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Roles of acetogens and methanogens in carbon capture and storage

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Roles of acetogens and methanogens in carbon capture and storage

by

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Abstract

Carbon capture and storage (CCS) aims to reduce atmospheric CO_2 by capturing emitted gas for storage in subsurface locations. Microorganisms such as methanogens and acetogens, can utilize H_2 and CO_2 as a carbon and energy source in the subsurface. In enrichments with produced water (PW) from a potential CCS site, high concentrations of acetic acid were observed followed by methane production. Microbial community compositions of enrichments showed the percentage of methanogens increased as enrichment period progressed while the percentage of acetogens decreased. Methanogens of the order *Methanomicrobiales* were mostly present when enrichment pH was acidic or neutral. Mixing PW with source water (SW) at the site also affected the types of microbes present in PW samples. Mixing PW with SW resulted in an increase of CFU/mL indicating some additional growth. Microbial community composition analysis indicates that oil field microorganisms may have originated from SW.

Acknowledgements

I would like to acknowledge the members of the Voordouw and Gieg lab for their support during the course of my program. I would also like to say a big thank you to the Dunfield, Hynes and Turner lab for their generosity in sharing equipment which was essential to the analysis of my work. To my parents, Adepoju Felix and Kehinde Mary Folarin, I say thank you for your love and support throughout the years.

Dedication

I would like to dedicate this thesis to Jesus Christ, my Lord, Saviour and Redeemer.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
PW	Produced water
SW	Source water
IW	Injection water
WP	Water plant
PWRI	Produced water re-injection
EOR	Enhanced Oil Recovery

Chapter 1 INTRODUCTION

1.1 Carbon capture and storage

The burning of fossil fuel accounts for about 60 percent of the global anthropogenic CO_2 emitted into the atmosphere. This is the result of an increasing demand in energy derived from fossil fuel (Neelis et al., 2005; Li et al., 2005; Princiotta, 2009). The storage of CO_2 into geological formations to reduce its emissions is currently practiced outside Canada, such as the In Salah CCS project in Algeria (Holloway, 1997; and Noble et al., 2012). Carbon capture and storage (CCS) is the process of reducing the carbon dioxide content of streams which would be released into the atmosphere by capturing and transporting CO_2 into permanent storage sites (Neelis et al, 2005; Lacis et al., 2010). The capture of CO_2 can be from different places such as process streams, heater and boiler exhausts, and vents from industries such as power generation, cement production, refining, chemicals, steel and natural gas processing (Karl and Trenberth, 2003; Princiotta, 2009).

1.1.1 **Effect of carbon dioxide on the atmosphere**

The Earth's atmosphere is a mixture of mostly N_2, O_2, water vapour and trace gases, including CO_2. Many of these gases contribute to the greenhouse effect on the Earth's atmosphere. Over the past two centuries, there have been significant changes in the concentration of some of these atmospheric gases. Human activities are the main causes driving this change (Princiotta, 2009; Li et al., 2005). These activities are dominated by an increasing reliance on the burning of fossil fuel to derive energy.

Anthropogenic causes of climate change are likely to continue for many centuries. Global warming results when this accumulation causes greenhouse gases to trap outgoing radiation from Earth to space (Raval and Ramanathan, 1989). The continuous increase leading to the high rate of increase of atmospheric CO_2 is particularly worrisome. The present CO_2 level of 385 ppm far exceeds the 315 ppm that was recorded in 1960 (Karl and Trenberth, 2003; Figure 1-1). The rate of increase of atmospheric CO_2 is much higher in recent years (Figure 1-1). Furthermore, the atmospheric residence time of CO_2 is exceedingly long, being measured in thousands of years (Karl and Trenberth, 2003). This makes the reduction of atmospheric CO_2 a serious and pressing issue, worthy of immediate attention. One of the methods currently being employed to reduce the emissions of CO_2 is by capturing and storing it in geological formations (Sen, 2008 & Holloway, 1997).

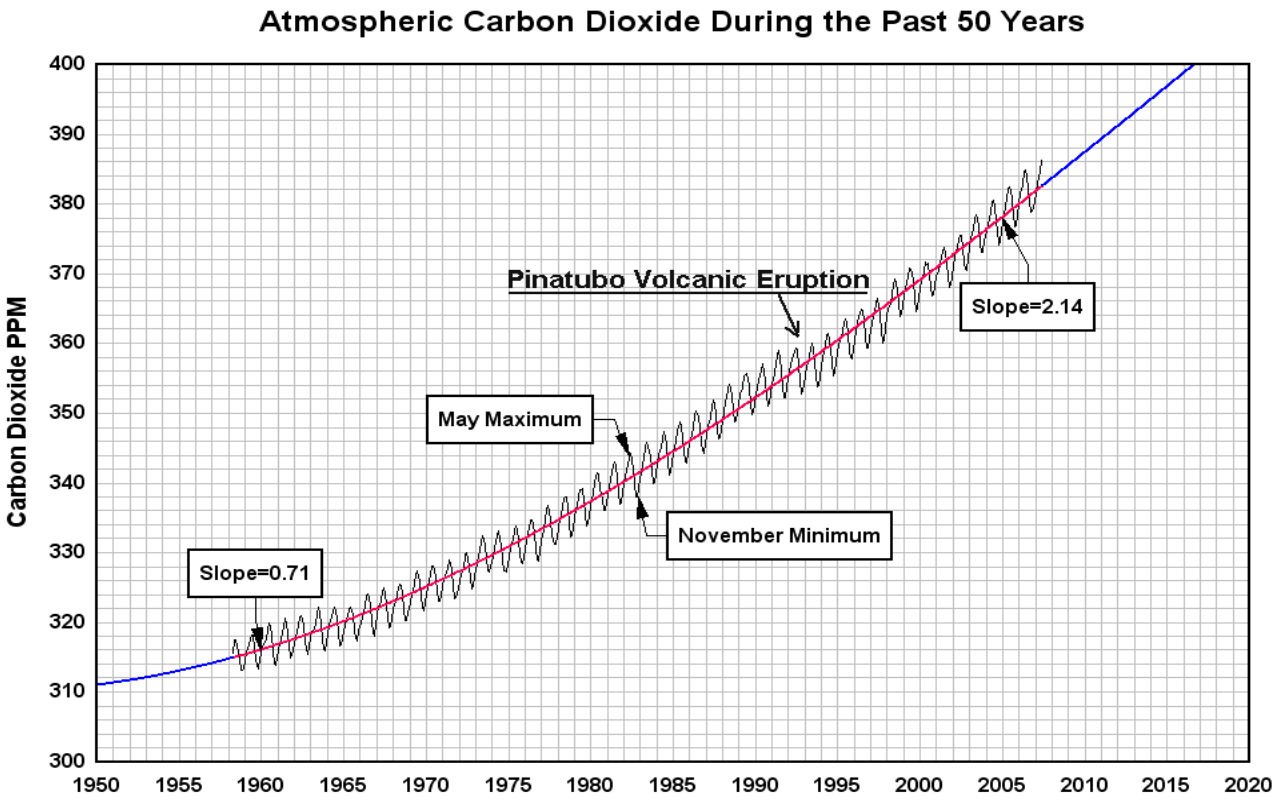


Figure 1-1: Annual and seasonal trend in atmospheric CO_2 from 1960 - 2010. Measurements were taken at Manua Loa Observatory, Hawaii. Atmospheric CO_2 levels decrease during the summer due to photosynthesis and increase over the winter months when photosynthesis is not taking place. The rate of increase (slope) is shown to be higher in the past few years. The Pinatubo volacanic eruption that occurred in the Philippines is thought to have affected the concentration of atmospheric CO_2. Source: http://scrippsco2.ucsd.edu/research/atmospheric_co2.html

1.1.2 **Process of carbon capture**

Different technologies (these are described below) are currently being employed for the capture of CO_2, and their application depends on the source of CO_2 (Holloway, 1997; Princiotta, 2009).

1.1.2.1 Pre-combustion technology

This process is used to separate CO_2 from syngas, a byproduct from gasified coal, prior to combustion. Syngas, which is a mixture of hydrogen and carbon monoxide is reacted with water to produce hydrogen and carbon dioxide (CO_2), which are separated.

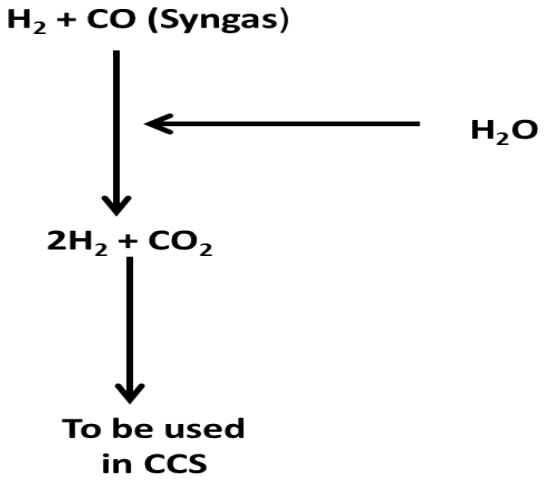


Figure 1-2: Overview of pre-combustion technology separation of CO_2 from syngas produced from gasified coal.

1.1.2.2 Post combustion technology

This process is used to capture CO_2 after fossil fuel has been burnt. CO_2 is captured from flue gas a byproduct from coal or gas power plants. Flue gas is composed of nitrogen in addition to CO_2.

$$\mathbf{80\%\ N_2 + 20\%\ O_2 + fossil\ fuel \rightarrow 80\%\ N_2 + 20\%\ CO_2}$$

1.1.2.3 Oxyfuel combustion technology

This process is also used after fossil fuel has been burnt but it aims to capture pure CO_2 that can be used for CCS. Fossil fuel is burnt with pure oxygen to produce flue gas which contains > 80% CO_2. Nitrogen is separated from flue gas cryogenically to obtain almost pure CO_2 for storage.

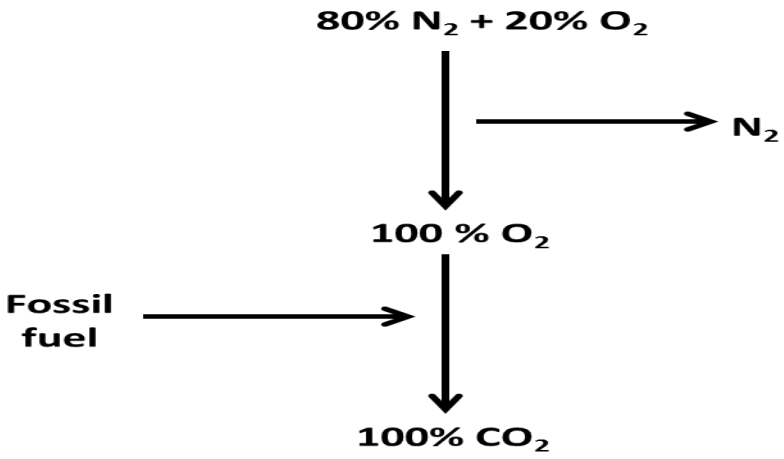


Figure 1-3: Overview of oxyfuel combustion technology for separation of CO_2 from fossil fuel.

Once CO_2 has been captured, using any of the above technologies, it is compressed, dried and transported and then injected into subsurface storage locations (Holloway, 1997; Princiotta, 2009 and Figure 1-2).

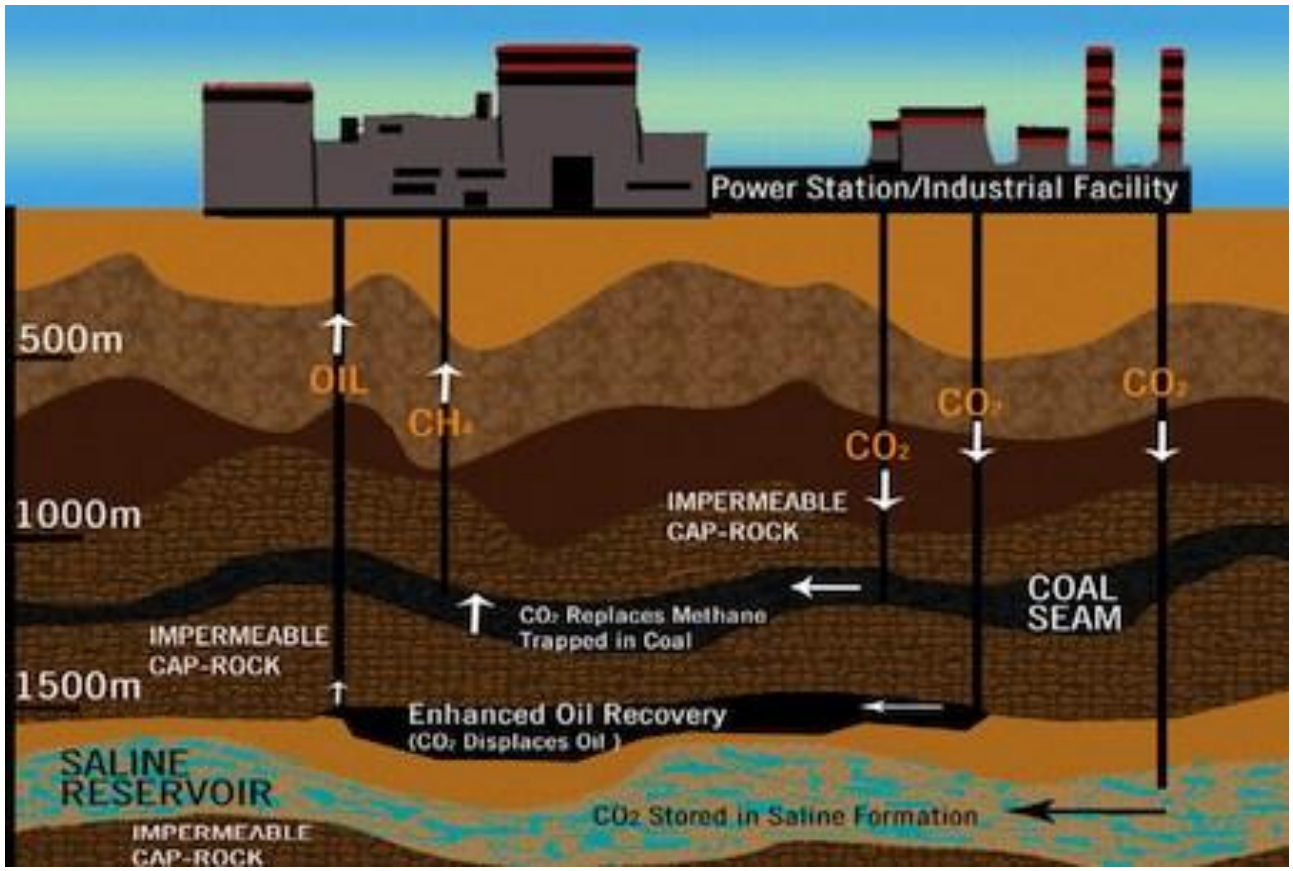


Figure 1-4: Relative depth of subsurface storage sites for CO_2 storage. CO_2 can be stored in saline reservoirs, depleted oil reservoirs or depleted coal bed methane reservoirs. The storage depths for all three reservoirs are all above 1000 m. Source: http://www.wri.org/project/carbon-dioxide-capture-storage/ccs-basics.

1.1.3 **Properties of CO_2 as a function of temperature and pressure**

The process of stable storage of CO_2 into subsurface storage sites requires certain conditions. J.W. Gibbs came up with an explanation for the different phase changes experienced by chemical compounds (Figure 1-5; Mladek et al., 2007). The Gibb's phase rule is given by the following equation:

$$\mathbf{F = C-P +2}$$

where F - # of degrees of freedom; P - # of phases and C - # of components.

A phase is defined as "a form of matter that is homogenous in chemical composition and physical state". Hence if we have only CO_2 (C=1) and we have two phases P = 2 (e.g. gas and liquid), we find F=1. This means that when we set the temperature e.g. 20°C, the pressure is fixed (Figure 1-5: ~ 50 atm). At certain temperature and pressure, CO_2 exhibits different states. For example at a pressure of about 5 atm and temperature of - 60°C, it is at the triple point where it exhibits properties of solid, liquid and gaseous states (Figure 1-5). In CO_2 storage, the critical point properties are of great importance since storage is in the form of a supercritical fluid. The supercritical fluid occurs at a temperature and pressure that is above that of the critical point, therefore the "CO_2" acts as a liquid solvent (Figure 1-5). An average depth of 1 km is required to achieve storage of supercritical CO_2 in geological sites with temperature and pressure of approximately 31.5°C or 304.7K and 73 atm (Mitchell et al., 2008). In oil fields injected CO_2 becomes miscible with oil under these conditions during the process of enhanced oil recovery (EOR). Therefore, residual oil in the reservoir swells and the viscosity is lowered (Emberley et al., 2004).

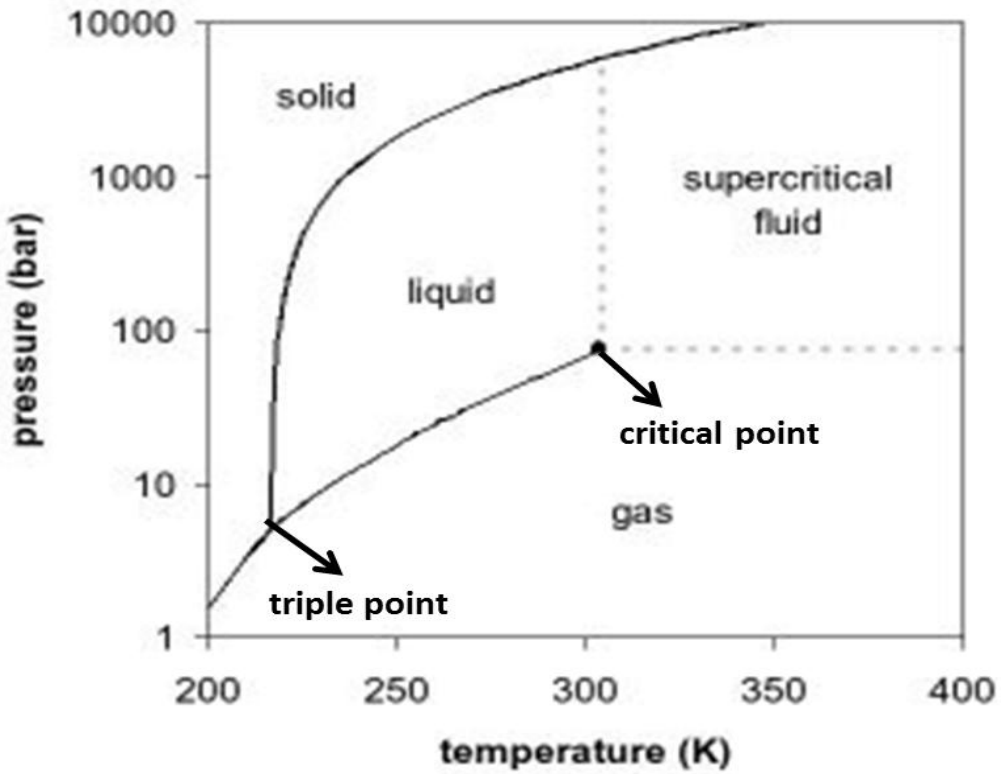


Figure 1-5: Pressures and temperatures that define the different phases of CO_2. The critical point properties are important for carbon capture and storage. CO_2 is injected in the supercritical state which occurs when temperature and pressure are above the 304.7K and 73 bars respectively. Note that 1 bar = 0.99 atm. Source: http://en.wikipedia.org/wiki/File:Carbon_dioxide_pressure-temperature_phase_diagram.jpg#filelinks

1.2 Secondary conventional oil production

Secondary conventional oil production is practiced when the initial pressure in a reservoir has been exhausted. This causes a reduction in oil production. Therefore water is injected (at injection wells - IW) to maintain reservoir pressure, pushing oil to the surface through production wells (Voordouw et al., 2009; Gieg et al., 2011; Korenblum et al. 2010). A major problem for continuous oil production on land is a steady supply of water. The problem of water availability is resolved by re-injecting produced water (PW) back into the reservoir after it is separated from produced oil (Tischler et al., 2010). This is referred to as produced water reinjection (PWRI). The shortfall in the volume of recovered PW requires the addition of make-up water, also referred to as source water (SW). The source water (SW) used for this purpose depends on availability and cost and may determine the microbial community that develops in an oil field (Gieg et al., 2011; Magot, 2005). This community can cause reservoir souring and corrosion of pipes used in water and oil transportation (Liamleam & Annachhatre, 2007). The process of secondary oil production is shown in Figure 1-6.

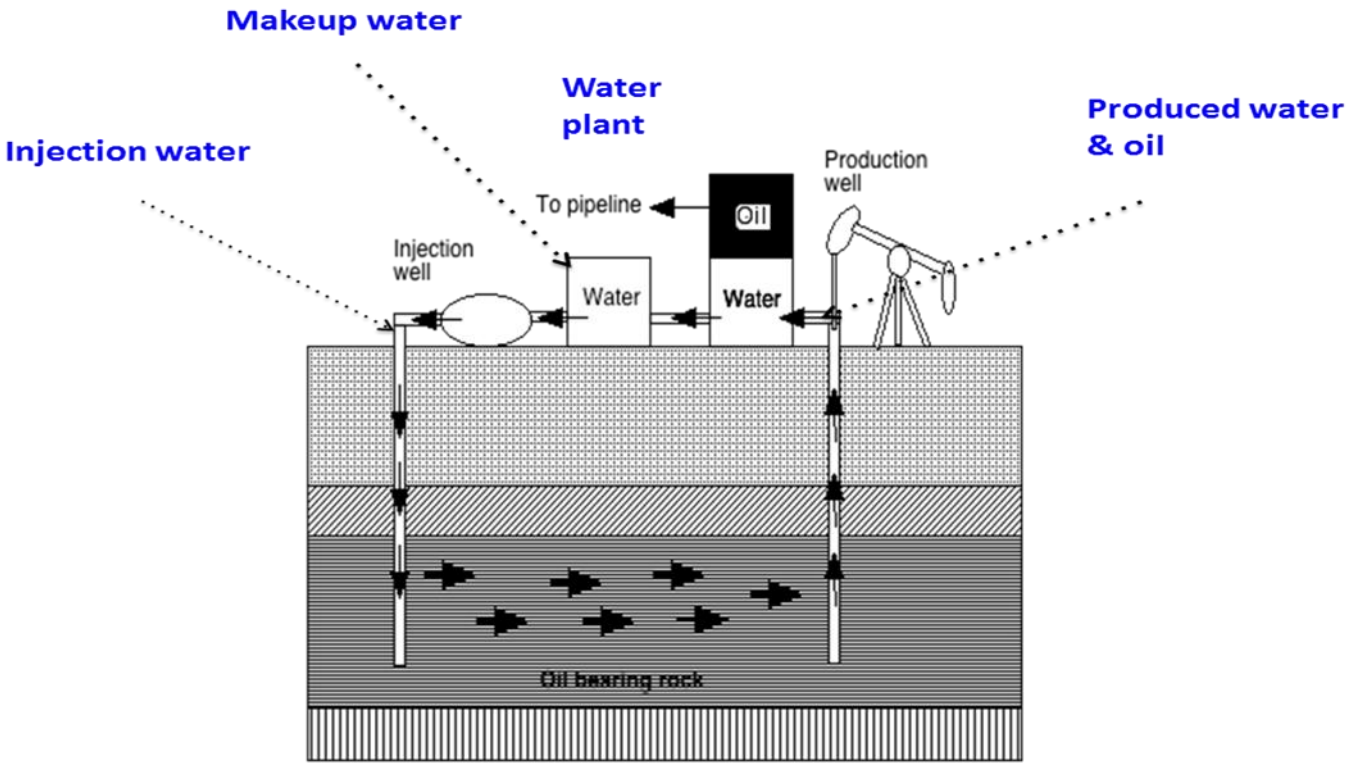


Figure 1-6: Schematic representation of oil production through PWRI. Water is injected into the reservoir at the injection well (IW) to increase reservoir pressure and push oil in oil bearing rock to the production well (PW). The oil-water mixture produced at PW is sent to the water plant, where is it mixed with makeup water (or source water) SW. The resulting IW is injected into the reservoir and the cycle continues.

