctive methane carbon isotopic signatures from marine sediments of –110 to -60‰ and those from freshwater sediments of –65 to -50‰ (Whiticar *et al.* 1986). Taking hydrogen isotopes into account further differentiates between these two types of methane generation. Methane from marine sediments generally has δD values of –250 to -170‰ while methane from freshwater environments has δD values between –400 to -250‰ (Whiticar *et al.* 1986). Combining measurements of both carbon and hydrogen isotopes allows for confident distinction between methods of methane generation in freshwater versus marine environments.

Research objective: To study the carbon isotopic fractionation in the methanogen *M.barkeri* when grown under autotrophic and heterotrophic conditions under both substrate-limiting and abundant substrate conditions. Specifically, carbon isotopic fractionation for methane, biomass, lipids and lipid biomarkers during growth on different substrates (H_2/CO_2, methanol, acetate, and TMA) was investigated under limiting and abundant substrate supply.

1.4. Hypersaline Environments

Astrobiologists are interested in halophilic microbes because understanding the microbiological processes that occur in unique and/or extreme environments on Earth is key to both helping preserve these systems and expanding our knowledge of life's possibilities. Those seeking to study early life are also interested because many of these extreme environments are believed to represent conditions present on the early Earth. For instance, it is believed that the ocean may have been much saltier when life first emerged than it is today (Hinrichs *et al*. 2000). Therefore learning how life survives under highly saline conditions may help us to understand how early microorganisms lived. In addition to learning about these processes, novel biochemical pathways and enzymes may be found through these endeavours. As with the discovery of Taq polymerase from *Thermus aquaticus* found in a Yellowstone hot spring (Brock and Freeze 1969), some of these studies may potentially lead to advances in related fields such as biotechnology and medicine, as well as enhancing our basic knowledge of these life forms.

Hypersaline conditions impose osmotic stress upon microorganisms, which they must overcome in order to maintain the integrity of their cellular membranes. Some species, designated as halotolerant, can adapt to increasing salinity while others, termed halophilic, actually require high salt levels. Microorganisms inhabiting these environments must maintain their cytoplasm in a hyperosmotic state to prevent loss of cytoplasmic fluid to the surrounding environment. However, hypersalinity exacts a high energetic toll upon all microbes living in these environments. The

energy required to maintain membrane integrity is expensive and organisms must have an abundant energy supply in order to survive (Oren 1999).

Halotolerant and/or halophilic organisms have two different strategies for coping with osmotic stress. One strategy involves maintaining high intracellular salt levels. Several Archaea and anaerobic heterotrophic Bacteria use inorganic ions (K^+) to balance their cytoplasm with extracellular salt levels (Oren 1999). This is also known as the “salt-in strategy”. While this system has been found to be more energetically efficient, it is less frequently observed.

The second, more widely observed strategy, requires microorganisms to accumulate organic osmolytes, which allow them to maintain their cytoplasm isoosmotic with the extracellular environment (Oren 1999). These compounds are referred to as compatible solutes because they are capable of accumulating to high intracellular concentrations without negatively interfering with metabolism. This strategy is used by a large variety of organisms including Archaea, Bacteria, yeasts and filamentous fungi (Santos and da Costa 2002). The salt-in strategy requires that organisms have cellular systems adapted to high salt concentrations while the more common compatible solute system does not require such adaptations. Those organisms using compatible solutes are able to tolerate a larger range of salinity as a result.

Of the known compatible solutes, glycine betaine (N,N,N-trimethylglycine) (figure1.2) is among the most widely used by microorganisms (Oren 1990). It is accumulated under hypersaline conditions by Bacteria as well as Archaea and Eukarya. In addition to helping create an osmotically balanced internal environment,

it offers other advantages to organisms. Glycine betaine may function as a molecular chaperone, providing protection to some important cellular molecules under salt stress conditions (Bourot *et al*. 2000, Diamont *et al.* 2001). It had also been shown to prevent inhibition of malate dehydrogenase by NaCl in *Horleum vulgare* (Pollard and Wyn Jones 1979). In natural environments, heat and salt stresses on microorganisms are often combined. Glycine betaine has been shown to protect cellular enzymes from thermodenaturation as well. Interestingly, an increased tolerance to cold attributed to the presence of this compatible solute has also been documented (Nyyssöla *et al*. 2000). Glycine betaine can also be used as a carbon and/or nitrogen source by some heterotrophic organisms (Oren 1990).

Table 1.1 identifies many microorganisms that accumulate glycine betaine for use in osmoregulation. It is not an exhaustive list of all Bacteria and Archaea that use this compound, however it shows how widespread its use as an osmoprotectant is. It is likely that many other microorganisms also utilize this compound since even the non-halophilic *E. coli* will use it for osmoprotection (Culham *et al*. 1993).

Due to its many advantageous properties in saline environments, most microorganisms inhabiting saline environments are able to take up betaine from their surroundings. Some can oxidize choline from the environment to glycine betaine as well. Despite its use by so many microbes, only a few can synthesize this compound *de novo* from simple compounds like glycine or glucose. These organisms include phototrophic bacteria, halophilic methanogens, halotolerant and halophilic cyanobacteria and two actinomycetes. Among those microorganisms that are able to

Table 1.1 Several glycine betaine-accumulating microorganisms

Bacteria	Actinobacteria	*Corynebacterium glutamicum*	Rubenhagen *et al.* 2000
		Streptomyces griseolus	Severin *et al.* 1992.
		Micrococcus halobius	Severin *et al.* 1992.
		Micrococcus varians	Severin *et al.* 1992.
	Rhodospirilli	*Sinorhizobium meliloti*	Boncampagni *et al.* 2000
	(α-Proteobacteria)	*Rhodospirillum salexigens*	Severin *et al.* 1992.
		Rhodospirillum salinarum	Severin *et al.* 1992.
		Azorhizobium brasilense	Riou and Le Rudulier, 1990.
	Zymobacteria	*Salmonella typhimurium*	Stirling *et al.* 1989.
	(γ-Proteobacteria)	*Escherichia coli*	Culham *et al.* 2001.
		Halomonas elongata	Cánovas *et al.* 1996.
		Halomonas halmophila	Severin *et al.* 1992.
		Halomonas variabilis	Severin *et al.* 1992.
		Chromatium purpuratum	Severin *et al.* 1992.
		Chromatium salexigens	Severin *et al.* 1992.
		Ectothiorhodospira halophila	Severin *et al.* 1992.
		Deleya halophila	Severin *et al.* 1992.
		Pseudomonas halosaccharolytica	Severin *et al.* 1992.
		Vibrio alginolyticus	Severin *et al.* 1992.
		Vibrio costicola	Severin *et al.* 1992.
	Bacilli	*Listeria monocytogenes*	Sleator *et al.* 2001.
		Lactobacillus lactis	Bouvier *et al.* 2000.
		Staphylococcus aureus	Vilhelmsson and Miller, 2002.
		Bacillus subtilis	Boncampagni *et al.* 2000.
		Marinococcus albus	Severin *et al.* 1992.
		Marinococcus halophilus	Severin *et al.* 1992.
Archaea	Methanosarcinales	*Methanosarcina thermophila*	Proctor *et al.* 1997.
		Methanosarcina mazei	Roeßler *et al.* 2002.
		Methanohalophilus portucalensis	Lai *et al.* 2000.
		Methanohalophilus zhilinae	Robertson *et al.* 1990.
		Methanohalophilus mahii	Robertson *et al.* 1990.
	Methanomicrobiales	*Methanogenium cariaci*	Robertson *et al.* 1990.
		Methanogenium anulus	Robertson *et al.* 1990.
	Methanococcales	*Methanococcus voltae*	Robertson *et al.* 1990.

synthesize their own glycine betaine, most have mechanisms for accumulating it from their environment as well (Nyyssöla and Leisola 2001, Lai *et al.* 2000). Table 1.2 indicates those organisms able to synthesize glycine betaine *de novo*.

All *de novo* synthesizer*s* known to date are believed to use the same pathway, which involves a series of methylations:

It is thought that the source of glycine for these microorganisms is 3-phosphoglycerate from glycolysis, indicating that it is linked to central cell metabolism (Nyyssöla *et al.* 2000). S-adenosyl methionine (SAM) is the methyl group donor for these reactions. Using SAM is very energetically expensive to the organisms, as each activated methyl group costs them 12 ATP equivalents. This may explain why most glycine betaine producers are also able to accumulate this compound when it's available in the environment (Nyyssöla and Leisola 2001).

Those organisms able to synthesize their own glycine betaine likely use uptake systems when this compound is available, because although this does require energy expenditure, it is less than that required for *de novo* synthesis (Oren 2001). This can partially explain why certain organisms are found in hypersaline environments while others are not. Due to the high cost of survival, those organisms that produce only small amounts of energy (by fermentation or respiration) are not expected to be found. The high energy demand on microbes inhabiting saline environments means that certain substrates may not be utilised by microbes at high salt levels. For example, methanogenesis from acetate proceeds at salt concentrations under 50g/L while methanogensis from H_2 and CO_2 is limited to salt concentrations

Table 1.2 Microorganisms capable of *de novo* glycine betaine synthesis.

Bacteria	Actinobacteria	*Actinopolyspora halophila*	Severin *et al*. 1992.
		Corynebacterium sp.U-3	Matmeeva *et al*. 1997.
	Rhodospirilli (α-Proteobacteria)	*Rhodospirillum salexigens*	Severin *et al*. 1992.
		Rhodospirillum salinarum	Severin *et al*. 1992.
	Zymobacteria (γ-Proteobacteria)	*Chromatium purpuratum*	Severin *et al*. 1992.
		Chromatium salexigens	Severin *et al*. 1992.
		Ectothiorhodospira marismortui	Severin *et al*. 1992.
		Ectothiorhodospira halochloris	Severin *et al*. 1992.
		Ectothiorhodospira abdelmalekii	Severin *et al*. 1992.
		Thiocapsa halophila	Severin *et al*. 1992.
		Chromohalobacter marismortui	Severin *et al*. 1992.
	Cyanobacteria	*Aphanothece halophytica*	Waditee *et al*. 2003.
Archaea	Methanosarcinales	*Methanohalophilus portucalensis* FDF1	Lai *et al*. 1991.
		Methanohalophilus strains SF1, SF2, SD1, Z7301, Z7401, Z7302, Z7404	Lai *et al*. 1991.

under 150g/L and methanogensis from methylated amines occurs up to approximately 250g/L (Oren 2001). Meanwhile, those organisms that use aerobic respiration or denitrification, phototrophs, as well as those using the "salt-in" strategy, are often able to survive in high salt conditions (Oren 2001).

Actinopolyspora halophila has been observed to "recycle" exogenously provided glycine betaine when additional betaine is provided in culture (Nyyssöla and Leisola 2001). This has also been observed in *E.coli* cultures (Lamark *et al.* 1992). It

was suggested at this time that this may represent a way for organisms to adjust the osmolarity of their cytoplasm. This may also be a major source of betaine for other organisms in their environment, which cannot synthesize their own glycine betaine.

For those organisms that need to accumulate exogenous glycine betaine, many use single, high affinity transporters. These have been found in *Methanohalophilus portucalensis* (Lai *et. al* 2000) and *Methanosarcina thermophila* (Proctor *et al.* 1997). Roeßler *et al.* (2002) set out to see if they could identify any salt-induced genes or proteins in methanogenic Archaea. *Methanosarcina mazei* Gö1 was chosen as their model organism because of its metabolic diversity and the fact that its genome had been sequenced. It was shown experimentally that this organism has a salt-induced glycine betaine transport system, and through genome screening they found a gene cluster, designated as *ota*, which codes for an ABC (ATP binding cassette)-type transporter. This includes an ATP binding protein, a transmembrane protein and a substrate binding protein, which were salt-induced. A search for microbes with genes expressing proteins similar to this substrate binding protein revealed that other members of the *Methanosarcinaceae* also produced similar proteins.

So, methanogens use glycine betaine, whether they synthesize it *de novo* or accumulate it from their environment. The *Methanosarcinacae* can use the breakdown product of glycine betaine as a major substrate for methanogenesis in hypersaline environments. The extent to which these two competing processes determine the distribution of methanogens (or carbon isotopic fractionation by methanogens) in the environment is unknown.

1.6 Microbial mats in Manitoba salt springs

The adaptation of microorganisms to survive under harsh environmental conditions can allow them to grow in abundance. Life in extreme conditions has the advantage that most other organisms are excluded; therefore competition for nutrients and predation are less significant factors, and layered microbial communities known as microbial mats are able to develop. These communities are believed to be modern day analogues of those formed on the early Earth before competition and predation by plants and animals limited their growth. Microbial mats often consist of an aerobic cyanobacterial photosynthetic upper layer overlying anoxygenic phototrophic bacteria (which require reduced compounds), with anaerobic chemoorganotrophic bacteria like SRB and methanogens in the bottom layers. Many of Earth's harshest environments are home to microbial mats, such as thermal hot springs, deserts, hypersaline water bodies, and the Antarctic.

Microbial mats offer an opportunity to study how microbes interact with each other under certain environmental conditions. While much work has concentrated on the photosynthetic mat members, less is known about the anaerobic members of microbial mats. As mentioned earlier, sulphate reducers and methanogens compete for substrates in many anaerobic environments. This includes microbial mats. While SRB can out-compete methanogens for substrates such as acetate and hydrogen, non-competitive methylated substrates like TMA and low sulphate conditions favour methanogenesis. Therefore, methanogens are often found in microbial mats, even when sulphate is abundant (Ormeland and Taylor 1978, Kosiur and Warford 1979, Mountfort and Asher 1981, Ormeland *et al.* 1982, Ormeland and Polcin 1982).

Anaerobic members of microbial communities in hypersaline environments interact and influence mat dynamics. For example, SRB are important anaerobic members of microbial mats in the hypersaline ponds of Guerrero Negro, Mexico, which consume dissolved organic matter, produce sulphide and support many phototrophic and chemotrophic bacteria within the mat community (Des Marais 1995). Methanogens have also been found in these mats, supporting a methanotrophic community in the upper layers (L. Jahnke, personal communication). In addition, a study examining the anoxic sediments underlying hypersaline microbial mats in Mediterranean salterns found evidence of fermentative bacteria, SRB, and methanogens at salinities of 25-32% (Moune *et al.* 2003).

Underground saline waters exist in the Manitoba lowlands west of the Rat, Red and Assiniboine rivers and lakes Winnipegosis and Manitoba (Grasby 2000). Several salt springs exist along the western boundaries of these lakes from The Pas in the North to Dauphin in the south (Figure 1.3). These springs were first described in La Verendrye's journals in the early 1700s and were once the source for salt production operations. The Monkman Saltworks was a highly productive Métis establishment on the Red Deer Peninsula, which produced over 1000 bushels of salt in a season at its peak in the 1800s. Just northwest of the town of Winnipegosis lies the Winnipegosis Salt Flat which was once home to a salt works operation in the late 1800s (Petch 1987), and the Northern Salt Syndicate was once located just north of the present-day town of Swan River at the McArdle Salt Flat in the 1930s (Petch 1987). Anthropological studies suggest that unique ceramics discovered throughout

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Figure 1.3 Saline spring locations along western shore of Lake Winnipegosis, Manitoba. From Dr. S. Grasby.

the lake Winnipegosis and Lake Manitoba region, known as Duck Bay Ware, may have been associated with even earlier salt production by First Nations people.

These springs have been studied by geologists over the last century and several theories have been used to explain the source of these brines. Tyrrell (1892) proposed that the salinity resulted from the dissolution of salt in rocks from the late Silurian or early Devonian age. Cole (1915) later suggested that the brines were either ancient residual sea water or they originated from meteoric waters dissolving a salt bed nearby to the west. Later, others examined the regional groundwater system and suggested that the spring water flowed from the Manitoba Uplands to the west of Lake Winnipegosis and from the upland areas of the Western Canadian Sedimentary Basin and the Northern Great Plains Region of the United States (Hitchon 1969, Hitchon *et al.* 1969, Hitchon *et al.* 1971, McCabe and Barchyn 1982, Simpson *et al.* 1987).

The geochemistry of the spring water has been studied by Cameron (1949), van Everdingen (1971) and Stephenson (1973). The Br/Cl ratios were studied by Wadien who concluded that the brines were formed by the dissolution of halite from the Devonian Prairie Evaporite formation by meteoric waters (Wadien 1984), but suggested that isotopic analysis should be done. More recently, the water system supplying these springs has been studied by Dr. Stephen Grasby of the Geological Survey of Canada in co-operation with Manitoba Water Resources and the Manitoba Geological Survey (Grasby *et al.* 2000, Grasby 2000). Stable isotope data suggests the source of these spring waters is indeed halite dissolution by meteoric water that originated as basal glacial meltwater. Grasby has suggested that different salinities in

the springs are likely due to differences in the degree of mixing with shallow groundwaters and subsurface residence time (Grasby 2000). While the average salinity of seawater is 35‰, the salinity of some springs reaches 61‰ (McKillop 1992, Grasby and Chen in review). They have also been found to have high levels of Ca and Fe (Grasby, personal communication).

A concentration of several northern springs is located at 52˚ 45' 10" latitude north and 100˚ 52' 50" longitude west (NAD 87). Here the salinity is so great that springs are surrounded by large salt pans that do not support plant life. These pans consist of a spongy, brainy-textured material, with iron stained sinters also found in some parts of the pans, whose colours vary from orange to red to purple (see Figure 1.4). Plants grow in concentric zones surrounding the salt pans according to their salt tolerance. Depending on the flow rate of the springs, these pans can be any range of sizes. Larger springs are raised and consist of deep cauldrons filled with clear brines from which small streams overflow (Figure 1.5). While plants are excluded at the springs, there is evidence of life in the form of microbial mats floating on the surface of some spring pools (Figure 1.6). Interestingly, some pools are completely covered by microbial mat growth while others contain none at all and some have an intermediate amount of mat growth. It may be that differences in temperature or sedimentation and deposition rates are responsible for the varying growth.

These mats represent a wonderful opportunity to study a unique continental hypersaline microbial community. These mats are somewhat unusual in that the upper layer is dominated by the marine alga *Percursaria percursa* (Londry *et al.* submitted), which has only been found in one other continental spring system in the Netherlands

Figure 1.4 Salt pan sinter colour variations at East German Creek in May 2002, from purple to red to orange.

Figure 1.5 Large spring overflowing into small stream at East German Creek in August 2003.

Figure 1.6 Microbial mat floating on spring pool at East German Creek in May 2002.

(Kornmann 1956). In fact, the area surrounding the western shore of Lake Winnipegosis is home to several marine species not found in other continental environments. Fossils of two marine microinvertebrates (*Cyntheromorpha fuscata* and *Cribroelphidium gunteri*) were found by Neilson *et al.* (1987). Burchill and Kenkel (1991) also identified the sea plantain *Plantago maritima* which is rarely found outside of coastal regions.

While plant and animal species have been examined at some of these spring sites, the microbial communities have not previously been studied. Given the findings of other marine species, it would be advantageous to know what kinds of microorganisms inhabit this unique environment. Microbial mats growing in saline conditions are usually studied in salt lakes and salterns, not in spring ecosystems.

Research objective: To explore the anaerobic microbial community of these continental hypersaline springs and their microbial mats through culturing and lipid analyses approaches. More specifically, to find methanogens and study the extent to which they are able to use the common compatible solute glycine betaine as a substrate source, as well as its break down product TMA. To examine the salt tolerance of methanogens in the springs growing on different substrates and microbial community diversity during different years and seasons through lipid analysis.

Chapter 2
Carbon isotopic fractionation in *Methanosarcina barkeri*

2.1 *Methanosarcina barkeri* studies

Microorganisms known as methanogens are a large, diverse group of strictly anaerobic Archaea that produce methane as a product of their metabolism (Whitman *et al.* 1992). The major substrates for methanogens in the environment are acetate, formate and H_2/CO_2, although some can also use other C-1 compounds like methanol and trimethylamine (TMA)(Whitman *et al.* 1992). Methanogens are believed to have existed for millions, perhaps billions of years (Woese 1987), and in that time they have had a significant impact on the Earth. With its role as a greenhouse gas, the methane produced by these microorganisms may have allowed Earth's early atmosphere to warm above today's average temperatures despite evidence of a young sun that was 20% dimmer (Pavlov *et al.* 2000). This may have made the early Earth's temperature more hospitable and allowed for other life forms to emerge.

In terms of isotopic fractionation, methanogens are capable of producing extremely light carbon (as light as -110‰, Botz *et al*. 1996), making them ideal organisms to use in studying carbon isotopic fractionation. As mentioned in chapter 1, this fractionation can be affected by different types of organic substrates and the availability of those substrates to microorganisms. Understanding how these factors impact the carbon isotopic signatures of methanogen biomass, lipids and methane may help interpretations of isotopic signatures in naturally occurring methanogenic populations. For instance, evidence of methanogens in modern and ancient environments may help us determine if these microbes are involved in anaerobic

methane oxidation, how long they have been present on Earth, and which substrates they have utilized.

One of the most well characterised methanogens is *Methanosarcina barkeri*, which is found in animal waste lagoons, the rumen of ungulates, as well as both freshwater and marine sediments (Whitman *et al.* 1992). It is known to grow autotrophically on H_2/CO_2, as well as heterotrophically on organic substrates such as acetate, methanol and trimethylamine (TMA). Unlike many other methanogens however, it is incapable of growth on formate. *M.barkeri* produces two unique isoprenoids that can be used as biomarkers: (1) pentamethyleicosane (PMI)(Figure 2.1A), unique to marine methanogens (Summons *et al.* 1998) and (2) sn-2 hydroxyarchaeol (Figure 2.2B), which is unique to the *Methanosarcinales* order (Hinrichs *et al.* 2000).

As mentioned in chapter 1, *M.barkeri* expresses the most carbon isotopic fractionation between substrate and methane when methanol is used as the substrate, followed by CO_2 and finally acetate (Krzycki *et al.* 1987, Summons *et al.* 1998). Isotopic signatures of methanogen biomass and biomarkers are relatively unknown, although one study indicated that *M.barkeri* had an isotope effect (ε) between TMA and biomass of 20.2‰ (Summons *et al.* 1998). This study also examined the carbon fractionation among lipid components and found that the isotopic effect from biomass to phytanyl chains and PMI was 18‰.

It is important to know exactly how much substrate availability would affect the carbon fractionation by comparing abundant substrate conditions with limited availability on all of these organic substrates. In most environments, substrates are

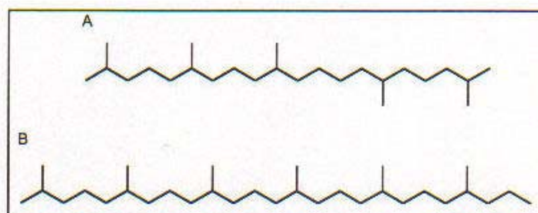

Figure 2.1 Chemical structures of PMI (pentamethyleicosane) (A) and squalane (B). From K.Dawson.

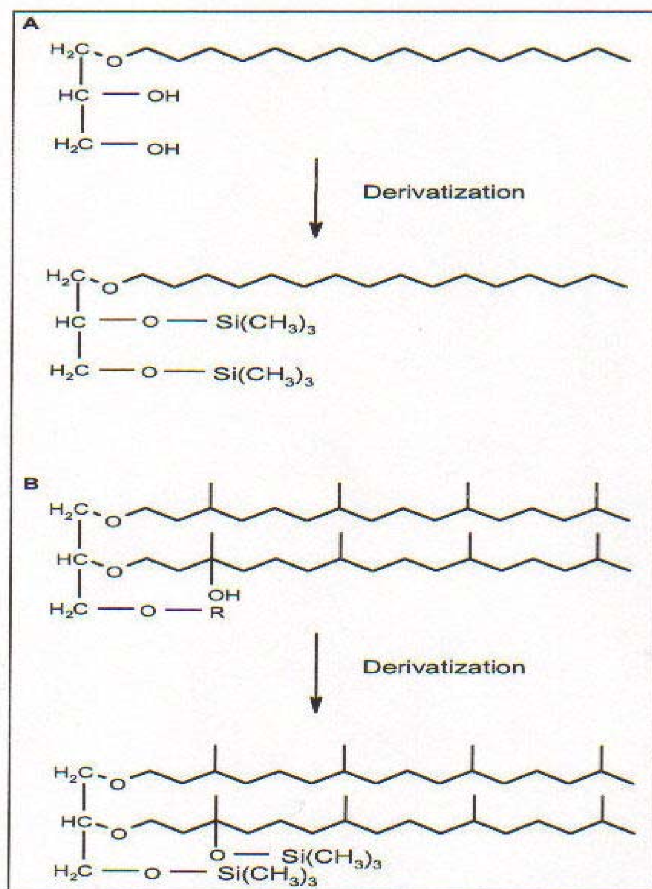

Figure 2.2 Chemical structures of HDG (hexadecylglycerol) (A) and sn-2 hydroxyarchaeol (B) before and after derivatization with BSTFA + TMCS. From K.Dawson.

available to microorganisms in limited quantities. Therefore, while it is important to know the maximal carbon fractionation a microbe is capable of under ideal, abundant substrate conditions in the lab, isotopic values seen in most environments will be much less dramatic.

The objectives of this study were to examine the effects of different substrates on the carbon isotopic signatures of methane, biomass and lipids produced by *M.barkeri* under conditions of different substrate availability. The approach was to provide *M.barkeri* with methanol, acetate, and TMA under both limited and abundant conditions. The hypothesis was that *M.barkeri* would fractionate carbon to a greater extent under abundant substrate conditions as compared to limited substrate conditions, and this fractionation would be greatest when cultures were supplied with methanol, followed by TMA, and acetate. In addition, it is possible that growth rate may affect carbon fractionation. Therefore, cultures were supplied with H_2/CO_2 at different rates in order to determine the impact that this had on fractionation. In the environment, growth rates are usually much less than in the lab, due to limiting substrates and other factors, so fractionation may be greater.

2.2 Materials and Methods

Culturing

The methanogen *Methanosarcina barkeri* strain Fusaro was provided by Dr. R. Sparling and grown on medium modified from Kandler and Hippe (1977). The modifications included: pH adjustment to 6.7, 0.05g/L yeast extract (except acetate cultures- see below), 0.125mg rezasurin and 2mM Na_2S. *M.barkeri* stock cultures

were grown in 165mL glass bottles which contained approximately 100mL culture and a N_2/CO_2 (80/20) headspace at a pressure of 75 kPa (before inoculation) and sealed with butyl rubber stoppers (Appendix 2.1). *M.barkeri* aggregates in very viscous clumps when growing on acetate. In order to ensure that each culture received the same inoculum, a similar, non-clumping, medium was used (see Appendix 2.2) (Summons *et al.* 1998). This media was used only for the inoculum as clumping was not a concern once cultures were inoculated.

Inoculum cultures

Inoculum cultures of *M.barkeri* were grown on all four substrates: H_2/CO_2 (80/20- which replaced N_2/CO_2 in the headspace at a pressure of 100 kPa), methanol (250mM), sodium acetate (200mM) and TMA (100mM). In an effort to minimize additional carbon sources, which could affect carbon isotope signatures, *M.barkeri* was grown with different amounts of yeast extract on each substrate in order to determine the minimum quantity required. It was determined that 1g/L yeast extract was required for consistent growth with acetate, whereas only 0.05g/L was needed for *M.barkeri* growth on methanol, H_2/CO_2 or TMA.

Cultures were maintained by monthly transfers of 10% volume/volume (v/v) inoculum except those grown on acetate (20% v/v). All cultures were incubated stationary at 37ºC in the dark. During incubation of the cultures to be used as inoculum, the pressure was monitored via a pressure gauge with needle adapter (TIF Instruments). If pressures exceeded 200 kPa, excess gas was vented to avoid stopper failure.

Isotope fractionation experiments

The isotope experiments with TMA and H_2/CO_2 were carried out by Kathleen Dawson, a previous undergraduate student in Dr. Londry's lab. In order to obtain sufficient biomass for determining isotopic effects on methane, biomass, total lipid extract (TLE) and biomarkers, three sizes of culture vessels were needed for these experiments. Small cultures were grown in 25mL Balsh tubes (for methane collection), medium cultures in 165mL serum bottles (for biomass, TLE and limited substrate biomarkers) and large cultures in 1.14L custom anaerobic culture bottles (Figure 2.3) (for abundant substrate biomarkers). Due to lack of inoculum, it was not possible to grow the large size cultures for biomarker samples during the acetate isotopes experiment (medium size cultures were used) and total lipid extract cultures were not done. All cultures were kept at a liquid/vessel ratio of 0.6 after inoculation. All cultures were inoculated with 10% v/v (20% for acetate cultures) and grown in triplicate.