1.2.1 **Medicine Hat Glauconitic C Field**

The Medicine Hat Glauconitic C (MHGC) field is located near Medicine Hat, in southeastern Alberta. The sampling map of the field is shown in Appendix A (Figure A-1). The reservoir in this field is at a depth of ~ 850 m with an in situ temperature of 30°C. The effluent of the Medicine Hat sewage treatment plant is used as the SW in a large part of this field (Agrawal et al., 2012). The untreated SW, 22-SW-U (Voordouw et al., 2009) is subjected to daily treatment with hypochlorite (bleach) and to quarterly treatment with the biocide acrolein, then filtered (22-SW-F) and further treated with the oxygen scavenger ammonium bisulphite. The treated water (22-SW-T) is then mixed with PW in the water plant. Treatment with ammonium bisulphite (NH_4HSO_3) removes oxygen before it is sent to the water plant:

$$2NH_4HSO_3 + O_2 \rightarrow H_2SO_4 + (NH_4)_2SO_4$$

The mixture of PW and produced oil is treated with demulsifier and subjected to high temperature (50^oC) to separate PW and produced oil. The oil produced in this field is heavy oil (API 16°). The American Petroleum Institute (API) scale is commonly used by oil producers to grade oil (Planckaert, 2005). The lower the API value, the heavier the oil and vice versa. It is calculated from the equation:

$$\mathbf{API = (141.5/Sg) – 131.5}$$

The specific gravity (Sg) is the density of oil at standard pressure of 15°C and pressure of 100 kPa (Planckaert, 2005). PW is also treated with corrosion inhibitors to limit pipeline corrosion (Fazal et al., 2011). At the water plant SW and PW are mixed in an approximate ratio of 1:3 and the resulting injection water is distributed to numerous

injection wells (Fig. 1-7). Previous work has shown that sulfate present in SW contributes to souring, which is being treated by injection of nitrate (Voordouw et al., 2009).

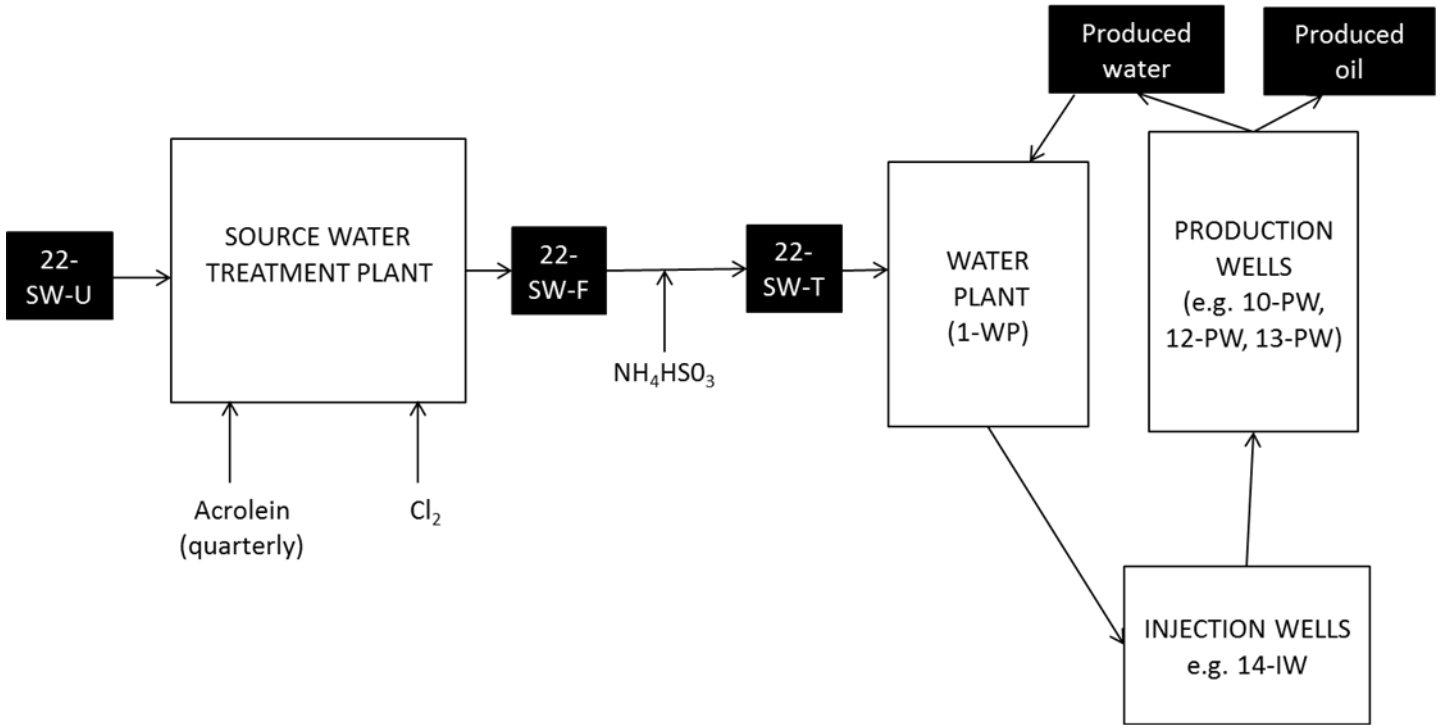


Figure 1-7: Schematic representation of water flow in the MHGC field. Untreated SW (22-SW-U) is the effluent from the Medicine Hat sewage treatment plant. 22-SW-U is treated quarterly with acrolein and daily with chlorine and passes through filtered beds and becomes filtered SW (22-SW-F). 22-SW-F is reacted with ammonium bisulfite to remove oxygen and becomes treated SW (22-SW-T). This is mixed with produced water (PW) in the field at the water plant (1-WP) in a 3:1 ratio.

1.2.1.1 Water chemistry data

Source water samples, SW-U, SW-F and SW-T had low salt (~ 230 mg/L of NaCl), low bicarbonate (65-110 mg/L) and relatively high sulfate (250-400 mg/L), as indicated in Table 1-1. The sulfate in 22-SW is the primary source of sulfate in the injection water (Voordouw et al., 2009). Water from the water plant and injection well (1-WP and 14-IW) had 3-5 g of NaCl/L, higher bicarbonate (285-2905 mg/L), but lower sulfate (14-88 mg/L), due to mixing with produced water. The water chemistry of PW can be inferred from that of the WP and IW because they are a mixture of PW and SW in 3:1 ratio. The PW had higher salt (~ 5 g/L NaCl) and lower sulfate concentrations than the SW. Although the water chemistries are different they may be able to support the growth of similar microbes. This is because the down-hole reservoir temperature of 30°C is comparable with what may be expected in the sewage treatment plant. The question now is whether mixing of the different waters can improve conditions for microbial growth. Microbial growth is determined by the available carbon and energy source (e.g. residual oil) and the presence of nutrients, such as ammonium and phosphate (Kuijvenhoven et al., 2006). Some of these could be specifically present in the SW. Hence the combination of SW and PW to create injection water (IW) for oil recovery could create favorable conditions for microbial growth in an oil reservoir (Patton, 1990; Gieg et al., 2011).

Table 1-1: Water chemistry of samples used in this study, except for produced water samples for which no data were available. All numbers, except for pH, are in mg/L (ppm). Average values are represented in cases where data for multiple samples were available.

	Date (month, year)	3, 2011	2, 2011	2, 3, 4, 7, 2011	3, 2009	3, 7, 2011
	Component\Sample	**22-SW-U**	**22-SW-F**	**22-SW-T**	**14-IW**	**1-WP**
Anions	Chloride	126	122	144	1520	2500
	Bicarbonate	65.5	106	106.76	285	2905
	Sulfate	249	248	410.2	87.5	13.6
Cations	Sodium	104	115	137.5	1640	2465
	Magnesium	29.5	33	43.6	16.6	11.4
	Calcium	77.8	76	88.8	97.9	44.2
	Strontium	0	0.16	0.12	1.4	1.76
	Barium	0	0	0.004	0.32	0.28
	Iron	0.16	0.04	0.018	1.04	3.9
	Potassium	17.7	16.6	17.6	16.3	16.1
	Manganese	0	0	0.01	0	0
pH		6.0	6.0	6.2	7.5	7.5
TDS		670	717	949	3666	7961

*All numbers, except for pH, are in mg/L (ppm). Average values are represented in cases where data for multiple samples were available. Data was provided by Baker Hughes.

1.2.2 **Enhanced oil recovery with CO_2 injection.**

In the petroleum industry, secondary techniques are usually employed to extract the remaining oil trapped in geological formations when primary production, driven by the initial reservoir pressure declines (Sen, 2008). About 60 percent or more of original oil in place (OOIP) remains trapped in reservoirs at the end of primary oil production (Holloway, 1997). These secondary techniques which includes injection of CO_2, lead to enhanced oil recovery (EOR) in oil reservoirs (Li et al., 2005; and Planckaert, 2005). The injection of CO_2 into a mature reservoir causes an increase in the saturation and relative permeability of oil which allows oil to flow more freely (Planckaert, 2005). The injected CO_2 dissolves in oil making it less viscous and allows trapped oil in the injected well to flow into a production well (Planckaert, 2005). This technique helps to recover additional OOIP (Figure 1-8; Karl and Trenberth, 2003). Produced CO_2 is re-compressed and re-injected. Because only a fraction of originally injected CO_2 is recovered, the process requires a constant input of liquid "make-up" CO_2, e.g. as produced in a coal-fired power plant. CO_2-EOR is practiced in Alberta and Saskatchewan. An example is the Weyburn field which is located ~ 130 km southeast of Regina, Saskatchewan (Emberley et al. 2004). In Figure 1-8, liquid CO_2 is injected into a well site, which helps to mobilize the residual oil so that it can be extracted. Oil and CO_2, as well as produced water, are separated above ground.

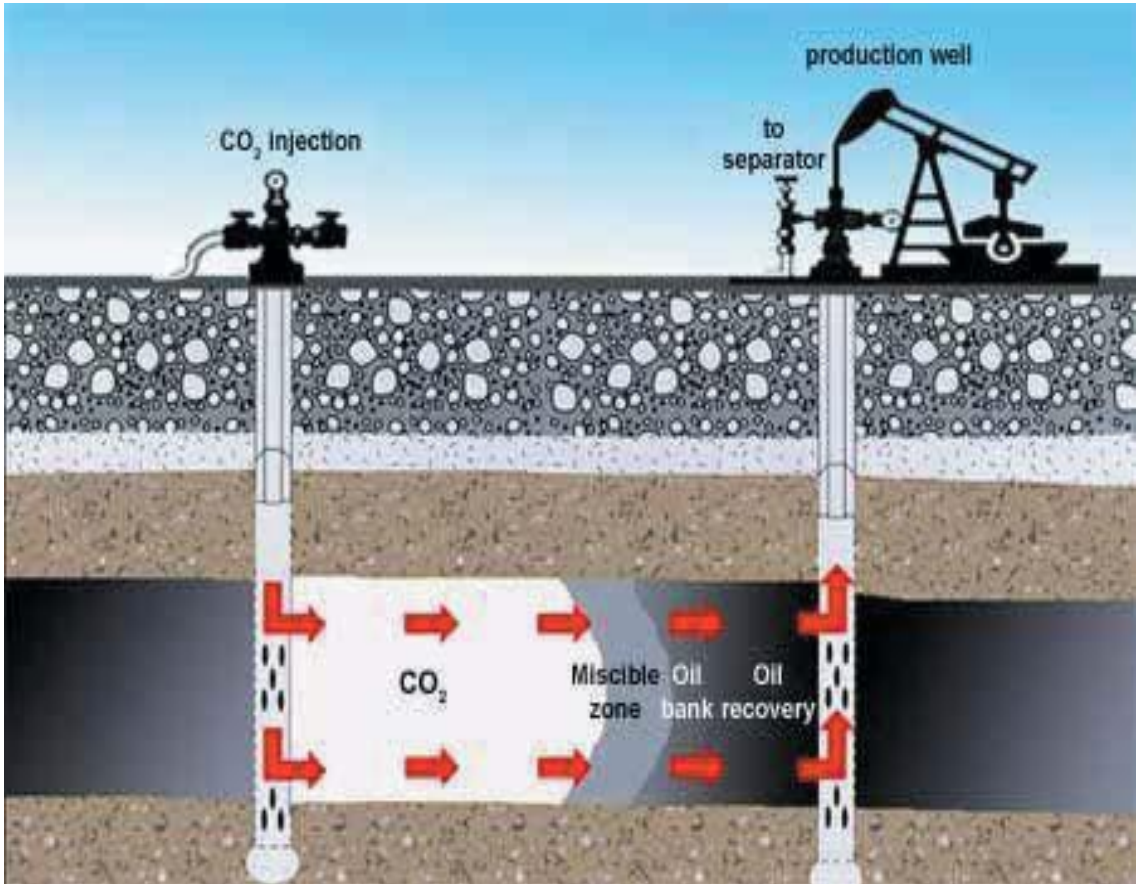


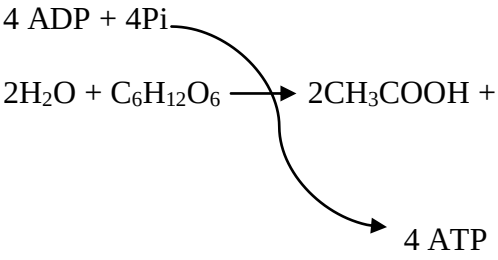
Figure 1-8: Injection of CO_2 for EOR. Injected CO_2 reduces the viscosity of oil in the reservoir and induces the oil to flow freely to the production well. Make-up CO_2 is needed for re-injection as not all the initial CO_2 that was injected is produced. Source: http://www.wri.org/publication/content/8355.

1.2.3 **Microorganisms in the subsurface**

Microorganisms have been found in all biospheres of the earth. This includes deep subsurface environments such as continental sedimentary rocks, igneous rock aquifers, as well as in fluid inclusions in ancient salt deposits from salt mines (Pedersen, 2000). Research in subsurface microbiology has increased our understanding of microbial activity in these locations. Microbial life plays a significant role in the ecology of the subsurface (Pedersen, 2000). In the paper by Basso et al., (2009) molecular techniques were used to identify microbial populations from different subsurface locations. Microorganisms belonging to the phyla *Firmicutes* and *Bacteroidetes*; and the class *Epsilonproteobacteria*, *Deltaproteobacteria*, and *Gammaproteobacteria* were identified in samples obtained from different subsurface locations (Basso et al., 2009). Petroleum reservoirs also contain anaerobic microorganisms which are responsible for biodegradation of oil (Grabowski et al., 2005). Experiments involving DNA isolation from samples obtained from both low and high temperature reservoirs have revealed the presence of anaerobic sulphate-reducing, fermentative, iron reducing and acetogenic *Bacteria*, as well as methanogenic *Archaea* (Grabowski et al., 2005). In the process of CCS, subsurface locations will contain high amounts of CO_2. This means the growth of microorganisms that use CO_2 in their energy metabolism might be favoured. These microorganisms include the acetogenic *Bacteria* (Braun and Gottschalk, 1981) and methanogenic *Archaea* (Basso et al., 2009).

1.2.3.1 Homoacetogenic *Bacteria*

An important product of the anaerobic degradation of organic material is acetic acid (Braun and Gottschalk, 1981). Homoacetogenic *Bacteria* convert one molecule of glucose to 3 acetic acid molecules. This involves formation of acetic acid from hydrogen and carbon dioxide (Diekert & Wohlfarth, 1994).



$\Delta G'^{\circ} = -216$ kJ **equation 1**

$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$ $\Delta G'^{\circ} = -95$kJ **equation 2**

Overall equation: $C_6H_{12}O_6 \rightarrow 3CH_3COOH$ $\Delta G'^{\circ} = -311$kJ **equation 3**

Acetic acid can also be produced from alcohols, other organic acids, amino acids and purines (Fuchs, 1986; Braun and Gottschalk, 1981). Formation of acetic acid from hydrogen and carbon dioxide as energy source is shown in equation 2 (Braun and Gottschalk, 1981). A net gain in ATP accompanies the reduction of CO_2 to acetic acid (equation 3; Braun and Gottschalk, 1981; Fuchs, 1986). Microorganisms in this group are mostly Gram positive *Bacteria* (Fuchs, 1986). Examples include *Clostridium aceticum* and *Acetobacterium woodii* (Heise et al., 1993).

Because homoacetogens use hydrogen for the production of acetic acid, they are important consumers of hydrogen in the subsurface (Conrad et al., 1989). In enrichment cultures of soil and sediment samples where hydrogen is the energy substrate and CO_2 is

the carbon source, mostly acetogenic activity was observed (Conrad et al., 1989). This provides evidence of the biogeochemical importance of acetogens in anaerobic systems.

1.2.3.2 Methanogenic *Archaea*

Methanogens are strictly anaerobic *Archaea* that produce methane as a product of their energy metabolism (Peters and Conrad, 1995; Brauer et al., 2004). Substrates which can be used for the formation of methane include H_2/CO_2, formate, methanol, methylamines, methyl sulphides, and carbon monoxide (Brauer et al., 2004; Braun and Gottschalk, 1981). In subsurface environments, acetate and H_2/CO_2 are the most important precursors in the formation of methane (Brauer et al., 2004). Hydrogenotrophic methanogens use H_2 and CO_2 to produce methane (equation 4) while acetotrophic methanogens use acetic acid (equation 5) a product of anaerobic metabolism (e.g. by homoacetogens; equation 2) to produce methane.

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

$$CH_3\text{-}COOH \rightarrow CH_4 + CO_2 \qquad \Delta G'^\circ = -37\ kJ \qquad \textbf{equation 5}$$

Methane production is one of the most important steps in the anaerobic degradation system, primarily because the formation of methane is irreversible under anaerobic conditions. Production of methane helps to break down the acidic organic compounds produced by fermentative bacteria. Acetate is a very important intermediate used by methanogens to produce methane (Conrad et al., 1986). Some methanogens however, prefer neutral pH conditions and are known as acidophilic methanogens (Takoni et al., 2008). These methanogens are therefore not capable of growth in

environments which are poorly buffered against acidification (Takoni et al., 2008). Other physicochemical conditions in a given environment such as temperature, concentration of H_2 and CO_2, formate and acetate aid the dominance of methanogens (Leybo et al., 2006).

The stable storage of carbon dioxide with microorganisms hopes to turn stored CO_2 into other products. The conversion of CO_2 to acetic acid is more favourable for the process of CCS because two molecules of CO_2 are required for acetogenesis to occur (equation 2). The possibility of increasing methane production from sequestered CO_2 stored in subsurface locations such as coal beds and oil reservoirs is currently being explored (Fujiwara et al, 2006; Sugai et al., 2008; Sugai et al., 2010). However, the production of methane from H_2/CO_2 incorporates just one molecule of CO_2 (equation 4), the conversion of acetic acid to methane also produces CO_2 which is not good for CCS (equation 5).

Chapter 2 OBJECTIVES

A diverse array of research is currently being done to improve the process of capturing CO_2 and ensure the stability of permanently stored CO_2 in the subsurface. The overall stability of the process has been a subject of great debate and this problem is being tackled by experts in areas of science such as chemistry, engineering and geology. However overlooking the impact microorganisms will have on subsurface storage of CO_2 will not provide us with a well-informed understanding of CCS. The microbial communities present in subsurface locations such as depleted oil fields are capable of enabling the capture of CO_2 under anaerobic conditions.

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \qquad \Delta G'^\circ = -95kJ$$

$$2H_2 + CO_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G'^\circ = -131\ kJ$$

$$CH_3\text{-}COOH \rightarrow CH_4 + CO_2 \qquad \Delta G'^\circ = -37\ kJ$$

In the MHGC field, the SW is chemically treated to reduce viable microbial counts in the field. The chemical treatment may produce carbon nutrients suitable for microbial metabolism. The produced water obtained from producing wells, is not being treated to reduce microbial counts. Microbial growth may be boosted in the field since only the SW is treated but not the PW. It would be interesting to determine the source of microbial population present in an oil field (using the MHGC as a model) and their impact on microbial activity in relation to CCS.

In view of this, the overall objectives of this project are:

1. **Determine the microbial community composition of PW samples obtained from a subsurface oil reservoir.**

It is important have a full understanding of the different types of CO_2 utilizing microorganisms such as acetogens and methanogens, which may be present in subsurface locations like oil fields. This will enable us to determine whether microbes that may impact CCS are present. Community analysis using 454 sequencing targeting 16S rRNA genes can be used for this purpose.

2. **Determine the activity of methanogens and acetogens in samples of oil field produced water.**

Because acetogens and methanogens are potentially important for CCS, their activity must be determined by incubations with H_2 and CO_2 and monitoring the production of acetic acid and methane.

3. **Determine conditions that affect and/or favour the growth of methanogens versus acetogens in enrichments with CO_2 and H_2.**

Production of high concentrations of acetic acid by acetogens may cause a reduction in the pH of the medium. Therefore the effect of pH on acetogenesis and methanogenesis must be determined. Secondly, the percentage of hydrogen in the headspace may favour either acetogenesis or methanogenesis. The effect of different mixing ratios of H_2 and CO_2 in the headspace must therefore, also be investigated.

4. **Determine if the mixing of SW and PW creates favorable conditions for microbial growth.**

In section 1.2.2., I stated that the combination of SW and PW to create IW may create favorable conditions for microbial growth in an oil reservoir. If SW contains carbon and other nutrients an increase in the amount of viable microorganisms present may result following mixing of SW and PW.

5. **Determine if the microbial community present in SW is similar or different from that found in PW.**

This will help us understand if microbes are introduced into the field from the SW or if they are actually indigenous to the oil reservoir. This objective has been addressed by others (Magot, 1996 and 2005). However, new sequencing methods like pyrosequencing have not yet been used to obtain a more detailed answer to this question. The microbial community present in SW and PW samples will also be compared with those of IW and WP during this investigation.