Parallel sets of *M.barkeri* cultures were established for each organic substrate. One set was allowed to consume only 5% of the substrate, which was all provided at the beginning of the experiment (unlimited availability). The substrate concentrations used during these experiments were determined previously to be the highest possible concentration that could be used without causing growth inhibition. The other set was allowed to consume 100% of the substrate provided in 10mM intervals throughout the experiment (limited availability). Increments of 10mM were added when cultures

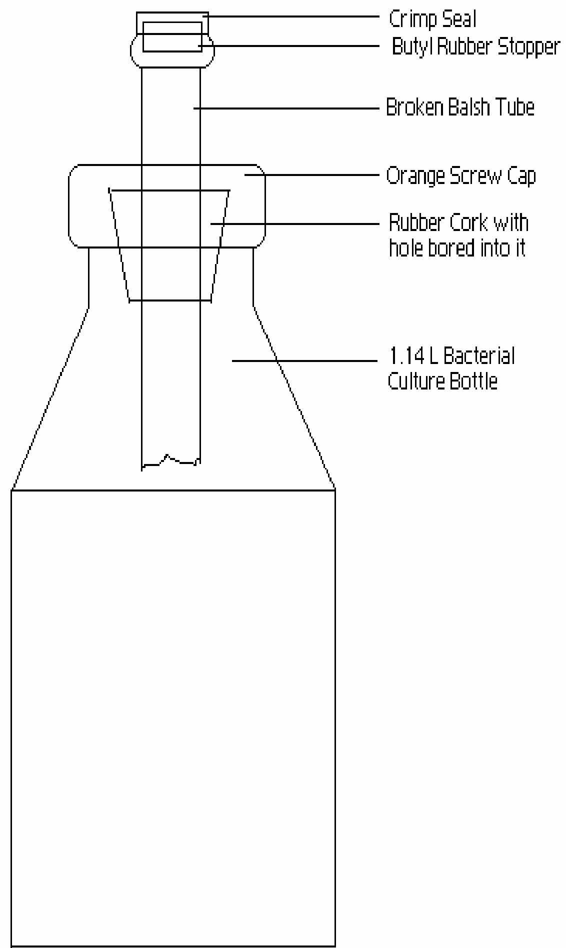

Figure 2.3 Diagram of custom "large" 1.14L anaerobic culture bottle used for culturing *M.barkeri* during isotopes experiments. (From K. Dawson.)

were determined to have used most of the substrate available from earlier additions, based on methane production in the substrate monitoring cultures. In the case of the hydrogen cultures, the effect of growth rate on carbon isotopic fractionation in *M.barkeri* was examined. Fast cultures were provided with 100 kPa H_2/CO_2 and shaken rapidly, while slow cultures were provided with H_2/CO_2 at 5% of headspace volume at two-day intervals. These cultures were also maintained at a smaller liquid to volume ratio of 0.3 in order to supply larger amounts of gaseous substrate. All TLE and biomarker cultures were grown in large bottles in order to supply large amounts of substrate because growth on hydrogen is less abundant.

Growth monitor cultures

It was not possible to measure the course of substrate conversion to methane in the experimental cultures because any sampling of the culture bottles could lead to isotope fractionation, so growth monitor cultures were also inoculated (medium-sized cultures). Upon inoculation, both the abundant and limited substrate growth monitor cultures pressures were measured and methane amounts determined. Methane sampling from the growth monitor cultures continued throughout the experiment in order to approximate substrate usage in the experimental cultures.

Methane analysis

Methanogenic inoculum cultures (and later, growth monitor cultures) were regularly tested for methane production. All needles and syringes used to sample from cultures were first flushed with N_2 to prevent any oxidation. Gas samples (0.1mL) taken from culture headspaces in triplicate were analyzed by gas chromatography on a Shimadzu mini-gas chromatograph (GC) with a flame

ionization detector, and quantified by comparing peak area values to those of a standard curve of known methane concentrations. The average amount of methane produced was then calculated taking temperature and atmospheric pressure into account (Appendix 2.3). Buswell's equation (Sulfita *et al.* 1997) was used to determine the amount of organic substrate that had been consumed (Appendix 2.4). Inoculum for these experiments was not used until the inoculum culture's methane production reached a plateau, in order to avoid substrate carry-over as much as possible. Following inoculation, the remaining culture was harvested in order that the isotope composition of the inoculum cells could be determined.

Immediately following inoculation, a triplicate set of medium cultures was autoclaved as sterile controls. In addition, the first triplicate set of small tube cultures was autoclaved to determine initial CH_4 $\delta^{13}C$ values. Later methane tube samples were then autoclaved at intervals during the experiments in order to observe any changes in the carbon isotopic values of methane produced at different points of the growth phase. Following autoclaving, these tubes were kept upside down to prevent any potential gas loss and/or exchange. Methane samples were only collected at the beginning of some isotopes experiments because at later times too much gas had accumulated and stoppers failed in the autoclave. These cultures could not be vented because this could result in isotopic carbon fractionation of the methane. However, as many methane samples as possible were collected.

Harvesting of experimental cultures

Once experimental cultures were estimated to have reached the intended substrate consumption (through observing the monitor cultures), the methane, pH and

pressure for each culture were recorded. The culture biomass was then collected via centrifugation at 6 000 x g for 30 minutes in a Sorvall Superspeed RC2-B automatic refrigerated centrifuge. Centrifuge bottles were acid washed in 1N HCl and rinsed with distilled water prior to use to avoid contamination. Following centrifugation, the supernatant media was then decanted and discarded. This was followed with a wash of the biomass in phosphate buffer (pH 7.4) (White and Ringelberg, 1998) and a second centrifugation. Again the supernatant was discarded, and the cell material was frozen at -20˚C. Samples were then lyophilized with a FreeZone 6 Litre Benchtop Labcono Dry System. Following lyophilization, dry weights of biomass were recorded.

Lipid Analysis

All glassware used for lipid analysis was baked in a muffle furnace at 450°C for 5 hours to destroy any contaminating lipids. For lipid and biomarker samples, 10-15 mg dry cell mass was extracted for total lipids by accelerated solvent extraction (ASE) as described in Macnaughton *et al*. (1997). *M.barkeri* lipid extracts were analysed for the presence of the expected biomarkers (PMI and sn-2 hydroxyarchaeol). In order to analyse the *M.barkeri* biomarker sn-2 hydroxyarchaeol, it was necessary to derivatize the samples so that the two hydroxyl groups present were made less polar (Hinrichs *et al.*, 2000). The derivatizing agent used was N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) + 1% timethylchlorosilane (TMCS). To each biomarker extract, 20µL of each of the internal standards, hexadecylglycerol (HDG, 0.1mg/mL) and squalane (210µg/mL) were added, followed by derivatization with 50µL BSTFA + 1% TMCS and 50µL pyridine. Lipid extracts were derivatized

to give the di-trimethylsilyl (TMS) derivatives of sn-2 hydroxyarchaeol and HDG (Figure 2.2). Following derivatization, the samples were again dried under N_2 gas and resuspended in 100µL chloroform.

Lipid biomarkers were analysed on a Saturn 2000 gas chromatograph/ mass spectrometer (GC/MS) with a 30m DB5-MS column. The oven temperature was set to 70°C for 1 minute, raised to 165°C at a rate of 25°C/minute, held there for 4 minutes, then raised to 320°C at a rate of 20°C/minute and held there for 43 minutes. The presence of methanogen biomarkers (PMI and sn-2-hydroxyarchaeol) were confirmed by retention times as well as mass spectra (Hinrichs *et al*. 2000)(Figure 2.4).

Isotope analysis of *M.barkeri* biomass, biomarkers, and methane

Frozen samples of *M.barkeri* biomass, total lipid extract (TLE), biomarkers, and cultures containing methane and CO_2 gas were sent to Dr. Roger Summons at the Massachusetts Institute of Technology for isotope analysis, as he is a leading expert in the field of carbon isotopes and the required instruments are not available at the University of Manitoba. Biomass samples consisted simply of harvested cells. The TLE and biomarker samples were separate ASE lipid extracts that were extracted from harvested cells. The internal standards HDG and squalane were added to these (except solid biomass and gas samples) before they were completely dried down under N_2 gas. Methane and CO_2 were analyzed by GC-IRMS conducted by Dr. R. Summons and A. Bradley. Gas compounds were separated on a 30m X 0.32mm ID Alltech AT-Q column with helium carrier flow at 3.0mL/minute, at a column temperature of 40˚C. Organic samples were converted to CO_2 at 950˚C, and isotopic

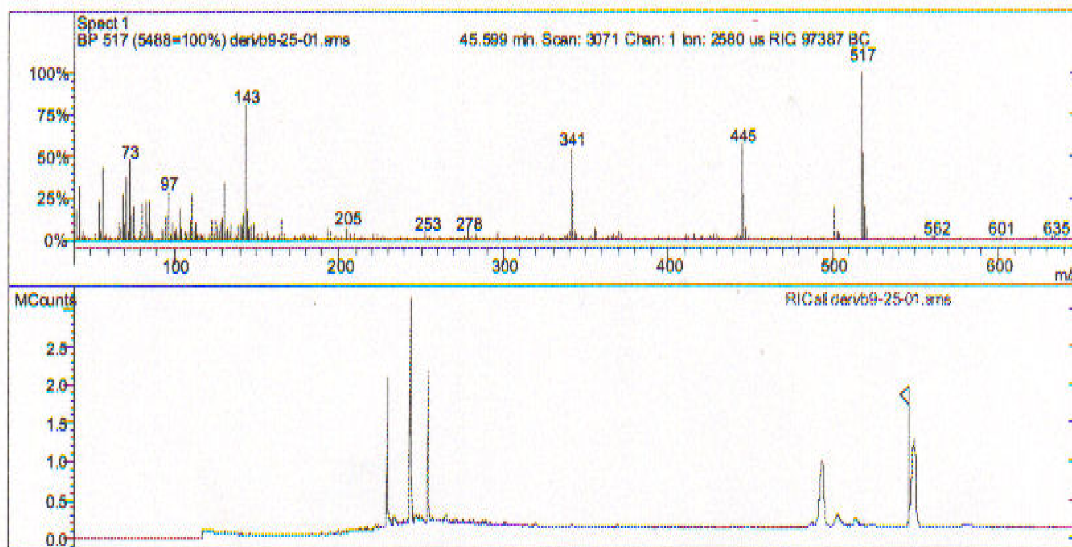

Figure 2.4 Chromatogram of derivatized *M.barkeri* cells. Sn-2 hydroxyarchaeol is indicated by marker and mass spectrum is shown above.

data was processed on a Finnigan MAT DeltaPlus isotope ratio monitoring mass spectrometer using the IsodatNT data package. Accuracy of isotope results were confirmed by independent analysis, and were found to be better than 1‰. Some substrates (acetate and CO_2) were also analysed in order to allow for $\Delta\delta^{13}C$ value calculations. Carbon isotope values of methanol and TMA were not available at this time, so commonly reported values of these compounds were used for calculations. Results for biomarker samples were not yet available at this time for the same reasons.

2.3 Results

M.barkeri grew on all four substrates, with corresponding production of methane and biomass. Results are presented for the methane, biomass and total lipid extract for each substrate. Analysis of biomarker samples is expected in the near future, as the delay was caused by instrument maintenance problems with equipment in Dr. R. Summons' lab. Carbon isotope measurements of acetate and H_2/CO_2 substrates were found to be -30.9‰ ± 0.6 for the former and -31.19‰ ± 0.19 for the latter. Actual values of methanol and TMA are pending, therefore common methanol ($\delta^{13}C$= -39‰) and TMA ($\delta^{13}C$= -35‰) isotope values were assumed for these substrates.

Methanol-Methane

Methane samples from the methanol isotopes experiment indicated that initially the methane was extremely isotopically light (~ -120‰), but over time these closed system cultures produced isotopically heavier methane as the substrate was used (and approached -31‰ , the isotope value of methanol)(Figure 2.5A). Changes

in CO_2 isotopic composition also followed this trend; as *M.barkeri* cultures grew, the CO_2 content became isotopically lighter initially while the final measurements indicated much less carbon fractionation (Figure 2.5B). The N_2/CO_2 gas mixture added to the headspace of cultures accounted for the bulk of the isotopically heavier CO_2 present at the time of inoculation, while subsequent measurements reflected the carbon isotopic composition of CO_2 produced by the methanogen. The final measurements were isotopically heavy because the fractionation to methane from limited substrate led to isotopically heavy CO_2 (mass balance).

Methanol-Abundant substrate

There was some discrepancy between the growth monitor cultures and the cultures harvested for isotope measurements. The triplicate growth monitor cultures produced methane rapidly (Figure 2.6). As a result, the abundant substrate cultures were harvested after only three days of incubation. It was calculated that the growth monitor cultures had used up an average of approximately 8.5% of the total substrate available at that time (Table 2.1). When the experimental cultures were harvested, it was discovered that they had consumed, on average (except biomarker cultures), slightly less of the total methanol available (approximately 8% for biomass and TLE cultures) than the growth monitor cultures. While this was more methanol consumption than targeted, the percentage used was still under 10% for all cultures, and this was still acceptable as abundant substrate conditions. The average yield of these cultures was near 15mg dry weight/mmoles CH_4 with the exception of the biomarker cultures in which high variability between the triplicate cultures lead to a lower average yield.

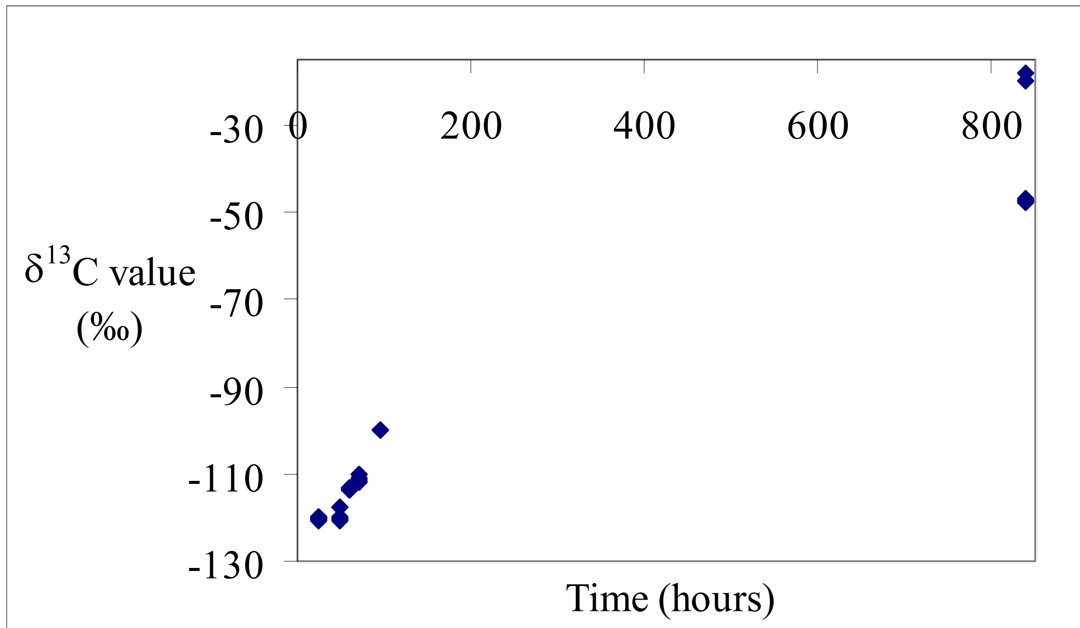

Figure 2.5A Changes in the δ^{13}C of methane over time as methanol was consumed by *M.barkeri*.

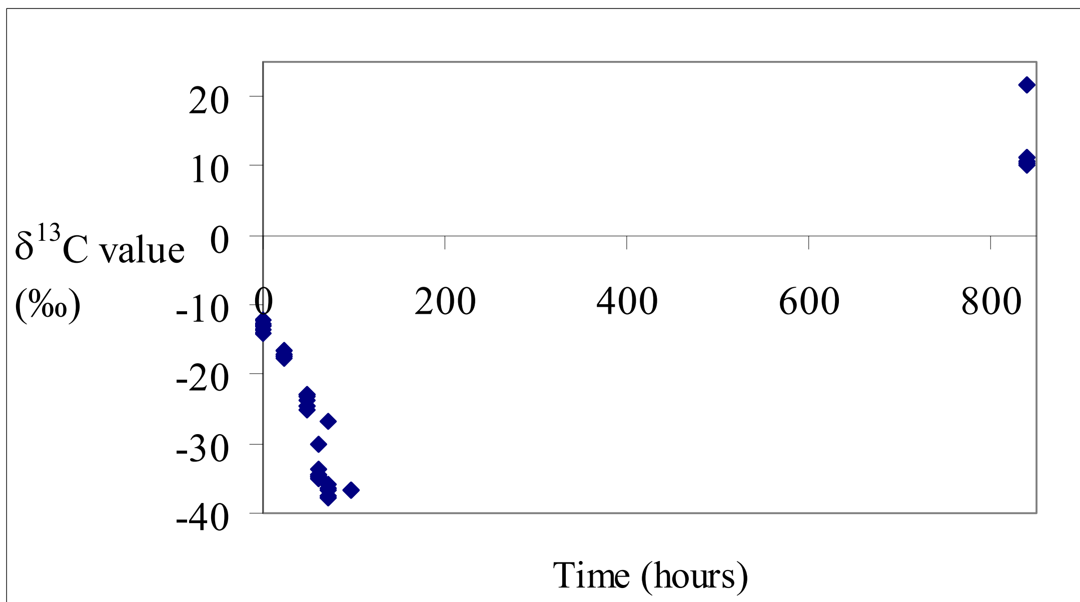

Figure 2.5B Changes in the δ^{13}C of carbon dioxide over time as methanol was consumed by *M.barkeri*.

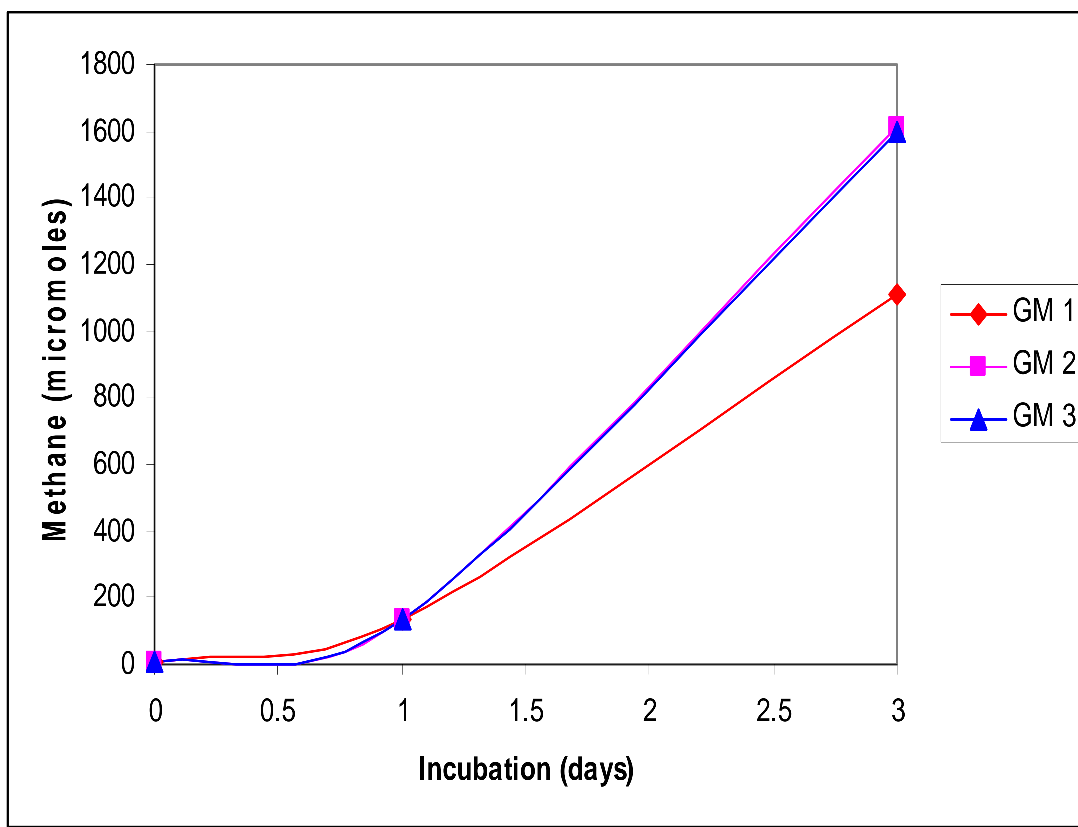

Figure 2.6 Methane production in growth monitor *M.barkeri* cultures supplied with abundant methanol.

Table 2.1 Average production of methane and biomass, decrease in pH in triplicate cultures with abundant methanol.

Culture	pH at harvest	Methane produced (millimoles)	Percent methanol consumed (%)	Cell mass produced (mg)	Yield (mg dry weight) / (mmoles CH_4)
growth monitor	6.38 ± 0.16	1.43 ± 0.28	8.5 ± 1.6	20.9	14.6 ± 0.3
biomass	6.25 ± 0.01	1.34 ± 0.15	7.9 ± 0.9	20.0 ± 5.5	14.9 ± 5.5
total lipid extract	6.25 ± 0.01	1.37 ± 0.10	8.1 ± 0.6	23.6 ± 14.7	17.2 ± 2.12
*biomarkers	6.50 ± 0.06	9.23 ± 3.10	7.2 ± 0.4	73.9 ± 27.0	8.0 ± 2.8

*biomarker cultures were in large bottles.

Carbon isotopic measurements of *M.barkeri* biomass and TLE are presented in Table 2.2. Surprisingly, biomass (-72.9‰) was much isotopically lighter than TLE (-38.2‰). Inoculum biomass (-59.6‰) was not as light as the experimental cultures (-72.9‰) because the inoculum did not have abundant substrate.

Table 2.2 Carbon isotope measurements of *M.barkeri* grown with abundant methanol.

Sample	Replicate	$\delta^{13}C$ value (‰)	$\Delta\delta^{13}C_{sample-substrate}$ value (‰)
biomass	1	-72.7	
	2	-72.7	
	3	-73.3	
average		-72.9 ± 0.4	-33.9 ± 0.3*
total lipid extract	1	-38.0	
	2	-40.6	
	3	-35.9	
average		-38.2 ± 2.4	+0.8 ± 2.4*
inoculum-biomass	1	-59.6	-20.6*
unamended-biomass	1	-59.6	-20.6*

*These values assume a $\delta^{13}C_{methanol}$ of -39‰, until actual measurements are known.

Methanol-Limiting substrate

The cultures with limiting substrate were grown for 30 days. The methanol substrate was added to 10mM concentrations at days 0, 4, 10, and 17. After thirty days, the growth monitor cultures did not show an increase in methane production (Figure 2.7) and had consumed, on average, 70% of the methanol provided (Table 2.3). The contribution of methane from the inoculum was minimal, the methane produced by unamended cultures accounted for only 0.1% of the substrate provided to the limited substrate cultures. The experimental cultures were harvested, and the methane values indicated that on average, 70% of the methanol had been consumed in these cultures as well. The apparent cessation of methane production could be due to the lowering of pH (Table 2.3) in spite of the bicarbonate buffering system, due to the production of large amounts of CO_2 gas. The average yield in these cultures was 6 mg biomass/mmole CH4, much less than with abundant substrate. Biomass production was lower in cultures supplied with limiting methanol (Tables 2.1 and 2.3), indicating that more carbon was allocated to methane production, rather than biomass production under these conditions.

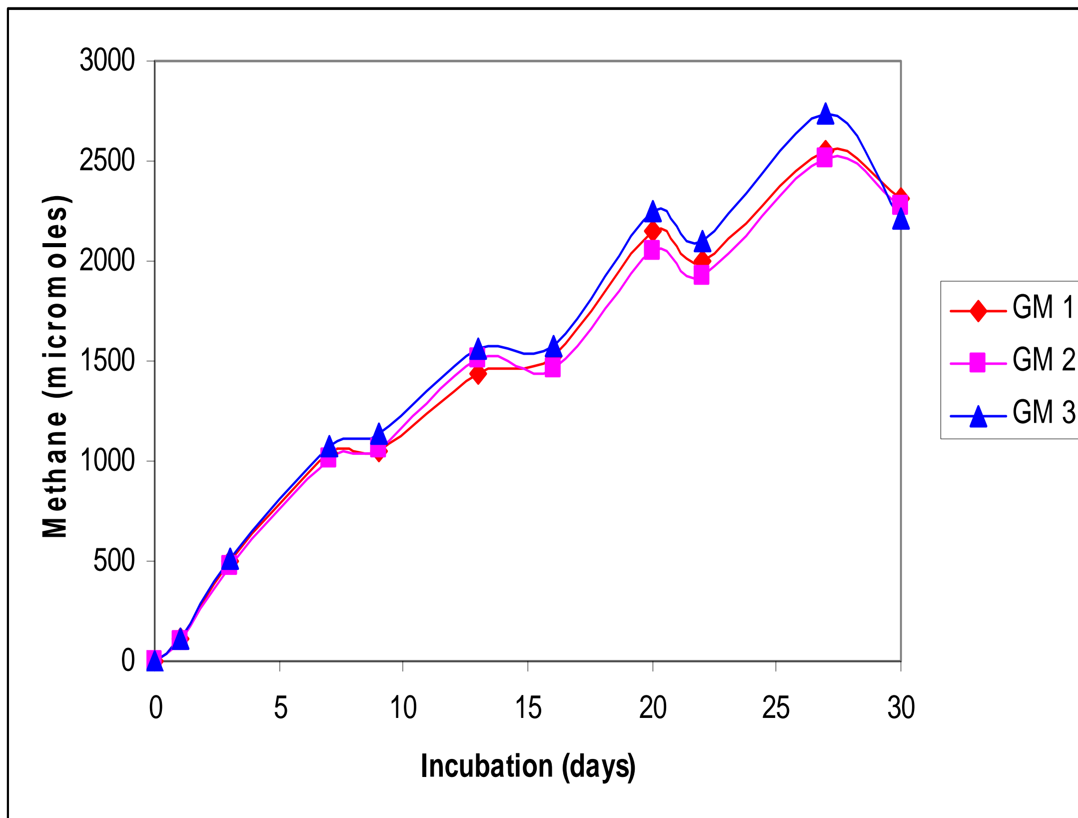

Figure 2.7 Methane production in growth monitor *M.barkeri* cultures with limiting supply of methanol.

Table 2.3 pH and methane measurements of triplicate limited substrate usage cultures grown on methanol.

Culture	**pH at harvest**	**Methane produced (millimoles)**	**Percent methanol consumed (%)**	**Cell mass produced (mg)**	**Yield (mg dry weight) / (mmoles CH_4)**
growth monitor	6.05 ± 0.02	2.59 ± 0.12	69.2 ± 3.2	16.7	6.5 ± 0.1
biomass	6.01 ± 0.02	2.55 ± 0.11	68.0 ± 2.9	14.4 ± 3.7	5.6 ± 3.7
total lipid extract	5.99 ± 0.01	2.61 ± 0.08	69.7 ± 2.2	15.8 ± 4.2	6.1 ± 4.2
biomarkers	5.99 ± 0.01	2.61 ± 0.04	69.5 ± 1.1	14.5 ± 3.2	5.5 ± 3.2
Extra (4 replicates)	6.05 ± 0.01	2.65 ± 0.04	70.6 ± 1.1	7.49	7.1 ± 0.1
unamended	6.41 ± 0.01	0.01 ± 0.00	0.1 ± 0.1	4.6	na

na = not applicable

The methanogen biomass harvested from the methanol-limited cultures was ^{13}C-depleted (-50.8‰) as compared to substrate, whereas the TLE appeared to experience very little fractionation ($\delta^{13}C_{TLE}$ = -33.2‰) (Table 2.4). The variation in $\delta^{13}C$ values between samples was due to differences between replicates, as measurements of $\delta^{13}C$ values were very precise.

Table 2.4 Carbon isotope measurements of *M.barkeri* growing with limiting methanol.

Sample	Replicate	$\delta^{13}C$ value (‰)	$\Delta\delta^{13}C_{sample-substrate}$ value (‰)
biomass	1	-50.9	
	2	-51.1	
	3	-50.2	
average		-50.8 ± 0.5	-11.8 ± 0.5*
total lipid extract	1	-30.9	
	2	-35.6	
	3	-33.0	
average		-33.2 ± 2.4	+5.8 ± 2.4*
inoculum-biomass	1	-59.6	-20.6*
unamended-biomass	1	-59.6	-20.6*

*These values assume a $\delta^{13}C_{methanol}$ of -39‰, until actual measurements are known.

Acetate-Methane

As with the methanol isotopes experiment, $\delta^{13}C$ values of methane produced by *M.barkeri* growing on acetate increased as substrate was consumed in the closed system cultures (Figure 2.8A). The initial methane samples were isotopically heavier than other early methane samples, probably due to carry-over from the inoculum. *M.barkeri* cultures grown with limiting acetate produced isotopically heavier CH_4 ($\Delta\delta^{13}C_{CH4-acetate}$ = -22.9 ± 0.8‰) compared to initial conditions ($\Delta\delta^{13}C_{CH4-acetate}$ = -35.1‰ ± 7.9) where acetate was abundantly available. Again, the initial carbon isotopic measurements of CO_2 were isotopically heavier than subsequent samples (Figure 2.8B) because CO_2 added to the headspace prior to inoculation would have dominated gas samples at this time. Following this however, CO_2 became isotopically lighter with acetate (-30.9 ± 0.6‰) consumption.

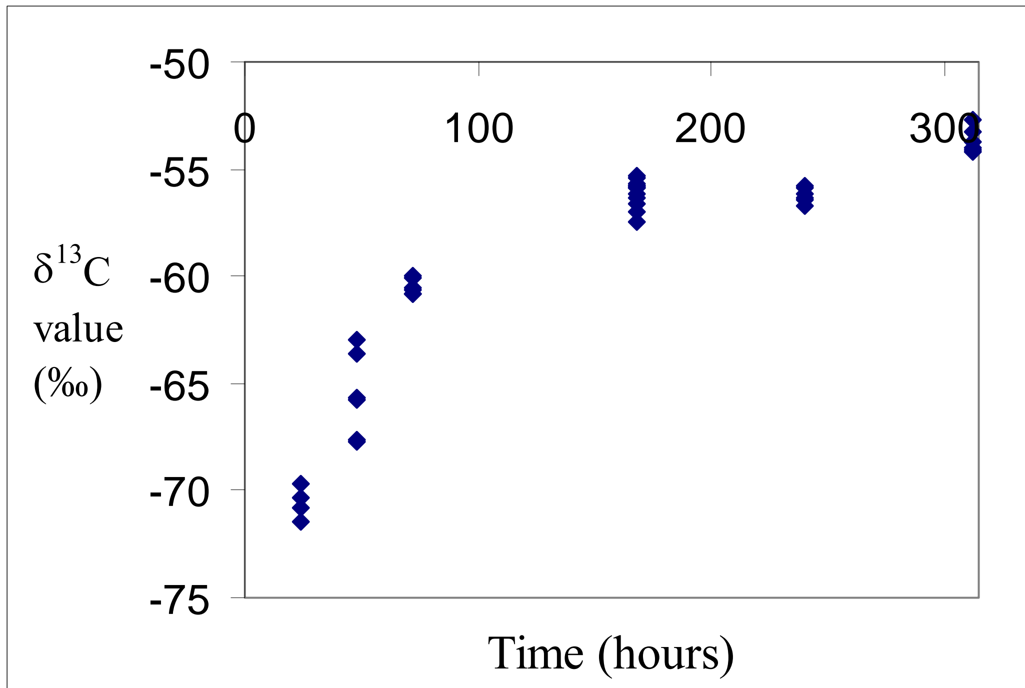

Figure 2.8A Changes in the $\delta^{13}C$ of methane over time as acetate was consumed by *M.barkeri*.

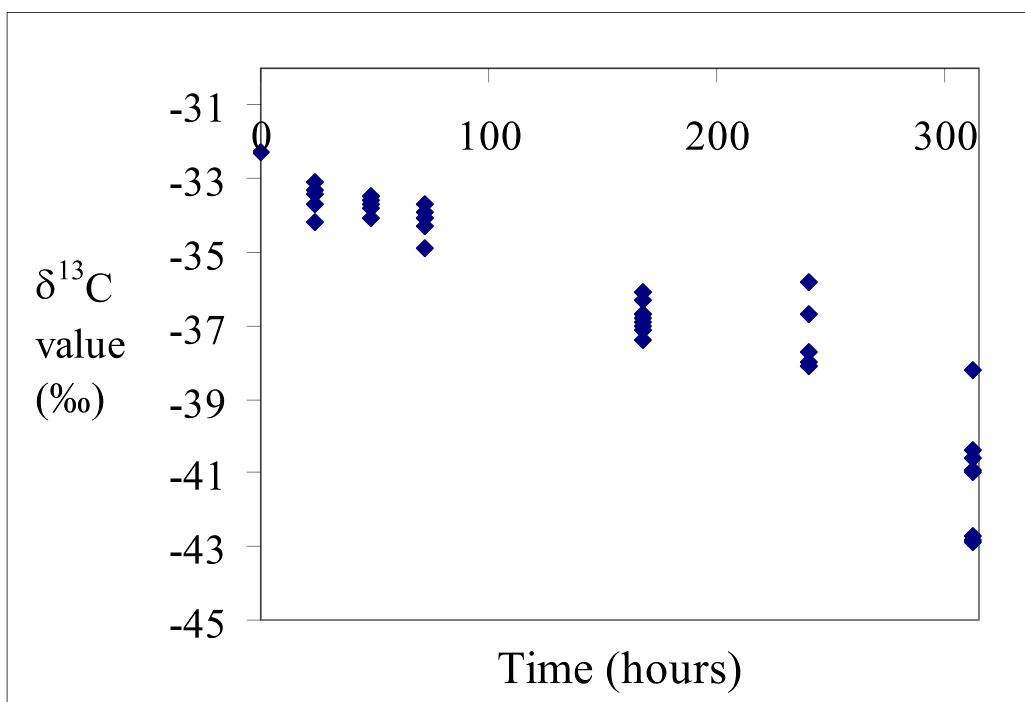

Figure 2.8B Changes in the $\delta^{13}C$ of carbon dioxide over time as acetate was consumed by *M.barkeri*.

Acetate Abundant substrate

Methane production by the growth monitor cultures is shown in Figures 2.9 A and B. The abundant substrate usage cultures were harvested after two weeks of incubation because it was calculated that, on average, the abundant substrate growth monitor cultures had consumed just over 6% of the available substrate at this time (Table 2.5). In this case, it appeared that the experimental cultures had consumed slightly more of the substrate, with an average of 8.4% consumption, with the exception of the large biomarker cultures, which were highly variable. There was not a large drop in pH as compared to the methanol experiment. As with the methanol experiment, *M.barkeri* cultures supplied with abundant acetate produced more biomass than the acetate-limited cultures (Tables 2.5 and 2.6).

Table 2.5 pH and methane measurements of triplicate limited substrate usage cultures grown on acetate.

Culture	**pH at harvest**	**Methane produced (millimoles)**	**Percent acetate consumed (%)**	**Cell mass produced (mg)**	**Yield (mg dry weight) / (mmoles CH_4)**
growth monitor	nd	0.87 ± 0.14	6.2 ± 1.0	nd	nd
biomass	6.94 ± 0.01	1.18 ± 0.12	8.4 ± 0.9	9.9 ± 0.6	8.4 ± 0.6
*biomarkers	7.41 ± 0.10	3.39 ± 3.93	2.4 ± 0.7	37.3 ± 8.0	11.0 ± 8.9

nd = not determined.
*biomarker cultures were in large bottles

Carbon isotope values of *M.barkeri* biomass from abundant acetate cultures and sterile controls are shown in Table 2.6. Measurements for TLE could not be done for this experiment due to lack of sufficient inoculum. Inoculum (-30‰) was slightly

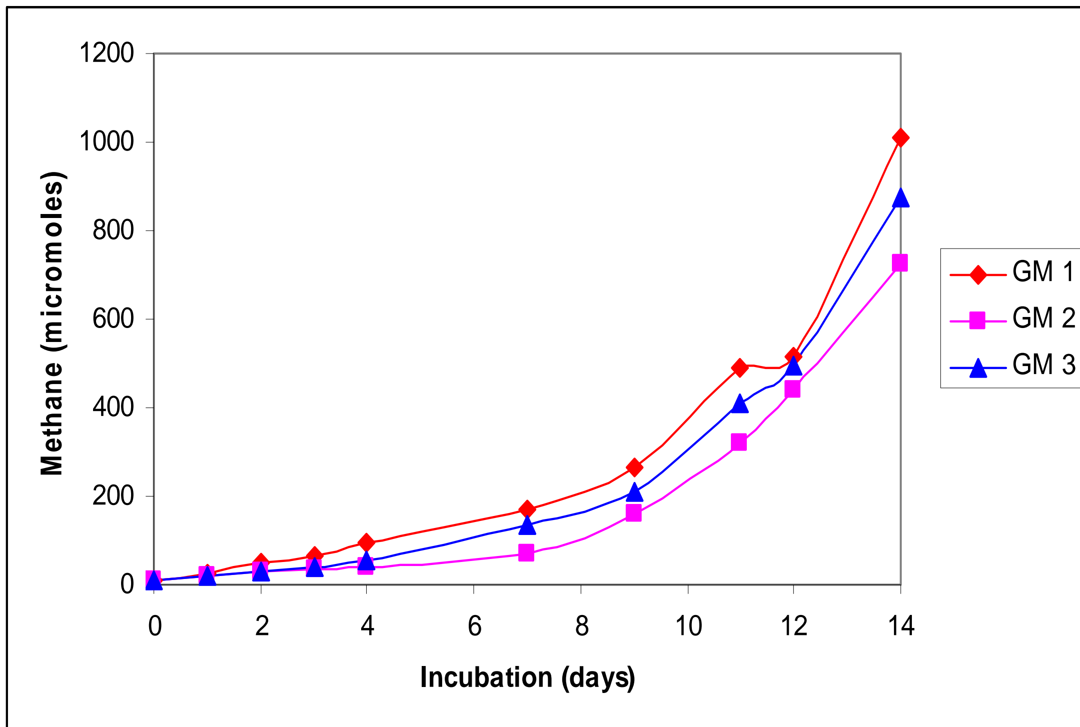

Figure 2.9A Methane production in growth monitor *M.barkeri* cultures supplied with abundant acetate.

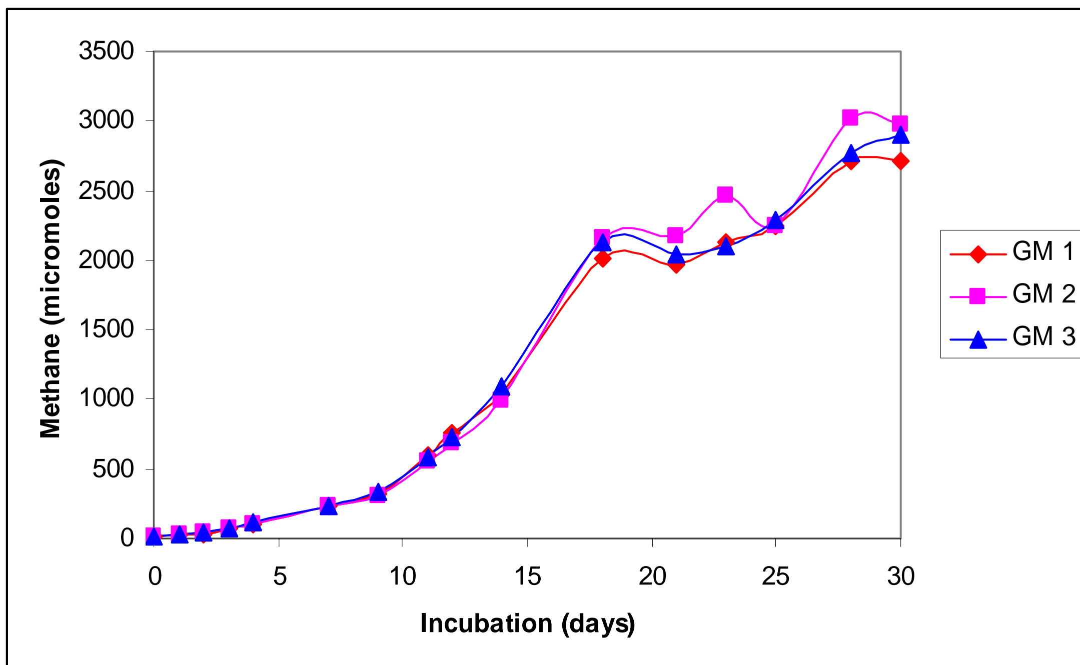

Figure 2.9B Methane production in growth monitor *M.barkeri* cultures supplied with limiting acetate.

isotopically heavier than the experimental biomass (-35‰), because this inoculum had been allowed to consume most of the substrate.

Table 2.6 Carbon isotope measurements of *M.barkeri* growing with abundant acetate.

Sample	Replicate	$\delta^{13}C$ value (‰)	$\Delta\delta^{13}C_{sample-substrate}$ value (‰)
biomass	1	-35.3	
	2	-35.8	
	3	-35.4	
average		-35.5 ± 0.2	-4.6 ± 0.6
unamended	1	-30.2	
	2	-29.9	
	3	-30.1	
average		-30.0 ± 0.2	0.9 ± 0.6

Acetate-Limited substrate

Acetate was added five times (on days 1, 16, 21, 28 and 32) (10mM each) to the limited substrate usage cultures before harvesting. When growth monitor cultures had consumed this acetate, all experimental cultures were harvested (Table 2.7). It was then discovered that there was substrate carry over from the inoculum culture despite efforts to prevent this. The first calculations of acetate consumption indicated more than 100% usage. After taking into account the average carry-over methane production in the unamended cultures, these values were then recalculated. These new values indicated an average consumption of 90% of the acetate (from carry over as well as additions) among the limited substrate cultures. Yield values were lower in these cultures than in those supplied with abundant methanol.

Table 2.7 pH and methane measurements of triplicate limited substrate availability cultures grown on acetate.

Culture	**pH at harvest**	**Methane produced (millimoles)**	**Percent acetate consumed (%)**	**Cell mass produced (mg)**	**Yield (mg dry weight) / (mmoles CH_4)**
growth monitor	nd	4.13 ± 0.18	94.50 ± 4.03	nd	nd
biomass	7.20 ± 0.05	6.00 ± 0.41	87.80 ± 9.51	12.0 ± 2.3	2.0 ± 2.3
biomarkers	7.17 ± 0.01	6.20 ± 0.39	92.20 ± 8.95	13.9 ± 0.6	2.2 ± 0.7
sterile control	6.78 ± 0.02	0.03 ± 0.00	0.80 ± 0.10	3.3 ± 1.4	nd
unamended	6.96 ± 0.03	2.16 ± 0.02	nd	6.6 ± 1.3	nd

nd= not determined.

Table 2.8 displays the biomass carbon isotopic measurements of cultures that received a limited supply of acetate. In this case, there was less carbon fractionation than in the abundant substrate cultures, as expected. Biomass of the limited substrate cultures (-31‰) was only 1‰ lighter than the unamended cultures (-30‰).

Table 2.8 Carbon isotope measurements of *M.barkeri* growing with limiting acetate.

Sample	Replicate	$\delta^{13}C$ value (‰)	$\Delta\delta^{13}C_{sample-substrate}$ value (‰)
biomass	1	-30.8	
	2	-30.9	
	3	-31.3	
average		-31.0 ± 0.3	-0.1 ± 0.6
unamended	1	-30.2	
	2	-29.9	
	3	-30.1	
average		-30.0 ± 0.2	0.9 ± 0.6

TMA-Methane

Isotopic values of methane produced by *M.barkeri* cultures during the TMA experiment indicated that methane became isotopically heavier as the substrate was consumed under both abundant and limiting substrate conditions (Figure 2.10A and B). Although both sets of *M.barkeri* cultures began the experiment with methane $\delta^{13}C$ values close to -100‰ from carry-over in the inoculum, the different growth conditions produced isotopically distinct methane as the experiment progressed. The abundant substrate cultures produced methane with $\delta^{13}C$ values of -86.81 ± 0.46‰. In this case, the initial methane carried over with the inoculum was very light, but became diluted by the methane produced by *M.barkeri*, which did not become heavier over time because substrate was abundant. In the case of limited substrate cultures, methane $\delta^{13}C$ values became as isotopically heavy as -28.52 ± 0.22‰ at the final time point. Isotopic values of CO_2 in these cultures were not measured.

TMA –Abundant substrate

Abundant TMA *M.barkeri* cultures were harvested after 5 days of growth, as they had consumed 5% of the available substrate. Experimental cultures had actually consumed less than this amount (Table 2.9), however sufficient biomass for isotope analysis was produced. As with the other organic substrates, *M.barkeri* cultures grown with abundant TMA produced more biomass than under limiting conditions (Tables 2.9 and 2.11).

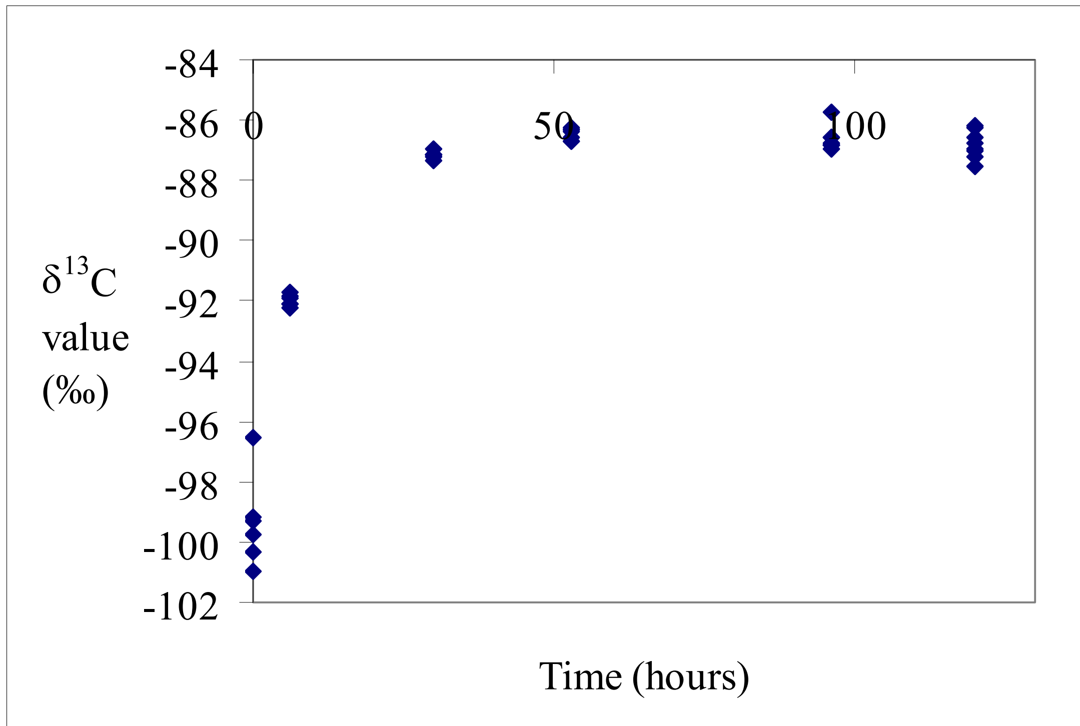

Figure 2.10A Changes in the δ^{13}C of methane over time as abundant TMA was consumed by *M.barkeri*.

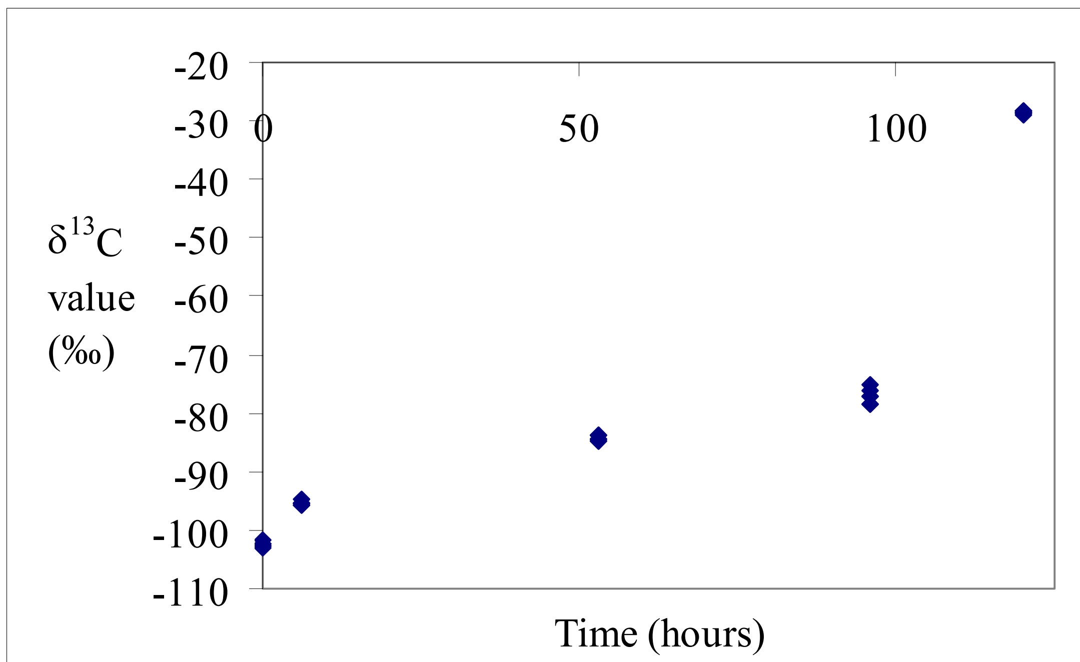

Figure 2.10B Changes in the δ^{13}C of methane over time as limiting TMA was consumed by *M.barkeri*.

Table 2.9 pH and methane measurements of triplicate abundant substrate availability cultures grown on TMA.

Culture	pH at harvest	Methane produced (micromoles)	Percent TMA consumed (%)	Cell mass produced (mg)	Yield (mg dry weight) (mmoles CH_4)
biomass	6.28 ± 0.04	0.73 ± 0.02	1.30 ± 0.00	22.7 ± 6.9	31.3 ± 9.0
total lipid extract	6.31 ± 0.02	0.65 ± 0.04	1.20 ± 0.10	18.1 ± 6.8	26.4 ± 8.7
*biomarker	6.33 ± 0.01	4.03 ± 0.27	1.00 ± 0.10	80.1 ± 3.7	19.9 ± 2.0
sterile control	6.40 ± 0.03	0.00 ± 0.00	nd	nd	nd
unamended	6.41 ± 0.04	1.10 ± 0.06	nd	25.8 ± 6.9	23.5 ± 7.5

nd= not determined. *biomarker cultures were in large bottles

Carbon isotopic analysis of abundant TMA *M.barkeri* cultures is presented in Table 2.10. Once again, biomass was isotopically lighter (-54.2‰) than TLE (-37.9‰). Interestingly, there was not a large difference in carbon isotopic values between the experimental biomass and the inoculum biomass. However, inoculum TLE samples were unexpectedly isotopically lighter (-48.2‰) than experimental TLE.

Table 2.10 Carbon isotope measurements of *M.barkeri* growing with abundant TMA.

Sample	Replicate	δ^{13}C value (‰)	$\Delta\delta^{13}C_{sample\text{-}substrate}$ value (‰)
biomass	1	-54.3	
	2	-53.7	
	3	-54.8	
average		-54.2 ± 0.55	-19.2 ± 0.55†
total lipid extract	1	-37.3	
	2	-38.6	
	3	*	
average		-37.9 ± 0.92	-2.9 ± 0.92†
inoculum-biomass	1	-54.3	nd
inoculum-TLE	1	-47.0	
	2	-48.1	
	3	-49.4	
average		-48.2 ± 1.20	nd

* Low sample response, not reliable value. nd = not determined.
† These values assume a $\delta^{13}C_{TMA}$ of -35‰ until actual measurements are known.

TMA-Limiting substrate

The limited substrate cultures of the TMA experiment were harvested after twelve days of growth, when methane production of growth monitor cultures had leveled off (data not shown). When experimental cultures were harvested, it was determined that *M.barkeri* had consumed only 80% of the TMA provided (Table 2.11). Lowered pH may have been a factor with these cultures, as with the methanol experiment. In this experiment, unamended cultures produced a lot of methane when inoculated into TMA-free media. In fact, the methane produced by these *M.barkeri* cultures would have accounted for approximately 14% of the total methane production expected from the limited substrate experimental cultures. Yield values indicate that *M.barkeri* directed more carbon into biomass with abundant TMA (average of 26 mg/mmole CH_4) than abundant methanol or acetate.

Table 2.11 pH and methane measurements of triplicate limited substrate availability cultures grown on TMA.

Culture	pH at harvest	Methane produced (millimoles)	Percent TMA consumed (%)	Cell mass produced (mg)	Yield (mg dry weight) / (mmoles CH_4)
growth monitor	nd	5.20 ± 0.30	66.2 ± 3.8	4.9 ± 6.1	9.4 ± 1.3
biomass	6.06 ± 0.02	6.34 ± 0.11	80.6 ± 1.4	4.9 ± 2.3	7.8 ± 0.3
total lipid extract	6.14 ± 0.04	6.31 ± 0.33	80.2 ± 4.2	4.7 ± 2.9	7.5 ± 0.9
biomarker	6.14 ± 0.04	5.96 ± 0.73	75.8 ± 9.3	5.6 ± 4.5	9.3 ± 0.4
sterile control	6.40 ± 0.03	0.00 ± 0.00	nd	nd	nd
unamended	6.41 ± 0.04	1.10 ± 0.06	nd	25.8 ± 6.9	23.5 ± 7.5

nd= not determined

Carbon isotopic measurements of *M.barkeri* cultures grown with limiting TMA are shown in Table 2.12. Biomass (-42.1‰) was once again isotopically lighter than TLE (-37.8‰). As with the abundant substrate TMA *M.barkeri* cultures, inoculum TLE (-48.2‰) was isotopically lighter than the experimental culture TLE samples.

Table 2.12 Carbon isotope measurements of *M.barkeri* growing with limiting TMA.

Sample	Replicate	$\delta^{13}C$ value (‰)	$\Delta\delta^{13}C_{sample\text{-}substrate}$ value (‰)
biomass	1	-42.1	
	2	-42.2	
	3	-42.2	
average		-42.1 ± 0.1	-7.1 ± 0.1
total lipid extract	1	-36.1	
	2	-38.3	
	3	-39.0	
average		-37.8 ± 1.5	-2.8 ± 1.5
inoculum-biomass	1	-54.3	nd
inoculum-TLE	1	-47.0	
	2	-48.1	
	3	-49.4	
average		-48.2 ± 1.2	nd

nd = not determined.

Hydrogen- Slow and fast growth methane

Methane produced by autotrophic *M.barkeri* cultures was fractionated to different extents depending on growth rate. Initial measurements of methane in slow growth cultures were very isotopically light (-114.66 ± 2.10‰), while even the initial fast growth cultures measurements were heavier (-81.86 ± 0.37‰)(Figure 2.11A and B). Given that both types of cultures had the same inoculum, the fast growth cultures probably produced methane more quickly, diluting the initial methane to a value

closer to the representative final value of –70.37 ± 0.30‰. The final value for slow growth cultures was lower at –79.10 ± 0.08‰. Assuming no carbon limitation, the $\Delta\delta^{13}C_{CH4\text{-}CO2}$ would be –39 and –48 for the fast and slow growth cultures respectively.

Hydrogen-Slow and fast growth biomass, TLE and biomarkers

Slow-growing *M.barkeri* hydrogen cultures were harvested after 23 days growth, while fast-growing cultures were harvested after 14 days (medium) and at 25 days (large). Consumption of substrate was not considered in this experiment because both sets of cultures were supplied with CO_2 in excess. However, the large TLE and biomarker cultures were incubated for a long time, such that vacuum developed in the flasks and methane could not be accurately measured. Carbon limitation may have become a factor in these cultures.

Table 2.13 pH and methane measurements of triplicate slow and fast growth cultures grown on hydrogen.

Culture	pH at harvest	Methane produced (millimoles)	Cell mass produced (mg dry weight)	Yield (mg dry weight) / (mmoles CH_4)
slow GM	6.42 ± 0.03	0.48 ± 0.02	3.3 ± 1.8	6.9 ± 1.8
slow biomass	6.30 ± 0.07	0.50 ± 0.00	4.3 ± 1.0	8.6 ± 1.0
*slow TLE	6.28 ± 0.03	2.91 ± 0.04	14.5 ± 1.5	5.0 ± 1.5
*slow biomarker	6.37 ± 0.04	2.83 ± 0.04	15.0 ± 3.8	5.3 ± 3.8
fast GM	6.70 ± 0.15	0.49 ± 0.00	3.0 ± 0.6	6.1 ± 0.6
fast biomass	6.59 ± 0.02	0.53 ± 0.03	2.9 ± 1.2	5.5 ± 1.2
*fast TLE	7.23 ± 0.07	na	78.5 ± 1.7	na
*fast biomarker	7.18 ± 0.13	na	88.2 ± 9.1	na

*TLE and biomarker cultures were in large bottles

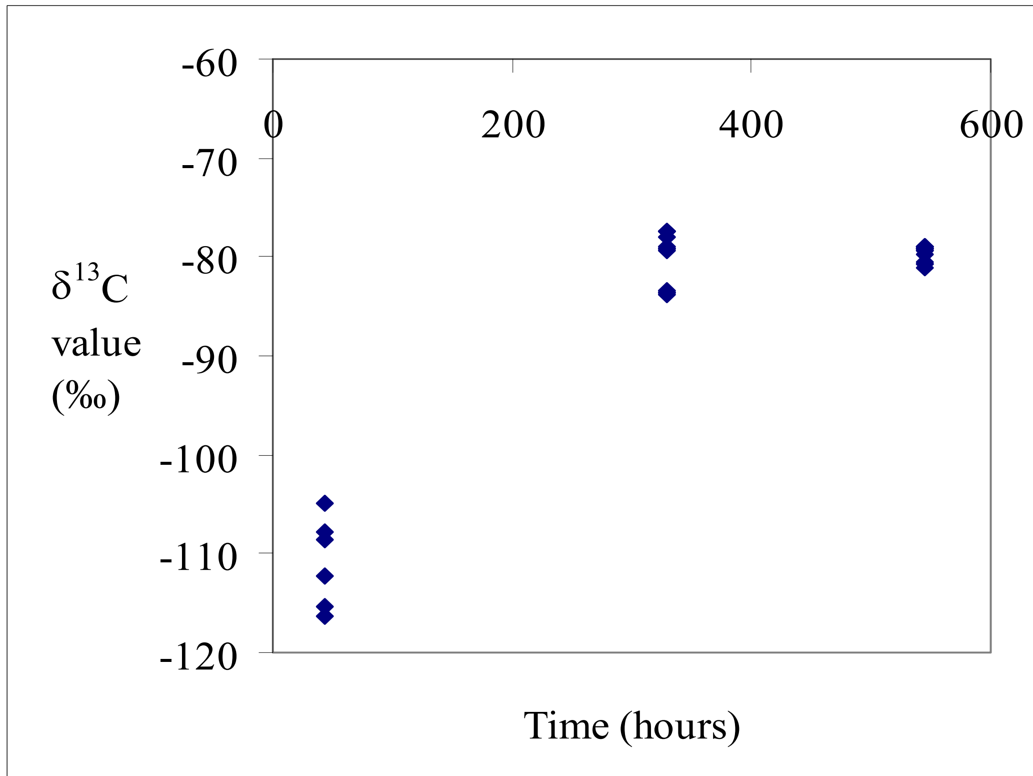

Figure 2.11A Changes in the $\delta^{13}C$ of methane over time as H_2/CO_2 was consumed by slow-growing *M.barkeri* cultures.