Chapter 3 Materials and Methods

3.1 Obtain sample from potential CCS site

Produced water (PW) containing 5-10% heavy oil (API 16°) from site 3-PW in the MHGC field was obtained in October, 2010 and September, 2011. Samples were collected in sterile1-L Nalgene bottles, filled to the brim to exclude air as much as possible and were received in the lab within 5 hours of collection. The bottles were transferred to an anaerobic hood containing 10% CO_2 and 90% N_2 for storage.

3.2 Enrichment media preparations

Three types of media were used to evaluate microbial activity. Medium X is a defined medium with 0.7g of NaCl/L. It was prepared as stated below:

1. To a 2-L Widdel flask, 1 L of deionized water was added followed by 0.7 g of NaCl, 0.14 g of KH_2PO_4, 0.14 g of $MgCl_2.6H_2O$, 0.14 g of $CaCl_2.2H_2O$, 0.7 g of NH_4Cl and 2-3 drops resazurin.
2. The flask was autoclaved together with medium, serum bottles and rubber stoppers.
3. Once the medium had cooled down while equilibrating with N_2/CO_2 (90:10) at the gas station on a lab bench, the remaining ingredients: 2 mL trace element solution, 1 mL selenite tungstate, 30 mL 1 M $NaHCO_3$ and 1 mM 1 M $Na_2S.9H_2O$ were added.
4. The pH was adjusted to 7.0.
5. 45 mL medium was distributed into autoclaved serum bottles which were sealed with autoclaved stoppers and crimp sealed.

X5 is a defined medium with 5 g NaCl per litre of the medium and was prepared like the X medium. Medium A is a rich medium with 2 g of yeast extract made according to a recipe from DSMZ catalogued as *Acetobacterium* medium 135. It was prepared as stated below:

1. To a 2-L flask, 1 L deionized water was added followed by 1.0 g of NH_4Cl, 0.33 g of KH_2PO_4, 0.45 g of K_2HPO_4, 0.10 g of $MgSO_4.7\ H_2O$, 20 mL of trace element solution, 20 mL vitamin solution, 2 g of yeast extract and 2-3 drops of resazurin.
2. Added a magnetic stir bar into the flask and placed it on the stirrer to stir while heating.
3. Placed the flask in an ice bucket to cool down while equilibrating with N_2/CO_2 (90:10) at the gas station on a lab bench.
4. The remaining ingredients: 30 mL 1M $NaHCO_3$, 3 mL 1 M cysteine, and 1 mL 1 M Na_2S x 9 H_2O were added to the medium.
5. The pH was adjusted to 7.4 by adding HCl and 45 mL of medium was distributed into serum bottles, capped with a rubber stopper and autoclaved.
6. The headspace was changed to H_2/CO_2 (80:20) by flushing with the gas.

The chemical compositions of the trace element and Balch vitamin solutions are shown in Tables B-1 and B-2 of Appendix B.

3.3 Enrichment experiments

3.3.1 Experiments with enrichment media X, X5 and A

The activity of methanogens and acetogens in media – X, X5 and A, was done with 3-PW obtained in October, 2010. This enabled us to study the activity of the microorganisms under different conditions. Triplicate volumes of 45 mL of each media and 5 mL of 3-PW were distributed into 150 mL serum bottles. The starting pH was 7.4 and gas phase was H_2/CO_2 (80:20). Produced water (5 mL) or a culture of *Acetobacterium woodii* (DSMZ 1030) was added. Bromoethane sulfonic acid (BESA) was added at a concentration of 20 mM in some cases. Rifampicin (10 mg/mL) was also added in some cases. BESA and rifampicin inhibit growth of methanogenic archaea and of bacteria respectively (Bleicher and Winter, 1994; Brauer et al., 2004). Some of the enrichments were amended with acetate (20 mM) and a headspace with N_2/CO_2 (90:10) to monitor methane formation from acetate (Beckmann et al., 2011). Incubations were done at 30°C with gentle agitation to keep the enrichment homogenous.

3.3.2 Experiments with different pH and H_2 concentrations.

Enrichments were done with a different headspace $H_2/CO_2/N_2$ (5:10:85) and/or a pH 5.5 (by amending pH of media with acetate) to test the effect of headspace H_2 concentration or the pH on activity of acetogens and methanogens. These experiments were done with 3-PW obtained in September, 2011 and prepared as above. The microbial community composition analyses of this sample were also determined.

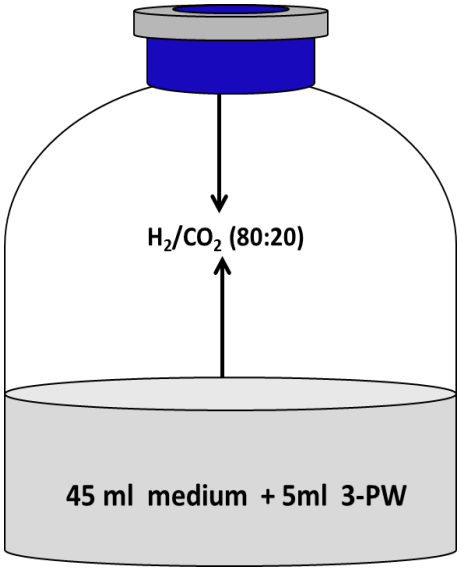


Figure 3-1: A sample enrichment bottle. Headspace is filled with H_2/CO_2 (80:20). A volume of 45 mL of any of the three media X, X5 or A is distributed into 120 mL or 159 mL serum bottles. A volume of 5 mL of the 3-PW sample is inoculated into the bottle.

3.4 Methane and acetic acid measurements

The concentration of methane was measured with a Hewlett Packard Model 5890 gas chromatograph (GC) equipped with a thermal conductivity detector using helium as the carrier gas and a packed stainless steel column, Poropak R, 80/100 (0.0049 cm by 5.49 m). Injector and detector temperatures were 150°C and 200°C, respectively. Sterile 1.0 mL syringes were used to inject 0.2 mL of headspace gas of samples or of standards into the GC. A standard line is shown in Table 3-1 and Figure 3-2. Standard bottles containing methane and nitrogen gas at different concentrations were prepared as follows.

1. Closed 159 mL sterile serum bottles were flushed with 100% N_2 or with 100% CH_4.

2. Methane (100 %, x mL) was then added to bottles containing 100% N_2.
3. The percentage of methane was calculated as 100 *[x/(x + 159)], where x = volume of CH_4 in each bottle. For example, a standard bottle containing 20% methane was made by injecting: [20/100] = [x mL/(x + 159 mL) = 39.75 mL of 100% methane.

Gaseous concentrations of methane in mM or µM were calculated by considering that 1 L of gas is 24.52 L/mol at 22°C. This gives the relation between the mixing ratio (% CH_4) and the concentration (µmol/L) as indicated in Table 3-1 and Figure 3-2.

Table 3-1: The peak areas obtained for different methane concentrations. The plot of the peak area versus concentration is used to extrapolate the concentration of methane in the headspace of enrichment bottles.

Mixing ratio (% CH_4)	Gas concentration (µmol/L)	Peak area
0.13	51	446030
0.22	90	755050
0.50	204	2007600
1.12	456	5390300
2.09	853	8853000
5.02	2045	22357000
10.17	4144	44450000

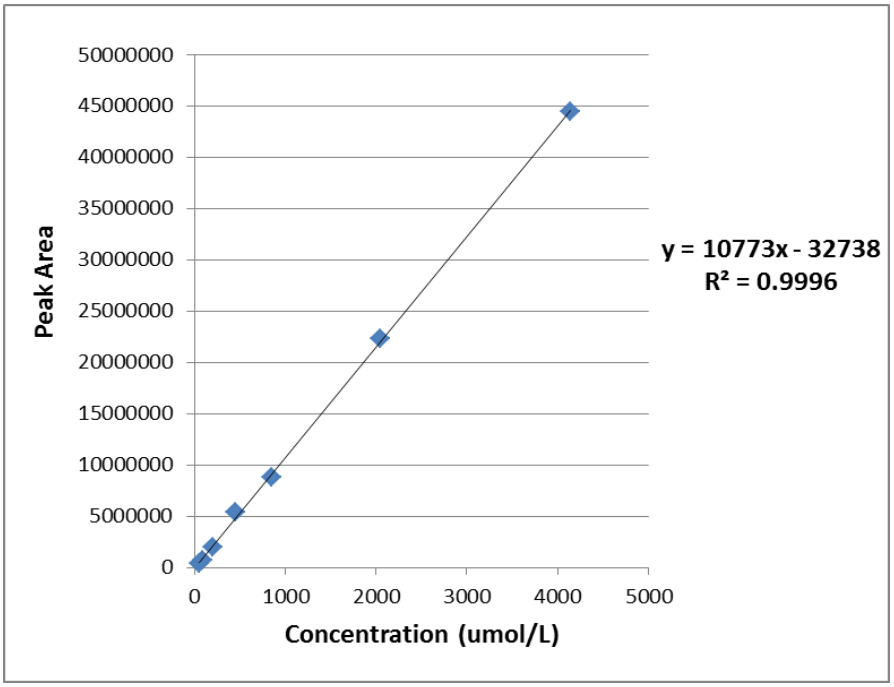


Figure 3-2: A sample methane standard line. The equation of the line is used to calculate the concentration of methane formed in the headspace of serum bottles.

The concentration of acetic acid was measured as acetate with a high pressure liquid chromatograph (HPLC). The instrument was equipped with a Waters 600E system controller and a Waters 2487 UV detector at 201 nm, using a Prevail Organic Acids 5µ column (250 by 4.6 mm; Alltech) with a mobile phase of 85 % (vol/vol) of 25 mM KH_2PO_4 (pH 2.5) and 15% (vol/vol) acetonitrile at 2.0 mL/min. A volume of 1.0 mL was obtained from an enrichment bottle with the aid of a sterile syringe and transferred into sterile 2.0 mL microcentrifuge tubes. The syringe was flushed with N_2/CO_2 (90:10) gas before it was used and 1 mL of anaerobic gas was injected to obtain a sample to avoid introducing oxygen into enrichments. Samples were then centrifuged at 13,300 x g for 30 minutes to separate solid particles from the liquid. The supernatant was transferred to

another sterile micro centrifuge tube and the pH and acetic acid concentration were measured. The pellet was used later for DNA extraction. Acetate standards were prepared from dilutions of 1 M sodium acetate. A volume of 300 µL of the standard or supernatant was transferred into HPLC tubes along with 20 µL of KH_2PO_4 buffer. The HPLC run for each sample was set for 40 minutes to allow it to detect other compounds such as propionate and butyrate (Grigoryan et al., 2008). The peak areas for different concentrations of acetate were used to generate a standard line (Table 3-2). The concentration of acetate in the samples was calculated from the equation of the standard line (Figure 3-3).

Table 3-2: The peak areas obtained from different acetate concentrations. The plot of the peak area versus concentration was used to extrapolate the concentration of acetate formed in enrichment bottles.

Acetate (mM)	Peak area
0.5	3430
1	7713
2	16309
5	34323
10	65089
20	151988
50	370003
60	437573
70	526384
80	597190
90	681453
100	780239

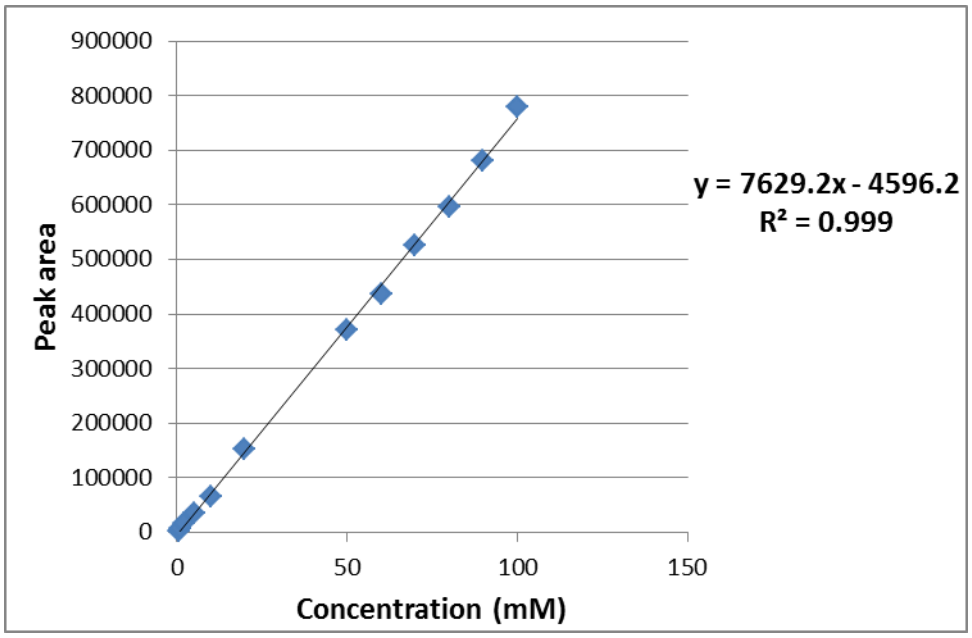


Figure 3-3: Sample acetate standard line. The equation of the line is used to calculate the concentration of acetic acid formed in enrichments.

3.5 Calculation of gas replenishment

Headspace gas was replenished periodically in most enrichment bottles by inserting a sterile 30-mL syringe containing the headspace gas into the serum bottle. The volume of gas (mL) taken up by the bottle from the syringe was then determined. The accumulated volume of gas for each replacement day was calculated and graphed and compared to the production of methane and acetate. A sample calculation for H_2 and CO_2 consumption by an enrichment is shown in below:

Total volume of gas used = 421 mL and headspace gas is H_2/CO_2 (80:20)

We can then say that: 0.8 * 421 mL = 337 mL H_2 and 0.2 * 421 mL = 84 mL CO_2 were used. We know that 1 L of gas at 30°C = 25.1 mL/mmol (30°C was the incubation temperature), therefore the total amount of H_2 and CO_2 used = 337/25.1 = 13.43 mmol.

The equation of methane and acetic acid formations shows that: $4H_2 + CO_2 \rightarrow CH_4 + 2H_2O$ or $4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$. That is, 80% H_2 with 20% CO_2 can be used to form CH_4 or 40% H_2 with 20% CO_2 can be acetic acid. Hence, we expect a total amount of 3.36 mmol CH_4 or 1.68 mmol CH_3COOH in the serum bottle (if either of these are the only product). The maximum concentration of methane or acetic acid would therefore be 48 mM or 24 mM, not correcting for the volume withdrawn during sampling. The expected concentrations of methane or acetic acid at different sampling time during the enrichment period are show in Tables 3-3 and 3-4.

Table 3-3: Expected concentrations of methane in enrichments with H_2/CO_2 (80:20) inoculation with 3-PW for the different sampling dates. Produced water sample was obtained in September, 2011.

Time (days)	8	9	10	12	18	23	25	31	37	40
Total volume of gas consumed (mL)	62	89	119	209	260	305	331	361	391	421
Volume of H_2 used (mL)	49	71	95	167	208	244	265	289	313	337
Methane concentration (mM)	7.0	10.1	13.5	23.8	29.6	34.7	37.6	41.1	44.5	47.9

Table 3-4: Expected concentrations of acetic acid in enrichments with H_2/CO_2 (80:20) inoculation with 3-PW for the different sampling dates. Produced water sample was obtained in September, 2011.

Time (days)	8	9	10	12	18	23	25	31	37	40
Total volume of gas consumed (mL)	62	89	119	209	260	305	331	361	391	421
Volume of H_2 used (mL)	25	36	48	84	104	122	132	144	156	168
Acetic acid concentration (mM)	3.5	5.0	6.8	11.9	14.8	17.4	18.8	20.6	22.3	24.0

3.6 Experiments with make-up water

3.6.1 Sample description and collection

Nine sites in the MHGC field were sampled at different dates. SW samples obtained were untreated SW (22-SW-U), chlorinated and filtered SW (22-SW-F) and ammonium bisulphite treated SW (22-SW-T). Initially, 22-SW-U and 22-SW-T samples were obtained from January to May 2010. After a review of the treatment that occurs in the field, all three SW were then sampled in September 2011, October 2011, December 2011 and January 2012. Pictures of the SW site and collection points are shown in Appendix A (Figure A-2, A-3, A-4, A-5 and A-6). Samples were also obtained from the central water plant 1-WP and injection water 14-IW received in August 2011, as well as from production wells 2-PW, 10-PW, 12-PW and 13-PW received in September and October 2011 (Voordouw et al., 2009). The PW samples contained 5-10% heavy oil (API gravity 16°). Samples were collected in sterile 1-L Nalgene bottles, filled to the brim to exclude air as much as possible and were received in the lab within 5 hours of collection. The

bottles were transferred to an anaerobic hood containing 10% CO_2 and 90% N_2 for storage.

3.6.2 Medium preparation

Viable anaerobic heterotrophs were enumerated on tryptone-yeast agar. Tryptone-yeast extract (TY) agar is a rich medium used to enumerate aerobic and anaerobic heterotrophs. TY agar plates were prepared as follows:

1. In a 2 L Erlenmeyer flask add 10 g of Bacto-tryptone, 5 g of yeast extract and 8 g of NaCl to 1 L of deionized water.
2. Stir for 20 minutes to dissolve.
3. Adjust the pH to 7.4, with a few drops of 2 M NaOH
4. Add 15 g of Bacto-agar and autoclave
5. Pour TY-agar into sterile petri dishes (plates) and allow to solidify. Dry overnight at a temperature of 30°C.
6. The next day, the plates are transferred to the anaerobic hood with an atmosphere of 90% N_2 and 10% CO_2.
7. Plates were stored in the anaerobic hood at least three days before they are used.

3.6.3 Anaerobic and aerobic counts of PW and SW with nutrients addition.

A mixture containing 40 uL of 100 mM KH_2PO_4 and of 100 mM NH_4Cl solution were added to a mixture of 3 mL 2-PW and 1 ml of 22-SW-U or 22-SW-T. A control experiment was performed by replacing SW with sterile deionized water. Ten-fold dilutions from 10^1 to 10^6 were made by addition of 100 µL to 900 µL of liquid TY

medium. 100 µL of each dilution were transferred to anaerobic TY agar plates. The samples were evenly distributed with a sterile glass spreader on the surface of the agar plates until they were completely absorbed in the agar. Plates were incubated at 23°C for 3 days in the anaerobic hood. Aerobic incubation at 23°C in the dark was also done. The number of colony-forming units per mL (CFU/mL) was calculated at different times over a period of 5 days.

3.6.4 Anaerobic and aerobic counts of water samples

Samples 22-SW-U and 22-SW-T were serially diluted (10^0 to 10^4) and 100 µL of each dilution were spread on anaerobic TY agar plates. Plates were incubated anaerobically and aerobically as in section 3.6.3. The CFU/mL was monitored at different times over a period of 10 days.

3.6.5 Anaerobic counts of water samples without nutrients addition

Injection water was mimicked by mixing 10 mL of one of the SW samples with 30 mL of 2-PW without nutrients addition. A control experiment was performed by replacing SW with sterile deionized water. Dilutions from 10^1 to 10^4 were made and 100 µL of these were plated and incubated at 23°C for 3 days. CFU/mL was monitored at various times over a period of 10 days.

A plate counter (Darkfield QUEBEC® Colony Counter) was used to count the number of CFU/ml for all experiments involving microbial counts. Data reported are averages for two to three dilutions as a function of time. Statistical analysis of the standard error was performed for averages obtained.

3.7 454 sequencing of 16S rRNA genes (pyrosequencing)

3.7.1 DNA extraction, PCR amplification and quantification

DNA was extracted from 3-PW obtained in October, 2010 and enrichments with H_2/CO_2 (80:20) headspace in experiments with X, X5 and A media (section 3.3.1). DNA was also extracted five primary and five secondary enrichments from experiments with different pH and H_2 (section 3.3.2) and for 11 field samples used in experiments with SW (section 3.6.1). Because the same procedure was used in all cases, a detailed description is given for DNA extraction from 40 mL of water samples. DNA from the water samples was extracted with the Fast DNA® Spin Kit using the FastPrep® Instrument (MP Biomedicals, Santa Ana, CA), as follows:

1. 40 mL of the water sample were centrifuged in an IEC Centra GP 8R centrifuge at 10,000 x g for 12 min.
2. The pellet was transferred into a Lysing Matrix A tube, 1.0 of Cell Lysis solution was added.
3. The contents of the Matrix A tube were then homogenized in the FastPrep® Instrument for 40 seconds at a speed setting of 6.0.
4. The Matrix A tube was placed in ice to cool and then centrifuged in a Fisher Scientific Accuspin Micro 17 centrifuge for 5-10 min at 13,000 x g.
5. 800 µL of the supernatant was discarded and was replaced by 800 µL of Binding Matrix. The tube was closed and inverted to mix its contents.
6. The microcentrifuge tube was incubated with gentle agitation for 5 min 23°C on a rotating wheel.

7. It was then centrifuged at 13,000 x g for 1 minute to pellet the Binding Matrix, supernatant was discarded.
8. 500 µL of already prepared SEWS-M (containing ethanol) was added to the microcentrifuge. The pellet was resuspended using the force of the liquid with a pipette tip.
9. The solution was centrifuged for 1 minute at 13,000 x g. The supernatant was discarded.
10. It was then centrifuged again for 1 minute at 13,000 x g to remove residual liquid.
11. DNA was eluted by gently resuspending the Binding Matrix in 100 µL of DES and incubating in a water bath at 55°C for 5 minutes.
12. The Binding Matrix was centrifuged at 13,000 x g for 1 minute. Eluted DNA was transferred to a clean microcentrifuge tube.

The first step in the DNA isolation was not done for enrichment sample because pellets for step 2 were obtained by centrifuging 1 mL of enrichment with a Fisher Scientific Accuspin Micro 17 centrifuge for 5 min at 13,000 x g.

Isolated DNA was subjected to PCR amplification prior to pyrosequencing. Pyrosequencing of 16S rRNA amplicons was done for all samples. PCR amplification was for 25 cycles with 16S primers 926Fw and 1392R, followed by 10 cycles with FLX titanium primers 454T_RA_X and 454T_FwB (Park et al., 2011) in a PCR machine. The forward primer 454T_FwB is bar-coded. Purified 16S amplicons (~125 ng) were sequenced at the McGill University and Genome Quebec Innovation Centre, Montreal,

Quebec with a Genome Sequencer FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation) (Agrawal et al., 2012).

It is important to determine the concentration of DNA that is obtained after the DNA isolation and PCR amplification. Amplicon concentrations less than 20 ng/µL usually produce poor results during the process of sequencing the 16S rRNA genes of the samples. The concentration of DNA was determined using the Qubit fluorimeter (Invitrogen). The protocol is listed below:

1. A working solution was made by diluting the Quant-iT™ reagent with the Quant-iT™ buffer in a ratio of 1:200.
2. Standard assay tubes were prepared by adding 190 µL of the working solution to 10 µL of standards (0 ng and 10 ng) provided in the Invitrogen Quant-iT™ kit.
3. The sample assay tubes were then prepared by adding 198 µL of the working solution to 2 µL of our final PCR amplified sample.
4. The standard and sample assay tubes were mixed by vortexing for 3 seconds and incubated for 2 minutes at room temperature. The concentration of DNA in the tubes was read in the fluorimeter and multiplied by 100 to correct for dilution.

3.7.2 Bioinformatics analysis

Data analysis was conducted with Phoenix 2, a 16S rRNA data analysis pipeline (Park et al., 2011). High quality sequences, which passed the quality control and chimeric sequence removal stages of the pipeline, were clustered into Operational Taxonomic Units (OTUs) at 5% distance by using the average linkage algorithm (Schloss and

Westcott, 2011). The sequences were then grouped into OTUs, rarefaction curves and additional alpha diversity indices including the estimated number of OTUs with Chao and Shannon's H index using the Mothur software package (Schloss et al., 2009).

The maximum number of OTU calculated using the Mothur software package and takes into account the number of singletons and doubletons present in the samples to obtain an estimate of the sample richness (Chao and Shen, 2003). The Chao OTU is calculated from the equation:

$$S_{chao} = S_{obs} + [n_1 (n_1 - 1)/ 2 (n_2 + 1)] \qquad \text{where,}$$

S_{chao} = the estimated richness of the sample; S_{obs} = the observed number of species; n_1 = the number of OTUs with only one sequence (singletons) and n_2 = the number of OTUs with only two sequences (doubletons).

The Shannon's H index of diversity also calculated with the Mothur software package estimates diversity based on abundance (Shannon, 1948). The equation for the Shannon's H index is:

$$H_{shannon} = -\sum_{i=1}^{S_{obs}} \frac{n_i}{N} ln \frac{n_i}{N} \qquad \text{where,}$$

S_{obs} = the number of observed OTUs; n_i = the number of individuals in OTU from hypothetical sample i and N = the total number of individuals in the community. The higher the Shannon's H index value the more diverse the sample is.

Amplicon libraries were clustered into a Newick-formatted tree using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm and visualized using Dendroscope (Huson et al., 2007). The distances between libraries were

calculated with the Bray-Curtis dissimilarity index in Morthur (Schloss et al., 2009). The non-metric Bray-Curtis dissimilarity index measures the beta diversity between the sample and only takes into consideration the evenness but not abundance of the OTUs (Bray and Curtis, 1957). A Bray-Curtis value of 0 will depict that the sample OTUs are very dissimilar while 1 means the OTUs are similar.

The differences between the PWs, SWs, WP and IW were also analyzed by plotting the values obtained from the Bray-Curtis dissimilarity Non-Metric Multidimensional Scaling (NMDS) in Mothur, which is based on the majorization algorithm method (Borg and Groenen, 2010). The NMDS plot helps us to visualize how distinct or similar the samples are from/to each other. A taxonomic consensus of all representative sequences was derived from the most frequently recurring species within 5% of the best bitscore from a BLAST search against the small subunit (SSU) reference data set SILVA102 (Pruesse et al., 2007).

Chapter 4 RESULTS

4.1 Enrichment of produced water in X, X5 and A media

4.1.1 Microbial community compositions of 3-PW

In order to evaluate the potential of microorganisms in oil field produced water to convert CO_2 and H_2 to methane or acetic acid, the community composition for samples obtained from producing well 3-PW received in October, 2010 and September, 2011 from the MHGC field was determined by 454 sequencing of 16S rRNA genes.

The results indicating the distribution of pyrosequencing reads over the most abundant taxa for October, 2010 and September 2011 are shown in Tables 4-1 and 4-2. The top 5 taxa identified at the genus level present in the October, 2010 sample included *Methanoculleus* (66.5%), *Methanosaeta* (22.9%), *Methanobacterium* (1.4%), *Methanocalculus* (1.2%), and *Methanofollis* (0.9%). The top 5 microbial genera present in the sample received in September, 2011 included *Methanoculleus* (58.8%), *Methanosaeta* (9.3%), *Thauera* (5.4%), *Methanolinea* (4.6%) and *Pseudomonas* (4.2%). Microorganisms which produce methane from H_2/CO_2 (*Methanoculleus*, *Methanobacterium*, *Methanocalculus*, *Methanifollis* and *Methanolinea*) or acetate (*Methanosaeta*) were found to be present in 3-PW samples obtained in October, 2010 and September, 2011 (Tables 4-1 and 4-2). Acetogens (e.g. of the genus *Acetobacterium*) were however not detected in either of the 3-PW samples obtained (Tables 4-1 and 4-2).

Table 4-1: Microbial community composition of 3-PW received in October, 2010. Average distribution of reads (%) obtained from 454 sequencing of 16S rRNA genes over taxa are shown.

Number of reads	12787	
Taxon (Phylum; class; order; family; genus)	**Reads**	**% Reads**
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanoculleus	8500	66.5
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosaetaceae_Methanosaeta	2927	22.9
Euryarchaeota_Methanobacteria_Methanobacteriales_Methanomicrobiaceae_Methanobacterium	184	1.4
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanocalculus	150	1.2
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanofollis	120	0.9
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosarcinaceae	105	0.8
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Thauera	92	0.7
Proteobacteria_Alphaproteobacteria_Rhodobacterales_Rhodobacteraceae_Rhodobacter	70	0.5
Proteobacteria_Deltaproteobacteria_Syntrophobacterales_Syntrophaceae_Smithella	53	0.4
Candidate division OP3	52	0.4
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae	49	0.4
Candidate division OP11	45	0.4
Euryarchaeota_Methanomicrobia_Methanomicrobiales _candidatus Methanoregula	34	0.3
Proteobacteria_Alphaproteobacteria_Rhizobiales	24	0.2
Actinobacteria_Actinobacteridae_Micrococcineae_Micrococcaceae	22	0.2
Proteobacteriac_Deltaproteobacteria_Desulfuromonadales_Geobacteraceae_Geobacter	20	0.2
Spirochaetes_Spirochaetes_Spirochaetales_Spirochaetaceae	20	0.2
Proteobacteria_Deltaproteobacteria_Syntrophobacterales_Syntrophaceae_Syntrophus	14	0.1
Thermotogae_Thermotogae_Thermotogales_Thermotogaceae_Kosmotoga	14	0.1
Euryarchaeota_Thermoplasmata_Thermoplasmatales	13	0.1
Proteobacteria_Alphaproteobacteria_Rhodospirillales	11	0.1
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Diaphorobacter	10	0.1
Euryarchaeota_Thermoplasmata_Thermoplasmatales	8	0.1
Chloroflexi_Anaerolineae_Anaerolineales_Anaerolineaceae	8	0.1
Proteobacteria_Deltaproteobacteria_Desulfuromonadales_Desulfuromonadaceae	8	0.1
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanospirillaceae_Methanospirillum	7	0.1
Synergistetes_Synergistia_Synergistales_Synergistaceae_Thermanaerovibrio	7	0.1
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae_Acetobacterium	0	0

Table 4-2: Microbial community composition of 3-PW received in September, 2011. Average distribution of reads (%) obtained from 454 sequencing of 16S rRNA genes over taxa are shown.

Number of reads	1120	
Taxon (Phylum; class; order; family; genus)	**Reads**	**% Reads**
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanoculleus	658	58.8
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosaetaceae_Methanosaeta	104	9.3
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Thauera	60	5.4
Euryarchaeota_Methanomicrobia_Methanomicrobiales_ Incertae Sedis_Methanolinea	51	4.6
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Pseudomonadaceae_Pseudomonas	47	4.2
Firmicutes_Clostridia_Clostridiales_Peptococcaceae	39	3.5
Candidate division OP3	33	2.9
Spirochaetes_Spirochaetes_Spirochaetales_Spirochaetaceae	13	1.2
Proteobacteria_Deltaproteobacteria_Syntrophobacterales_Syntrophaceae_Smithella	9	0.8
Candidate division OP11	9	0.8
Proteobacteria_Alphaproteobacteria_Rhodobacterales_Rhodobacteraceae_Pannonibacter	8	0.7
Proteobacteria_Deltaproteobacteria_Syntrophobacterales_Syntrophaceae_Syntrophus	7	0.6
Bacteroidetes_Bacteroidia_Bacteroidales_Bacteroidaceae_Bacteroides	7	0.6
Chloroflexi_Anaerolineae_Anaerolineales_Anaerolineaceae	5	0.4
Thermotogae_Thermotogae_Thermotogales_Thermotogaceae_Kosmotoga	4	0.4
Euryarchaeota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_Methanobacterium	4	0.4
Firmicutes_Clostridia_Thermoanaerobacterales_Thermoanaerobacteraceae_Gelria	4	0.4
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Parabacteroides	3	0.3
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanofollis	2	0.2
Euryarchaeota_Methanomicrobia_Methanomicrobiales_ Incertae Sedis_Methanocalculus	2	0.2
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosarcinaceae_Methanolobus	2	0.2
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosaetaceae_Methanosaeta	2	0.2
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Proteiniphilum	1	0.1
Candidate division OD1	1	0.1
Synergistetes_Synergistia_Synergistales_Synergistaceae_Aminiphilus	1	0.1
Proteobacteria_Deltaproteobacteria_Syntrophobacterales_Syntrophobacteraceae_Syntrophobacter	1	0.1
Proteobacteria_Deltaproteobacteria_Desulfuromonadales_Desulfuromonadaceae_Desulfuromusa	1	0.1
Chloroflexi_Anaerolineae_Anaerolineales_Anaerolineaceae_Leptolinea	1	0.1
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae_Acetobacterium	0	0

4.1.2 Acetogenic activity of *Acetobacterium woodii*

Acetobacterium woodii (DSMZ 1030) was used as a positive control to test the growth of acetogens in defined X medium X and amended with H_2/CO_2 (80:20). Acetate was observed within 2 days after inoculation of *A. woodii* in this medium and reached a concentration of 44 mM after 48 days of incubation (Figure 4-1). Methane production was not observed in enrichment bottles with *A. woodii*, in agreement with the fact that it is a pure culture lacking methanogens. There was also no acetate or methane produced in a negative control without the addition of *A. woodii*. The X medium is therefore able to support the growth of acetogenic *Bacteria* that may be active in 3-PW.

4.1.3 Primary enrichments of 3-PW in medium X

Medium X contains 0.7 g/L of NaCl which is much lower than that of produced water. Acetic acid production was observed in X medium enrichments in the presence of the methanogenesis inhibitor BESA (Figure 4-2A and B). After 20 days of incubation, the acetate concentration in enrichment bottles without BESA (Figure 4-2A) was 32 mM and stayed constant up to 56 days of incubation. In enrichments with H_2/CO_2 (80:20) + BESA the acetate concentration was 31 mM after 20 days of incubation but then decreased to 22 mM at 56 days of incubation (Figure 4-2B). This was because one of the experimental replicates showed a decrease in the concentrations of acetate (standard error bars Figure 4-2B). The decrease in acetate concentration could possibly be due to the formation of other volatile fatty acids such as propionate or butyrate from acetate. These products were tested but were not detected in the experiments with either H_2/CO_2 (80:20) only or

H_2/CO_2 (80:20) with addition of BESA (data not shown). The methane concentration in enrichment bottles with H_2/CO_2 (80:20) was 27 mM at the end of the 56 day incubation period (Figure 4-2A). The negative control without the addition of 3-PW for both experiments (Figure 4-2) did not produce acetate or methane. Therefore, methanogenic *Archaea* and acetogenic *Bacteria* present in 3-PW were capable of growing in X medium.

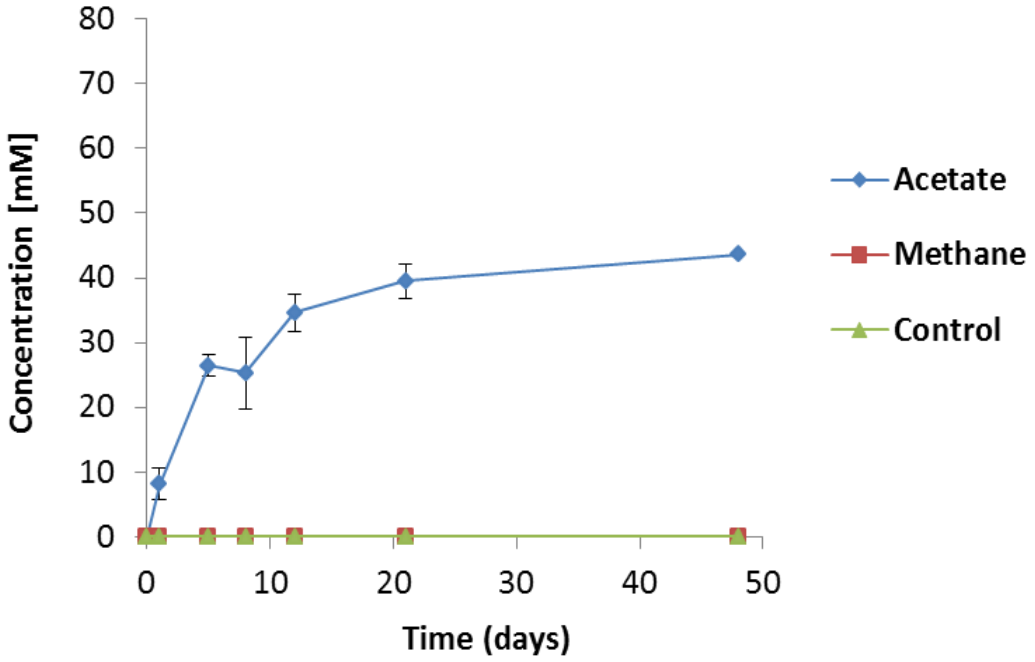


Figure 4-1: Acetate concentration produced by pure culture of *Acetobacterium woodii* (DSMZ 1030) in X medium (0.7 g/L NaCl) with headspace gas H_2/CO_2 (80:20). The negative control experiment did not contain *A. woodii*. The data shown are averages of 2 replicates; standard error bars are shown where these exceeded the size of the symbols.

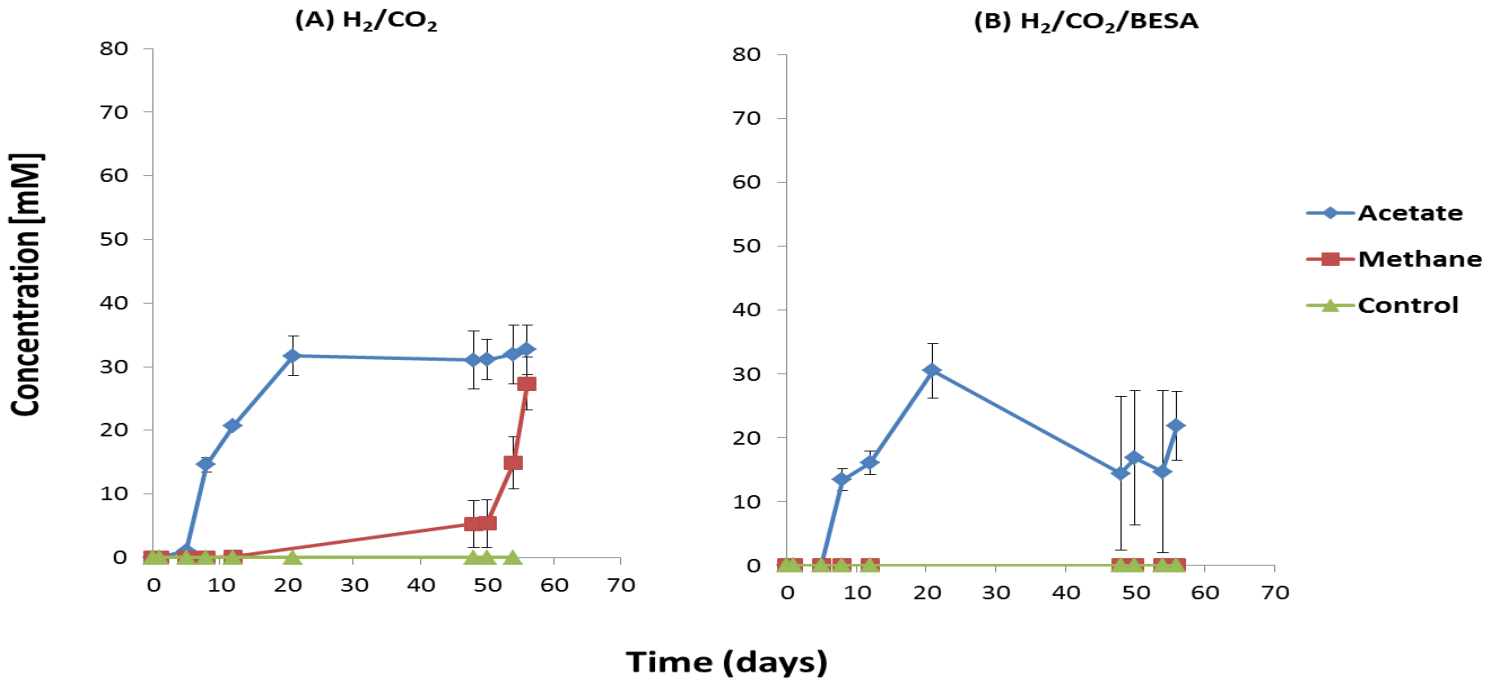


Figure 4-2: Acetate and methane concentration produced by primary enrichments of 3-PW in X medium (0.7 g/L NaCl) during 56 days of incubation with (A) H_2/CO_2 (80:20) and (B) H_2/CO_2 (80:20) with 20mM BESA added. The 3-PW sample obtained in October, 2010 was used as inoculum. The negative control experiments were done without the addition of 3-PW. The data shown are averages of 2 replicates; standard error bars are shown where these exceeded the size of the symbols.

4.1.4 Primary enrichments of 3-PW in X5 medium

Four enrichments were done with X5 medium which had a salt concentration (5 g/L NaCl) similar to that of produced water (Figure 4-3 and 4-4). Enrichments with a headspace of H_2/CO_2 (80:20) produced an acetate concentration of 48 mM and a methane concentration of 5.5 mM after 56 days of incubation (Figure 4-3A). After 56 days of incubation, the headspace gas was replenished with H_2/CO_2 (80:20). This caused a further increase in product concentration. After 64 days of incubation, the acetate concentration was 54 mM and the methane concentration was 15 mM (Figure 4-3A). There were no acetate or methane produced in the negative control without 3-PW. Production of methane was not observed in a second set of incubations with headspace gas of N_2/CO_2 (90:10) with 20 mM acetate added to the medium (Figure 4-3B). The acetate concentration in this enrichment was similar to that of the negative control experiment which did not contain 3-PW; microbial activity was therefore not detected (Figure 4-3B). Acetotrophic methanogens, if active in the 3-PW sample (October, 2010), would have used the available acetate present to make methane.

Enrichments with the methanogenesis inhibitor BESA and the antibiotic rifampicin which targets *Bacteria* only are shown in Figure 4-4. Acetic acid concentration in the incubation with headspace gas H_2/CO_2 (80:20) with 20 mM BESA added was 47 mM after 56 days of incubation. Acetate concentration however decreased to 40 mM at the end of the 64 day incubation period (Figure 4-4A). Methane production was not observed in the enrichment with 20 mM of BESA added. In enrichment with the addition of 10 mg/L rifampicin, the concentration of methane was 12 mM after 56 days

of incubation (Figure 4-4B). After replenishing the headspace at day 56, the methane concentration increased to 23 mM at the end of the 64 day incubation period. No acetic acid production was observed throughout the incubation period (Figure 4-4B). It thus appears that BESA inhibits methanogenesis but not acetogenesis (Horn et al., 2003), whereas rifampicin inhibits acetogenesis but not methanogenesis (Brauer et al., 2004). Also methanogenesis was delayed, and did not start until day 20, similar to enrichments with addition of rifampicin (Figure 4-4). This indicates that methanogenesis is from H_2/CO_2 (80:20) and not from acetate. The negative control without the addition of 3-PW for both experiments (Figure 4-4) did not produce acetate or methane.

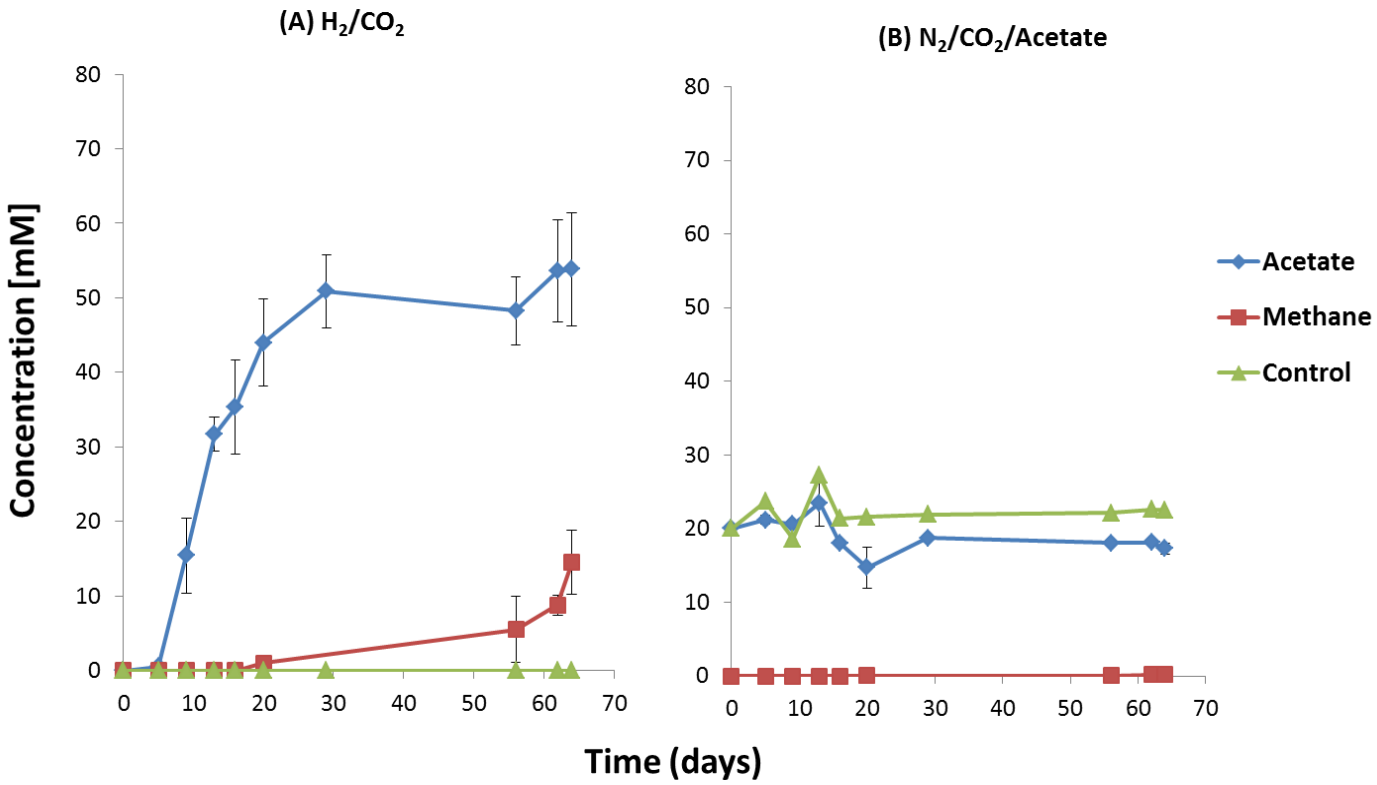


Figure 4-3: Acetate and methane concentration produced by primary enrichments in X5 medium (5 g/L NaCl) during 64 days of incubation with (A) H_2/CO_2 (80:20) and (B) N_2/CO_2 (90:10) with 20 mM acetate. The 3-PW sample obtained in October, 2010 was used as inoculum. The negative control experiments were done without the addition of 3-PW. The data shown are averages of 3 replicates; standard error bars are shown where these exceeded the size of the symbols.