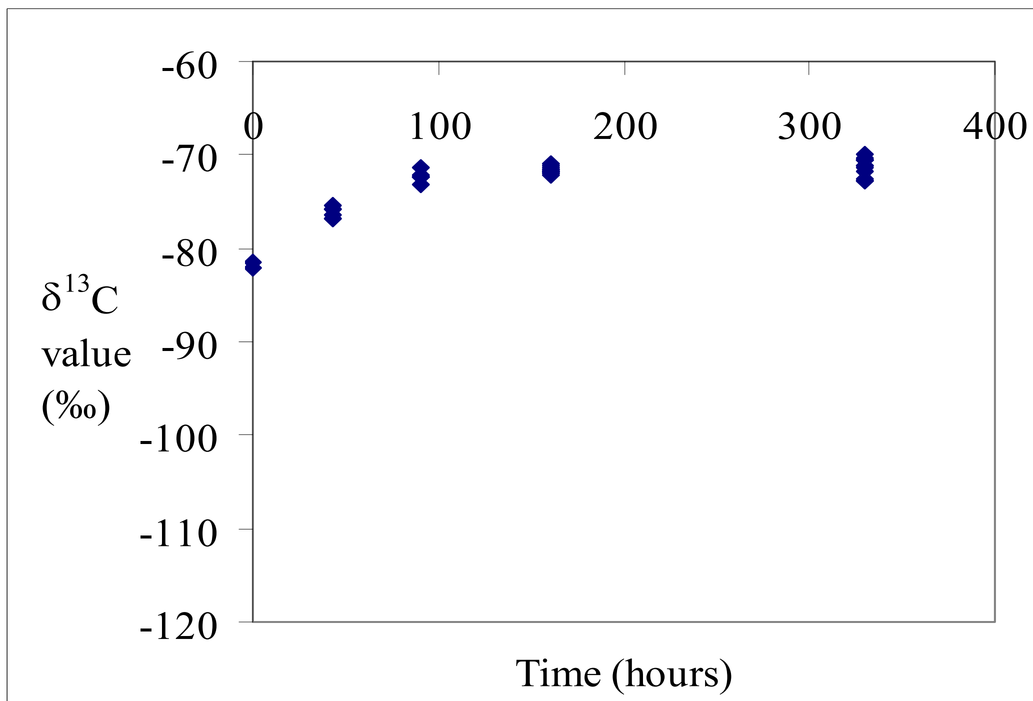

Figure 2.11B Changes in the $\delta^{13}C$ of methane over time as H_2/CO_2 was consumed by fast-growing *M.barkeri* cultures.

Table 2.14 displays carbon isotopic measurements of biomass and TLE from the *M.barkeri* hydrogen experimental cultures. While growth rate affected methane carbon isotopic values, fractionation from substrate to biomass was very similar between slow ($\Delta\delta^{13}C_{biomass-CO2}$ =-12.9 ± 2.1‰) and fast growth cultures ($\Delta\delta^{13}C_{biomass-CO2}$ = -13.6 ± 0.7‰). The different growth rates did result in distinct TLE $\delta^{13}C$ values, with slow growth samples displaying more fractionation ($\Delta\delta^{13}C_{TLE-CO2}$ = -7.5 ± 0.9‰) than fast growth cultures ($\Delta\delta^{13}C_{TLE-CO2}$ = -1.7 ± 0.9‰). However, this could have been due to substrate (CO_2) limitation in these cultures.

Table 2.14 Carbon isotope measurements of *M.barkeri* growing with hydrogen.

Sample	Replicate	$\delta^{13}C$ value (‰)	$\Delta\delta^{13}C_{sample-substrate}$ value (‰)
inoculum biomass	1	-43.7	nd
inoculum total lipid extract	1	-34.4	
	2	-36.3	
average		-35.4 ± 1.4	nd
slow growth biomass	1	-42.8	
	2	-42.9	
	3	-46.4	
average		-44.1 ± 2.0	-12.9 ± 2.1
slow growth TLE	1	-38.4	
	2	-39.5	
	3	-38.3	
average		-38.7 ± 0.7	-7.5 ± 0.9
fast growth biomass	1	-44.5	
	2	-45.1	
	3	-44.7	
average		-44.8 ± 0.3	-13.6 ± 0.7
fast growth TLE	1	-33.0	
	2	-32.2	
	3	-33.6	
average		-32.9 ± 0.7	-1.7 ± 0.9

nd = not detected

2.4 Discussion

Methanol isotopes experiment

The methanol isotopes experiment resulted in a large carbon isotope fractionation between substrate and methane at initial conditions with a $\Delta\delta^{13}C_{CH4-CH3OH}$ value of -81.3 ± 0.1‰ (assuming a $\delta^{13}C_{CH3OH}$ of -39‰). Krzycki *et al.* (1987) found $\Delta\delta^{13}C_{CH4-CH3OH}$ values of -74.8 and -72.5‰ under abundant substrate conditions. These results indicate an even greater fractionation to methane. Over time, as the substrate was consumed, methane became isotopically heavier and the final time point measured (30 days), gave a $\Delta\delta^{13}C_{CH4-CH3OH}$ value of +1.1 ± 16.3‰. This was the largest difference between initial and final methane values observed for any of the substrates.

For biomass, less fractionation was seen with limited substrate, as expected. The $\Delta\delta^{13}C_{biomass-CH3OH}$ value under these conditions was -11.8 ± 0.49‰, while the $\Delta\delta^{13}C_{biomass-CH3OH}$ value for abundant substrate availability was -33.9 ± 0.30‰. This pattern was expected, but had not been measured previously.

The TLE samples were found to be isotopically heavier than biomass under both substrate conditions (Figure 2.12). The heaviest TLE samples were those from abundant substrate conditions, with a $\Delta\delta^{13}C_{TLE-biomass}$ of +34.7 ± 2.36‰. The $\Delta\delta^{13}C_{TLE-biomass}$ values of samples grown under limited substrate conditions were still isotopically heavy, but less so, with a $\Delta\delta^{13}C_{TLE-biomass}$ of +17.6 ± 2.39‰. Under both conditions, the TLE samples were even isotopically heavier than the assumed value for the methanol substrate. Overall, for the methanol experiment samples in terms of

carbon fractionation, methane was isotopically lightest, followed by biomass, and finally TLE.

Acetate isotopes experiment

Over time, *M.barkeri* cultures grown on acetate under limited substrate conditions produced isotopically heavier methane ($\Delta\delta^{13}C_{CH4\text{-}substrate} = -22.9 \pm 0.78‰$) compared to initial (abundant) conditions ($\Delta\delta^{13}C_{CH4\text{-}substrate} = -35.1 \pm 7.9‰$). Krzycki *et al.* (1987) found a $\Delta\delta^{13}C_{CH4\text{-}substrate}$ of -21.2‰ with abundant substrate conditions and Gelwicks *et al.* (1994) reported an $\varepsilon_{CH4\text{-}substrate}$ of -21.3‰. These findings are near the values reported here for limiting substrate conditions, but once again there was even greater fractionation under abundant substrate conditions.

Interestingly, biomass was not much isotopically lighter than the substrate for the acetate experiment samples. The $\Delta\delta^{13}C_{biomass\text{-}substrate}$ values were -4.6 ± 0.65‰ (abundant substrate) and -0.1± 0.65‰ (limited substrate). Knowing that acetate use results in the least carbon fractionation of all four substrates, this was not entirely surprising. Figure 2.13 clearly shows that biomass of *M.barkeri* with abundant substrate was isotopically lighter than that of limiting substrate cultures. Overall, for *M.barkeri* growing on acetate, methane was isotopically lightest, followed by biomass, which was slightly lighter than the substrate.

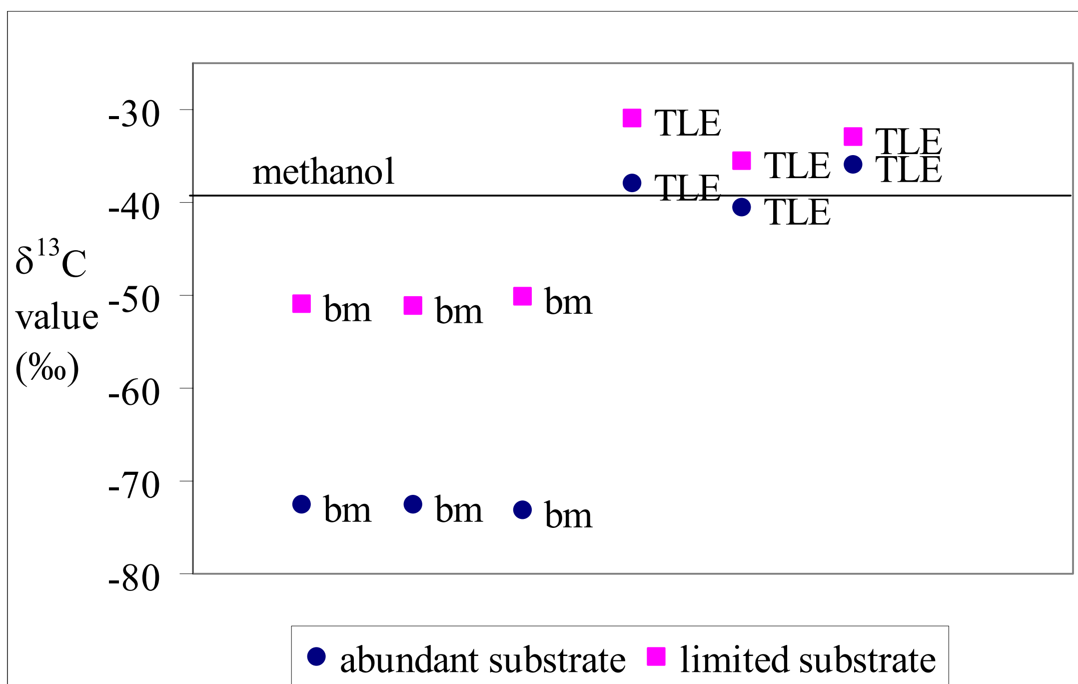

Figure 2.12 Biomass (bm) and total lipid extract (TLE) $\delta^{13}C$ values of triplicate *M.barkeri* cultures grown with abundant and limiting supplies of methanol.

TMA isotopes experiment

Cultures for methane samples were set up slightly differently for the TMA isotope experiment. In this case, two parallel sets of culture tubes were grown in which one set was supplied with abundant substrate and the other set with limited substrate. Therefore the values obtained for both types of cultures can be compared at each time point. Initial readings exhibited similar carbon fractionation between the two sets, with a $\Delta\delta^{13}C_{CH4\text{-substrate}}$ of -60.44 ± 0.38‰ for limited substrate and -56.98 ± 0.19‰ for abundant substrate samples. Methane became isotopically heavier as the substrate was used, in both cases (see note in results section). Summons *et al.* (1998) found an $\varepsilon_{CH4\text{-substrate}}$ of 50.2‰ among early growth samples. Again, these values indicate even more fractionation.

Less carbon isotope fractionation was seen in *M.barkeri* biomass grown with limited TMA substrate conditions (Figure 2.14), where the $\Delta\delta^{13}C_{biomass\text{-substrate}}$ = -7.1 ± 0.05‰, compared to -19.2 ± 0.54‰ under abundant substrate conditions (as expected). Even with limited substrate conditions, biomass was lighter than the substrate. In their study, Summons *et al.* (1998) reported an $\varepsilon_{biomass\text{-substrate}}$ of 20.2‰ among early growth (abundant substrate), which is very close to our value.

Once again, TLE samples were found to be very isotopically heavy relative to substrate and biomass. In fact, it was unexpectedly heavier than biomass under both growth conditions (Figure 2.14); abundant substrate conditions resulted in a $\Delta\delta^{13}C_{TLE\text{-biomass}}$ of +16.3 ± 1.08‰ while limited substrate conditions gave a $\Delta\delta^{13}C_{TLE\text{-biomass}}$ of +4.3 ± 1.53‰. Similar $\delta^{13}C$ values for biomass (-51.7 ± 4.9‰) and TLE (-54.85 ± 8.7‰) were reported by Summons *et al.* (1998). For the *M.barkeri*

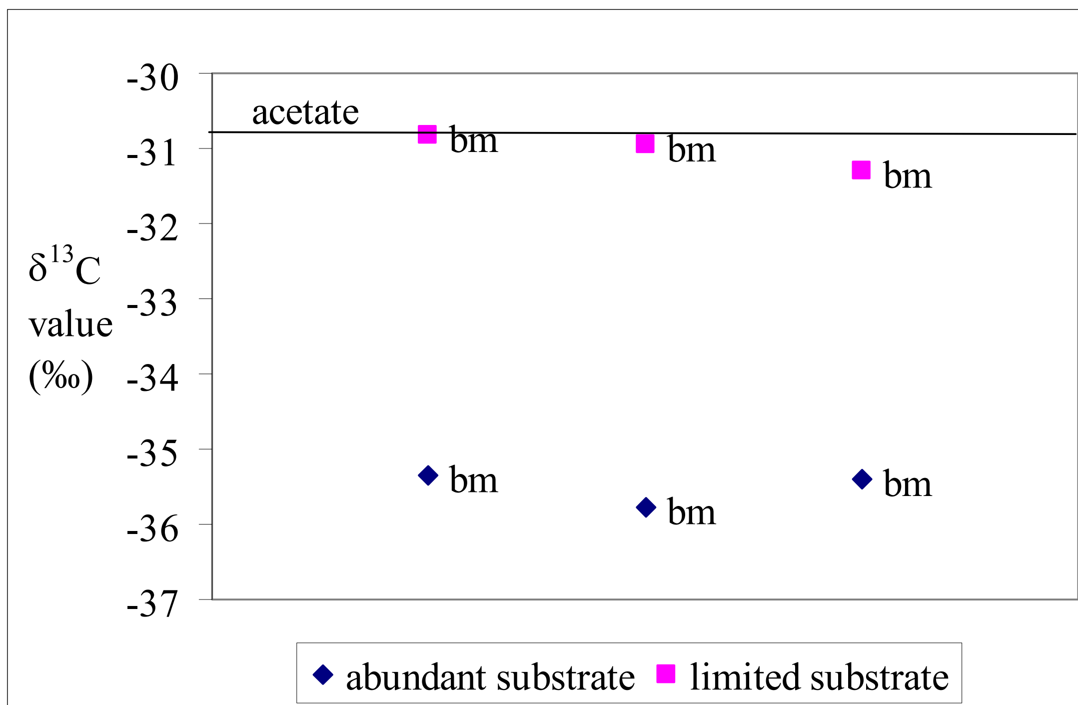

Figure 2.13 Biomass (bm) $\delta^{13}C$ values of triplicate *M.barkeri* cultures grown with abundant and limiting supplies of acetate.

TMA experiment samples, methane was isotopically lightest, followed by biomass, then TLE, and all were lighter than the substrate.

H_2/CO_2 isotopes experiment

More carbon fractionation was seen in the methane produced by *M.barkeri* when grown on H_2/CO_2 under slow growth conditions ($\Delta\delta^{13}C_{CH4\text{-}substrate}$ = -79.69 ± 4.55‰) than under fast growth conditions ($\Delta\delta^{13}C_{CH4\text{-}substrate}$ = -44.95 ± 0.85‰). This comparison had not been previously described. In 1978, Games and Hayes reported $\Delta\delta^{13}C_{CH4\text{-}CO2}$ values of *M.barkeri* cultures of -41.2 and -41.4‰. Later, Krzycki *et al*. (1987) found $\Delta\delta^{13}C_{CH4\text{-}CO2}$ values of -46.3 and -45.6‰. Clearly, there was much more carbon fractionation observed than either of those studies in these cultures. However, these new values are not unreasonable, as other methanogens have exhibited similar fractionation between CO_2 and CH_4. *Methanococcus vannielii* was found to have a fractionation factor (α) between CO_2 and CH_4 of as high as 1.0790, which corresponds approximately to a $\Delta\delta^{13}C_{CH4\text{-}substrate}$ of -76‰ (Botz *et al.* 1996). The same study also reported *Methanococcus thermolithotrophicus* exhibited an approximate $\Delta\delta^{13}C_{CH4\text{-}substrate}$ of as much as -67‰ and *Methanococcus igneus* displayed an approximate $\Delta\delta^{13}C_{CH4\text{-}substrate}$ of as much as -66‰.

Biomass produced by *M.barkeri* in this experiment had similar carbon isotopic signatures whether under fast ($\delta^{13}C_{biomass}$ = -44.8 ± 0.3‰) or slow ($\delta^{13}C_{biomass}$ = -44.1 ± 2.0‰) growth conditions (Figure 2.15). It was expected that biomass would be isotopically lighter with slow growth conditions, however growth rate did not

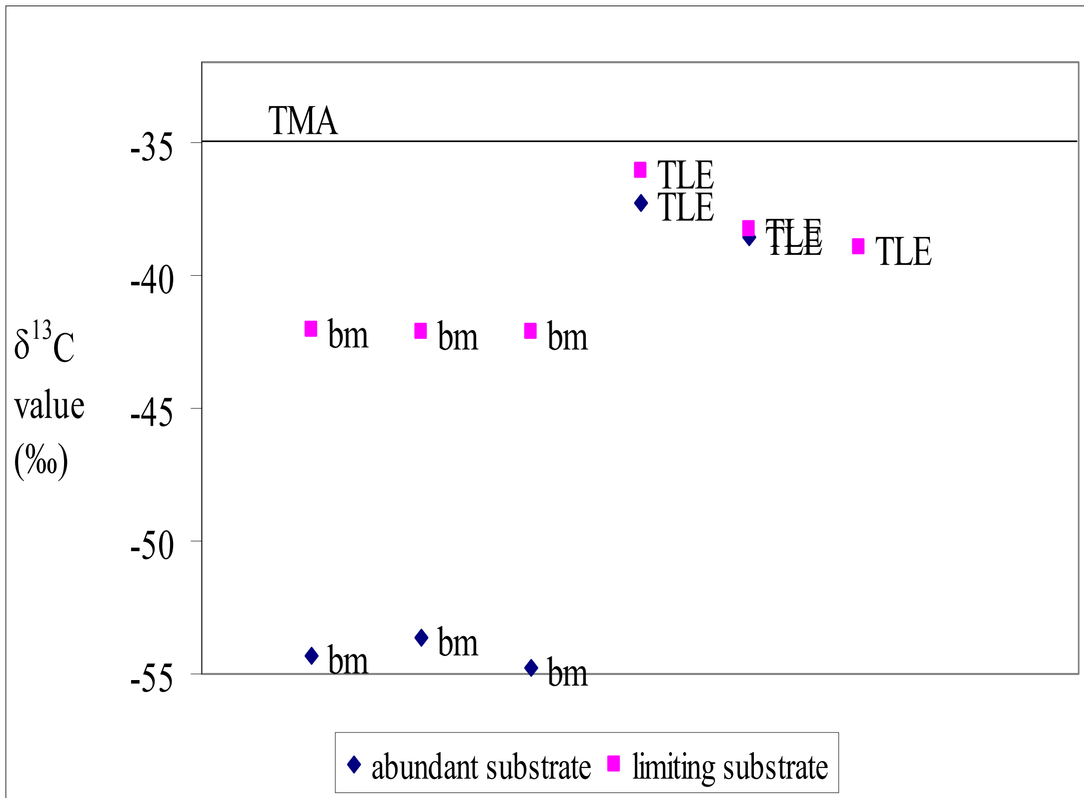

Figure 2.14 Biomass (bm) and total lipid extract (TLE) $\delta^{13}C$ values of triplicate *M.barkeri* cultures grown with abundant and limiting supplies of TMA.

affect these values. House *et al.* (2003) found more fractionation from CO_2 substrate in *M.barkeri* biomass with an approximate $\Delta\delta^{13}C_{biomass\text{-}substrate}$ value of -20‰.

As with the TMA and methanol samples, TLE was isotopically heavier than biomass for *M.barkeri* cells grown on H_2/CO_2 (Figure 2.15). This was regardless of growth conditions; slow growth resulted in a $\Delta\delta^{13}C_{TLE\text{-}biomass}$ of +5.4 ± 2.1‰ and fast growth gave a $\Delta\delta^{13}C_{TLE\text{-}biomass}$ of +11.9 ± 0.79‰. For *M.barkeri* cultures grown autotrophically, methane was isotopically lightest, followed by biomass, then TLE, and all were lighter than the CO_2.

Overview observations of isotopes experiments

Comparisons of the carbon isotopic values of methane, biomass and TLE produced by *M.barkeri* grown on all four substrates are shown in Table 2.15. In all cases, methane was found to be isotopically lighter than biomass and TLE. In terms of biomass, the availability of abundant substrate resulted in more carbon fractionation in all cases. The isotopically lightest biomass carbon was produced by *M.barkeri* grown on methanol, followed by TMA, H_2/CO_2, and finally acetate (Figure 2.16). This is in good agreement with methane data Krzycki *et. al* reported in 1987, but also includes TMA in direct comparison. In general, carbon isotopic fractionation between substrate and methane was greatest when *M.barkeri* was grown on methanol, followed by H_2/CO_2, then TMA and finally acetate.

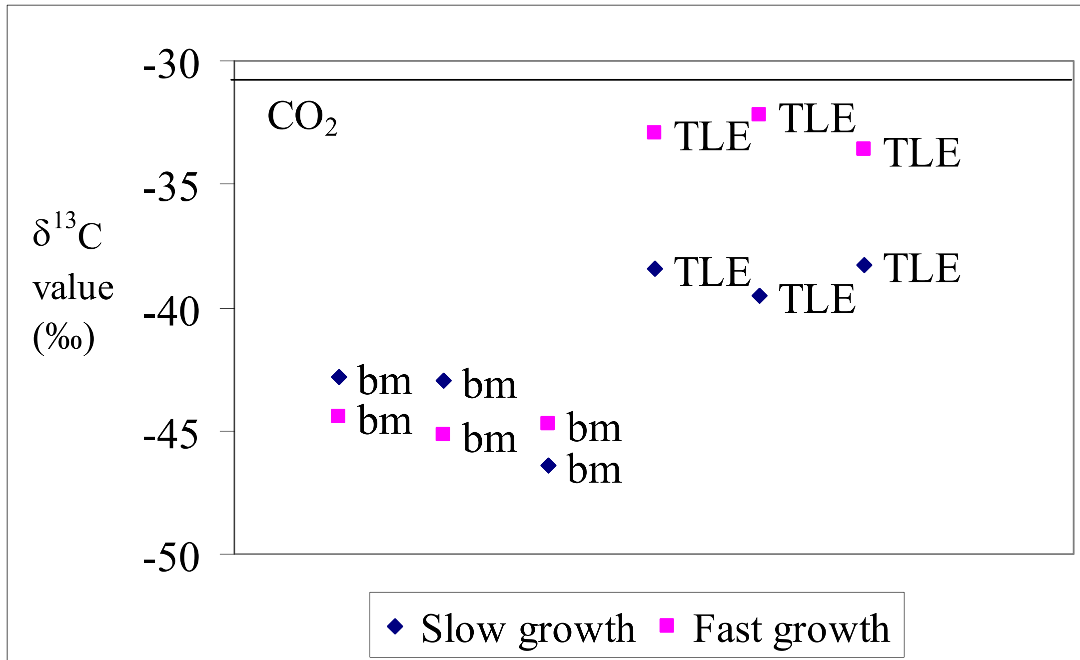

Figure 2.15 Biomass (bm) and total lipid extract (TLE) $\delta^{13}C$ values of triplicate *M.barkeri* cultures grown with abundant and limiting supplies of H_2/CO_2.

Table 2.15 *M.barkeri* isotope experiment carbon isotope value comparisons.

		$\delta^{13}C$ values (‰)			
Substrate condition	**organic substrate**	**CO_2**	**initial CH_4**	**final biomass**	**final TLE**
H_2/CO_2					
fast	na	-31.19 ± 0.58	-76.14 ± 0.14	-44.8 ± 0.34	-32.9 ± 0.71
slow	na	-31.19 ± 0.58	-110.88 ± 4.51	-44.1± 2.03	-38.7 ± 0.68
acetate					
limited	-30.9 ± 0.6	-33.5 ± 0.3	-71.0 ± 7.9	-31.0 ± 0.25	nd
abundant	-30.9 ± 0.6	-41.2 ± 0.7		-35.5 ± 0.24	nd
TMA					
limited	-35*	-28.52 ± 0.22	-95.44 ± 0.38	-42.1 ± 0.05	-37.8 ± 1.51
abundant	-35*	-28.52 ± 0.22	-91.98 ± 0.19	-54.2 ± 0.54	-37.9 ± 0.93
methanol					
limited	-39*	14.3 ± 6.4	-120.3 ± 0.1	-50.8 ± 0.49	-33.2 ± 2.34
abundant	-39*	-33.9 ± 1.2		-72.9 ± 0.34	-38.2 ± 2.34

*Due to instrument delays, isotopic values of these organic substrates are estimates until actual measurements can be done.
na= not applicable.
nd = not determined.

The most unexpected finding was that in all cases, $\delta^{13}C$ values of TLE samples were relatively isotopically heavy. This seemed to be consistent among all the different substrate experiments. It is noteworthy that TLE samples from all substrates growing under both conditions were isotopically heavier than biomass. This was not expected, as lipid carbon is generally somewhat isotopically lighter than the total biomass (Abelson and Hoering 1961, Hayes 1993). All abundant substrate conditions resulted in $\delta^{13}C_{TLE}$ values of approximately -38‰. All limited substrate conditions gave $\delta^{13}C_{TLE}$ values of approximately -33‰, except those from the TMA experiment where the values were again approximately -38‰. There are several possible explanations for this finding. It may indicate contamination of the TLE samples, possibly from the lipid extraction process. This can be determined by the $\delta^{13}C$ measurements of biomarker samples. Another possibility is that some carbon

fractionation occurred during the ASE extraction process, although this is unlikely. More likely, it may be explained by the fact that biomass accumulation occurs through a different pathway than methanogenesis, and there may be a physiological explanation.

M.barkeri cultures grown on all three organic substrates produced more biomass when grown under abundant substrate conditions, allocating more of the additional carbon into growth than methane production, and this (biomass) carbon was fractionated to a greater extent. Both the flow through the methyl-corrinoid to acetyl-CoA (Figure 2.16) and the carbon fractionation were affected, with acetate being the least affected (based on yield and isotopic values). This makes sense in terms of carbon flow in *M.barkeri*, as acetate carbon does not flow through the methyl-corrinoid as methanol and TMA do, but rather joins in at acetyl-CoA. This also explains why *M.barkeri* grown with methanol and TMA give similar isotopic values.

In the case of *M.barkeri* grown with H_2/CO_2, when more hydrogen was available (with shaking in fast cultures), more carbon was put into biomass, and the pH in these cultures rose. With less available hydrogen in the slow growth cultures, proportionally more CO_2 went to methane production, even though the amount of methane produced in the end was the same as the fast growth cultures. Substrate availability did not affect carbon isotopes of biomass, but did affect the methane produced. This was the only experiment in which this was the case, and it may also be explained in terms of carbon flow, as CO_2 enters the carbon pathway through acetyl-CoA in a different manner than the organic substrates (Figure 2.16). Enzymes

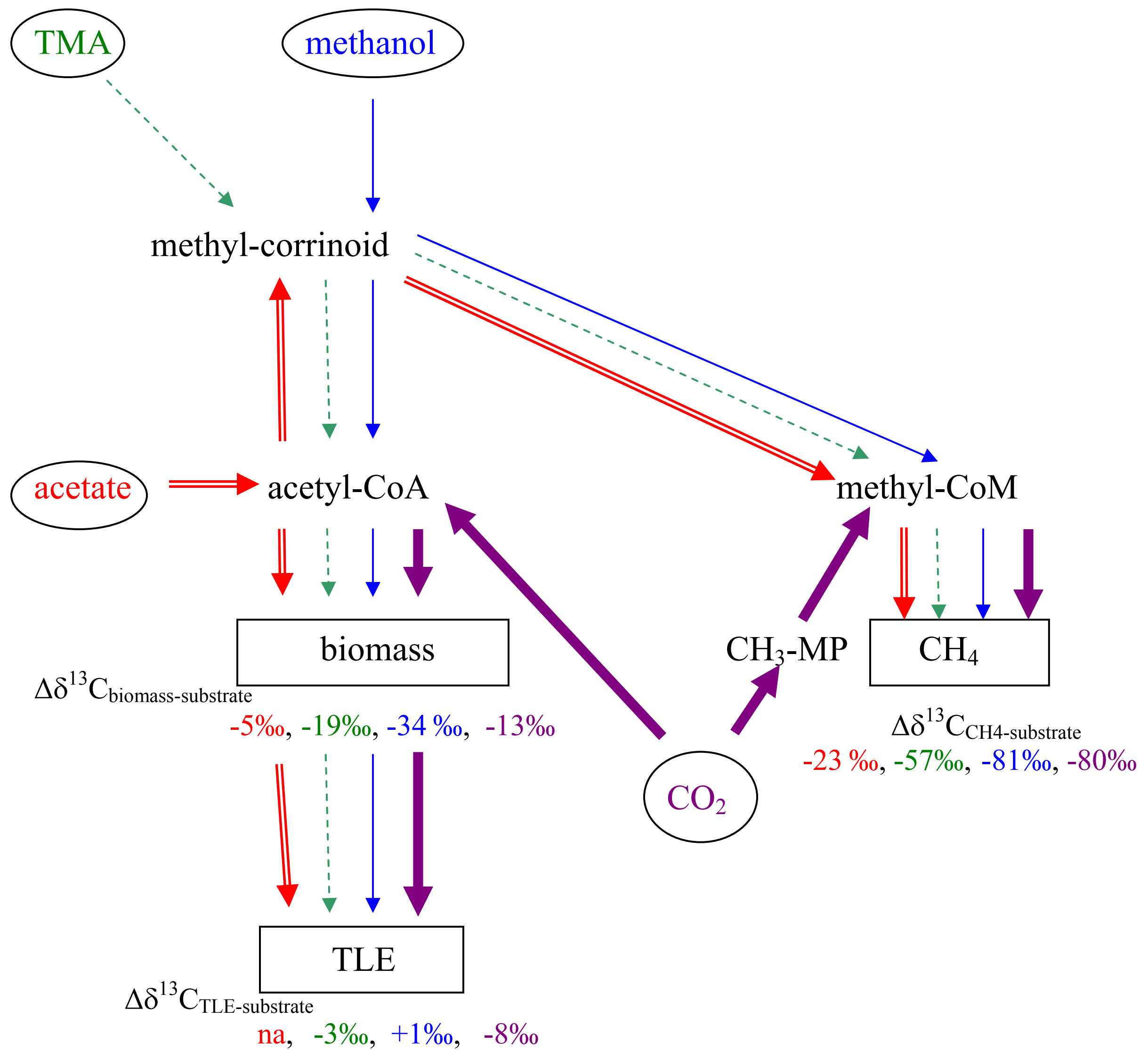

Figure 2.16 Carbon isotope fractionation in *M.barkeri* growing with abundant supplies of acetate, methanol and TMA, as well as H_2/CO_2 under slow growth conditions.
MP = methanopterin, CoM = coenzyme M, na = not available.

catalyzing the conversion of CO_2 to biomass did not fractionate carbon based on greater hydrogen availability, however enzymes leading to methane production fractionated more during slow growth, when less biomass was produced.

2.5 Application of Results

In 1986, Whiticar *et al.* proposed that methanogenesis from CO_2 reduction could be distinguished from methanogenesis via acetate fermentation based on the carbon and hydrogen isotope composition of the methane produced. They proposed that methanogenesis in marine sediments is dominated by the former process, while methanogenesis in freshwater sediments is dominated by the latter. This led them to suggest that methane in marine sediments (CO_2 reduction) can be defined by $\delta^{13}C$ values of -110 to -60‰, and methane from freshwater (acetate fermentation) by $\delta^{13}C$ values of -65 to -50‰. However, *M.barkeri* growing on TMA produced methane with carbon isotope values near -100‰ (within the "CO_2 reduction range"). The presence of this substrate in marine environments would be expected, as the break down product of a common compatible solute. This indicates that assumptions of dominance by a methanogenic pathway based on the criteria presented by Whiticar *et al.* (1986) may not be valid. The incorporation of hydrogen isotopes would help to clarify this.

Carbon isotopic signatures of methanogen biomarkers in natural environments may offer insights into both substrate type and substrate availability of natural anaerobic microbial populations. For instance, these biomarkers may help solve the mystery of anaerobic methane oxidation (AMO). It has been proposed that methanogens, in close association with SRB, have reversed their metabolism and

consume methane to produce H_2 and CO_2 (DeLong 2000). This has been reinforced by the discovery of extremely isotopically light methanogen biomarkers from sediments where this process occurs. These biomarkers are so isotopically light that they must have been formed from a very light carbon source, such as methane (Pancost *et al.* 2000). Pure culture investigations of methanogen carbon isotopic fractionation may indicate that methanogens are capable of producing biomarkers with carbon isotopic signatures as low as those observed on the ocean floor, from substrates other than methane.

2.6 Future Research

In addition to the effects of different substrates and substrate availability, carbon isotopic fractionation in methanogens is affected by other factors, including temperature. It is well known that isotopic fractionation decreases as temperature increases (Hoefs 1997). The experimental results presented here were performed at 37˚C because this is the optimal growth temperature for *M.barkeri*. However, environmental conditions where *M.barkeri* exists will, in many cases, be much lower. This may mean that natural populations of *M.barkeri* are capable of larger fractionations than those observed in these experiments. Therefore, studies of *M.barkeri* growth at environmental temperatures could allow for better interpretation of carbon isotopic values in methane and biomarkers. However, distinguishing the carbon isotope fractionation effects of lower temperatures from that of lower growth rates due to other factors, such as limiting substrate, would be very difficult in environmental samples.

In terms of pure culture research, a simple bioreactor was attempted in order to study autotrophic *M.barkeri* growth. The goal was to increase biomass and remove growth phase effects. However, recurrent problems were encountered with maintaining a reduced medium. A succession of reductants was used including Na_2S and dithionate in combination with a pre-culture palladium catalyst. Unfortunately this system was not able to remain consistently reduced. In the future, such a system would be useful in studying carbon fractionation in *M.barkeri*, perhaps with the addition of stronger reducing agents. In addition, as mentioned in chapter 1, hydrogen can be subject to extremely large fractionations because it is such a small, light molecule. Therefore, a chemostat could be used to study not only the carbon fractionation effects of growth rate, but also the hydrogen/deuterium fractionation effects as well. Development of an accurate method for detecting the amount of biomass produced (taking the tendency of *M.barkeri* cells to clump into consideration) would be helpful in this work.

Previous studies have proposed that combining carbon and hydrogen isotopic signatures of methane in marine and freshwater environments can indicate whether methanogenesis originates from acetate or H_2/CO_2 (Whiticar *et al.*1986). In this way, hydrogen isotopes may also be diagnostic of methanogenic substrates. For this reason, it would be advantageous to use pure culture studies of methanogens to discover how they fractionate hydrogen into methane when grown on different substrates, as well as under different growth conditions, just as was done for the carbon isotopes.

Chapter 3 Hypersaline Spring Studies

3.1 Introduction

The hypersaline springs discharging along the western shore of Lake Winnipegosis in West Central Manitoba create a very unique continental environment. The spring waters can reach salinities of 61‰ (McKillop *et. al* 1992), almost double that of seawater (approximately 35‰). These hypersaline springs have been flowing for at least 5,500 years (Patterson *et. al* 1997), and may have emerged with the retreat of the continental ice sheet from this region approximately 10,000 years ago. Previous geochemical and stable-isotope analysis of the springs have indicated they originated as meteoric water (Grasby *et al.* 2000). During the Pleistocene a large influx of glacial meltwater dissolved evaporate beds below, and with the retreat of the ice sheets, this water resurfaced as springs with high salinity (Grasby *et al.* 2000, Grasby and Chen in review).

An entire ecosystem including marine species of animals (Chordata, Protozoa, Rotifera, and Anthropoda) (McKillop *et al.* 1992), plants (Burchill and Kenkel 1991) and algae (Londry *et al*. submitted) exists in the middle of the continent due to these hypersaline waters. Salt pans form around discharge areas, and vegetation surrounds the pans in concentric circles dependant on salt tolerance (Burchill and Kenkel 1991). It has been suggested (Patterson *et al.* 1997) that the presence of these marine species is due to transportation via birds (perhaps through fecal matter) from coastal areas, including Hudson Bay in Northern Manitoba.

Microbial mats growing on the surface of spring waters have been observed in the Dawson Bay area (north-western shore of the lake) as well as further east along

the Red Deer River and near Pelican Bay (east of Dawson Bay) (Londry *et al.* submitted). These microorganisms appear to be the only life forms capable of surviving in this extremely saline water. While more complex life forms are unable to survive in the springs or their surrounding salt pans, microbial communities flourish. Microbial mats growing in continental saline lakes and salterns have been well studied, but those of saline springs are not well understood (particularly the anaerobic microbes present). These mats contain important information about microbial life in extreme conditions and serve as modern analogues for microbial mats, which were the dominant life forms during the Precambrian. This may also aid in the understanding of how life may potentially exist in extraterrestrial saline environments.

Due to the high salinity of these spring waters, the presence of the compatible solute glycine betaine is expected, as many microorganisms employ this method of salt tolerance. Therefore, its breakdown product trimethylamine (TMA) was also expected to be available. While this compound is believed to be a "non-competitive" substrate for methanogens (even in the presence of sulphate reducing bacteria) (Oremland and Polcin 1982), it was not known whether methanogenesis was an important process within the springs. The aim was to study the microbial composition of the springs, focusing on the anaerobic communities of methanogens and sulphate reducing bacteria (SRB) present. Specifically, the goal was to learn which substrates these microorganisms were able to use through culturing, the potential rates of methanogenesis and sulphate reduction in the spring sediments though the use of added substrates and inhibitors, and to study community structure within the

microbial mats through lipid analysis. Samples were collected in October of 2000, May of 2002 and August of 2003 in order to compare microbial mat growth between different seasons and years. As this was a primary investigation of this site and some samples were collected prior to starting my work on this area, changes in the microbial mats throughout different time points during the same year were not done, and interannual changes were also expected when comparing the mats during different seasons.

3.2 Materials and Methods

Field Sampling

An aggregation of several salt springs is located near Dawson Bay at 52˚ 45' 10" latitude north and 100˚ 52' 50" longitude west (NAD 87). The springs are located at East German Creek, near a gravel road leading to the Sapotaweyak Cree Nation off of provincial highway no.10 approximately 100 km north of Swan River past Mafeking. From the road, a small expanse of bush opens up to a grassy meadow from which the salt pans emerge. Of the discharge sites in the Dawson Bay area, this site contains the most springs and the salt pan is by far the largest (Figure 3.1A). Field trips were conducted on May 16-19, 2002 and August 5-7, 2003.

At this site, salinity was so great that springs were surrounded by a large salt pan, which did not readily support plant life (Figure 3.1B). Only two small halophilic *Salicornia* plants (Figure 3.1C) were observed growing on the pans. The salt pan consisted of a spongy, brainy-textured orange to reddish-brown tufa material immediately overlying a viscous black layer (Figure 3.2), which may have been iron

Figure 3.1A East German Creek salt spring discharge site in May 2002.

Figure 3.1B Lack of plant life around salt springs and salt pan at East German Creek in May 2002.

Figure 3.1C *Salicornia* growing near edge of spring at East German Creek in August 2003.

Figure 3.2 Tufa material covering salt pan surrounding springs throughout the salt pan at East German Creek.

Figure 3.3 Colourful gravel-like sinters surrounding some springs at East German Creek. White material is precipitated, crystallized salt.

sulphide formed from sulphide produced by SRB. Gravel-like sinters composed of calcium carbonate were present around several springs and colours varied from brown to red to purple (Figure 3.3). Boulders were commonly found throughout the pan as has been described at many spring sites in the area (Bezys *et al.* 1997).

Many of the larger springs were raised and had small streams flowing over the sides of their pools. Often these streams joined each other and flowed together. The brine waters of the springs were very clear and a gray deposit was easily visible along the bottom of the pools as seen in Figure 3.4. Gas bubbles were frequently seen rising from the springs as well as some spots along the streams (Figure 3.5). Today, gas bubbles frequently rise from the springs and consist of mainly N_2 (95%), with traces of CO_2 (1.8%), He (1.5%), Ar (0.8%), O_2 (0.9%), and CH_4 (0.02%) (McKillop *et. al* 1992).

During the first field trip in May of 2002, three springs were selected for sampling based on the relative amounts of microbial mat growth. One spring with a low amount of mat growth was used (Figure 3.6A), as well as one with a high amount (completely covered pool) (Figure 3.6B) and one with an intermediate amount (partially covered pool) (Figure 3.6C). An undisturbed, "pristine" spring of each type was also chosen for physical measurements to ensure that readings were not affected by sampling. Temperature, pH and pool size measurements were taken at all six of these springs. Temperature readings were taken at different times throughout the day as well as at different locations within the pools to see how much spatial and temporal variation existed. Edge and centre surface temperatures were taken within a few cm of the water surface, while deep centre temperature readings were taken from as close

Figure 3.4 Gray deposit observed in spring pool basins at East German Creek.

Figure 3.5 Gas bubbles released in springs at East German Creek.

Figure 3.6A Low growth spring at East German Creek in May 2002.
Tent stake provided for scale.

Figure 3.6B High growth spring at East German Creek in August, 2003.
Spring pool is completely covered by microbial mat growth.

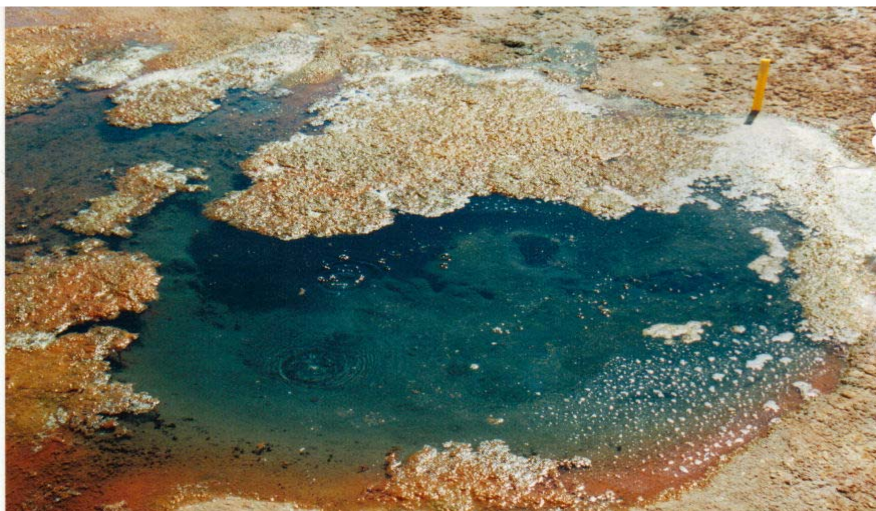

Figure 3.6C Medium growth spring at East German Creek in May 2002.
Tent stake provided for scale.

as possible to the spring source, therefore, this depth depended on the individual spring.

On each field trip, microbial mat samples were collected from various salt springs at the site. Mat material was collected on both occasions from pools with low, medium and high growth development and transferred to aluminum foil with the use of a sterilized trowel. These mat samples were kept in aluminum foil on ice in a cooler for transportation back to the lab where they were transferred to a freezer (-20˚C). Mat samples collected by Dr. S. Grasby (Table 3.1) were preserved the same way.

Table 3.1 Locations, temperatures, and pH readings of mat and sediment spring samples collected on October 18, 2000.

sample number	Location (NAD 87) latitude	longitude	temperature (˚C)	pH
M993010	52°52.603'	101°02.886'	9.3	6.94
M003004	52°45.266'	100°53.429'	4.7	6.91
M003005	52°47.977'	100°49.430'	6.9	6.70
M003006	52°47.919'	100°53.118'	6.0	6.56

Enrichment cultures

On each field trip, sediment and salt water samples were also taken from springs containing well-developed microbial mat growth to inoculate anaerobic enrichment cultures. Just beneath the top layer of the salt pan surface, an ink black sediment was present, which most likely contained iron sulphide, indicating that anaerobic conditions were likely present there. High growth pool sediment was used as inoculum because it was assumed to contain the most organics and the most

microbes as well. One third of a serum bottle (with a total volume of 165mL) was filled with sediment from the edge of the pool and brine water was added to bring the volume up to approximately two thirds full. The slurries were flushed with nitrogen and sealed with butyl rubber stoppers. Five mL of slurry was then inoculated into prepared anaerobic methanogen and sulphate reducing bacteria enrichment media in glass serum bottles (165mL) (Appendices 3.1 and 3.2). These media were designed to mimic the chemical environment found in the springs as closely as possible based on chemical analysis obtained from Dr. S. Grasby.

Core samples

Two sediment cores of approximately 20cm length and 5cm width were taken from the edge of a high growth spring on May 19 of 2002. The top of the tube was stoppered once the corer was in place to provide suction, then the tube was lifted up out of the sediment and finally the bottom was stoppered once the corer was completely out. Then the top stopper was taken off and subcoring was done via a plastic syringe approximately 10cm in length and 2cm in width which had been cleaved off at the end. The syringe plunger was inverted and used to force the sample to the lip of the tube where sectioning could be done. One core was taken for microscopy and 2mm sections were stored in 2% NaCl:ethanol for preservation. The second core was taken for sulphide analysis and sections from this core were stored in 10% zinc acetate to prevent oxidation. These samples were kept in a cooler on ice for transportation back to the lab where they were transferred to a freezer until analysed.

***In situ* activity measurements**

Two experiments were conducted to determine *in situ* rates of methanogenesis and sulphate reduction in the springs. Approximately 7.5mL of sediment was transferred from the edge of a high growth spring to small (9mL) glass serum vials. Different inhibitors and substrates were then added. Inhibitors used included molybdate (50mM), 2-bromo-ethanesulfonic acid (BESA) (50mM) and ethylene (0.1%) while substrates added were glycine betaine (10mM), TMA (0.1,1 and10mM) and H_2 (100% of headspace). In addition, combinations of substrates and inhibitors were also prepared: betaine with ethylene, molybdate or BESA, and TMA with molybdate or BESA. Before crimp sealing, each vial was flushed with nitrogen for thirty seconds. Vials were then incubated upside down in the sediment around the edge of the spring as seen in Figure 3.7. In this way, vials were kept as close as possible to natural conditions.

For each experimental condition, nine replicates were set up in order to determine microbial activity at different time points throughout the experiment (although only seven were used at spring 2 due to time constraints). The following time points were used for spring 1: 0, 0.5, 1, 2, 3, 17.75, 19.75, 22 and 23 hours. Spring 2 incubation times were: 0, 0.5, 1, 15, 16, 19 and 20.25 hours. At the designated times, one vial of each experimental condition was arrested with the addition of 50-100µL of a saturated mercuric chloride solution and stored at 0-4°C until analysed. Methanogenesis and sulphate reduction were assessed in the cultures. Each culture was analysed for pressure, methane concentration (by GC as described

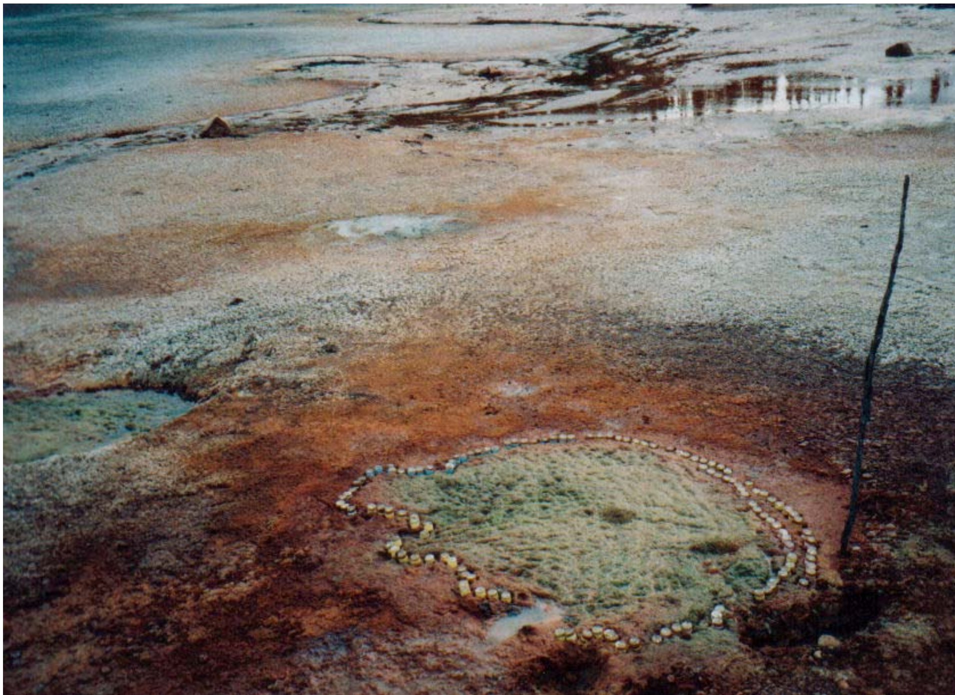

Figure 3.7 Vials incubating during *in situ* methanogenesis and sulphate reduction rate experiment at East German Creek August 6 and 7, 2003.

in chapter 2), and for sulphide. Sulphide was quantified by a colourimetric assay (Cline 1969).

Sulphate-reducing bacteria cultures

Cultures were established with microbial mat and sediment from high growth springs to determine the presence of SRB and determine their response to different substrates. On the first field trip in May of 2002, five substrates were supplied to SRB cultures: H_2/CO_2 (80%:20%) 10mM methanol, 10mM acetate, 10mM TMA and 10mM formate. Cultures were incubated at room temperature in the dark. Pressure readings were taken as described in chapter 2 once every few weeks and those growing on H_2/CO_2 were provided with more gas when pressures fell below 20 kPa.

For SRB cultures initiated on the second field trip in August of 2003, TMA was the only substrate provided. These cultures were inoculated with a slurry obtained from a high growth spring edge, just as was done in May of 2002. In this case, cultures were inoculated to determine whether SRB in this hypersaline environment were able to use TMA as a substrate. Despite reports that TMA is considered a non-competitive substrate for methanogens co-existing with SRB (Oremland and Polcin 1982), the original SRB cultures from the first field trip indicated possible use of this substrate by these organisms. These new cultures were supplied with a series of different TMA concentrations: 0.1mM, 0.5mM, 1mM, 10mM, 25mM and 50mM (see Appendix 3.2 for media composition). Cultures were again incubated at room temperature in the dark, and pressure readings were taken weekly. Expired SRB cultures were kept in the fridge following transfers, until cells were harvested (as described in chapter 2).

Sulphate reduction in enrichment cultures was monitored. Loss of sulphate in weekly samples was measured by ion chromatography performed with a Dionex DX 500 chromatography system equipped with a GP50 gradient pump and ED40 electrochemical detector. The eluent of 1.8mM bicarbonate and 1.7mM carbonate was used at 2mL/min with an AS4A column or with a PRP-X100 column (Hamilton), in which case phenol was added (1mM) and the eluent was adjusted to pH 10.0. Samples were centrifuged for 5 minutes at 9300 x g, then diluted to 1 in 50 in milli-Q water. Concentrations were determined by comparison of peak areas to standard curves.

Methanogen cultures

Five substrates (H_2/CO_2 (80%:20%), 10mM methanol, 10mM acetate, 10mM formate and 10mM TMA) were supplied to methanogen cultures on the first field trip (see Appendix 3.1 for media composition). The second field trip to the site in August of 2003 involved inoculating enrichment cultures in order to investigate if extreme halophilic methanogens from the springs were able to survive at even greater salinities. Methanogenic enrichment cultures were inoculated with a slurry created from sediment in an area slightly removed from edge of the spring (Figure 3.8). This area had visible salt crystals forming on the surface and sediment here was assumed to be more saline than sediment at the pool edge.

The substrates chosen for the second field trip salinity experiment were 100 kPa H_2/CO_2 (80%:20%) + 10mM betaine, and 10mM TMA. A series of eight enrichment cultures under methanogenic conditions with salinities ranging from 1% to 30% were inoculated from the mat/sediment slurry (Appendix 3.3). NaCl was

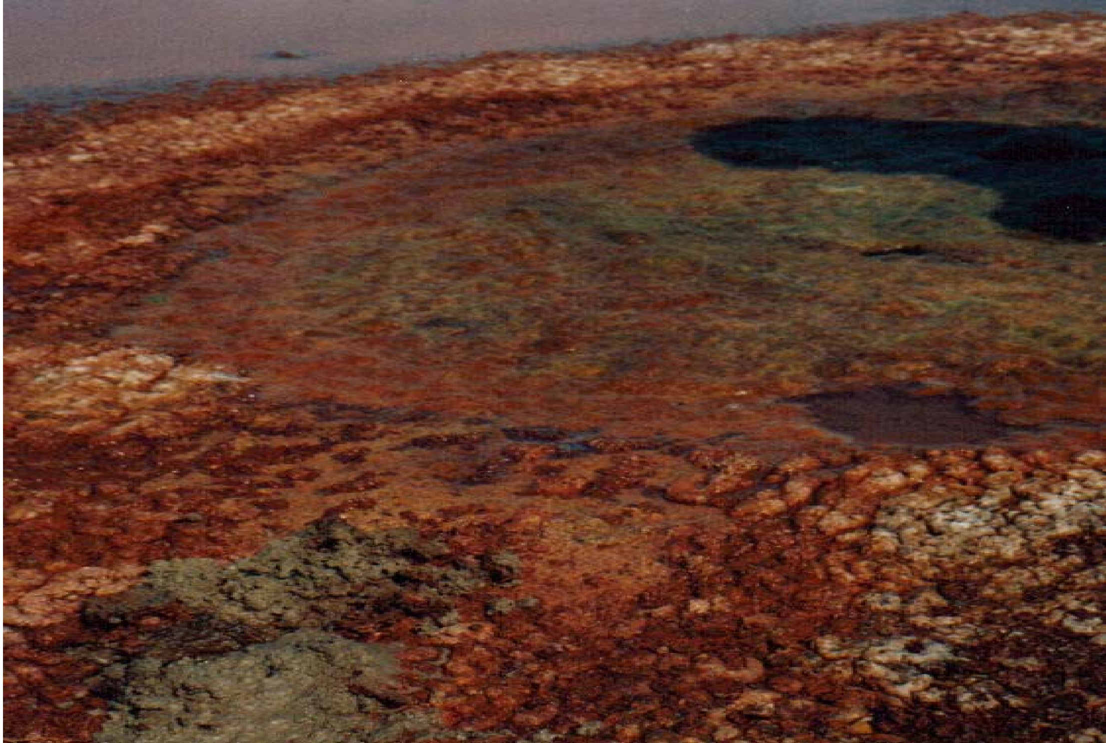

Figure 3.8 Sediment source for methanogen salinity series experiment. Sediment was taken from beyond edge of spring where salt crystals were observed at East German Creek on August 7, 2003.

primarily used to manipulate the salinity of these cultures because these were the dominant ions in the spring water. Six additional methanogen enrichment cultures supplied with 10mM glycine betaine (hereafter referred to as betaine) were inoculated on the second field trip in order to determine if the compatible solute would stimulate methanogenesis, as its break down product TMA does.

All methanogen cultures were also incubated in the dark at room temperature. Pressure readings were taken on a weekly basis along with methane measurements (as described in chapter 2) to monitor methanogenic activity. Methane yield was calculated based on expected production from the amount of substrate added, using Buswell's equation to calculate molar ratios. Monthly transfers of 10% v/v were performed to maintain viability and enrichment of methanogens in cultures. Following transfers, cultures were harvested and biomass was then collected and lyophilized for lipid analyses.

Microbial mat lipids

Several microbial mat samples were analysed for lipid composition. Samples were collected during field trips in May of 2002 and August of 2003 as described above. Some mat samples from nearby springs had also been taken previously by others working in the area. Dr. Steve Grasby, a hydrogeologist with the Geological Survey of Canada, collected mat and sediment samples on earlier field trips (Table 3.1) and Kathleen Dawson, a former student in Dr. Londry's lab, extracted these samples for analysis. All samples were lyophilized, dry weights were determined, and samples were extracted in the ASE as previously described (chapter 2).

The lipid extracts were divided into two parts and prepared for analysis of both methanogen biomarkers and bacterial fatty acid methyl esters (FAME) by GC/MS. In addition, enrichment cultures were harvested after the completion of the growth amendment experiments. Cultures were centrifuged and pellets lyophilized as described in chapter 2, then extracted as per the mat samples. Lipid analysis was done as for the *M.barkeri* experiments (PMI, sn-2 hydroxyarchaeol). This was done to allow for the detection of both marine methanogens in general and *Methanosarcina* spp. specifically in the spring mats and enrichment cultures.

Samples for FAME analysis were prepared by silicic acid chromatography and mild alkaline methanolysis as previously described (White and Ringelberg 1998), except that the acetone elution from the silicic acid cleaning was not used because it resulted in the loss of FAME from the final recovery step (data not shown). Internal standards were added to samples immediately prior to methanolysis. Samples from the 2002 field trip and those collected earlier received 25µL of 1 mg/mL glyceryl tritridecanoate, while the 2003 field trip samples received 25µL of 1 mg/mL PC 20 (L-α-phosphatidyl choline, di-arachidoyl). The standards confirmed that methanolysis was working properly, because the fatty acids must be cleaved from its glycerol backbone to produce either tridecanoic acid (13:0) or eicosanoic acid (20:0). For each set of samples run through the silicic acid columns, a positive (internal standards only) and negative control were also prepared. All samples and controls were suspended in 400µL chloroform and stored frozen until analysed.

Culture lipid analyses

Methanogen biomarkers were analysed by GC/MS as previously described (chapter 2). Identity was confirmed by comparison to analyses of *M.barkeri* extracts through retention time and spectra. Internal standards were compared to external standards of hydrocarbons analysed on the same day (as per chapter 2). Peaks were quantified by peak areas.