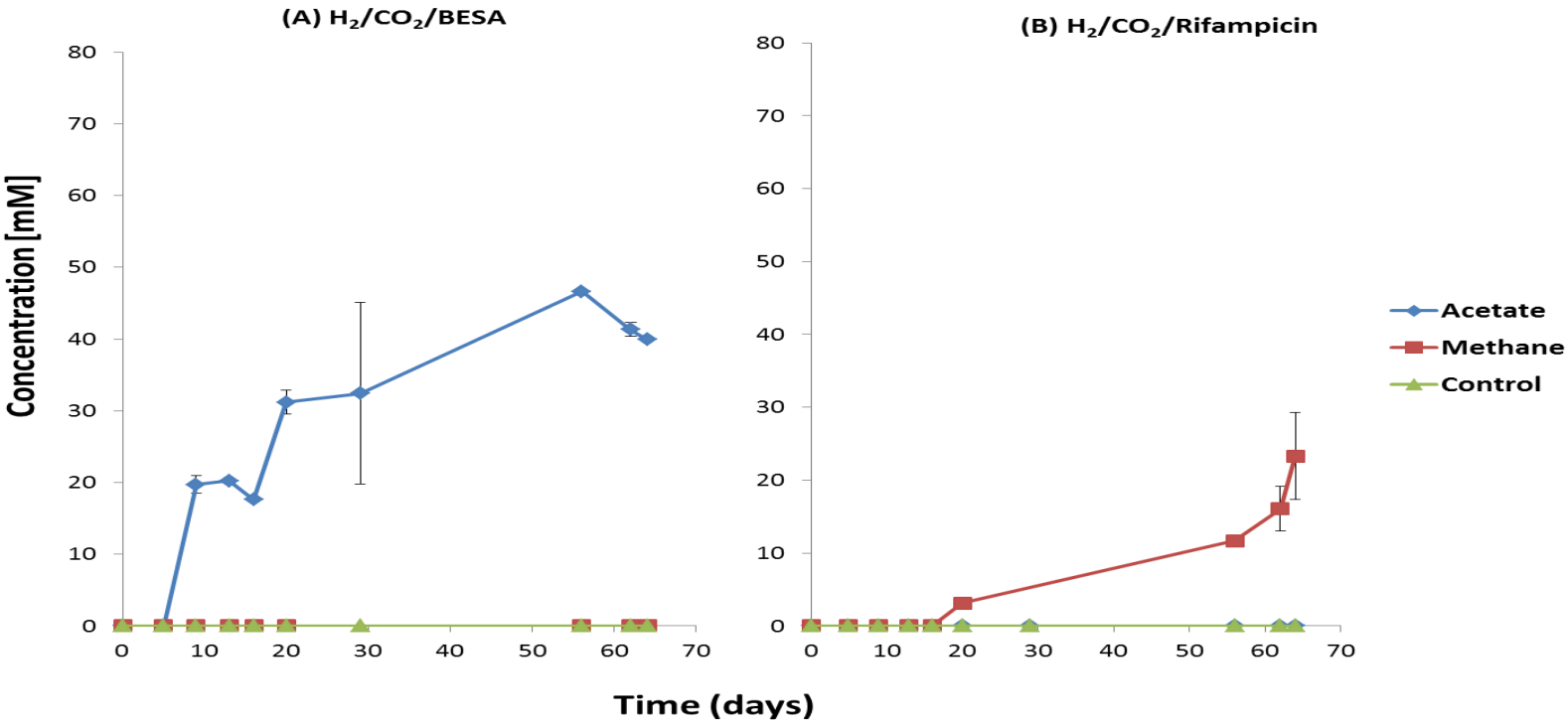


Figure 4-4: Acetate and methane concentration produced by primary enrichments in X5 medium (5 g/L NaCl) during 64 days of incubation with (A) H_2/CO_2 (80:20) with 20 mM BESA added and (B) H_2/CO_2 (80:20) with 10 mg/L rifampicin added. The 3-PW sample obtained in October, 2010 was used as inoculum. The negative control experiments were done without the addition of 3-PW. The data shown are averages of 3 replicates; standard error bars are shown where these exceeded the size of the symbols.

4.1.5 Microbial activities in defined X and X5 media and rich medium A.

Experiments with medium A (Figure 4-5) were done to observe the activity of methanogens and acetogens in a rich medium in comparison to the defined X and X5 media. Acetate production was observed the following day after inoculation of 3-PW, reaching a concentration of 54 mM after 56 days of incubation. Methane production was not observed until after 12 days of incubation with 11 mM of methane being present after 56 days of incubation (Figure 4-5). The negative control without the addition of 3-PW (Figure 4-5) did not produce acetate or methane.

All 3 growth media were compared to determine which would be best to use for further experiments with methanogens and acetogens (Figure 4-6). Comparison was done for primary enrichments with an H_2/CO_2 (80:20) headspace only. Enrichments in medium X produced 32 mM of acetic acid while enrichments in media X5 and A produced 54 mM acetic acid. Methane production was 27 mM in medium X, 14.5 mM in medium X5 and 11 mM in medium A (Figure 4-6).

At the end of the incubation period, pH measurements were taken for some of the enrichments (Table 4-3). All the pH values were acidic except that for enrichment in medium A with H_2/CO_2 (80:20). This is in agreement with the results observed in Figures 4-2, 4-3, 4-4 and 4-5 which shows that high concentrations of acetic acid were produced. The pH value of 7.71 measured in medium A may be because it is better buffered, since other microorganisms are capable of growing in the medium. Further experiments will be done to test if there is an effect of pH on growth of acetogens and methanogens.

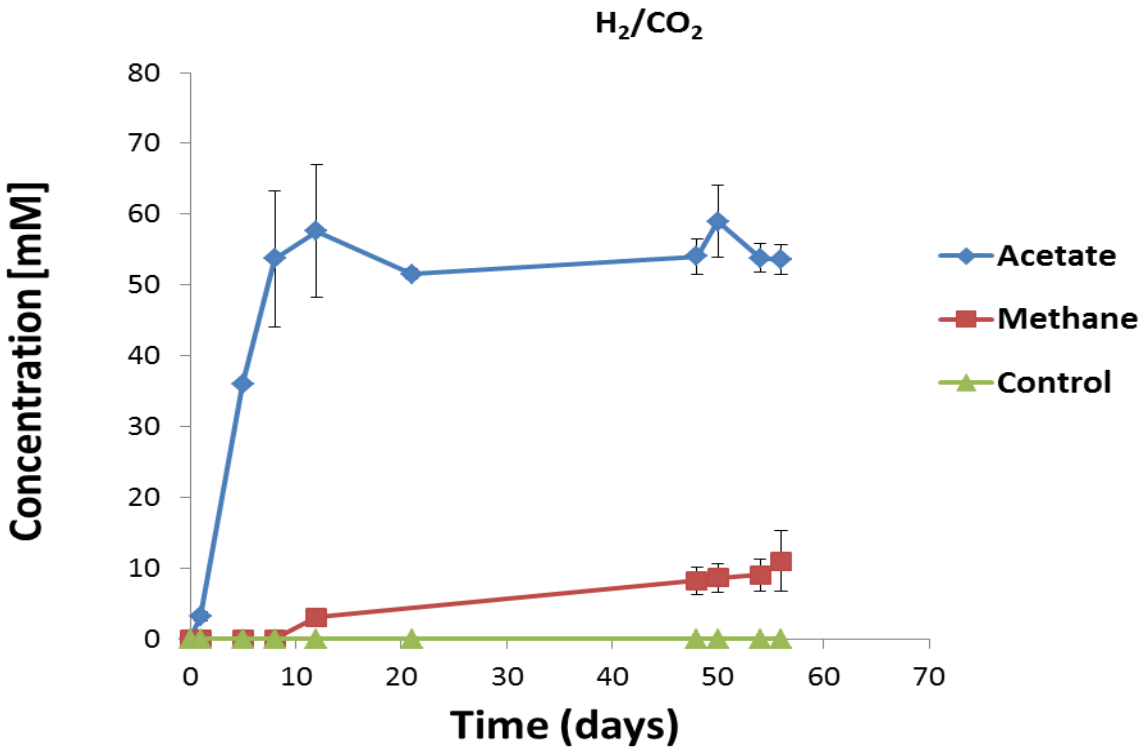


Figure 4-5: Acetate and methane concentration produced by enrichment of 3-PW in medium A (2 g/L yeast extract) during 56 days of incubation with H_2/CO_2 (80:20). The 3-PW sample obtained in October, 2010 was used as inoculum. The negative control experiment was done without the addition of 3-PW. The data shown are averages of 3 replicates; standard error bars are shown where these exceeded the size of the symbols.

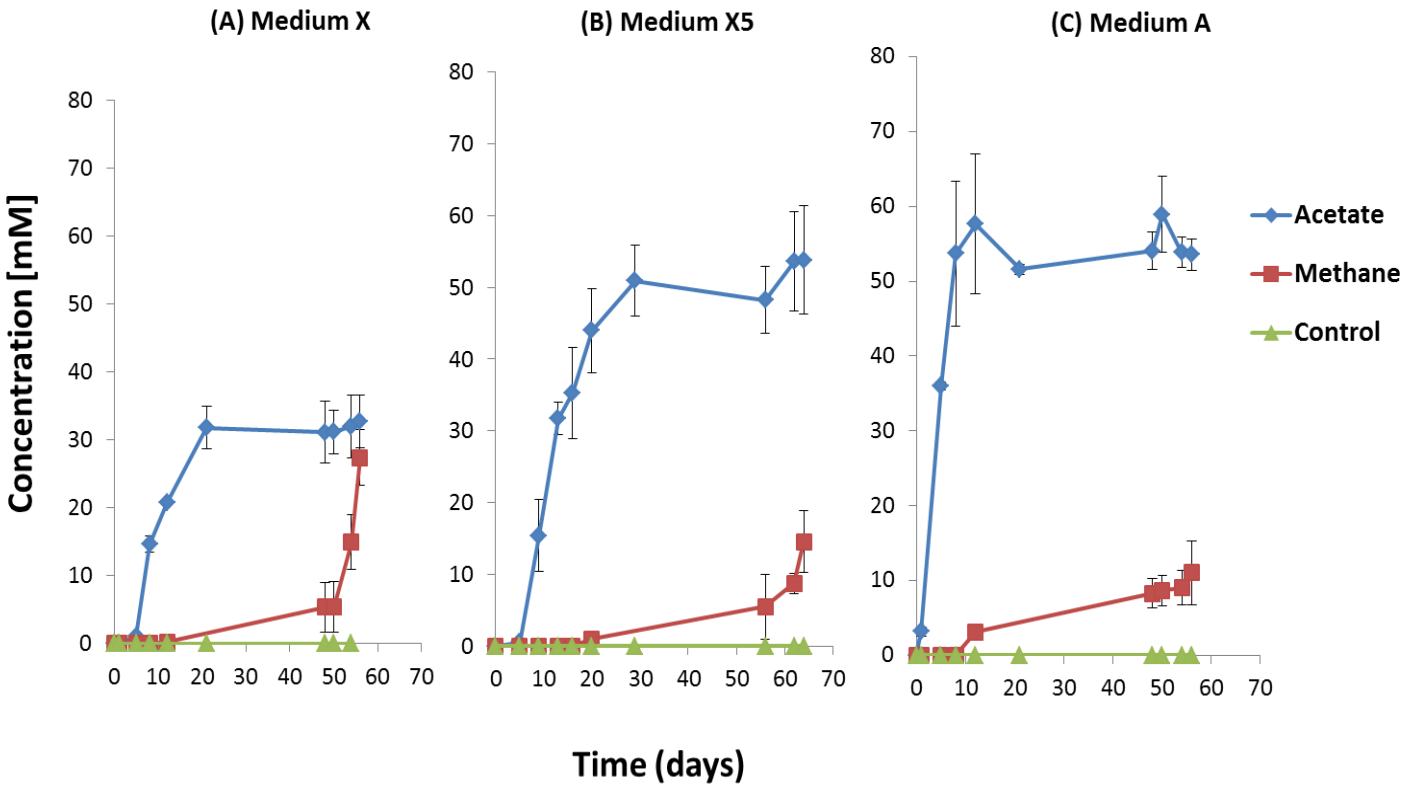


Figure 4-6: Comparison of acetic acid and methane production in (A) medium X with 0.7 g/L NaCl (B) medium X5 with 5 g/L NaCl and (C) medium A with 2 g/L yeast extract. The headspace gas was H_2/CO_2 (80:20) with 3-PW sample obtained in October 2010 used as inoculum. The negative control experiments were done without the addition of 3-PW. The data shown are averages of 2 or 3 replicates; standard error bars are shown where these exceeded the size of the symbols.

Table 4-3: The pH values of selected enrichment experiments. The defined media X and X5 contained 0.7 g/L and 5 g/L NaCl respectively, while rich medium A contained 2 g/L yeast extract. Concentration of methanogenic inhibitor BESA was 20 mM in cases when it was added. The pH values were obtained at the end of enrichment period. Headspace gas was H_2/CO_2 (80:20).

Sample	pH
Medium A + H_2/CO_2	7.71
Medium X5 + H_2/CO_2 + BESA	5.22
Medium X + H_2/CO_2 + BESA	5.17
Medium X5 + H_2/CO_2	5.33

4.1.6 Community composition of primary enrichments in X, X5, and A media.

The microbial community composition analyses were done for media X, X5 and A under the same enrichment condition (Figure 4-6 and Tables 4-4 & 4-5). A total of 55265 reads were obtained for all three media conditions (Table 4-4). The percentage of *Archaea* in media X and X5 was 90-92% while that in medium A was 78%. The operational taxonomic units (OTUs) for all three media were lower than the estimated OTUs (Chao). The Shannon index of diversity obtained in media X and X5 was low (0.4 -0.59) indicating little diversity in the community composition (Table 4-4).

The *Archaea* community in the X and X5 media was mostly composed of the genus *Methanobacterium* (90-92%) while that of medium A was mostly composed of genus *Methanoculleus* (78%). Other microbial genera present in smaller fractions in medium X

were *Methanoculleus* (0.2%), *Citrobacter* (3.1%), *Acetobacterium* (2.5%) and *Desulfovibrio* (1.3%) (Table 4-5). Other than the genus *Methanobacterium*, the community in medium X5 also contained members of the microbial genera *Acetobacterium* (0.5%), *Spirochaeta* (6.2%), and *Desulfovibrio* (0.1%) (Table 4-5). Other microbial genera present in enrichments with medium A were *Acetobacterium* (1.4%), *Methanobacterium* (0.6%), *Citrobacter* (8.7%) and *Petrimonas* (1.2%) (Table 4-5). The presence of the microbial genera *Acetobacterium* (Table 4-5) in these enrichments is in agreement with the fact that high acetogenic activity was detected with all three media (Figure 4-6). Although the methane concentration in medium X5 was lower than that of medium X (Figure 4-6), they both contained similar percentages of the methanogenic genus *Methanobacterium* (Table 4-5).

Since both acetic acid and methane are key products in the storage of CO_2 by subsurface microorganisms in depleted oil fields, medium X5 was chosen as the best medium for growth, because high concentrations of acetic acid (54 mM) were observed and analysis of its microbial community composition shows the presence of high fraction of methane producing microorganisms. This was most likely because medium X5 had similar salt concentration (5 g/L NaCl) to 3-PW.

Table 4-4: Statistics and bioinformatics analysis of pyrotag sequences obtained for the amplicon libraries of enrichments of 3-PW in media X, X5 and A. The defined media X and X5 contained 0.7 g/L and 5 g/L NaCl respectively, while rich medium A contained 2 g/L yeast extract. The headspace gas was H_2/CO_2 (80:20).

Enrichment medium	ID	Reads				5% cutoff		
		Chimeras removed	*Archaea*	*Bacteria*	% *Archaea*	OTUs	Estimated OTUs (Chao)	Shannon's index
X	382	20904	18759	2145	90	89	202	0.59
X5	385	20203	18498	1705	92	60	95	0.40
A	381	14158	11096	3062	78	92	159	1.05

Table 4-5: Microbial community composition of 3-PW enrichments in media X, X5 and A. The defined media X and X5 contained 0.7 g/L and 5 g/L NaCl respectively, while rich medium A contained 2 g/L yeast extract. The headspace gas was H_2/CO_2 (80:20).

	X	X5	A
Number of reads	20904	20203	14158
Taxon (Phylum; class; order; family; genus)	% Reads	% Reads	% Reads
Euryarchaeota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_Methanobacterium	89.5	91.5	0.60
Proteobacteria_Gammaproteobacteria_Enterobacteriales_Enterobacteriaceaer_Citrobacter	3.11	0.02	8.66
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae_Acetobacterium	2.54	0.52	1.41
Firmicutes_Clostridia_Clostridiales_Clostridiaceae	1.31	0.05	0.28
Proteobacteria_Deltaproteobacteria_Desulfovibrionales_Desulfovibrionaceae_Desulfovibrio	1.25	0.08	0.14
Spirochaetes_Spirochaetes_Spirochaetales_Spirochaetaceae_uncultured	0.63	0.01	0.16
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_uncultured	0.27	0	0.02
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanoculleus	0.20	0.01	77.6
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Proteiniphilum	0.15	0.03	0
Proteobacteria_Alphaproteobacteria_Rhizobiales_Methylobacteriaceae_Methylobacterium	0.13	0	0
Bacteroidetes_Bacteroidia_Bacteroidales_Rikenellaceae_vadinBC27	0.09	0	0.01
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanocorpusculaceae_Methanocorpusculum	0.05	0.06	0.02
Proteobacteria_Gammaproteobacteria_Enterobacteriales_Enterobacteriaceae	0.02	0	0.18
Bacteria_Synergistetes_Synergistia_Synergistales_Synergistaceae_Thermanaerovibrio	0.01	0	0.85
Firmicutes_Clostridia_Clostridiales_Peptostreptococcaceae	0.01	0.02	3.77
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Petrimonas	0.01	0.03	1.15
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Pseudomonadaceae_Pseudomonas	0.01	0.05	0.01
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanofollis	0.01	0	0.01
Proteobacteria_Gammaproteobacteria_Aeromonadales_Aeromonadaceae_Aeromonas	0.01	0.09	0
Firmicutes_Clostridia_Clostridiales_Anaerovorax	0	0	0.42
Firmicutes_Clostridia_Clostridiales_Clostridiaceae_Acidaminobacter	0	0.01	0.34
Bacteroidetes_Sphingobacteria_Sphingobacteriales_WCHB1-69	0	0.01	0.15
Synergistetes_Synergistia_Synergistales_Synergistaceae_Aminiphilus	0	0.01	0.09
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanocalculus	0	0.02	0.04
Spirochaetes_Spirochaetes_Spirochaetales_Spirochaetaceae_Spirochaeta	0	6.17	0.01

4.2 Effects of hydrogen concentration and pH

4.2.1 Primary enrichments with 80% H_2 and 20% CO_2

In addition to the production of acetate and methane it is important to record the volume of gas (H_2 and CO_2) used by microorganisms and the change in the pH of the enrichment medium due to microbial activity. As in previous experiments, acetogenesis was observed early in the incubation period (Figure 4-7A), whereas methanogenesis was observed later (Figure 4-7B). At day 18, the acetate concentration was 26 mM and decreased to 21 mM by day 40 (Figure 4-7A). The methane concentration was 1.3 mM at day 18 and increased to 7 mM by day 40 (Figure 4-7B). Figure 4-7A also shows the change of pH as a function of time. At day 0, the pH was 7.5 and decreased to 5.4 on day 40. The biggest drop in pH was observed during the peak of acetate production from days 8 to 23 (Figure 4-7A). The pH however remained unchanged during the peak of methane formation (Figure 4-7A & B). Methanogenic activity was thus observed when the enrichment pH was relatively low. The total amount of H_2/CO_2 (80:20) gas used during the 40 day enrichment period was 421 mL (Figure 4-7B). The calculations relating gas use to the concentration of acetate and methane formed can be found in the methods (Section 3.5). The headspace gas was initially used for the formation of acetate, whereas later gas use was mainly for methane formation (Figure 4-7A & B).

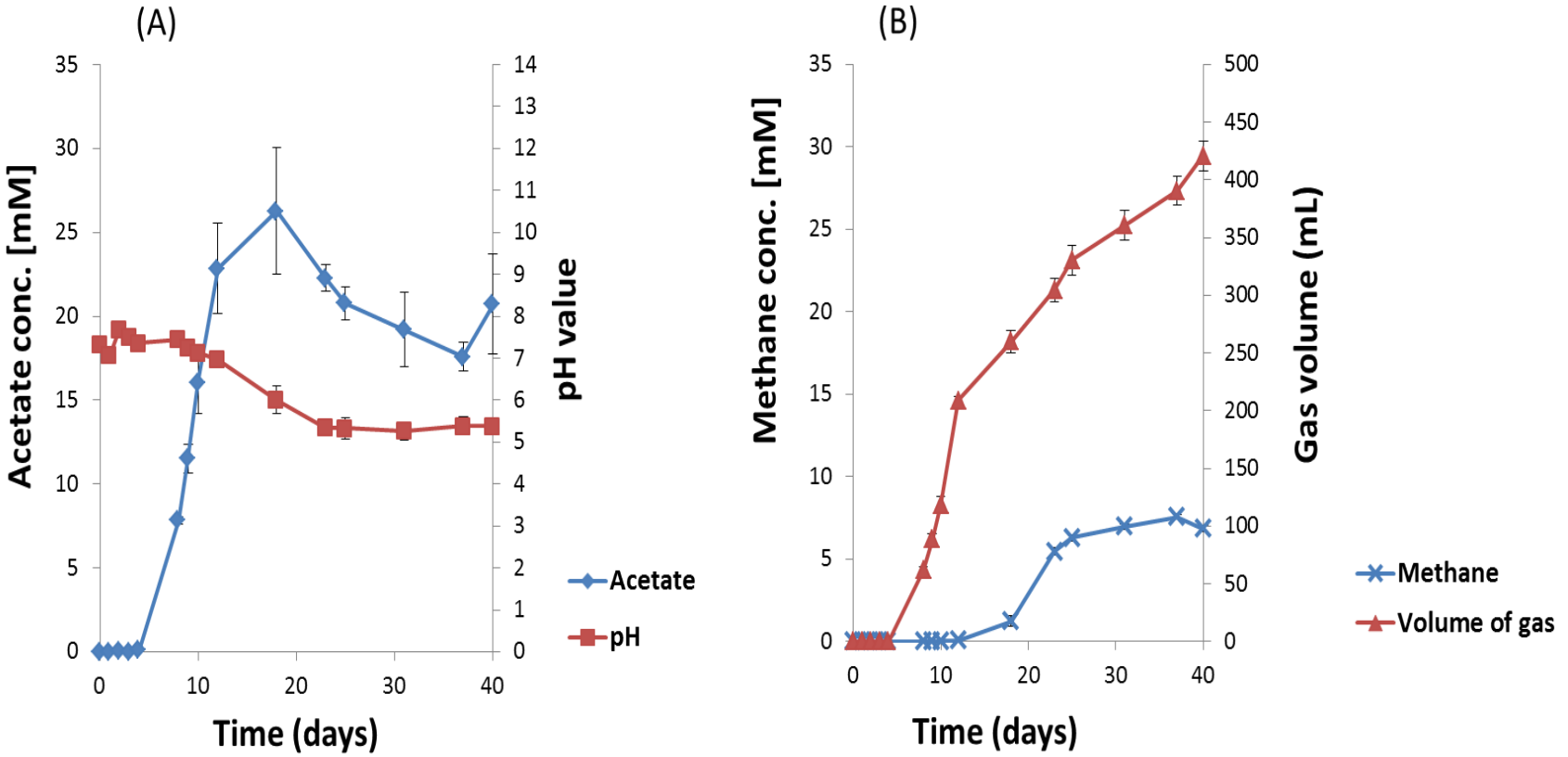


Figure 4-7: Acetate concentration with pH value (A) and methane concentration with volume of gas (B) shown as a function of time for primary enrichments of 3-PW with headspace gas H_2/CO_2 (80:20). The 3-PW sample obtained in September, 2011 was used as inoculum in defined medium X5 (5 g/L NaCl). The data shown are averages of 3 replicates; standard error bars are shown where these exceeded the size of the symbols.