FAME were analysed on the same GC/MS with slightly different conditions. The column oven was initially set at 150°C, then raised to 185°C at a rate of 1.5°C/ minute, and further increased to 325°C, where it was held for one minute for a total run time of 30 minutes. Ions were monitored after 3 minutes within the range of 40-400 mass/charge ratios. Identifications were by comparison of retention time and spectra to standards of FAME analysed on the same day (BAME, Sigma). PCA analysis was preformed on FAME data via the software program The Unscrambler.

3.3 Results

Physical Characteristics of Salt Springs

The springs sampled during the 2002 field trip varied greatly in size (Table 3.2). However, size did not appear to determine the amount of microbial mat growth present. Most spring pools were roughly oval in shape, so measurements given are those at the widest and longest points. Some medium growth springs were quite large, and many low growth pools observed at this site were very small (not recorded). The pH readings taken were near neutral at all the springs, with an average pH of 6.67.

Disturbed springs tended to have lower pH values, except in the low growth spring. This may have been caused by disturbance of these springs during sampling when sediment and spring water slurries were made (prior to pH measurements).

Table 3.2 Spring pH and pool size measurements taken at East German Creek on May 17, 2002.

Spring Type	pH readings	dimensions (cm)
Low growth pristine	6.78	60.8 X 80.8
Medium growth pristine	6.87	250.3 X 50
High growth pristine	6.70	20.6 X 30.4
Low growth disturbed	6.78	230 X 240.2
Medium growth disturbed	6.48	240.7 X 70.2
High growth disturbed	6.42	50.5 X 50.5

pH readings were taken at 15:20 on May 17, 2002. Disturbed springs were sampled prior to pH measurement. Dimensions indicate the longest and shortest diameters of the oval-shaped spring pools.

Temperature measurements were also taken at three times in different locations within the springs (Figures 3.9A and B). Daily temperatures fluctuated by as much as 10˚C and morning readings were generally the coldest, as expected. The least temperature variation was seen in the centre of the springs where the cool ground water enters, whereas the greatest variations were at the edge, which is more influenced by surface air temperature fluctuations and radiative heating. The highest temperatures tended to be at the edge, which is where mat growth was commonly seen. While high and medium growth springs had mat growth throughout, even most low growth springs displayed mat growth around the edges of the pools. In the pristine springs, the relative amount of mat growth seemed to correlate with spring temperature fluctuations at the centre surface of the pools (Figure 3.9B). This trend was not observed in the disturbed springs and required further investigation.

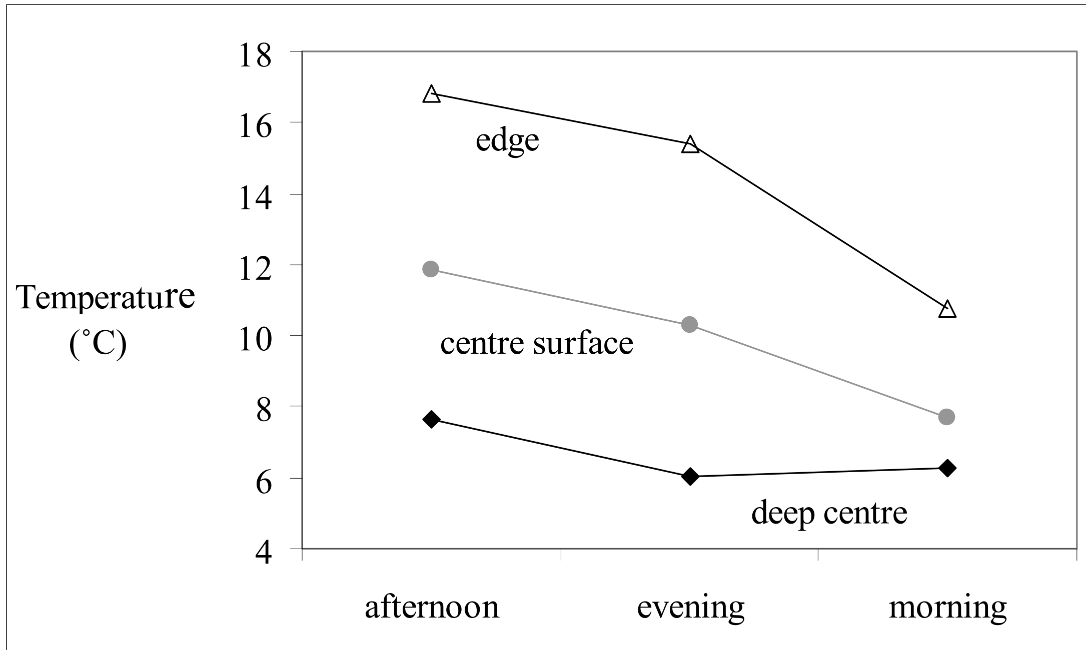

Figure 3.9A Temperature readings at different locations within spring pools at East German Creek on May 17 and 18, 2002.

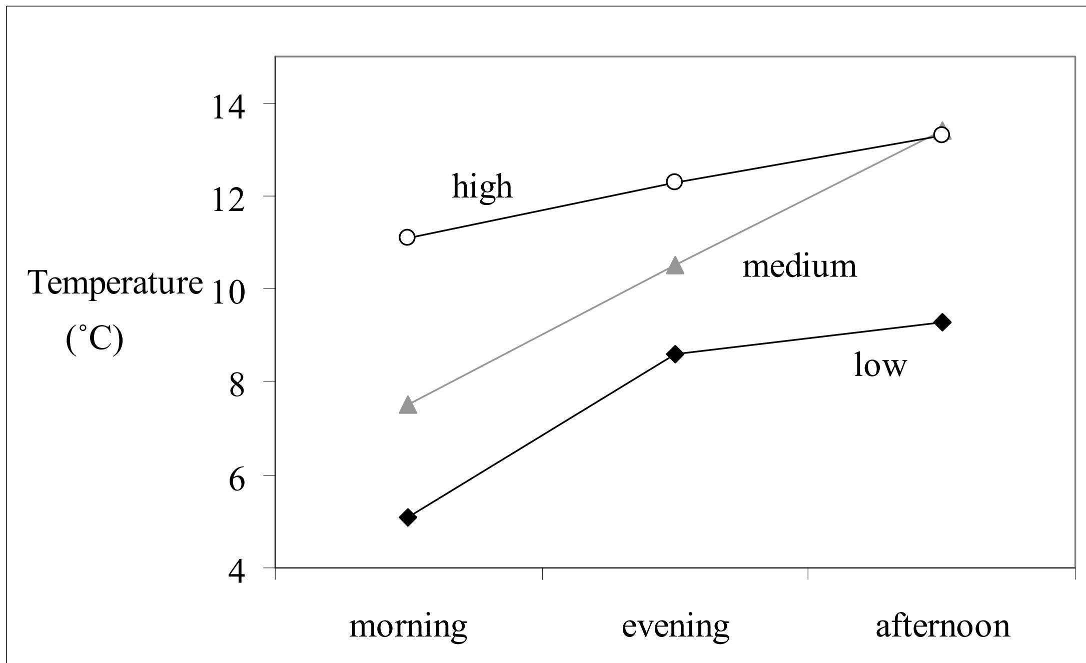

Figure 3.9B Temperatures of the centre surface waters in low, medium and high growth pristine springs at East German Creek on May 17 and 18, 2002.

Microbial mat samples collected during the May 2002 field trip were analysed by microscopy in order to determine whether the mats were of cyanobacterial or algal nature. Similar mat compositions were observed in all cases with diatoms, rotifers and bacteria present in addition to the dominant organism, which appeared to be an alga. Pascal Badiou (University of Manitoba, Botany department) was able to positively identify this dominant mat organism from a fresh mat sample collected in August, 2003, as the marine algae *Percursaria percursa*. Cyanobacteria also occurred sporadically in the mats, and many bacteria were visible in simple wet mounts with phase contrast microscopy. The differences in colour of the mats were apparently due to differences in the accumulation of rusty brown clumps around the dominant green-coloured algae.

Microbial mat lipids

Community lipid analysis of microbial mats and sediment was performed on samples taken at different times of the year from hypersaline springs in the Lake Winnipegosis area containing mat growth. FAME analysis of these samples collected prior to the spring 2002 field trip, are shown in Table 3.3. FAME of possible or likely bacterial origin are listed. This table does not include polyunsaturated FAME data, as the focus is on prokaryotic FAME, and the contribution of polyunsaturates was small. As seen in Figure 3.10, mat samples were dominated by monounsaturated fatty acids with varying proportions of normal and branched fatty acids. At each site, mat samples (except M003004) contained more monounsaturated and less normal FAME than the underlying sediment. Apart from M993010, sediment samples also contained

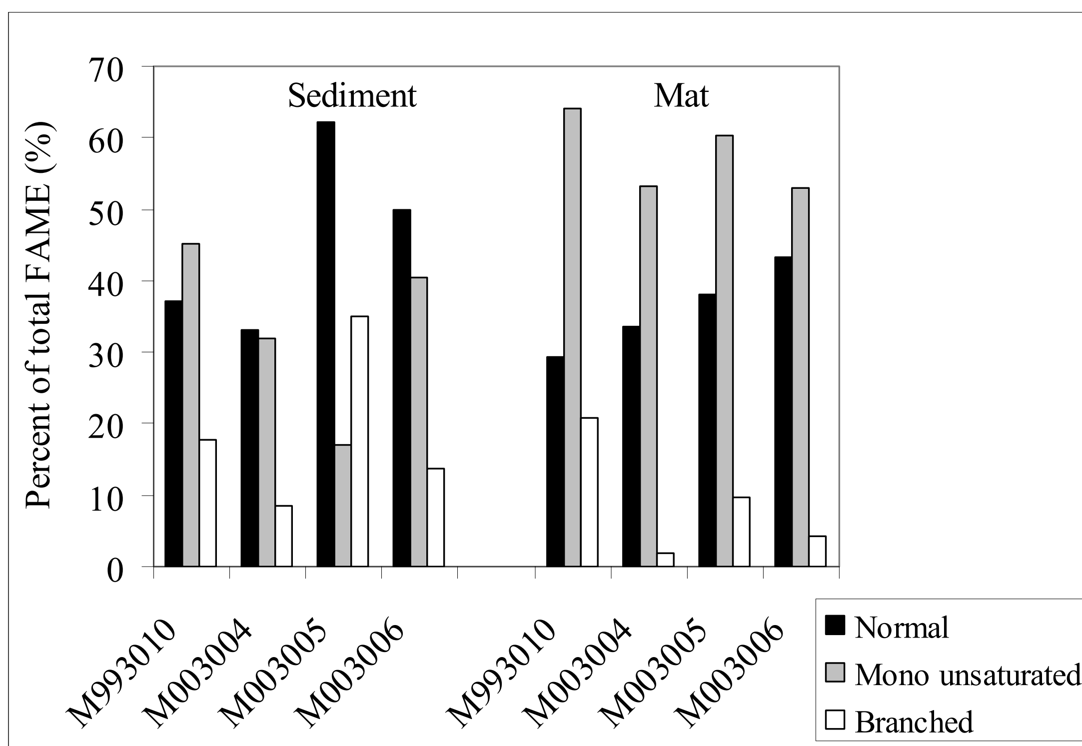

Figure 3.10 Comparison of normal, monounsaturated and branched FAME between mat and sediment samples collected from various locations* near the western shore of Lake Winnipegosis on October 18, 2000.
*See Table 3.1 for exact locations.

more branched fatty acids than mats. Interestingly, an unusual branched 15:1 fatty acid was found in only mat samples, and not in any of the sediment samples. It should be noted that these percentages add up to greater than 100%. This is because some fatty acids are both monounsaturated and branched.

Table 3.3 Microbial mat FAME of samples collected on October 18, 2000 from various locations* near western shore of Lake Winnipegosis.

Fatty acid Identity	Peak Area (% of total) M993010 sediment	M993010 mat	M003004 sediment	M003004 mat	M003005 sediment	M003005 mat	M003006 sediment	M003006 mat
br13:0	9.37	3.32	19.77	7.83	17.42	1.07	8.34	1.62
13:0	9.26	0.09	10.18	4.02	36.55	2.06	6.69	0.81
br14:0	3.39	2.54	0.99	1.74	0.00	0.00	0.42	1.44
14:0	3.92	0.22	3.04	1.34	1.94	13.20	0.66	1.15
br15:1	0.00	1.80	0.00	0.17	0.00	0.40	0.00	0.00
i15:0	0.87	0.00	1.58	1.42	0.00	0.10	0.21	0.35
a15:0	4.03	0.88	12.59	2.29	3.29	0.38	0.83	0.26
15:0	1.10	0.26	0.70	0.57	0.73	0.19	0.78	0.45
16:1 (cisΔ^{9})	31.70	42.98	18.99	32.29	3.56	40.40	12.00	31.57
16:0	22.02	28.07	17.67	25.46	20.93	21.76	41.19	38.78
17:1	0.33	0.00	2.07	2.19	0.34	0.81	0.00	0.00
17:1	0.23	0.00	1.11	1.45	0.19	0.26	0.00	0.00
17:0	0.15	0.04	0.37	0.73	1.32	0.13	0.20	0.07
18:1 (cisΔ^{9})	11.85	8.67	8.85	15.96	12.66	17.99	26.95	16.36
18:1 (cisΔ^{11})	0.07	9.09	0.04	0.10	0.03	0.00	0.00	3.54
18:0	0.62	0.57	0.73	1.15	0.77	0.57	0.30	1.99
br19:1	0.07	0.06	0.00	0.23	0.00	0.06	0.00	0.49
19:0	0.00	0.00	0.42	0.34	0.00	0.18	0.00	0.00
20:1	0.55	0.35	0.45	0.16	0.00	0.15	1.35	0.81
20:1	0.48	1.06	0.46	0.55	0.29	0.30	0.08	0.31
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00

* Locations of microbial mat samples are given in Table 3.1.

Some fatty acids are indicative of Gram negative and Gram positive bacteria. While i14:0, i15:0, a15:0, i16:0, i17:0 and a17:0 are found in Gram positive bacteria, Gram negatives contain cyclic 17, cyclic 19, 15:1, 16:1(cisΔ9), 17:1 and 18:1(cisΔ9 and 11) (Zelles 1997). In this case, however, 16:1(cisΔ9) found in the microbial mats and sediment samples was not considered indicative of Gram negative bacteria because it was present in such large quantities. It is unlikely that this group of organisms, which would make up a small percentage of the overall biomass, would be responsible for such a large percentage of the fatty acids in these samples. The mat and sediment samples collected in 2000 indicated that both were dominated by Gram negative bacteria, and that mats contain even greater proportions of these microorganisms than the sediments, with the exception of the M003006 samples (Figure 3.11). A larger proportion of 18:1(cis Δ9) in this sediment (M003006) over the others was responsible for the bulk of the increase in Gram negative-associated fatty acids.

Six microbial mat samples were collected during the 2002 field trip. One pristine spring and one disturbed spring for each growth condition (low, medium and high) were sampled. FAME analyses of these mats are shown in Table 3.4. As before, all mats from pristine springs were dominated by unsaturated fatty acids (Figure 3.12). Low growth mats were found to have the highest percentage of monounsaturated fatty acids. It appears there were slightly more normal FAME and less unsaturated FAME in the springs with higher growth. The same trend was observed in the disturbed springs (data not shown). As with the mat samples from 2000, FAME indicative of Gram negative bacteria were dominant over those of Gram

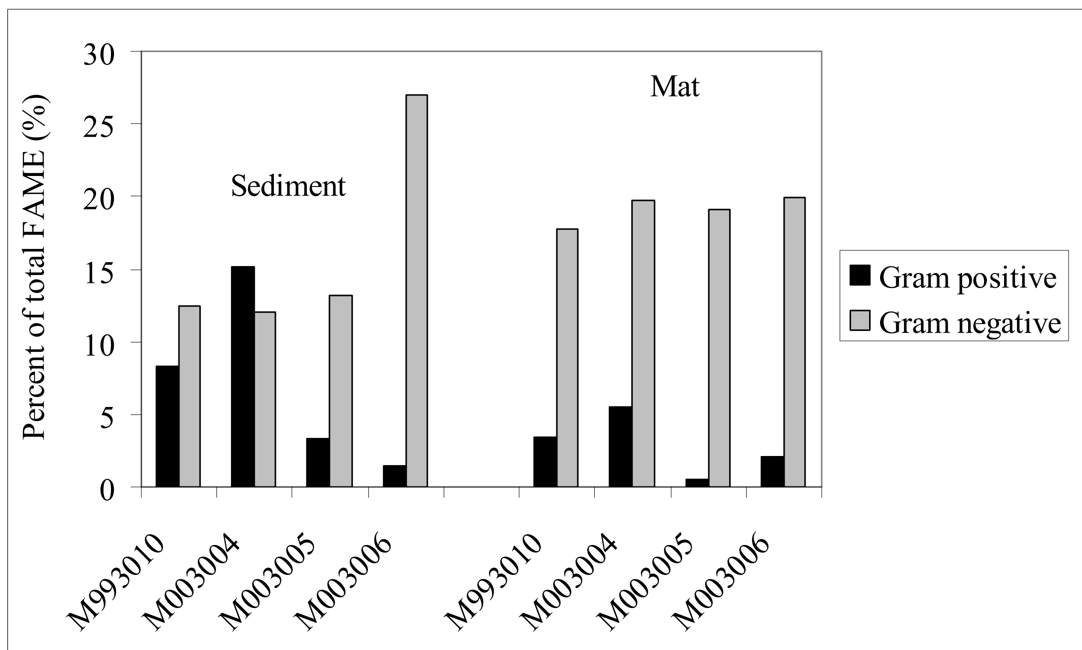

Figure 3.11 Comparison of Gram positive and Gram negative-associated FAME from mat and sediment samples collected from various locations* near the western shore of Lake Winnipegosis on October 18, 2000.
*See Table 3.1 for exact locations.

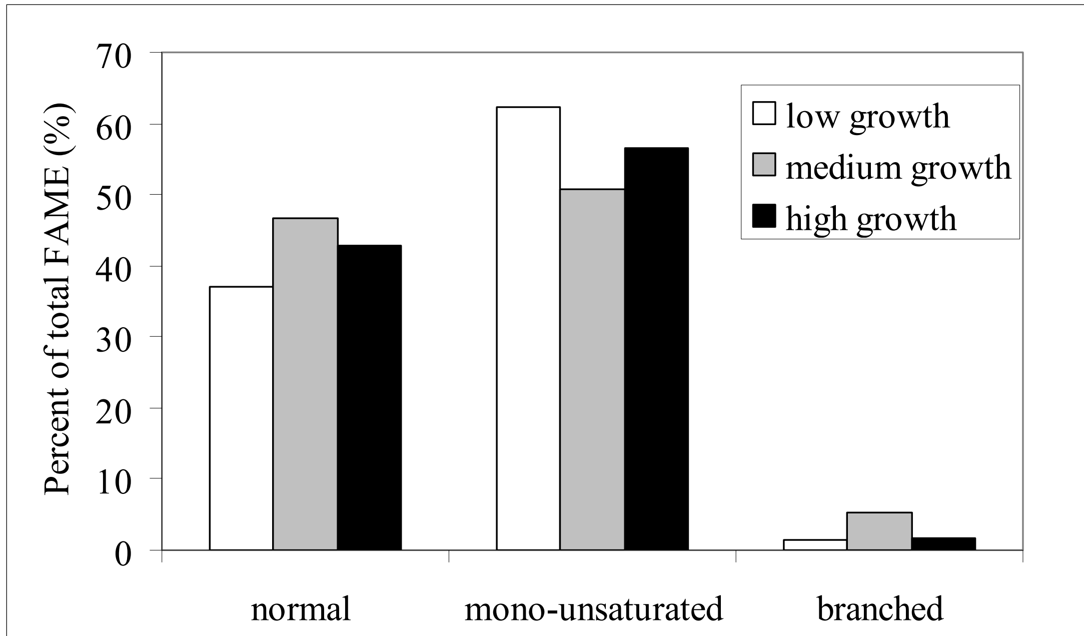

Figure 3.12 FAME profile of low, medium and high growth pristine spring mats sampled at East German Creek on May 18, 2002.

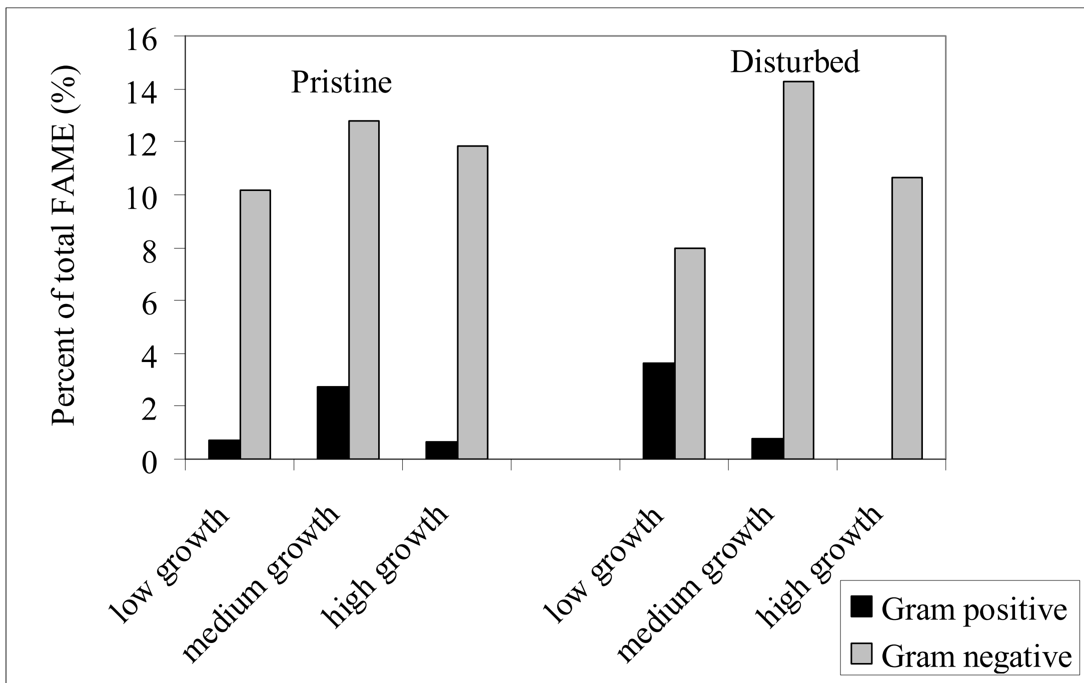

Figure 3.13 Comparison of Gram positive and Gram negative-associated FAME from pristine and disturbed springs collected at East German Creek on May 18, 2002.

positive bacteria (Figure 3.13). In both pristine and disturbed samples, the lowest proportion of Gram negative FAME were found in low growth mats.

Table 3.4 FAME analysis of microbial mat samples collected from East German Creek on May 18, 2002.

	Peak area (% of total)					
Fatty acid Identity	Pristine low growth mat	Disturbed low growth mat	Pristine medium growth mat	Disturbed medium growth mat	Pristine high growth mat	Disturbed high growth mat
14:0	5.80	5.32	7.97	6.89	5.52	10.68
br15:1	0.00	0.00	2.67	4.32	0.99	0.00
i15:0	0.12	3.63	2.71	0.76	0.31	0.00
a15:0	0.26	0.00	0.00	0.00	0.37	0.00
15:0	0.62	0.48	0.79	0.71	0.62	0.75
br16:0	0.35	0.00	0.00	0.00	0.00	0.00
16:1 (cisΔ^9)	50.12	55.26	34.86	46.74	41.97	41.56
16:0	30.03	25.44	36.75	25.35	35.51	33.93
17:1	0.72	1.48	0.08	5.62	0.23	0.00
17:1	0.28	0.32	0.04	0.00	0.10	0.00
17:0	0.23	0.41	0.15	0.10	0.09	0.08
18:1 (cisΔ^9)	5.83	4.59	6.37	6.56	6.06	6.30
18:1 (cisΔ^{11})	3.36	1.60	6.28	2.09	5.45	4.34
18:0	0.42	0.63	0.94	0.63	1.10	0.89
19:1	0.88	0.11	0.00	0.17	0.62	1.47
br20:1	0.78	0.48	0.00	0.00	0.00	0.00
20:1	0.05	0.07	0.16	0.04	0.20	0.00
20:1	0.22	0.18	0.22	0.04	0.87	0.00
Total	100.07	100.00	100.00	100.00	100.00	100.00

A representative of each type of growth condition was collected during the 2003 field trip. Table 3.5 contains the FAME analysis of these samples. Interestingly, while the low and medium growth mats were dominated by unsaturated fatty acids as seen with

most of the mats previously sampled, the high growth mats contained more normal fatty acids than monounsaturated (Figure 3.14). A noticeable trend of decreasing amounts of unsaturated FAME occurred with increasing mat growth. In all three mats, the proportion of branched FAME was much lower than monounsaturated or normal FAME, as seen in the other samples. Once again, FAME indicative of Gram negative bacteria were dominant in these mats, with very little FAME diagnostic of Gram positive bacteria present (Figure 3.15).

Table 3.5 FAME analysis of microbial mats collected from East German Creek on August 7, 2003.

Fatty acid Identity	Peak Area (% of total)		
	Low Growth Mat	Medium Growth Mat	High Growth Mat
13:0	0.07	0.00	0.25
i14:0	0.05	0.23	0.21
14:1	0.02	0.11	0.21
14:1	0.05	0.11	0.00
14:0	9.73	7.69	7.36
br15:1	0.59	0.89	0.84
i15:0	0.31	0.31	0.56
a15:0	0.50	0.39	0.68
15:0	0.47	0.52	0.44
16:1(cisΔ^9)	40.86	43.27	16.75
16:0	30.19	36.16	46.52
17:1	0.02	0.07	0.05
17:1	0.31	0.20	0.48
17:0	0.11	0.06	0.05
18:1(cisΔ^9)	5.65	4.90	10.57
18:1(cisΔ^{11})	7.75	3.47	12.42
18:0	0.93	0.43	0.68
br19:0	0.36	0.61	0.23
19:1	0.72	0.30	0.36
20.1	1.00	0.18	0.29
20:1	0.30	0.11	1.05
Total	100.00	100.00	100.00

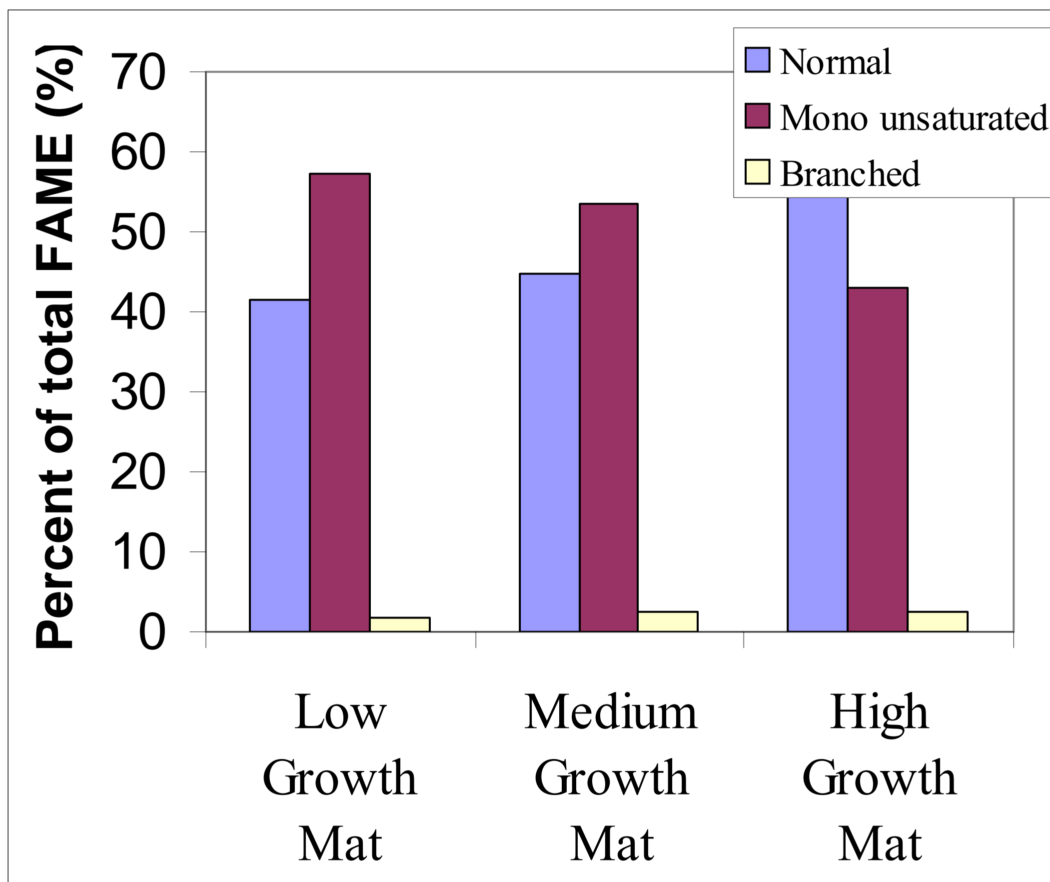

Figure 3.14 Comparison of normal, monounsaturated and branched FAME between mat and sediment samples collected at East German Creek on May 18, 2000.