4.2.2 Primary enrichments with 5% H_2, 10% CO_2 and 85% N_2

Acetate production was observed after 3 days of inoculation with 3-PW followed by methane production after 8 days (Figure 4-8A). Acetate and methane concentrations formed were lower than those observed with H_2/CO_2 (80:20) in the headspace (Figure 4-7). After 8 days of incubation, the acetate concentration was 0.4 mM while the methane concentration was 0.03 mM (Figure 4-8). There was a slight decrease in acetate concentration from days 10 – 21 to 0.3 mM (Figure 4-8A) with no apparent increase in methane concentration (Figure 4-8B). The gas in the headspace of the enrichments bottles was renewed by replenishing the entire headspace at each sampling period from day 21 onwards (black arrows in Figure 4-8). This increased the concentrations of acetate (Figure 4-8A) and cumulative methane (Figure 4-8B). At the end of the 40 day incubation period, the acetate concentration was 0.8 mM (Figure 4-8A) while the methane concentration was 0.35 mM (Figure 4-8B). There was also a slight drop in the enrichment pH from 7.5 to 6.7 (Figure 4-8).

Overall the primary enrichments with headspace gas of 5% H_2 showed similar trends to those with headspace gas of 80% H_2. Acetogenic activity was still observed first, followed by methanogenic activity. These results indicate that CO_2 injected into an oil field may be converted to acetic acid initially, followed by methane provided that H_2 is available.

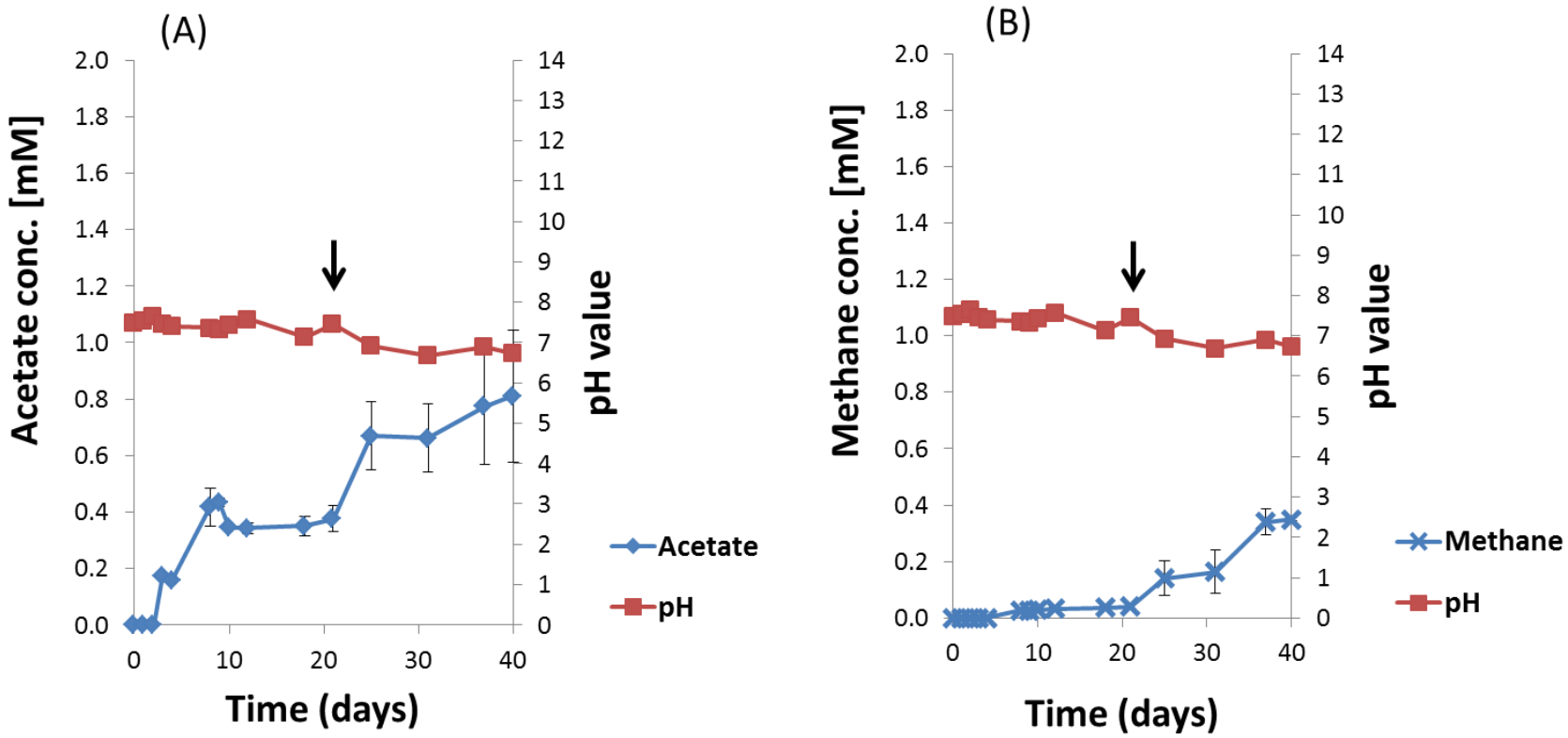


Figure 4-8: Acetate concentration (A) and methane concentration (B) shown as a function of time for primary enrichments of 3-PW with headspace gas $H_2/CO_2/N_2$ (5:10:85). The pH of the enrichment during the 40 day period is also shown. The 3-PW sample obtained in September, 2011 was used as inoculum in defined medium X5 (5 g/L NaCl). The arrow indicates the point at which the headspace gas was completely replenished. The data shown are averages of 3 replicates; standard error bars are shown where these exceeded the size of the symbols.

4.2.3 Secondary enrichment with 80% H_2 and 20 % CO_2

A volume of 1 ml of the primary enrichment of 3-PW with headspace gas H_2/CO_2 (80:20) was transferred into medium X5 with the pH set to 5.6, 7.6 and 8.5 (Figure 4-9). Because acetic acid was used to adjust the pH, acetate concentrations were 5 mM for pH 8.5, 16 mM for pH 7.6 and 28 mM for pH 5.5 (Figure 4-9A). In the course of the incubations there were no changes in the acetate concentrations of the secondary enrichment (Figure 4-9A). There was however an increase in the concentration of methane for each pH condition (4-9B). At 24 days of incubation, the methane concentration was 7.5 mM. The headspace was then renewed completely by replenishing the headspace gas at day 24 (black arrow Figure 4-9B). This caused an increase in the methane concentrations to 37-40 mM for all pH conditions. A total of 178- 199 mL of H_2/CO_2 (80:20) were used during the methanogenesis period (Figure 4-10).

It thus appears that conditions used in this experiment led exclusively to methanogenesis but not acetogenesis at all starting pH conditions (Figures 4-9 and 4-10). Starting pH was therefore not a factor in determining whether the enrichments are mainly acetogenic or methanogenic.

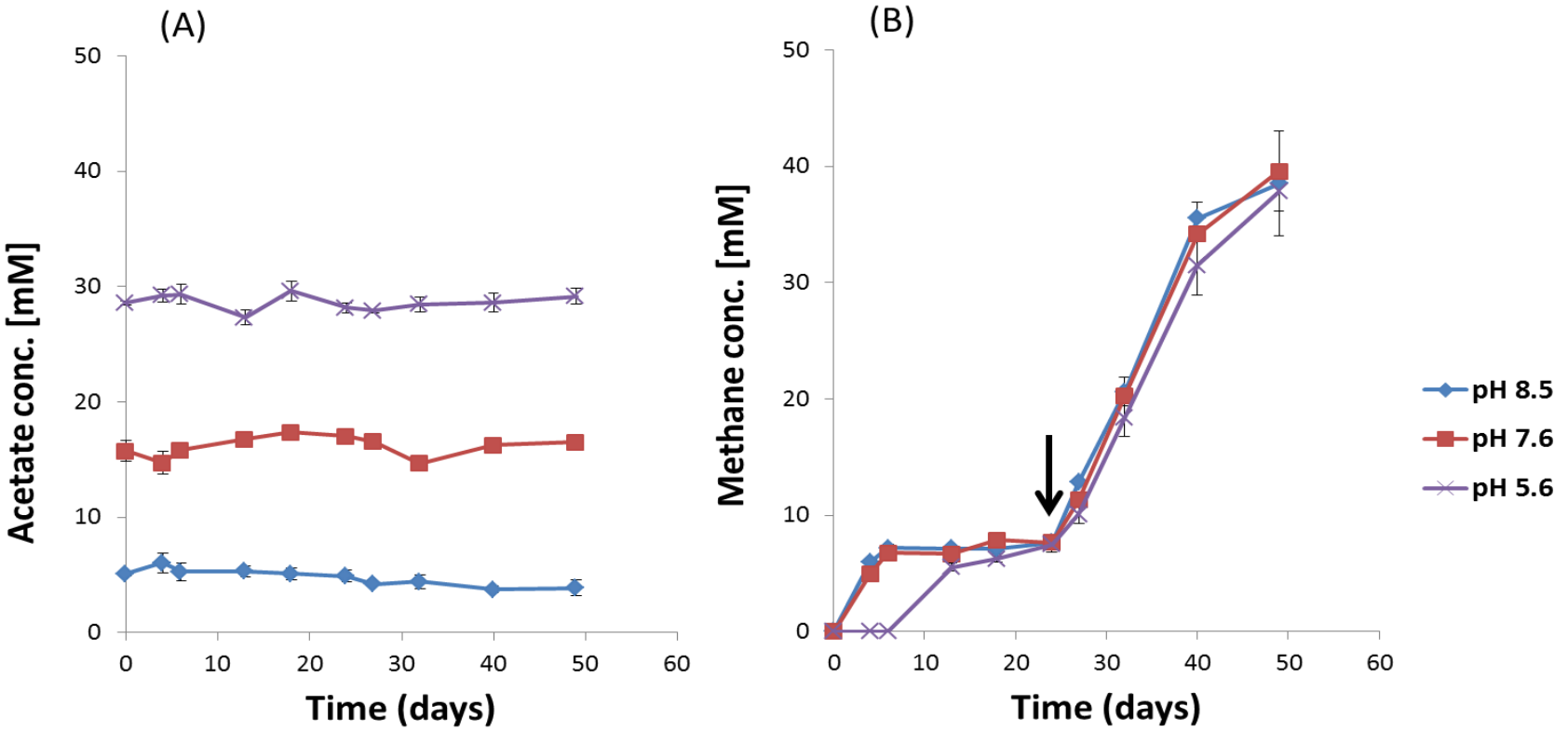


Figure 4-9: Acetate concentration (A) and methane concentration (B) shown as a function of time for secondary enrichments of 3-PW with headspace gas H_2/CO_2 (80:20). The pH of the enrichments was set to 8.5, 7.6 and 5.6 with addition of acetate in some cases. The black arrow indicates the point at which the headspace was completely replenished. The 3-PW sample obtained in September, 2011 was used as inoculum in defined medium X5 (5 g/L NaCl). The data shown are averages of 2 replicates; standard error bars are shown where these exceeded the size of the symbols.

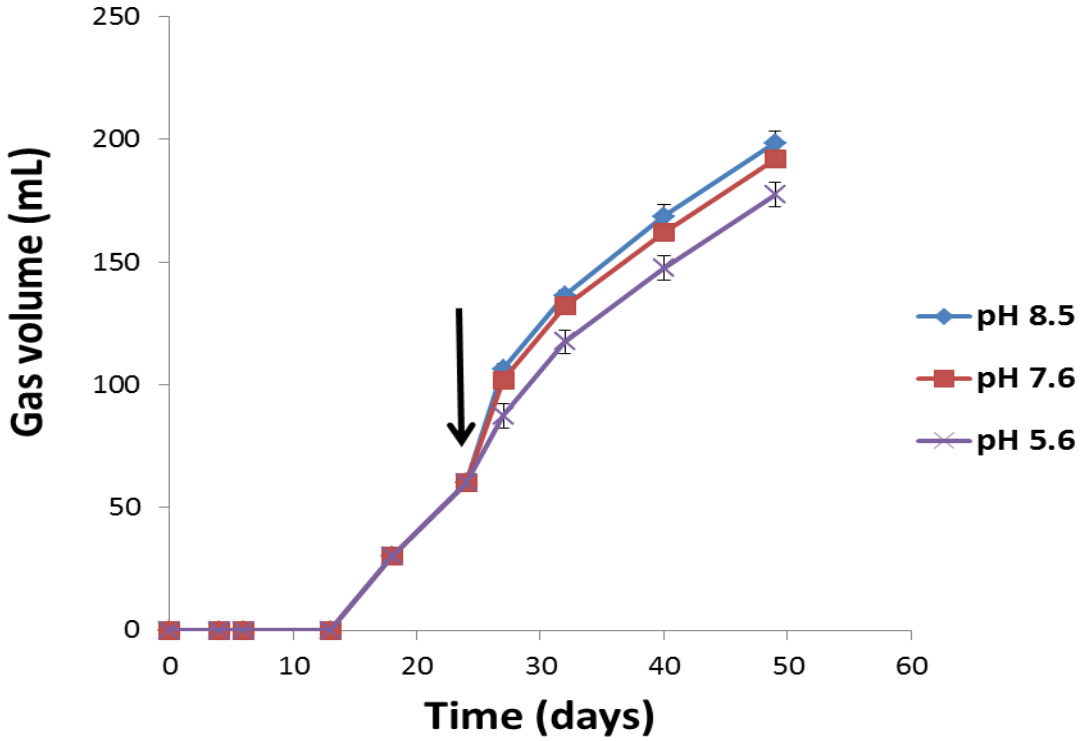


Figure 4-10: The volume of gas use shown as a function of time for secondary enrichments of 3-PW with headspace gas H_2/CO_2 (80:20). The pH of the enrichments was set to 8.5, 7.6 and 5.6 by addition of acetate. The black arrow indicates the point at which the headspace was completely replenished. The 3-PW sample obtained in September, 2011 was used as inoculum in defined medium X5 (5 g/L NaCl). The data shown are averages of 2 replicates; standard error bars are shown where these exceeded the size of the symbols.

4.2.4 Microbial community composition of primary and secondary enrichments

Samples of the primary enrichment of 3-PW with headspace gas H_2/CO_2 (80:20) for days 0, 8, 18, 25 and 40 (Figure 4-7) were used for DNA isolation. Likewise, samples were taken from two secondary enrichments in which 1 mL of the primary enrichment was transferred to X5 medium set at pH 7.6 and 5.5 (Figure 4-9). DNA isolation was done for pH 7.6 (samples collected on days 0, 24 and 49) and pH 5.5 (samples collected on days 24 and 49). In total 10 samples were collected: 5 from the primary enrichment and 5 from the secondary enrichment at pH 7.6 and pH 5.5. After DNA isolation, all 10 samples were PCR amplified and sent for 454 sequencing of the 16S rRNA genes.

A total of 113,976 reads were obtained for all 10 enrichments. A low number of reads (165) was obtained for the primary enrichment at day 0. These data will therefore not be discussed further. The numbers of reads for the other 9 samples are indicated in Table 4-6. The reads that correspond to *Archaea (*methanogens) and *Bacteria* are listed together with the fraction of *Archaea*. The primary enrichments had only 0.3% *Archaea* on day 8, 49% on day 18, and 68-74% on days 25 and 40. In the secondary enrichment with pH at 7.6, the fraction of *Archaea* was 74%, 95% and 92% on days 0, 24 and 49, respectively. Secondary enrichment with pH 5.5 gave 93% and 89% *Archaea* on days 24 and 49 respectively. The initial formation of acetate, followed by methane formation from day 8 onwards in the primary enrichment (Figure 4-7) is thus due to the absence of a significant fraction of active *Archaea*. They were however present from day 18 onwards. Also, when the primary enrichment is used as an inoculum much higher

fractions of *Archaea* were obtained (74% on day 0 and 89 – 95% on days 24 and 49). This resulted in the formation of methane but not acetic acid (Figures 4-9).

The estimated number of operational taxonomic units (OTUs) present in the 10 enrichments with H_2/CO_2 (80:20) was between 133 and 271. The largest number of OTUs was on day 25 of the primary enrichment (Table 4-6). In secondary enrichments with X5 medium at pH 7.6, the largest number of OTUs was on day 24 (188) and lowest on day 49 (150). The estimated number of OTUs for secondary enrichments with X5 medium at pH 5.5 was 143 for day 24 and 133 for day 49. Shannon's H index was also used to rate the diversity in community composition (Table 4-6). Generally the community in the primary enrichment was more diverse with higher values of Shannon's H index in comparison to the community of the secondary enrichment. The microbial community diversity in the primary enrichment also increased gradually with time.

Table 4-6: Statistics and bioinformatic analysis of pyrotag sequences obtained for the amplicon libraries from 4 primary enrichments and 5 secondary enrichments in medium X5 (5 g/L NaCl). Enrichments were analysed by 454 sequencing of 16S rRNA genes for all 9 enrichment samples with 3-PW sample obtained in September, 2011 used as inoculum. The headspace gas was H_2/CO_2 (80:20).

Enrichment	Sample	ID	Reads				5% cutoff		
			Chimeras removed	*Archaea*	*Bacteria*	% *Archaea*	OTUs	Estimated OTUs (Chao)	Shannon's index
Primary enrichment	Day 8	868	14427	40	14387	0.3	105	156	0.37
	Day 18	869	13629	6634	6995	49	153	207	1.56
	Day 25	870	15922	11766	4156	74	143	271	1.74
	Day 40	871	10238	6992	3246	68	125	196	2.22
Transfer to pH 7.6 *	Day 0	877	10099	7486	2613	74	116	169	1.48
	Day 24	878	12205	11569	636	95	110	188	1.47
	Day 49	879	11672	10680	992	92	85	150	1.57
Transfer to pH 5.5 *	Day 24	880	12885	12003	882	93	62	143	0.77
	Day 49	881	12734	11283	1451	89	76	133	1.15

* Samples from the primary enrichment were used to inoculate the secondary enrichment at pH 7.6 or 5.5.

A detailed description of the percentage of microbial taxa present in the 3 different enrichments analysed in Table 4-6 is shown in Tables 4-7 and 4-8 and also Figures 4-11 and 4-12. The results of the analysis were dependent on the day of incubation.

The top microbial genera present in the primary enrichments at day 8 were *Acetobacterium* (96%) (Table 4-7 and Figure 4-11). Top microbial genera present at days 18, 24 and 40 were *Acetobacterium, Methanofollis, Methanospirillum* and *Methanobacterium*. A decrease in the percentage of *Acetobacterium* was observed after day 8 to 49% on day 18, 24% on day 25 and 30% on day 40 but an increase in methanogens was observed from days 8 to 40. The percentage of *Methanofollis* on day 8 was 0.01% but this increased to 38 % on day 18, 50% on day 25 with a slight decrease to 28% on day 40 (Table 4-7 and Figure 4-11). *Methanobacterium* was also present at 4.7 % on day 18, 3% on day 25 and 26% on day 40 while *Methanospirillum* was 5% on day 18, 20.5% on day 25 and 14% on day 40 (Table 4-7 and Figure 4-11). This is in support of the results observed in Figure 4-7 where the concentration of methane increased as a function of time. Other microbial taxa present at very small percentages from days 0 to 40 included members of the microbial genera *Eubacterium, Geobacter, Methanolobus, Proteiniphilum* and *Methanosaeta*; and members of the microbial families *Lachnospiraceae, Methanobacteriaceae,* and *Rhodobacteraceae* (Table 4-7).

Table 4-7: Microbial community composition of primary enrichment of 3-PW in medium X5 (5 g/L NaCl) at days 8, 18, 25 and 40. The 3-PW sample was obtained in September, 2011. The headspace gas was H_2/CO_2 (80:20). Average distribution of reads (%) obtained from 454 sequencing of 16S rRNA genes over taxa are shown.