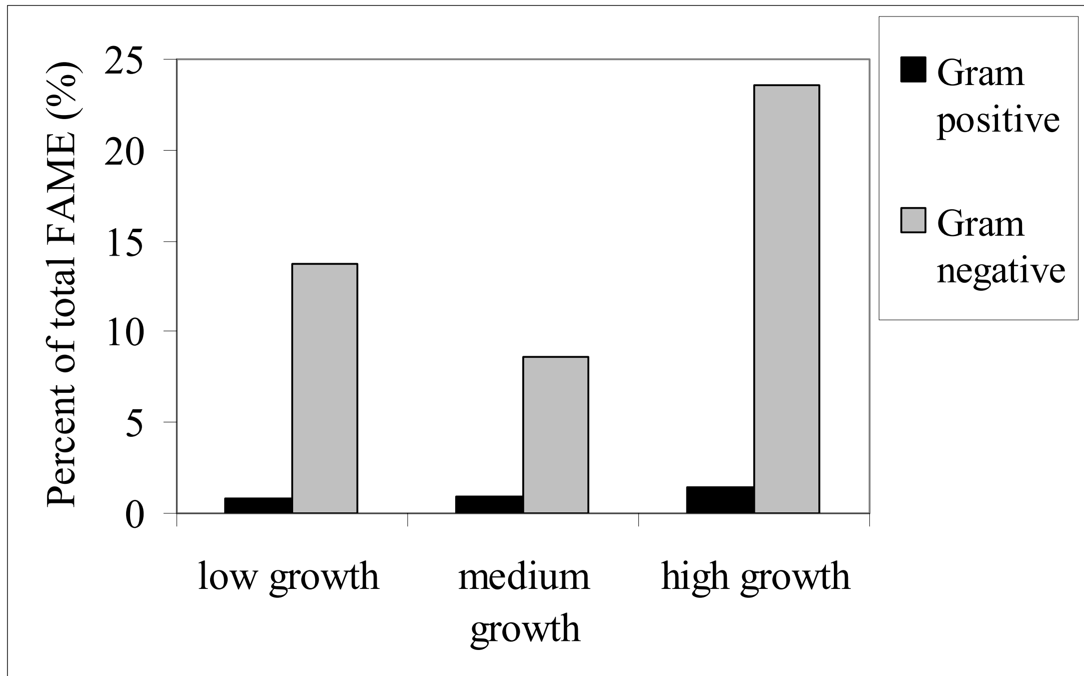

Figure 3.15 Comparison of Gram positive and Gram negative-associated FAME from low, medium and high growth springs collected at East German Creek on August 7, 2003.

In addition to FAME formed through methanolysis and derivatization of phospholipids, all mat samples analysed by GC/MS also contained the free fatty acids 16:1 and 16:0, which were likely products of mat decomposition. Noticeably absent in the FAME samples were the SRB biomarkers i17:1 (*Desulfovibrio*), and 10Me16:0 of the complete oxidizing bacteria as well as some iron-reducing bacteria. Despite the presence of suitable environmental conditions for these anaerobes, no evidence of these fatty acids were found. It may be that these microbes were simply not present in significant quantities for detection.

From the mats collected in 2002, the greatest amount of polyunsaturated fatty acids were found in a high growth spring (5.49% in the disturbed spring), although the low growth pristine spring contained 4.74%, and the pristine medium growth spring contained only 2.03%. Of the mats collected in 2003, the greatest amount of polyunsaturated FAME was found in the high growth spring with 7.25% of the total FAME. However, the low growth mat contained 6.68%, and the medium growth spring 4.96% polyunsaturated FAME. Therefore, although the greatest levels of polyunsaturated FAME were found in the high growth springs, low growth mats contained more of these FAME than the medium growth mats.

Principle component analysis (PCA) was preformed on microbial mat FAME data in order to investigate if differences and/or similarities in FAME could be seen between springs that had differing amounts of microbial mat growth. In the case of mat samples collected in 2000, although sediment (in black) and microbial mat (in white) samples from the same site did not cluster closely together, all mat samples tended to aggregate together despite being from different sites (Figure 3.16). Most of

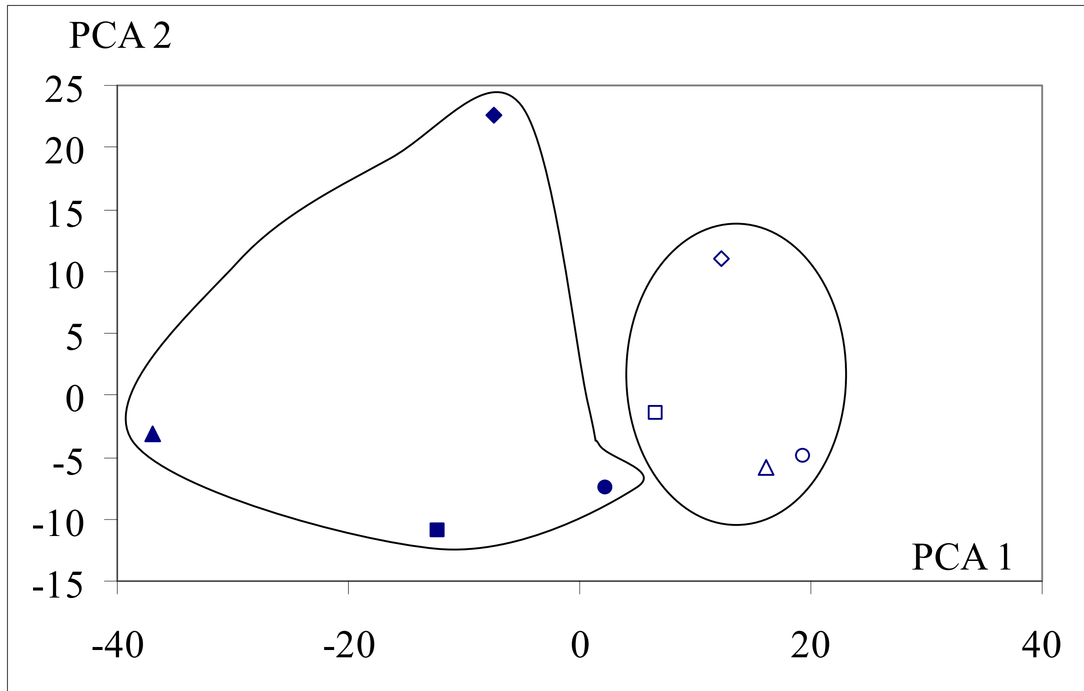

Figure 3.16 PCA analysis of sediment and mat samples collected at various locations* near the western shore of Lake Winnipegosis on October 18, 2000. Circles represent M993010, squares represent M003004, triangles represent M003005, and diamonds represent M003006. Dark symbols are sediments and light symbols are mats.
*See Table 3.1 for exact locations.

the variance on PCA1 was explained by the amount of fatty acids 16:1 (34%) and 13:0 (28%), while PCA 2 variance was mainly determined by 16:0 (30%), 18:1 (19%) and 16:1 (15%) (data not shown). PCA 1 accounted for 63%, and PCA 2 for 23%, of the overall variance.

PCA analysis of FAME from microbial mats collected from the springs in 2002 indicate distinct clustering of low growth mats (circles) from high growth mats (triangles) from pristine and disturbed springs (Figure 3.17). Medium growth mats (squares) did not aggregate closely; however, they were the only samples to plot on the positive side of PCA 2. In general, disturbed spring mats (in white) were slightly more positive along PCA 2 than their pristine counterparts. Variance along PCA 1 for this data set was explained for the most part by the amount of 16:1 (42%) and 16:0 (28%). Changes in the amounts of the fatty acids 17:1, br15:1, 16:0 and 16:1 were responsible for most of the variance along PCA 2 (22%, 21%, 20% and 17% respectively).

PCA analysis of 2003 mat samples did not result in the separation of samples based on other parameters provided (data not shown). However, when FAME data from all microbial mat samples collected were analysed by PCA, groups did emerge among the samples (Figure 3.18). There was a separation of mats collected in 2000 (fall) from those collected in 2002 (spring) and 2003 (summer). This may be due to different sampling locations since 2002 and 2003 samples were collected from springs in close proximity. On the other hand, it may be due to different sampling seasons and/or years. There was no clear separation of the 2002 and 2003 mat samples, although in general, the summer samples tended to be slightly more negative

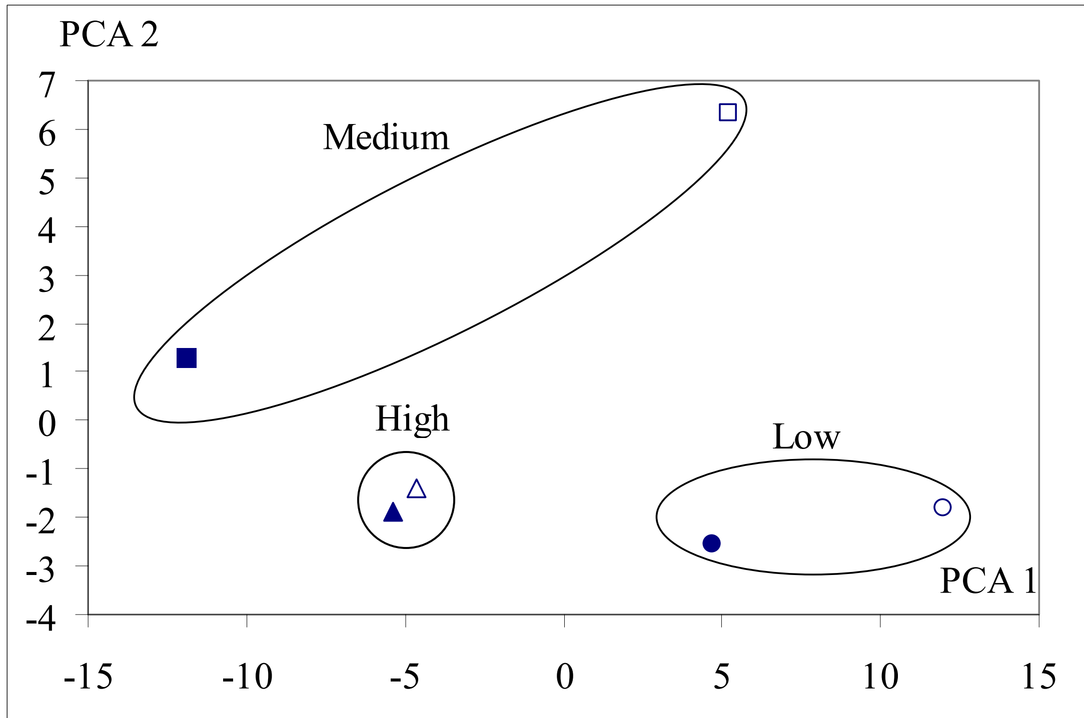

Figure 3.17 PCA analysis of FAME from microbial mats collected at East German Creek on May 18, 2002. Circles represent low growth mats, squares represent medium growth mats and triangles represent high growth mats. Dark symbols are pristine spring samples and light symbols are disturbed spring samples.

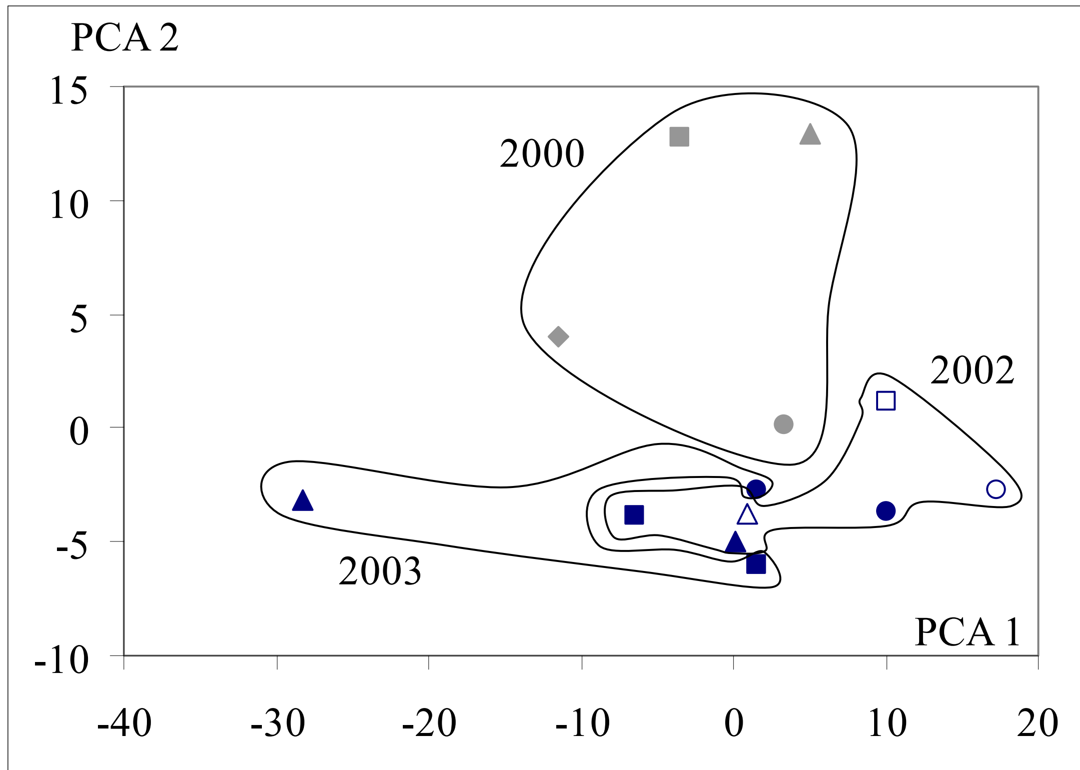

Figure 3.18 PCA analysis of FAME from microbial mats collected at various locations* near the western shore of Lake Winnipegosis on October 18, 2000 and at East German Creek on May 18, 2002 and on August 7, 2003. Grey symbols represent 2000 mat samples. Of these, the circle represents M993010, the square represents M003004, the triangle represents M003005, and the diamond represents M003006. For all 2002 and 2003 mat samples, circles represent medium growth mats, squares represent medium growth mats and triangles represent high growth mats. Of the 2002 mat samples, the dark symbols represent pristine spring mats and light symbols represent disturbed spring mats.
*See Table 3.1 for exact locations.

on PCA 1. Variance along this axis is mainly explained by 16:1 (45%) and 16:0 (26%), while amounts of 18:1 (30%) and 16:0 (26%) are responsible for variance along PCA 2. The axes explain 65% (PCA 1) and 20% (PCA 2) of the overall variance.

Analysis of Archaeal lipids in mat material collected in 2002 and 2003 did not provide evidence of archaeal populations, however derivatization of samples was not successful, and therefore sn-2 hydroxyarchaeol could not be seen whether it was present in the mats or not. Other peaks which could not be identified using molecular library searches, were also detected in most mat samples during Archaeal lipid analysis. These included compounds with molecular weights of 327, 374, 400 and 402, and relative peak areas did not correlate with spring type.

SRB enrichment culture studies

Cultures established for SRB from the 2002 field trip did result in sulphate reduction from samples taken from each of the springs (Figure 3.19). Along with methanol, hydrogen supported sulphate reduction from all springs, indicating autotrophic SRB may play an important role in these mats. Sulphate reduction was greatest with formate and acetate in low growth and high growth springs. Here, it appeared that sulfate reduction exceeded 100%, and this may have been due to endogenous substrate that was present in the slurry inoculum as these calculations were based on the amount of sulphate added to the media. Sulphate reduction was lowest from the medium growth spring.

Successive transfers of the SRB cultures were also analysed for sulphate reduction. By the third transfer of these cultures, it became obvious that methanol and

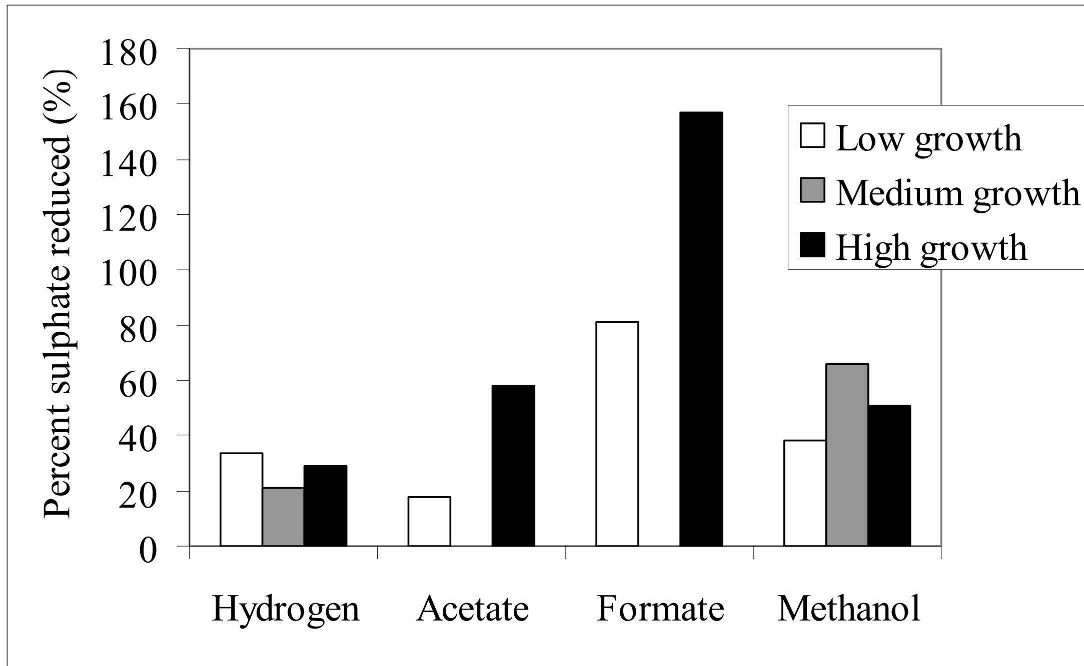

Figure 3.19 Sulphate reduction from four amended substrates of original SRB cultures inoculated at East German Creek on May 17, 2002.

acetate were no longer being used as substrates for sulphate reduction. After this point, only those cultures supplied with H_2/CO_2, formate and TMA were continued. By the sixth transfer, only those cultures provided with hydrogen as a substrate continued reducing sulphate.

The SRB cultures inoculated during the 2003 field trip supplied with TMA as a substrate, were also monitored for sulphate reduction. However, TMA did not support sulphate reduction in these cultures (data not shown). The small amount (<5mM) of sulphate loss in the cultures could be explained by analytical variations, and did not correlate with the amount of substrate added, which could have led to the reduction of 28mM sulphate.

FAME analysis of SRB cultures inoculated in May, 2002 from low and medium growth springs, and supplied with H_2/CO_2, indicated an enrichment of a branched 15:0 fatty acid between the original cultures and the eighth transfers, suggesting Gram positive bacteria were enriched. Typical SRB biomarkers (i17:1 and 10Me16:0) were not detected.

***In situ* rate experiments**

Unfortunately, *in situ* rate experiments for sulphate reduction and methanogenesis were not successful. During experimental set up, vials were prepared by first loading sediment, followed by treatments with inhibitors and substrates. All vials were loaded with sediment before additions were made so the experiment could be started for all vials at a similar time. The time required to load all vials was likely too long for the small sediment samples to remain anaerobic before gas flushing was done. Therefore, the activity was likely lost before the experiment was even started. A

sulphide assay was attempted to examine sulphate reduction; however, sulphide production was not seen in any of the samples. Similarly, methane production was not seen in the methanogenesis experiment vials.

Methanogenesis culture studies

Methanogenesis was observed in the cultures established on the 2002 field trip from all five substrates. Methanogenesis was greatest with methanol, followed by TMA, with lower amounts from formate and acetate (Figure 3.20). Of the cultures inoculated during this field trip, methanogenesis was primarily observed from the medium growth spring slurry. Small but measurable methane production from the other spring cultures was observed, so although they were not producing as much methane, methanogens were present in the low and high growth springs as well.

These cultures were analysed to see if any enrichment for methanogen biomarkers occurred. The biomarker sn-2 hydroxyarchaeol was found in a limited number of the cultures. Only the low growth spring TMA culture and the medium growth acetate and methanol cultures (Figure 3.21) contained this biomarker. Therefore, some of the methane production by the methanol and acetate-grown medium growth spring cultures must have been due to *Methanosarcinales*.

The impact of salinity on methanogenesis in cultures established from slurries from the outer edge of a high growth spring with TMA as a substrate, indicated that activity for these methanogens decreased above 5% salinity (Figure 3.22). A similar pattern was shown by the salinity series cultures provided with glycine betaine and

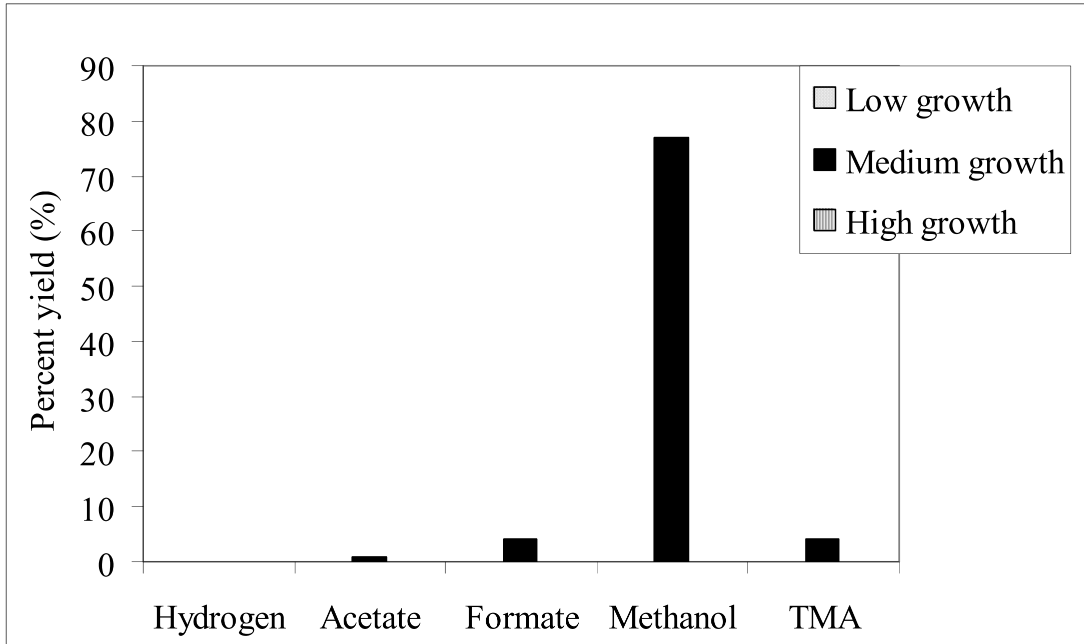

Figure 3.20 Methane production of original methanogen cultures inoculated at East German Creek on May 17, 2002 from five amended substrates after 25 days incubation, expressed as percentage expected from the amount of substrate added.

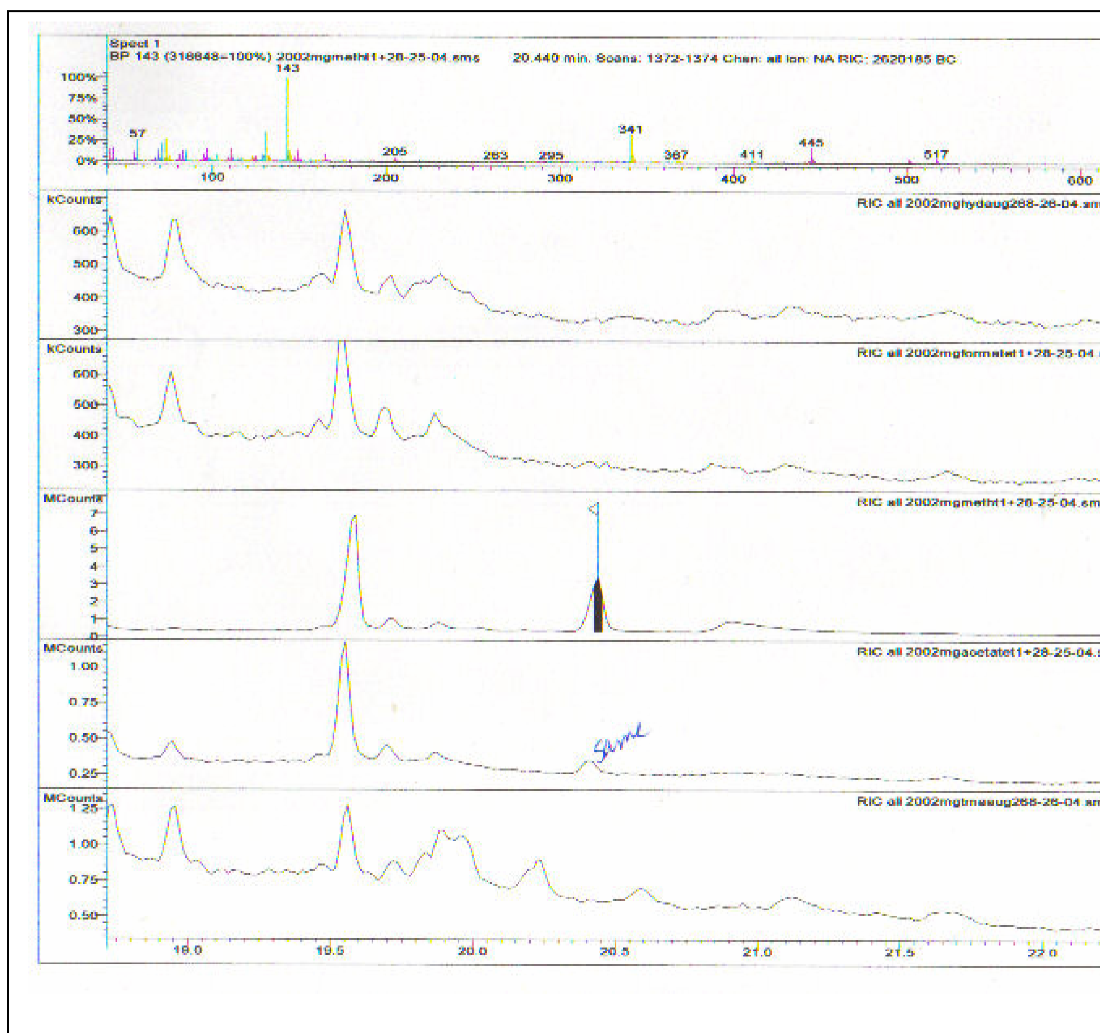

Figure 3.21 Chromatograms of methanogen cultures inoculated at East German Creek on May 18, 2002 and supplied with hydrogen (2nd panel), formate (3rd panel), methanol (4th panel), acetate (5th panel) and TMA (6th panel) as substrates. Mass spectrum (top panel) of sn-2 hydroxyarchaeol peak (indicated by arrow) found in medium growth methanol and acetate cultures.

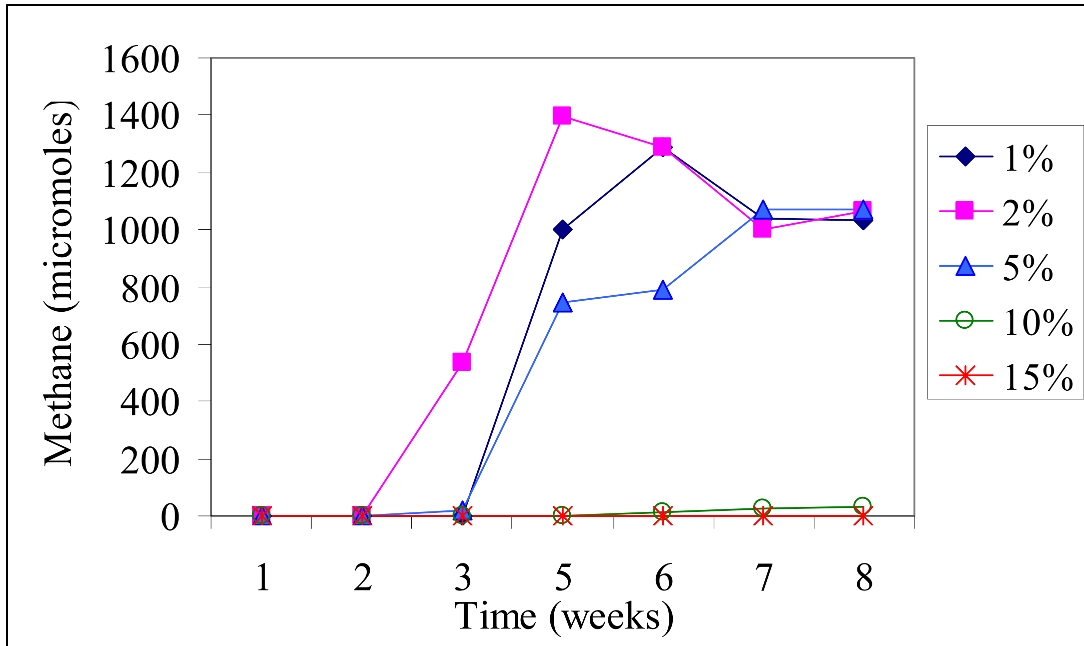

Figure 3.22 Methane production of cultures inoculated at East German Creek on August 7, 2003 and supplied with 10mM TMA over a range of salinities.