	Day 8	Day 18	Day 25	Day 40
Number of reads	14427	13629	15922	10238
Taxon (Phylum; class; order; family; genus)	% Reads	% Reads	% Reads	% Reads
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae_Acetobacterium	96.1	48.9	24.4	29.79
Proteobacteria_Betaproteobacteria_Burkholderiales_Alcaligenaceae_Castellaniella	0.92	0	0	0.01
Bacteroidetes_Bacteroidia_Bacteroidales_Rikenellaceae_vadinBC27	0.69	0.31	0.13	0.04
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Proteiniphilum	0.45	0.23	0.04	0.16
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae_Eubacterium	0.42	0.16	0.07	0.08
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae	0.33	0.50	0.20	0.19
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosarcinaceae_Methanolobus	0.17	0.26	0.18	0.01
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae	0.08	0.03	0.04	0.02
Firmicutes_Mollicutes_Acholeplasmatales_Acholeplasmataceae_Acholeplasma	0.08	0.02	0.01	0
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosaetaceae_Methanosaeta	0.08	0.01	0.01	0
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_uncultured	0.06	0.03	0.02	0.02
Firmicutes_Clostridia_Clostridiales_Clostridiaceae_Clostridium	0.05	0.08	0.03	0.04
Bacteria_Firmicutes_Clostridia_Clostridiales	0.04	0.07	0.06	0.09
Proteobacteria_Alphaproteobacteria_Rhodobacterales_Rhodobacteraceae	0.01	0.04	0.11	0.65
Proteobacteria_Deltaproteobacteria_Desulfuromonadales_Geobacteraceae_Geobacter	0.01	0.10	0.20	0.21
Firmicutes_Clostridia_Clostridiales__Incertae sedis_Fusibacter	0.01	0.04	0.01	0
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanofollis	0.01	38.4	49.6	27.7
Firmicutes_Clostridia_Clostridiales_Clostridiaceae	0.01	0	0	0
Euryarchaeota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_Methanobacterium	0	4.7	3	25.9
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanospirillaceae_Methanospirillum	0	5.00	20.48	13.8
Euryarchaeota_Methanomicrobia_Methanomicrobiales	0	0.15	0.53	0.40
Euryarchaeota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_uncultured	0	0	0.01	0.35
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae	0	0.60	0.49	0.13
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Petrimonas	0	0	0.05	0.05
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosarcinaceae	0	0.02	0.01	0.01
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Paludibacter	0	0	0.01	0.01
Firmicutes_Clostridia_Clostridiales_Incertae sedis_Tissierella	0	0.02	0	0
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_Incertae sedis	0	0.02	0	0
Firmicutes_Clostridia_Clostridiales_Clostridiaceae_Acidaminobacter	0	0.01	0	0

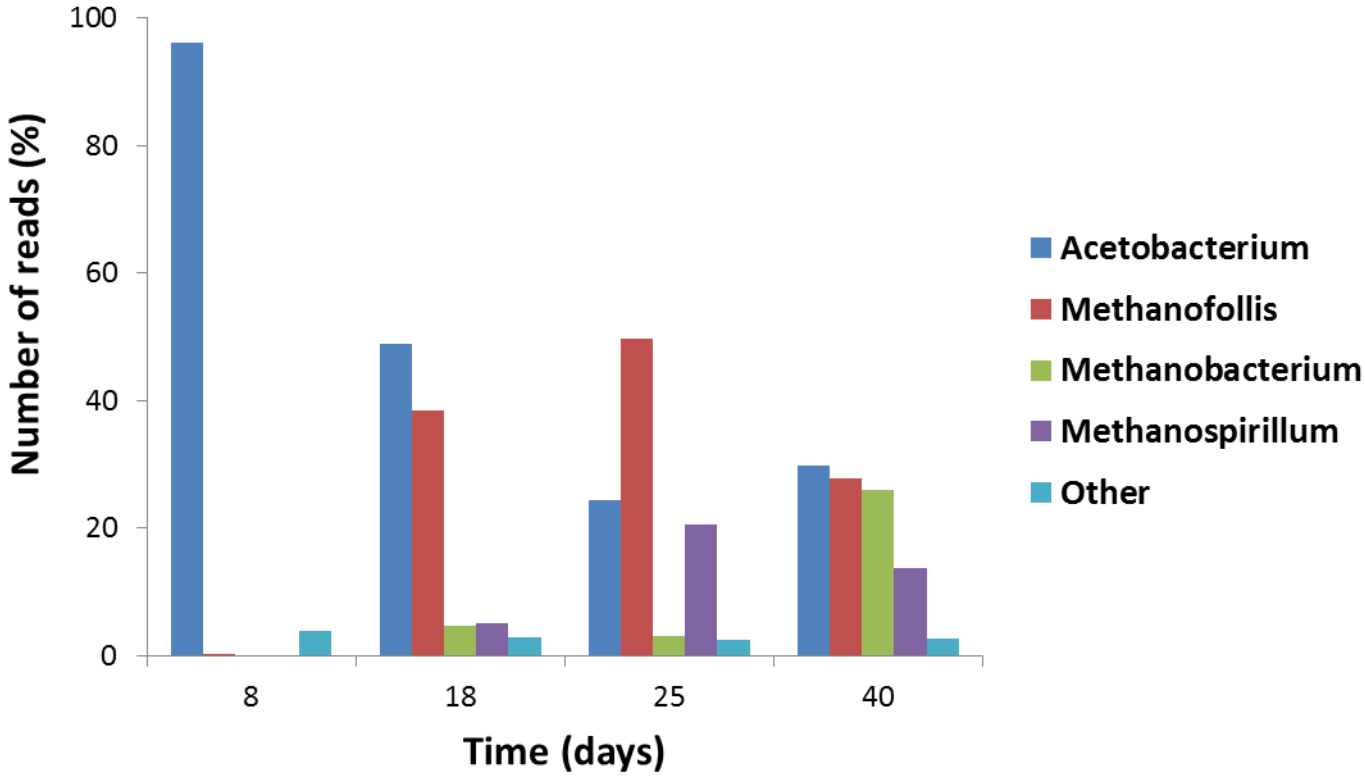


Figure 4-11: The top microbial genera present in the microbial community of primary enrichments of 3-PW in X5 medium (5 g/L NaCl) at days 8, 18, 25 and 40. Other refers to the other microbial taxa which were also detected as in Table 4-7.

Although similar microbial taxa were present in secondary enrichments at pH 7.6 at days 0, 24 and 49 their percentage composition differed (Table 4-8 and Figure 4-12). The top taxa present were *Methanospirillum, Acetobacterium, Methanofollis* and *Methanobacterium*. The percentage of *Acetobacterium* decreased from 24% on day 0 to 2% on day 24 and then to 0.4% on day 49 (Table 4-8 and Figure 4-12). This is in support of results obtained in Figure 4.9A, where no increase in the initial concentrations of acetate in the medium was observed. An increase in the percentage of methanogens was also observed in secondary enrichment at pH 7.6 as shown in Figure 4-9B. The percentage of *Methanospirillum* was 60% on day 0, 69% on day 24 and 59% on day 49 while *Methanofollis* was 10% on day 0, 22% on day 24 and 30% on day 49 (Table 4-8 and Figure 4-12). The percentage of *Methanobacterium* however decreased from 4% on day 0 to 2.4% on day 24 and finally 0.9% on day 49 (Table 4-8 and Figure 4-12).

The community composition for secondary enrichment on days 24 and 49 are shown in Table 4-8 and Figure 4-12. Again, the percentage of *Acetobacterium* present decreased from 6.5% on day 24 to 0.3% on day 49. In the secondary enrichments at pH 5.5, a high percentage of *Methanobacterium* was observed on day 24 (88.4%) and day 49 (90%) while a low percentage of *Methanospirillum* was observed (1.6% on day 24 and 0.4% on day 49). *Methanofollis* was also present with 2.9% on day 24 and 1.2% on day 49 (Table 4-8 and Figure 4-12). Overall, different changes in microbial community composition were observed in both secondary enrichments at pH 7.6 and pH 5.5.

Table 4-8: Microbial community composition of secondary enrichment of 3-PW in medium X5 (5 g/L NaCl) at pH 7.6 and 5.5. Community at pH 7.6 was done at days 0, 24 and 49; and pH 5.5 was done at days 24 and 49. The headspace gas was H_2/CO_2 (80:20). Average distribution of reads (%) obtained from 454 sequencing of 16S rRNA genes over taxa are shown.

	pH 7.6			pH 5.5	
Day	**0**	**24**	**49**	**24**	**49**
Number of reads	10099	12205	11672	12885	12734
Taxon (Phylum; class; order; family; genus)	% Reads	% Reads	% Reads	% Reads	% Reads
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanospirillaceae_Methanospirillum	59.6	69.0	59.04	1.65	0.42
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae_Acetobacterium	23.9	2.02	0.39	6.50	0.28
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanofollis	9.93	22.20	30.3	2.95	1.25
Euryarchaeota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_Methanobacterium	4.02	2.38	0.86	88.4	86.9
Proteobacteria_Alphaproteobacteria_Rhodobacterales_Rhodobacteraceae	0.66	0.54	5.36	0.10	0
Euryarchaeota_Methanomicrobia_Methanomicrobiales	0.52	1.18	1.29	0.04	0
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae	0.24	0.07	0	0.07	0.01
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae	0.13	0.10	0.01	0	0.20
Bacteroidetes_Sphingobacteria_Sphingobacteriales_WCHB1-69	0.12	1.13	2.19	0	0
Firmicutes_Clostridia_Clostridiales_Eubacteriaceae_Eubacterium	0.11	0.00	0	0.02	0
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae_uncultured	0.10	0.01	0.02	0.01	0.02
Bacteroidetes_Bacteroidia_Bacteroidales_Rikenellaceae_vadinBC27	0.09	0.05	0	0	0
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Proteiniphilum	0.06	0.03	0.02	0.02	0.02
Proteobacteria_Deltaproteobacteria_Desulfuromonadales_Geobacteraceae_Geobacter	0.06	0	0	0.01	0.01
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosarcinaceae_Methanolobus	0.03	0.03	0.01	0	0.01
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae	0.02	0.02	0.01	0.01	0.41
Euryarchaeota_Methanobacteria_Methanobacteriales_Methanobacteriaceae_uncultured	0.02	0.02	0	0.15	0.06
Firmicutes_Clostridia_Clostridiales_Clostridiaceae_Clostridium	0.02	0.36	0.03	0	0
Chloroflexi_Anaerolineae_Anaerolineales_Anaerolineaceae	0.02	0.02	0.01	0	0
Spirochaetes_Spirochaetes_Spirochaetales_Spirochaetaceae_uncultured	0.01	0.10	0.15	0	1.23
Proteobacteria_Deltaproteobacteria_Desulfovibrionales_Desulfovibrionaceae_Desulfovibrio	0.01	0.52	0.01	0.01	0.11
Synergistetes_Synergistia_Synergistales_Synergistaceae_Thermanaerovibrio	0.01	0.03	0.03	0	0
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanoculleus	0.01	0	0.02	0	0
Synergistetes_Synergistia_Synergistales_Synergistaceae_Aminiphilus	0.01	0	0	0	0
Proteobacteria_Deltaproteobacteria_Desulfovibrionales	0	0.05	0.01	0.01	8.52
Proteobacteria_Gammaproteobacteria	0	0.07	0	0.08	0.39
Bacteroidetes_Bacteroidia_Bacteroidales_Porphyromonadaceae_Petrimonas	0	0.01	0.06	0	0.02
Actinobacteria_Actinobacteridae_Actinomycetales_Corynebacterineae_Rhodococcus	0	0	0	0	0.01
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanocalculus	0	0	0	0.02	0
Chloroflexi_Anaerolineae_Anaerolineales_Anaerolineaceae_Levilinea	0	0	0.03	0	0
Chloroflexi_Anaerolineae_Anaerolineales_Anaerolineaceae_uncultured	0	0.02	0.01	0	0
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosaetaceae_Methanosaeta	0	0	0.01	0	0
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosarcinaceae_Methanosarcina	0	0	0.01	0	0
Thermotogae_Thermotogae_Thermotogales_Thermotogaceae_Kosmotoga	0	0	0.01	0	0
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae	0	0.02	0	0	0

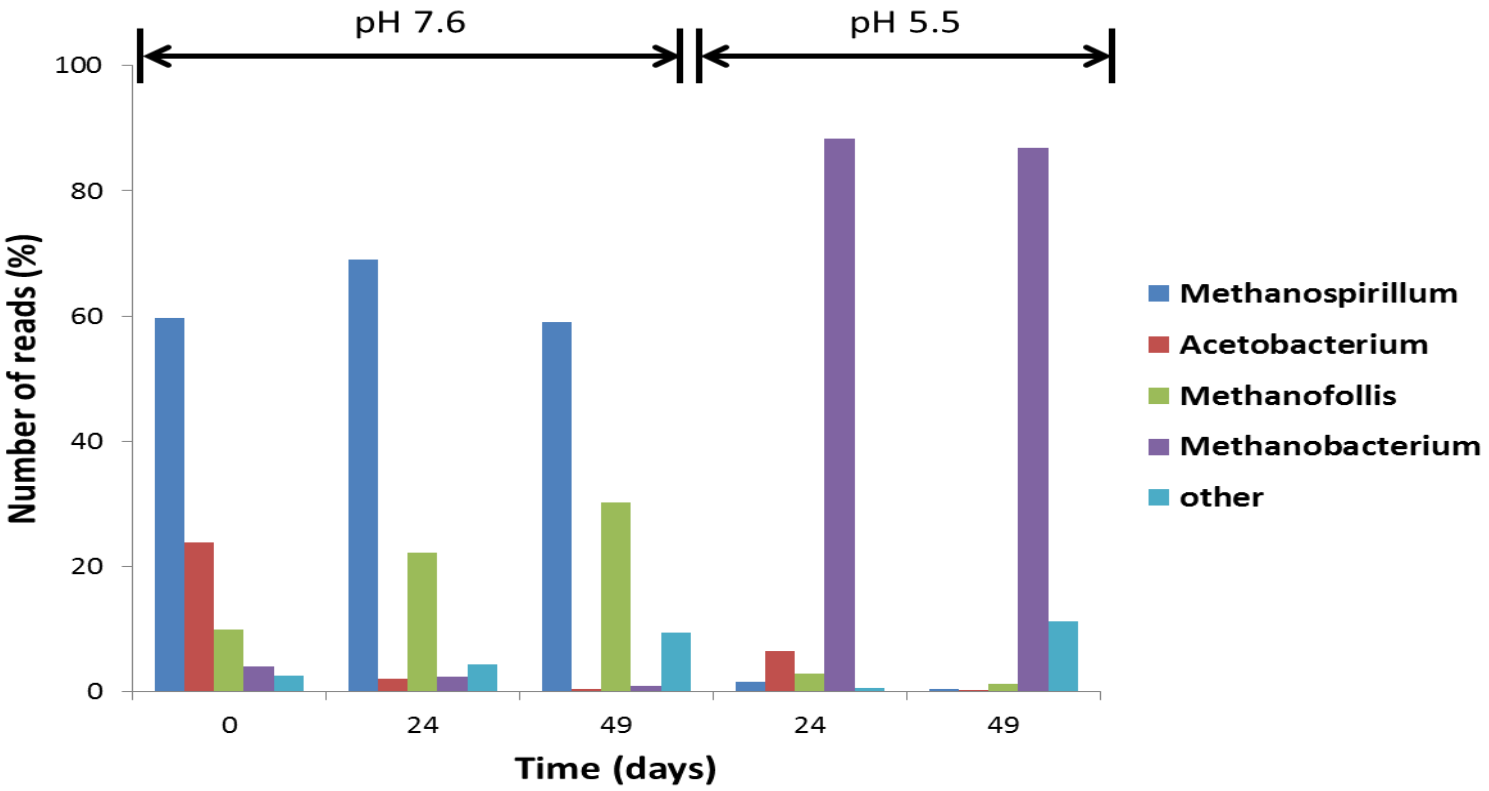


Figure 4-12: The top microbial genera present in secondary enrichment with X5 medium at pH 7.6 and 5.5. The community at pH 7.6 was done at days 0, 24 and 49; and pH 5.5 was done at days 24 and 49. The headspace gas was H_2/CO_2 (80:20). Other refers to the other microbial taxa which were also detected as in Table 4-8.

4.3 Growth by mixing of SW and PW

4.3.1 Effect of nutrients on mixing SW and PW

As indicated in section 3.63, source water (22-SW-U or 22-SW-T) is mixed with produced water (2-PW) to give injection water. SW is the output of the Medicine Hat sewage treatment plant. Samples of untreated and treated SW were collected (see Figure 1-5, Appendix A-3 and A-6), referred to as 22-SW-U and 22-SW-T. The objective was to determine whether addition of nutrients (phosphate or ammonium) or the mixing of SW and PW gave additional growth.

Results for mixing 22-SW and 2-PW in the presence of 100 mM Pi and 100 mM NH_4^+ are shown in Figure 4-13. Under aerobic conditions the log (CFU/mL) was seen to increase from 4.5 to 7.8 over a period of 5 days when mixing 22-SW-U or 22-SW-T with 2-PW (Figure 4-13A). The counts were a little lower when 2-PW was mixed with water. Because the increase in log (CFU/mL) was similar when using H_2O instead of 22-SW-U or 22-SW-T we can conclude that the increase observed of 3 log units was more likely caused by the addition of nutrients than by mixing of 2-PW with 22-SW.

Under anaerobic conditions a large difference was observed between initial log (CFU/mL) when mixtures of SW-U or SW-T and 2-PW were used, as compared to mixture of H_2O and 2-PW (Figure 4-13B). This indicates that the SW contributed a majority of the anaerobic count (10^8 CFU/mL), whereas 2-PW had only 10^5 CFU/mL. All three mixtures showed a similar increase of log CFU/mL with time (Figure 4-13B). It should be noted that insufficient dilutions were performed in some cases, making it hard for bacterial counts to be analyzed.

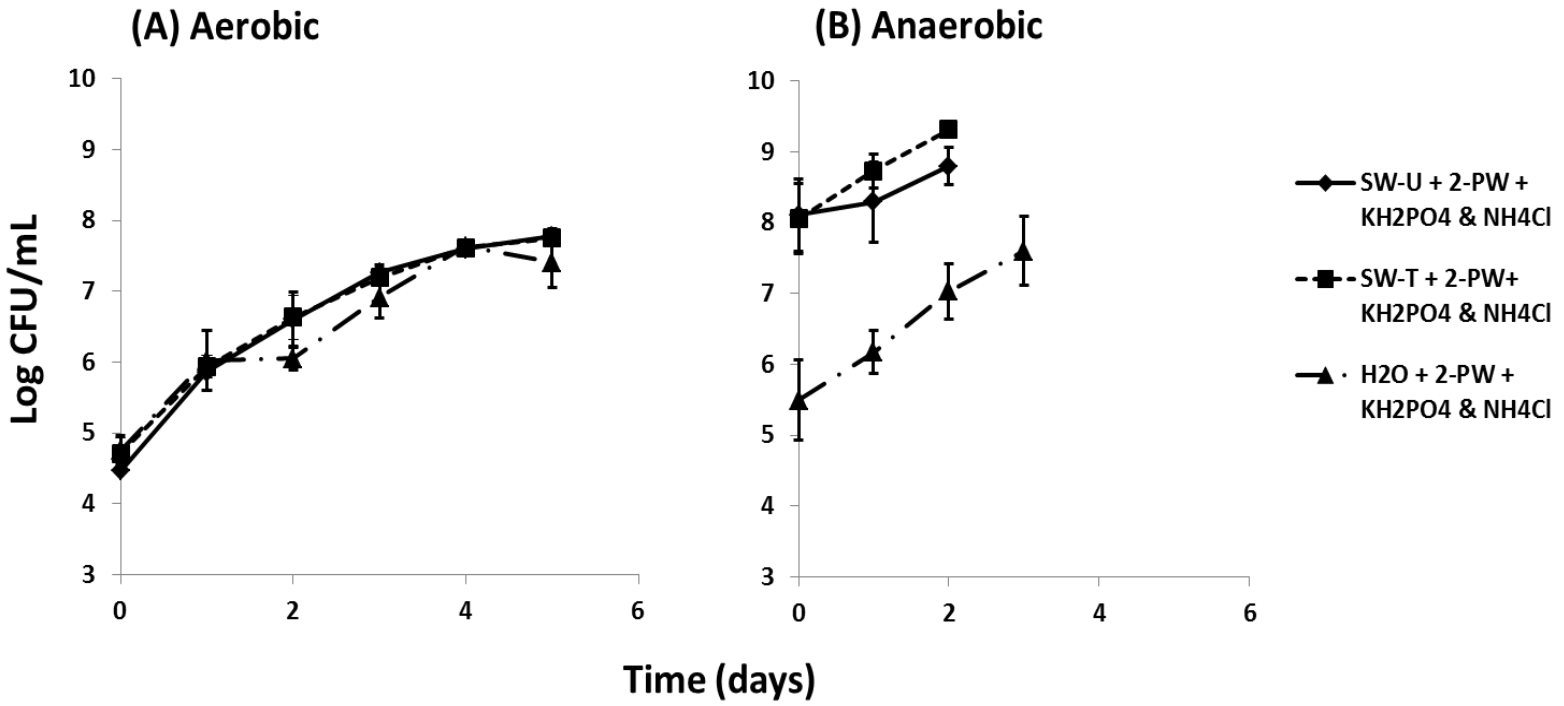


Figure 4-13: Microbial counts with nutrient addition for aerobic (A) and anaerobic (B) mixtures of source water samples (SW-U and SW-T) and produced water 2-PW with phosphate and ammonium nutrients. Water samples were obtained in March, 2010. The data shown are average counts for 2-3 dilutions; standard error bars are shown where these exceeded the size of the symbols.

Mixing of 22-SW and 2-PW without nutrients addition generally showed lower CFU/mL than mixing with nutrient addition. Microbial counts for aerobic incubation showed an increase of 1.7-2 log units, when 2-PW was mixed with 22-SW-U or 22-SW-T (Figure 4-14A). However, the experiment with deionized water showed no significant change in CFU/mL during the 9 days of incubation (Figure 4-14A). There was no distinct pattern for incubations without oxygen. Microbial counts were either constant or decreased for incubations of 2-PW with 22-SW, whereas they increased for incubations with H_2O (Figure 4-14B).

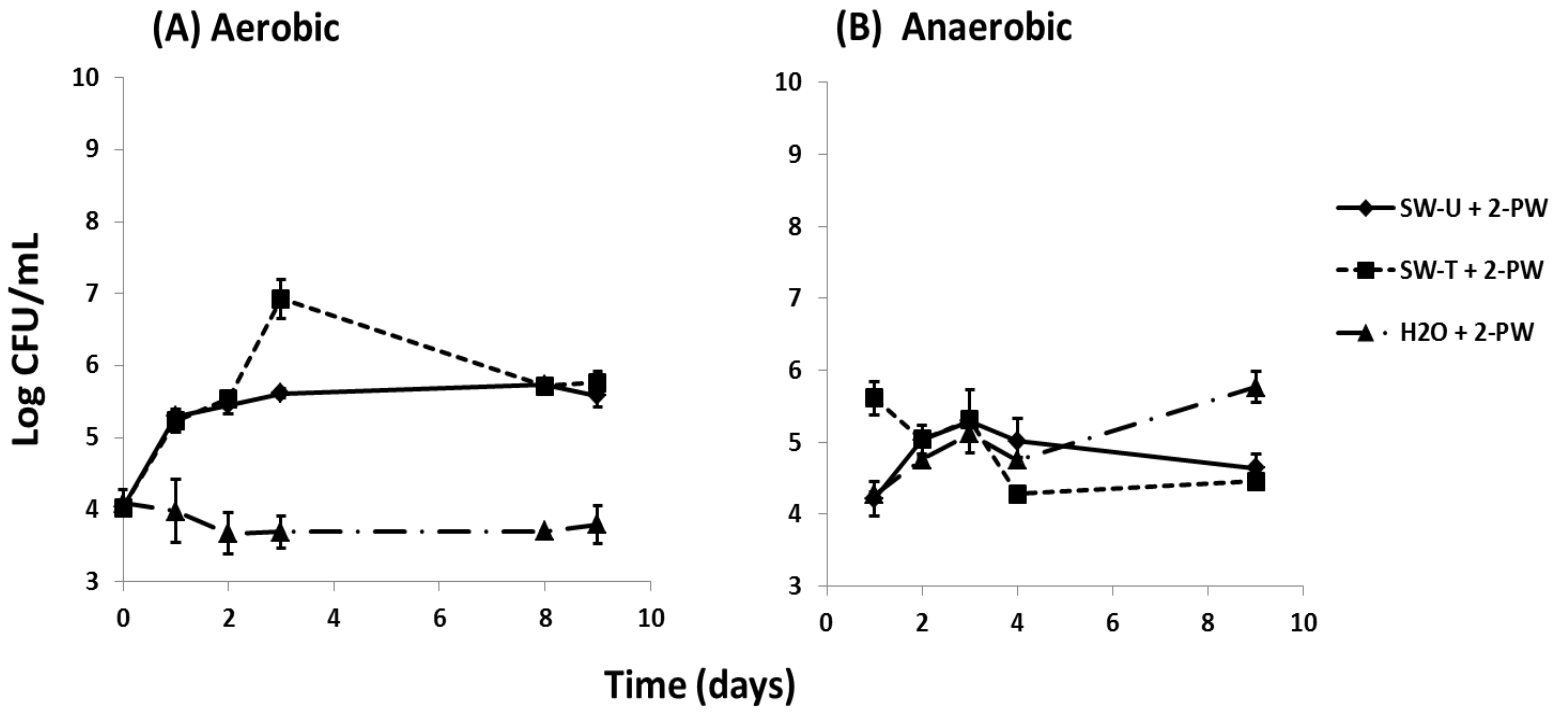


Figure 4-14: Microbial counts without nutrient addition for aerobic (A) and anaerobic (B) mixtures of source water samples (SW-U and SW-T) and produced water 2-PW without nutrient addition. Water samples were obtained in March, 2010. The data shown are average counts for 2-3 dilutions; standard error bars are shown where these exceeded the size of the symbols.