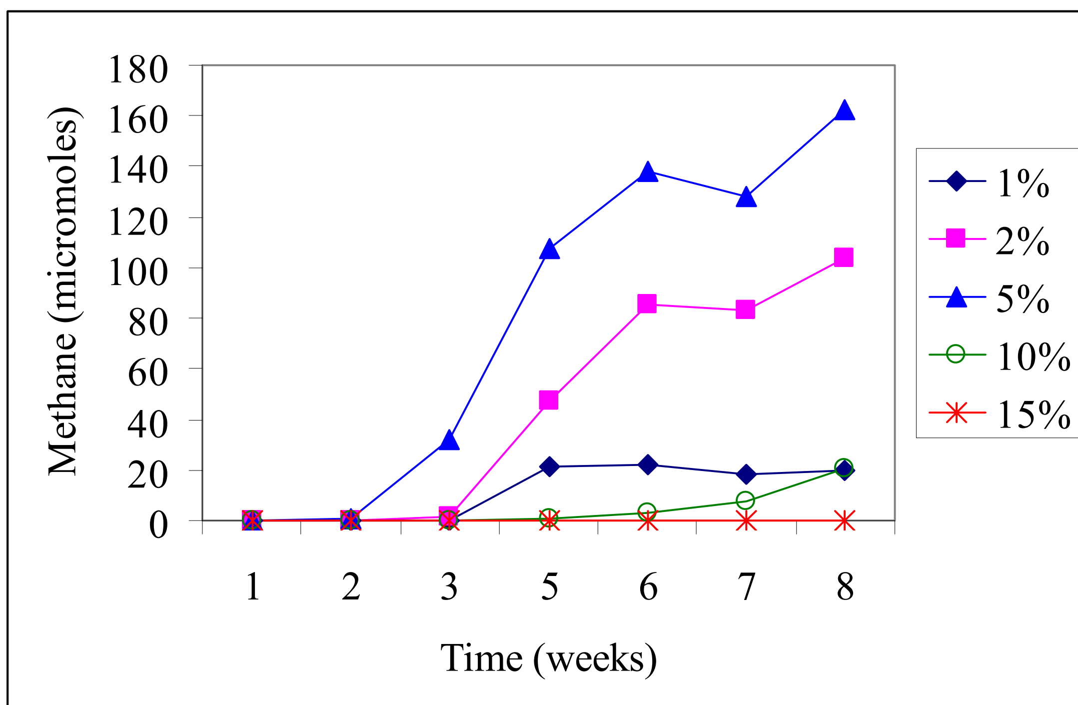

Figure 3.23 Methane production of cultures inoculated at East German Creek on August 7, 2003 and supplied with 10mM glycine betaine and 100 kPa H_2/CO_2 over a range of salinities.

H_2/CO_2 (Figure 3.23). In this case, maximal methane production occurred with 5% salinity. Methane production decreased both above and below this salinity. With both substrates, some methanogenesis was observed at 10%, but not at 15%. Sn-2 hydroxyarchaeol was also found in 1, 2 and 5% salinity TMA cultures (data not shown), but not in the other salinity series cultures.

3.4 Discussion

Physical measurements taken at the springs suggest that the spring waters remain very similar despite different discharge locations, as has been found by others studying the chemical make up of these waters (McKillop *et al.* 1992, Grasby and Chen in review). Spring size and pH measurements were unable to provide a pattern that could be linked to the amount of microbial mat growth present in a spring pool. Spring temperatures were highest at the edges, where microbial mat growth was most often seen. Even low growth springs had mat growth here. The temperature at the centre surface of springs may affect the extent of mat growth. The greatest amount of mat growth was seen with the highest temperatures at this location for the pristine springs (Figure 3.9B). While disturbed springs did not follow this pattern, these springs were sampled and highly disrupted before measurements were taken. This may have caused mixing with the cooler, deeper water, resulting in lower temperatures of the centre surface in medium and high growth springs. Perhaps mat growth can originate at the warm spring pool edges and extend further into the pool surface dependant on temperature. This is a possible explanation as to why some springs experience high amounts of microbial mat growth while others have very little. However, although average temperature readings suggest mats may grow in

springs that experience higher temperatures, the daily average temperatures were not significantly different.

Microbial mat studies

While the presence of algae in a hypersaline environment was not surprising, the discovery of *Percursaria percursa* as the specific dominant organism within the microbial mats of these springs was very interesting because this marine alga has only been reported in one other continental setting (Kornmann 1956). The presence of marine species was not entirely unexpected, as other researchers at these salt springs have reported the presence of several marine species (Patterson *et al.* 1997, McKillop *et al.* 1992).

FAME of microbial mats and sediment samples collected in the fall of 2000 indicated that the distribution of microbes in mats and sediments may differ. Mats contained proportionally more fatty acids associated with Gram negative microorganisms than sediment samples. In general, mat samples contained more monounsaturated and less normal FAME than the underlying sediment, and sediment samples contained more branched fatty acids than mats. While this could indicate a change in microbial community populations, it may also be the result of temperature differences between the two. The presence of more branched chain fatty acids would be expected in the sediments because of the cooler temperatures, since microbes adjust to colder external environments by increasing the amounts of these fatty acids. The greater proportion of monounsaturated FAME in the mat samples was mainly due to increases in 16:1. This fatty acid is produced by both prokaryotes and

eukaryotes; therefore, while it is likely *P. percursa* itself is responsible for this change, it is not conclusive.

The microbial mats collected during the May 2002 and August 2003 field trips were also dominated by monounsaturated fatty acids. There were more normal and less unsaturated FAME with increasing growth in both pristine and disturbed springs. Just as with the mat samples collected in 2000, FAME associated with Gram negative bacteria were much more prevalent than those associated with Gram positive bacteria. Interestingly, the highest proportions of these Gram negative FAME were consistently found in the low growth mats, suggesting Gram positive bacteria and/or *P.persursa* may play a larger role in mats with more growth.

The high levels of the fatty acid 16:1 (cisΔ9) found in the microbial mats samples were unusual, as it is normally found in quantities of under 10%, or in some cases up to half that of 16:0. In these spring mats, levels of 16:1 were most often greater than that of 16:0. Neither Ulvales or cyanobacteria are known to produce such large amounts of this monounsaturated fatty acid. In the study of a tropical coral island in Australia, the dominant fatty acids of sediment samples were 14:0 (10%), 16:0 (21%), 16:1 (cisΔ9) (12%), 18:1(cisΔ11) (6%) (Perry *et al.* 1979). However, when they cultured aerobic heterotrophs from this sediment, they found these were dominated by 16:1 (cisΔ9) (28%), 16:0 (14%) 17:1(cisΔ9) (13%) and 18:1(cisΔ11) (11%). This is also interesting in light of the presence of the two 17:1 fatty acids found in the spring mats. These and other odd chain monounsaturated fatty acids are uncommon in most bacteria; however, studies of marine organisms indicate they may be distinguishing features of marine strains (Perry *et al.* 1979). FAME profiles of

these spring mats, as well as local flora and fauna, are unusual and most closely resemble marine systems.

Sn-2 hydroxyarchaeol was not observed in any of the mats from either field trip, however derivatization of these samples was not successful. Therefore, further attempts to identify this biomarker in these microbial mats samples should be done. *Methanosarcinaceae* may be present, even if not in high numbers, since they were found in some methanogen cultures. It may also be that they are present in significant numbers only in the sediment, where the lower redox potential would make for a better environment for these anaerobes.

Although prokaryotic FAME were the real focus, some polyunsaturated FAME were also found in the microbial mats. The most notable of these was a branched 18:2 fatty acid which accounted for 1-7% of total FAME in the 2000 samples, approximately 1% of all 2002 samples, and between 1 and 3% of 2003 mat samples. These were likely from *P.percursa* but did not completely correlate with level of growth. The FAME profile for *P.percursa* has not been reported, but a study of three related green algae (Ulvales) organisms were found to contain several polyunsaturated fatty acids, including 16:4, 18:2, 18:3, and 18:4 (Li *et al.* 2002).

PCA analysis of all mat samples resulted in a clustering of those collected in 2000 (fall) and those collected in 2002 (spring) and 2003 (summer)(Figure 3.18). While there was not a clear separation between mats in the latter two years, the 2002 mat samples were plotted slightly more positive along PCA1, which was influenced strongly by 16:1. The separate grouping of the 2000 mats may suggest changes in microbial population between spring and summer are less dramatic than those which

happen during the very cold fall and winter months. However, the fall mat samples were collected from different springs within the Lake Winnipegosis area, while the spring and summer samples were all collected from the East German Creek discharge area. So it is also possible that location also plays a role in the microbial mat populations. On the other hand, each of the 2000 mat samples were taken from different sites. Therefore, it could be argued that these mats aggregate together despite variations in sampling site, and seasonal variation may be responsible for this aggregation, not location. Further sampling would be required to separate the effect of season and location.

Enrichment cultures

In providing organic substrates, acetate and formate were chosen because they were expected to be present within the springs as metabolic intermediates of many microorganisms. Methanol was used because it was a known substrate for many anaerobes, particularly methylotrophic methanogens. TMA was used because these microorganisms live in a hypersaline environment, and glycine betaine was assumed to be present. Hydrogen was also provided as an autotrophic substrate for these anaerobes as well.

While SRB cultures from the low and high growth springs sampled in 2002 supported sulphate reduction from H_2, acetate, formate and methanol, the medium growth spring only displayed sulphate reduction with H_2 and methanol. At the same time, methanogenesis was greatest from the medium growth spring. Methanogens also utilized acetate and formate as substrates in this spring, while the SRB did not.

For some reason, the medium growth spring was more hospitable to methanogens than the other two springs.

Further analysis also revealed the presence of *Methanosarcinales* in the low growth (TMA culture) and medium growth (acetate and methanol) springs. Since low levels of methane production were also seen in the methanogen cultures from low and medium growth springs, it is possible that *Methanosarcinales* are present in these springs as well, but larger amounts of biomass would be required to detect sn-2 hydroxyarchaeol.

Methanogen cultures supplied with glycine betaine (10mM) showed minimal methane production (data not shown). It seems that in these springs, another group of microorganisms is required to break down glycine betaine to TMA for methanogens to consume. As expected, methanogens from the springs supplied directly with TMA grew well in culture. Microbes in saline and hypersaline environments produce, accumulate and release glycine betaine, thereby maintaining a consistent supply of this precursor. In addition, TMA's role as a "non-competitive" substrate means that methanogens in the springs were likely accustomed to using this substrate, as they would not have to compete with SRB for this carbon source.

The experiment testing the effects of salinity on methanogenesis in cultures indicated that these methanogens were well adapted to the spring environment. These springs have a salinity of approximately 6% (McKillop *et al.* 1992) and for cultures provided with TMA, maximal activity decreased above 5% salinity (Figure 3.22). For H_2/CO_2 and glycine betaine cultures, methanogenesis decreased both above and below 5% salinity (Figure 3.23). Although provided with a hypersaline environment,

methanogenic extreme halophiles do not appear to be present in these springs. This may be indirect evidence that the salinity of these springs remains relatively stable. Evidence of methanogenesis through dissolved methane profiles have been observed in Great Salt Lake at salinities of 33.3% (Baedecker 1985, Schink *et al.* 1983) and another study mentions microbial methane production from methionine in sediments from this lake (Zeikus 1983).

At the central Manitoba salt springs, methanogenesis with TMA was much greater (up to ~1400µmoles CH_4) than that with H_2/CO_2 (up to ~170µmoles CH_4), even when considering that 1 mole of TMA yields more than twice as much methane as 1 mole of H_2/CO_2. This is in good agreement with Oren's hypothesis that substrates in saline and hypersaline environments are used based on their ability to provide sufficient energy to an organism for survival in these energetically costly surroundings, and TMA is more energetically productive as a substrate than H_2/CO_2.

While marine methanogens were present throughout, sn-2 hydroxyarchaeol was found only in 1, 2 and 5% salinity cultures of the TMA series (data not shown). It appears *Methanosarcinales* were enriched when supplied with TMA but not with H_2/CO_2, or they do not produce similar amounts of sn-2 hydroxyarchaeol when growing on the different substrates.

Future Work

While the presence of methanogens and sulphate reducers in the hypersaline springs of North-Western Manitoba have been confirmed, there is much more to learn about microbial life and diversity in this very unique ecosystem. Identification and diversity exploration focusing on the anaerobic community could reveal new

functions for known organisms and likely identify many marine species of anaerobes unknown in continental environments. In addition, some interesting trends were observed among the lipid profiles of mat samples collected; however, replication and statistical analysis of these would have to be done in order to determine whether changes in these profiles are significant. Lipid community analysis of microbial mat samples collected from the East German Creek site during the winter season (some large springs continue flowing year-round) would complete the seasonal mat lipid data, and perhaps better indicate how the microbial population changes throughout the year.

The *in situ* rates of both methanogenesis and sulphate reduction remain unknown, and should be investigated. A new experimental design could be developed that allows for faster substrate and inhibitor additions while maintaining an anaerobic environment.

While surface temperature may play a role in the extent of microbial mat development in the springs, other factors may also be important. Sedimentation rates remain a likely influence. Again, a new experimental design would be required that would involve a sturdier sediment collection vessel. In addition, although salinity series methanogen cultures were unable to reveal the presence of methanogenic extreme halophiles with either TMA or H_2/CO_2 + betaine as a substrate, it would be worth trying another substrate, perhaps methanol. It seems plausible that extreme halophiles could be present, given the hypersaline environment.

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Appendix 2.1

Methanosarcina barkeri culturing media

(adapted from Kandler and Hippe, 1977)

Component	Amount required (g/L)
Prior to autoclaving:	
K_2HPO_4	0.35
KH_2PO_4	0.23
NH_4Cl	0.5
*Yeast extract	0.05
$MgSO_4 \bullet 7H_20$	0.5
$CaCl_2 \bullet 2H_2O$	0.25
NaCl	2.25
$NaHCO_3$	0.85
Na_2CO_3	0.5
Mineral elixir (in this appendix)	10mL/L
Vitamin supplement (in this appendix)	10mL/L
Resazurin (5mg/100mL)	5mL/L

pH adjusted to 6.5

Headspace gases then exchanged with either H_2/CO_2 (80%:20%) or N_2/CO_2 (80 %:20%)

Following autoclaving:

Na_2S (0.01M) (immediately prior to inoculation)	10mL/L
Substrate addition	dependant on stock solution

*Cultures grown on acetate required 1g/L yeast extract.

Mineral elixir

Component	Amount required (g/L)
Distilled H2O	to 1L
Trisodium nitriloacetate*($C_6H_6NO_6Na_3$)	2.02
$FeCl_3 \bullet 6H_2O$	0.21
$CoCl_2 \bullet 6H_2O$	0.2
$MnCl_2 \bullet 4H_2O$	0.1
$ZnCl_2$ (anhydrous)	0.1
$NiCl_2 \bullet 6H_2O$	0.1
$CaCl_2 \bullet 2H_2O$	0.05
$CuSO_4 \bullet 2H_2O$	0.05
$Na_2MoO_4 \bullet 2H_2O$	0.05

- can also use nitrilotriacetic acid if pH is adjusted to 7.

Vitamin supplement

Component	Amount required (mg/L)
Distilled water	to 1L
Pyroxidine HCl	10
Riboflavin	5
Thiamine	5
Nicotinic acid	5
p-amino benzoic acid	5
Lipoic acid (thioctic acid)	5
Biotin	2
Folic acid	2
Cyanocobalamin	1

- This solution should be filter sterilized (not autoclaved) and stored at 4˚C in the dark.

Appendix 2.2

Methanosarcina barkeri non-clumping culturing media for acetate isotopes experiment

Component	Amount required (g/L)
Prior to autoclaving:	
K_2HPO_4	0.35
KH_2PO_4	0.23
$MgSO_4 \bullet 7H_20$	0.5
$CaCl_2 \bullet 2H_2O$	0.14
$Fe(NH_4)_2(SO_4)_2$	0.002
NaCl	5.8
KCl	0.76
*Yeast extract	0.1
$NaHCO_3$	0.85
Cysteine hydrochloride	0.5
Mineral elixir (in this appendix)	10mL/L
Vitamin supplement (in this appendix)	10mL/L
Resazurin	0.001g/L

pH adjusted to 6.5

Headspace gases then exchanged with N_2/CO_2 (80 %:20%)

Following autoclaving:

Na_2S (0.01M) (immediately prior to inoculation)	10mL/L
Substrate addition (acetate)	dependant on stock solution

*Again, acetate cultures required 1 g/L yeast extract.

Appendix 2.3 Calculating methane production

Methane standards of 5%, 10%, 20%, 30% and 100% were used to create a standard curve to compare methane readings for each sampling session. In order to make the appropriate standard concentrations, different aliquots of a pure methane sample were diluted to achieve the different standard concentrations. Standards were made up in 160mL glass serum bottles. The volume of pure methane required in each standard was calculated:

5%- 8mL of 100% methane (8mL/160mL = 5%)

10% -16mL of 100% methane

20%- 32mL of 100% methane

30%- 48mL of 100% methane

100%- stock bottle

Before methane was added, the standard bottles were crimp sealed. The total volume inside was then 160mL and some of this air was removed in order to maintain the total volume at 160mL after methane addition. Therefore, the volume corresponding to the amount of methane to be added was removed by needle and syringe. Finally, methane was taken from the stock (100%) bottle and added to the standards, again via needle and syringe.

Changing environmental conditions cause the exact volume in the standards to fluctuate. This is why a standard curve was necessary on each methane sampling session. As an example, if a 1% standard was made up with 10mL of methane in a total volume of 1L, several factors such as temperature and pressure would have to be taken into account. First, these parameters would have to be converted to standard

temperature and pressure (STP). Temperature would be reported on the Kelvin scale (0˚C = 273 K) and pressure as mmHg (1kPa = 7.5 mmHg). If this standard was made up at room temperature (22˚C) and atmospheric pressure (760 mmHg), then the volume of methane could be converted to STP (273K, 760mmHg):

10mL X [273/(273 + temp ˚C)] X (pressure kPa/760 mmHg)

= 10 X (0.925) X (1)

= 9.25 mL CH_4/L (µL CH_4/mL)

So this 1% standard would actually be 0.925% methane.

To convert to micromoles of methane, the gas constant (22.4L/mol or µL/µmol) and the amount injected (0.1mL) must be taken into account. In the case of the 1% standard example:

[CH_4 (µL/mL) X volume injected (0.1 mL)]/ 22.4 µL/µmol

= (9.25 µL/mL) X 0.1mL)/ 22.4 µL/µmol

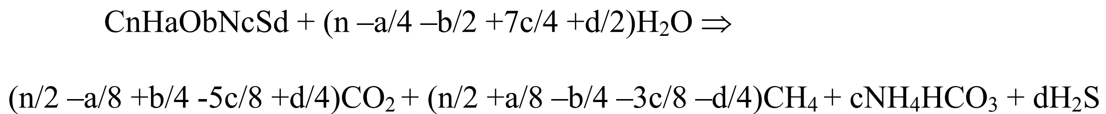
= 0.041 µmoles CH_4 (or 41 millimoles)

In this way, the amount of methane present in headspace of samples was determined by comparing values to the standard curve once the pressure and headspace volume of those samples were known.

Appendix 2.4 Buswell's equation

The amount of methane produced by methanogens from one mole of substrate is not the same for each of the different substrate. For this reason, Buswell's equation (Sulfita *et al.* 1997) is used to calculate how much substrate has been consumed based on the amount of methane that has been produced.

Buswell's equation:



$$CnHaObNcSd + (n - a/4 - b/2 + 7c/4 + d/2)H_2O \Rightarrow$$

$$(n/2 - a/8 + b/4 - 5c/8 + d/4)CO_2 + (n/2 + a/8 - b/4 - 3c/8 - d/4)CH_4 + cNH_4HCO_3 + dH_2S$$

The substrate is represented as CnHaObNcSd and subscript letters indicate the element's abundance.

Using methanol (CH_3OH) as an example, n =1, a =4, b =1, c =0 and d =0.

The amount of CH_4 produced will be determined by (n/2 +a/8 –b/4 -3c/8 –d/4)

= (1/2 +4/8 -1/4 -3(0)/8 -0/4)

= ¾ or 0.75 moles

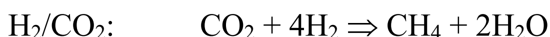
The next step is to calculate the amount of each of the products that will result with the consumption of one mole of methanol. Then the equation must be balanced.

For methanol, this becomes: $4CH_3OH \Rightarrow 2H_2O + CO_2 + 3CH_4$

The balanced equations for the other substrates studied are:

TMA: $4(CH_3)_3NH^+ + 6H_2O \Rightarrow 3CO_2 + 9CH_4 + 4NH_4^+$

Acetate: $8CH_3COOH \Rightarrow 8CO_2 + 8CH_4$



H_2/CO_2: $CO_2 + 4H_2 \Rightarrow CH_4 + 2H_2O$

Appendix 3.1

Field methanogen media

(adapted from Kandler and Hippe, 1977 taking spring chemistry into account)

Component	Amount required (g/L)
Prior to autoclaving:	
NaCl*	50.85
$MgCl_2 \cdot 6H_2O$	2.54
$CaCl_2 \cdot 2H_2O$	2.2
NH_4Cl	0.5
KH_2PO_4	0.2
Yeast extract	0.05
$NaHCO_3$	0.85
Na_2CO_3	0.5
Na_2SO_4	0.05
Mineral elixir (as in appendix 2.1)	10mL/L
Vitamin supplement (as in appendix 2.1)	10mL/L
Resazurin (5mg/100mL)	5mL/L

pH adjusted to 6.5

Headspace gases then exchanged with either H_2/CO_2 (80%:20%) or N_2/CO_2 (80 %:20%)

Following autoclaving:

Salt spring water (filter sterilized)	50mL/L
Na_2S (0.01M) (immediately prior to inoculation)	10mL/L
Substrate addition	dependant on stock solution

* Media for the second field trip's salinity series enrichment cultures used varying amounts of NaCl as shown in appendix 3.3.

Appendix 3.2

Field SRB media

Component	Amount required (g/L)
Prior to autoclaving:	
NaCl	50.85
$MgCl_2 \cdot 6H_20$	2.54
$CaCl_2 \cdot 2H_20$	2.2
Na_2SO_4	4.0
NH_4Cl	0.25
KH_2PO_4	0.2
KCl	0.5
Resazurin (5mg/100mL)	5mL/L
Distilled water	to 1L
pH adjusted to 6.5	

Headspace gases then exchanged with either H_2/CO_2 (80%:20%) or N_2/CO_2 (80 %:20%)

Component	Amount required (g/L)
Following autoclaving:	
SRB master mix (in this appendix)	5mL/L
Bicarbonate (1M)	30mL/L
Salt spring water (filter sterilized)	50mL/L
Na_2S (0.01M) (immediately prior to inoculation)	10mL/L
Substrate addition	dependant on stock solution

SRB Master Mix

Made from a mix of 10mL of each of the following five solutions:

1. Tungstate-selenite solution

Component	Amount required (mg/L)
Distilled water	to 1L
NaOH	400
$Na_2SeO_3 \cdot 5H_2O$	6
$NaWO_4 \cdot 2H_2O$	8

2. Vitamin solution

Component	Amount required (mg/100mL)
10mM $NaPO_4$ buffer @ pH 7.1	100mL
p-amino benzoic acid	4
D(+) biotin	1
Nicotinic acid	10
Calcium D(+) pantothenate	5
Pyroxidine dihydrochloride	15

- This solution should be filter sterilized (not autoclaved) and stored at 4˚C in the dark.

3. Thiamine solution

Component	Amount required (mg/100mL)
25mM $NaPO_4$ buffer	100mL
thiamine chloride dihydrochloride	10

pH adjusted to 3.4

- This solution should be filter sterilized.

4. Vitamin B12 solution

Component	Amount required (mg/100mL)
Distilled H_2O	100mL
Cyanocobalamine	5

- This solution should be filter sterilized.

5. Non-chelated trace element mixture

Component	Amount required (g/100mL)
Distilled H_2O	987mL
HCl	12.5mL
$FeSO_4 \cdot 7H_2O$	2.1
H_3BO_4	0.03
$MnCl_2 \cdot 4H_2O$	0.1
$CoCl_2 \cdot 6H_2O$	0.19
$NiCl_2 \cdot 6H_2O$	0.024
$CuCl2 \cdot 2H_2O$	0.002
$ZnSO4 \cdot 7H_2O$	0.144
$Na2MoO4 \cdot 2H_2O$	0.036

- pH adjustment should not be done to this solution as precipitates will form.

Appendix 3.3 Salinity series media calculations

Each ion in a solution adds to the salinity, therefore the overall salinity of the "regular" methanogen field media was calculated:

Component	Amount (g/L)	Ions	Atomic weight	Moles		g/L
NaCl	50.85	Na+	22.99	50.85g/58.44g/mol	0.87 X 22.99 =	20
		Cl-	+ 35.45	= 0.87 moles	0.87 X 35.45 =	30.84
			58.44			
$MgCl_2 \cdot 6H_2O$	2.54	Mg2+	24.31	2.54g/203.306g/mol	0.0125 X 24.31 =	0.304
		2 Cl-	2 X 35.45	= 0.0125 moles	0.0125 X (2) 35.45 =	0.886
		6 H2O	+ 6 X 18.016			
			203.306			
$CaCl_2 \cdot 2H_2O$	2.2	Ca2+	40.08	2.2g/147.012g/mol	0.015 X 40.08 =	0.6012
		2 Cl-	2 X 35.45	= 0.015 moles	0.015 X (2) 35.45 =	1.0635
		2 H2O	+ 2 X 18.016			
			147.012			
NH_4Cl	0.5	NH4+	18.042	0.5g/53.492g/mol	0.009 X 18.042 =	0.1624
		Cl-	+ 35.45	= 0.009 moles	0.009 X 35.45 =	0.3191
			53.492			
KH_2PO_4	0.2	K+	39.1	0.2g/136.086g/mol	0.0015 X 39.1 =	0.0587
		H2PO4-	+ 96.986	= 0.0015 moles	0.0015 X 96.986 =	0.145
			136.086			
$NaHCO_3$	0.85	Na+	22.99	0.85g/84.008g/mol	0.01 X 22.99 =	0.233
		HCO3-	+ 61.018	= 0.01 moles	0.01 X 61.018 =	0.6102
			84.008			
Na_2CO_3	0.5	2 Na+	2 X 22.99	0.5g/105.99g/mol	0.0047 X 2(22.99) =	0.2161
		CO3 2-	+ 60.01	= 0.0047 moles	0.0047 X 60.01 =	0.282
			105.99			
Na_2SO_4	0.05	2 Na+	2 X 22.99	0.05g/142.05g/mol	0.000352 X 2(22.99) =	0.0324
		SO4 2-	+ 96.07	= 0.000352	0.000352 X 96.07 =	0.0338 +
			142.05			
					Total =	55.787

The units of g/L convert to parts per thousand (ppt) or ‰. So the "regular" media has a salinity of approximately 56‰ or 5.6%. Ninety-one percent (50.84‰ of 55.787‰) of the salinity in this media is obtained from NaCl, therefore the salinity was adjusted for this series by manipulating the NaCl concentration. The contribution of the other media components to the salinity was not thought to be significant enough to require additional adjustment of these concentrations as well.

The addition of salt spring water to the media also contributes to the overall salinity and this was taken into account. Chemical analysis from McKillop *et al.* 1992 was used to determine just how much salinity was contributed from this water.

East German Creek water (as analysed by McKillop *et al*. 1992)

Component	Amount (mg/L)	g/L	g/50mL
Cl-	33500	33.5	1.675
Na+	21350	21.35	1.0675
K+	550	0.55	0.0275
Ca2+	1188	1.188	0.0594
Mg2+	250	0.25	0.0125
SO4 2-	3050	3.05	0.1525
HCO3-	365	0.365	0.0183
Fe2+	2.84	0.00284	0.000142
Mn2+	0.06	0.00006	0.000003
F-	1.11	0.00111	0.0000555
PO4 3-	0.03	0.00003	+ 0.0000015
		Total =	3.013

This indicates that when the spring water is added to the media at 50mL/L, the contribution to salinity is 3.013g. Therefore, a 1% saline solution which requires a final salinity of 10‰ (or 10ppt or 10g/L) only requires an additional 6.987g/L (10-3.013).
In this way, the media for the salinity series' were determined.

Final salinity	Media contribution without NaCl	After spring water contribution	NaCl required
1% (10ppt)	6.987	-4.947	2.04
2% (20ppt)	16.987	-4.947	12.04
5% (ppt)	46.987	-4.947	42.04
10% (ppt)	96.987	-4.947	92.04
15% (ppt)	146.987	-4.947	142.04
20% (ppt)	196.987	-4.947	192.04
25% (ppt)	246.987	-4.947	242.04
30% (ppt)	296.987	-4.947	292.04

Appendix 3.4 East German Creek spring temperature measurements

	Temperature (degrees Celsius)								
	Deep centre			Centre surface			Edge (in mat)		
Spring Type	15:00	17:20	10:40	15:00	17:20	10:40	15:00	17:20	10:40
LG disturbed	12.2	3.1	7.1	13.9	13.7	9	22.4	17.8	11.2
pristine	6.8	6.3	6.5	9.3	8.6	5.1	17.9	16	11.9
MG disturbed	5.6	5.5	5.6	11.8	5.6	5.6	13.5	9.1	6.8
pristine	9.5	9.1	6.7	13.4	10.5	7.5	16	15.3	13.1
HG disturbed	7	6.9	7.4	9.3	11.1	7.7	13.5	17.9	11.9
pristine	4.7	5.4	4.2	13.3	12.3	11.1	17.7	16.4	9.7
average	7.6	6.1	6.3	11.8	10.3	7.7	16.8	15.4	10.8
standard deviation at location			2.1			2.9			3.9

Temperature measurements were taken on May 17, 2002 for the 15:00 and 17:20 readings and on May 18, 2002 for the 10:40 readings.

LG, MG and HG represent low, medium and high growth springs.