4.3.2 Effect of mixing SW and PW

In order to determine whether mixing of SW and PW provides more favorable conditions for growth, we compared the log CFU/mL as a function of time for individual samples with those for mixtures. A theoretical count, calculated for mixtures from the counts of individual samples, assuming no growth, was also calculated. Because in the field no nutrients are added and SW and PW are kept anaerobic, all experiments were done without nutrients addition under anaerobic conditions.

Anaerobic bacterial counts for samples received in September, 2011 are shown in Figure 4-15. Individual samples for 22-SW-U and 22-SW-F showed an increase in CFU/mL by 2-3 log units after 9 days of incubation. The CFU/mL of 22-SW-T and of 2-PW remained constant at 10^3/mL and at 10^5/mL, respectively. At the end of the 9 day incubation period 2-PW, 22-SW-U and 22-SW-F gave counts of about 10^5/mL while 22-SW-T gave counts of 0.62 x 10^2/mL (Figure 4-15A). Results for experiments where SW is mixed with PW in a 1:3 ratio are shown in Figure 4-15B. Mixing of SW and 2-PW gave mostly increases in log CFU/mL with time, whereas mixing of H_2O with 2-PW did not. In order to determine whether mixing of SW and PW gave additional growth we compare the results in Figure 4-15B with those in Figure 4-15C, which show the theoretical counts obtained by addition of individual counts observed at various times in Figure 4-15A. The theoretical counts were an average 1 log unit lower than those for the actual experimental mixture. However, this is also observed for the mixture of H_2O and 2-PW. We therefore cannot conclude that mixing gives additional growth.

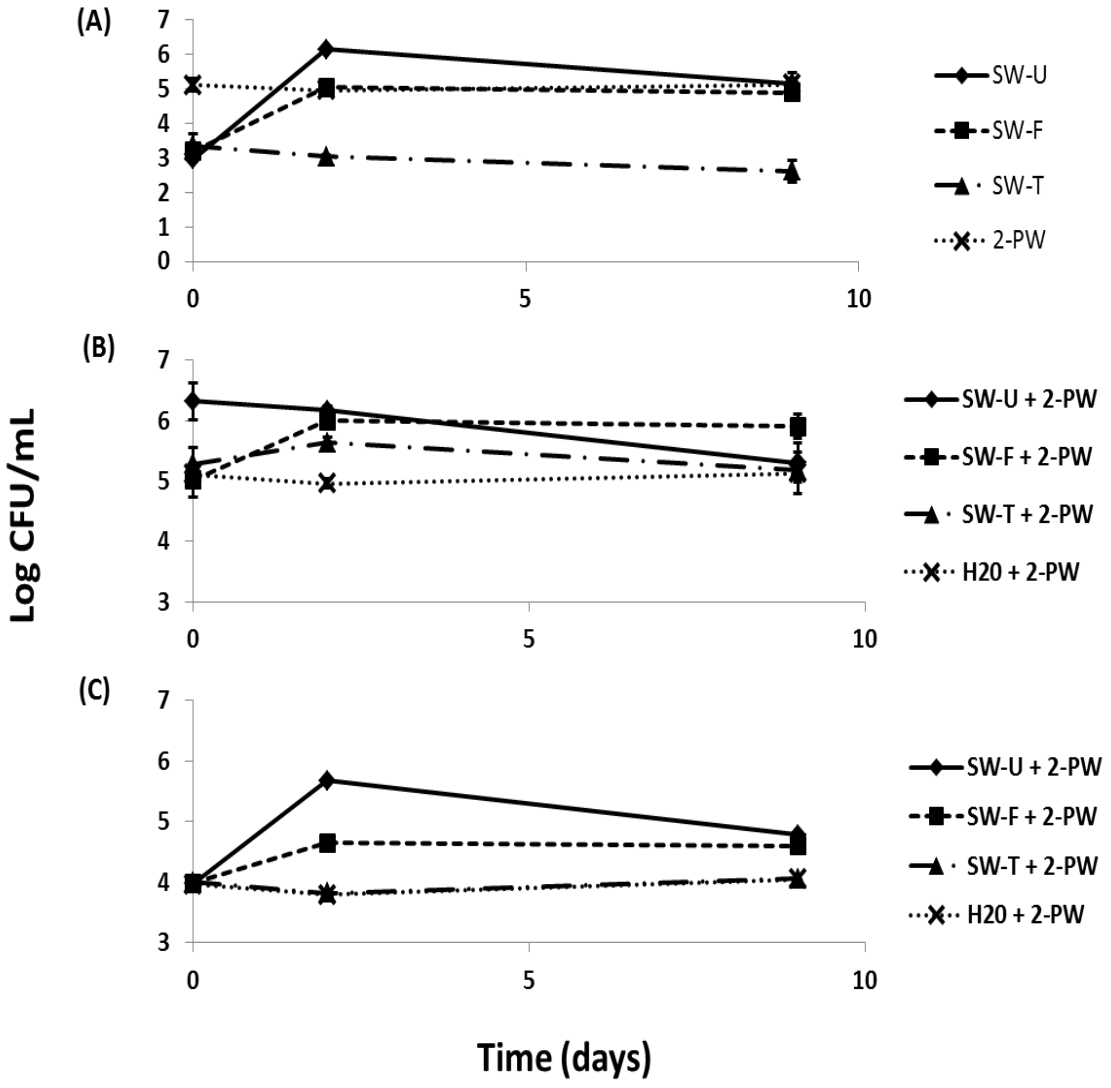


Figure 4-15: Anaerobic bacterial counts (CFU/mL) as a function of time (days) of samples obtained in September, 2011. Counts are for source waters SW-U, SW-F, SW-T and for produced water 2-PW samples obtained in September, 2011. Individual count of water samples (A), mixed samples imitating injection water (B) and theoretical counts obtained from individual sample counts (C) assuming no additional growth are shown. The data shown are average counts for 2-3 dilutions; standard error bars are shown where these exceeded the size of the symbols.

Anaerobic bacterial counts for samples received in October, 2011 are indicated in Figure 4-16. Individual samples SW-U and SW-T had low counts between 0 and 30/mL, whereas the CFU/mL in SW-F and 2-PW was higher at 10^5/ml (Figure 4-16A). Experiments in which a 1:3 ratio of each of SW samples was mixed with 2-PW gave an increase in the first two days of incubation by about 1 log unit to a log CFU/mL of 5.5-6.2, these numbers then declined somewhat (Figure 4-16B). The theoretical counts calculated for 1:3 mixtures of 22-SW with 2-PW, assuming no additional growth are shown in Figure 4-18C. Comparison with the data in Figure 4-16B supports the idea that mixing of the two waters gave additional growth of maximally 1 log unit.

Results obtained for samples received in December 2011 were comparable to those received in October 2011. SW-U lacked anaerobic heterotrophs, whereas SW-T had between 10-100/mL (Figure 4-17A). The log CFU/ml for SW-F and 2-PW was 4 initially, but increased by 1 to 1.5 log units upon incubation for 9 days (Figure 4-17A). Likewise, incubation of a 1:3 mixture of SW-U, SW-F or SW-T and 2-PW showed a strong increase in log CFU/mL with time of 1.5 log units (Figure 4-17B). Comparison with the theoretical counts, calculated as the 1:3 mixture of the counts of individual samples (Figure 4-17C), indicates that smaller increases in log CFU/mL occurred upon mixing of these SW and PW waters of maximally 0.3 log units.

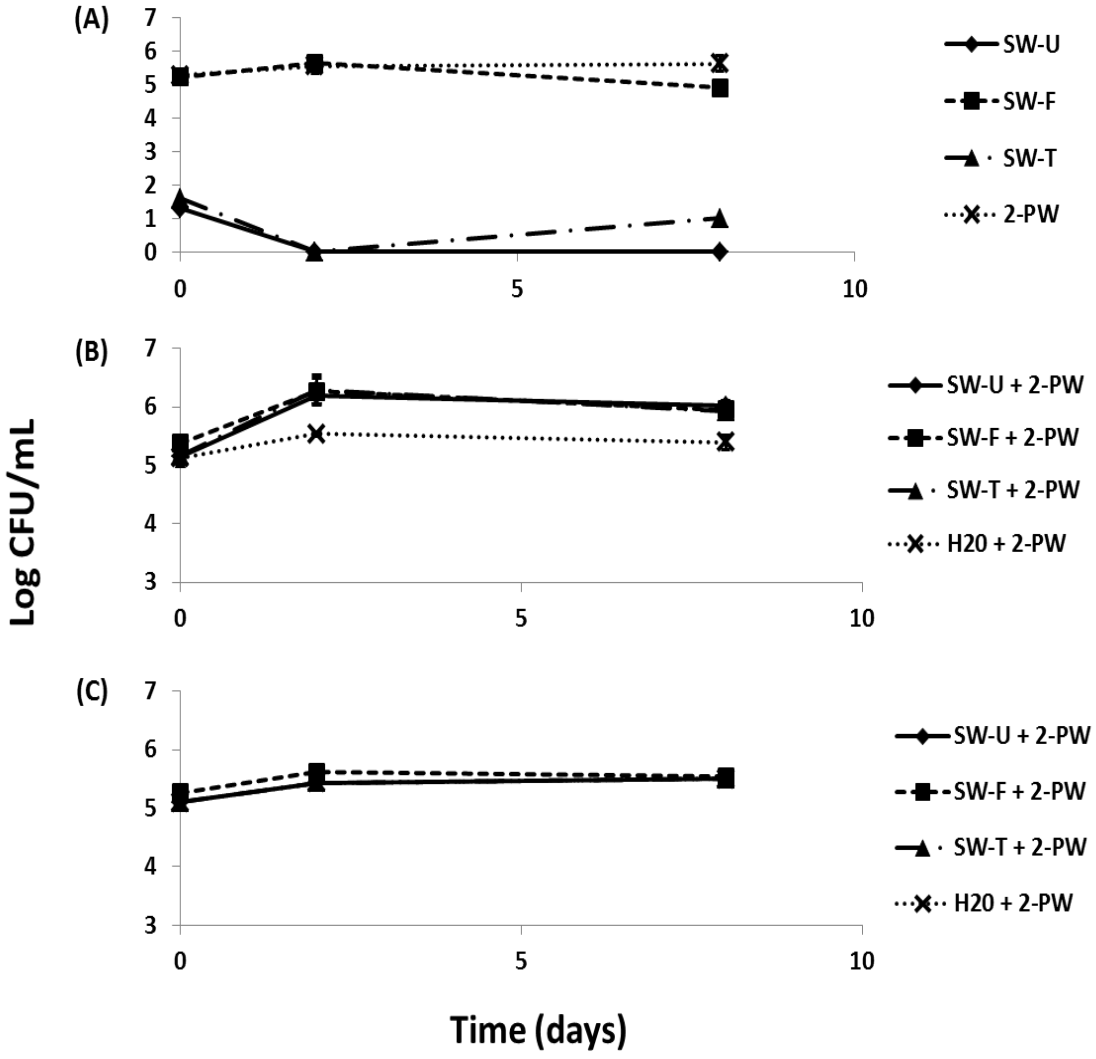


Figure 4-16: Anaerobic bacterial counts (CFU/mL) as a function of time (days) of samples obtained in October, 2011. Counts are for source waters SW-U, SW-F, SW-T and for produced water 2-PW samples obtained in September, 2011. Individual count of water samples (A), mixed samples imitating injection water (B) and theoretical counts obtained from individual sample counts (C) assuming no additional growth are shown. The data shown are average counts for 2-3 dilutions; standard error bars are shown where these exceeded the size of the symbols.

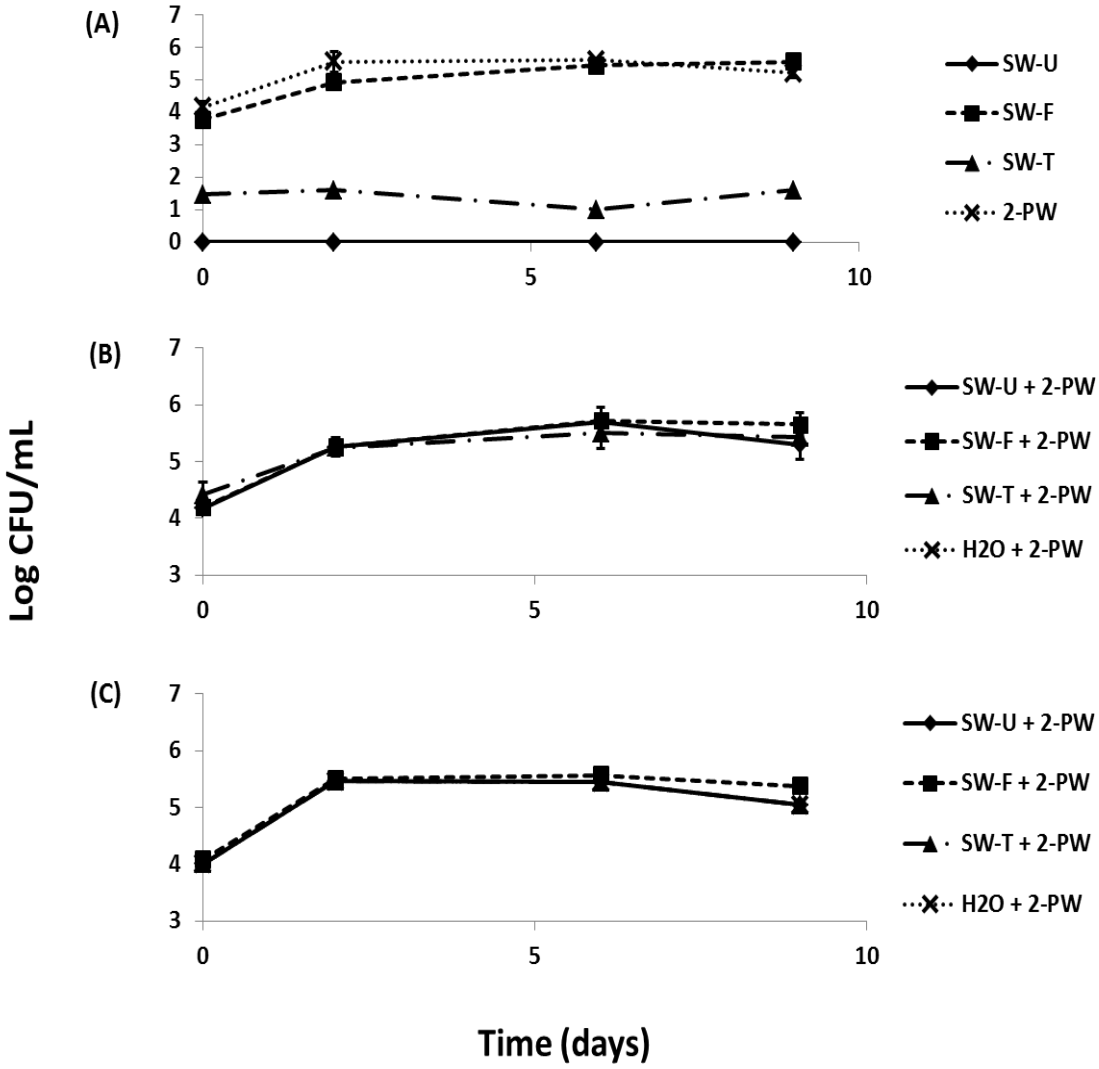


Figure 4-17: Anaerobic bacterial counts (CFU/mL) as a function of time (days) of samples obtained in December, 2011. Counts are for source waters SW-U, SW-F, SW-T and for produced water 2-PW samples obtained in September, 2011. Individual count of water samples (A), mixed samples imitating injection water (B) and theoretical counts obtained from individual sample counts (C) assuming no additional growth are shown. The data shown are average counts for 2-3 dilutions; standard error bars are shown where these exceeded the size of the symbols.

Anaerobic bacterial counts for samples received in January 2012 are shown in Figure 4-18. 22-SW-T had log CFU/mL = 5.5 while 2-PW had log CFU/mL = 6 after 9 days of incubation. No anaerobic heterotrophs were present in SW-U and SW-F (Figure 4-18A). Incubation of mixtures of SW-U, SW-F or SW-T and 2-PW gave bacterial counts between 5-5.3 log units (Figure 4-18B). Theoretical counts calculated from data for individual samples (Figure 4-18C) were comparable to those of Figure 4-18B. Hence, no evidence for additional growth for mixing was obtained.

Overall there was no strong evidence obtained for additional growth with mixing of SW and PW waters, except for samples collected in October, 2011 (Figure 4-16). An interesting observation was that CFU/mL for 22-SW-U (untreated source water) were generally lower than that for filtered and treated SW (22-SW-F and 22-SW-T). The benefits of treatment (chlorination, addition of bisulfite and acrolein) are thus not clear.

Anaerobic bacterial counts were also done for central water plant samples received from September 2011 – January 2012 (Figure 4-19). All samples showed an increase in bacterial counts with time, the largest increase occurring in October, 2011. Typical counts of water leaving the water plant to be injected in the reservoir are 10^5 to 10^6 CFU/mL.

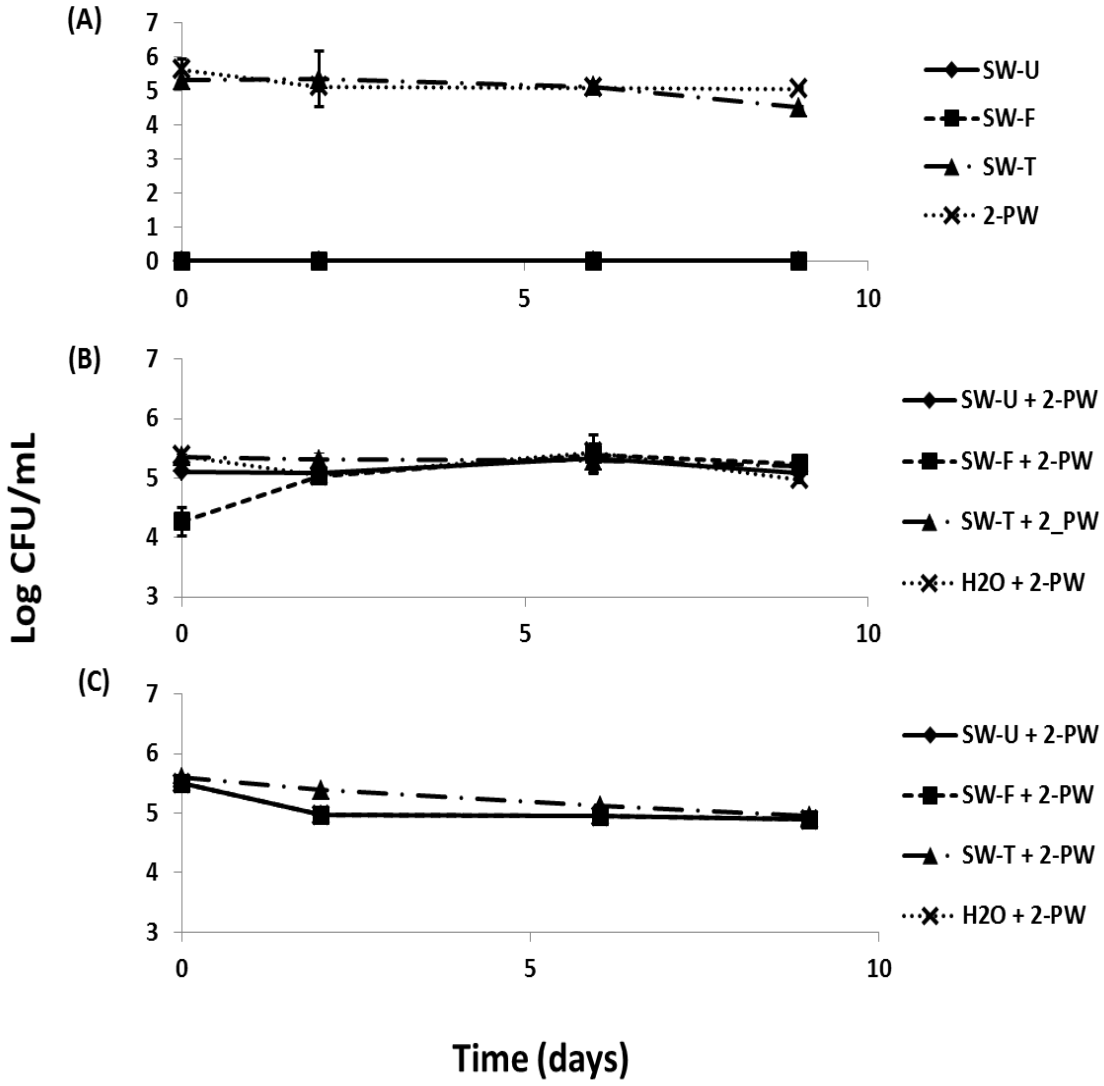


Figure 4-18: Anaerobic bacterial counts (CFU/mL) as a function of time (days) of samples obtained in January, 2012. Counts are for source waters SW-U, SW-F, SW-T and for produced water 2-PW samples obtained in September, 2011. Individual count of water samples (A), mixed samples imitating injection water (B) and theoretical counts obtained from individual sample counts (C) assuming no additional growth are shown. The data shown are average counts for 2-3 dilutions; standard error bars are shown where these exceeded the size of the symbols.

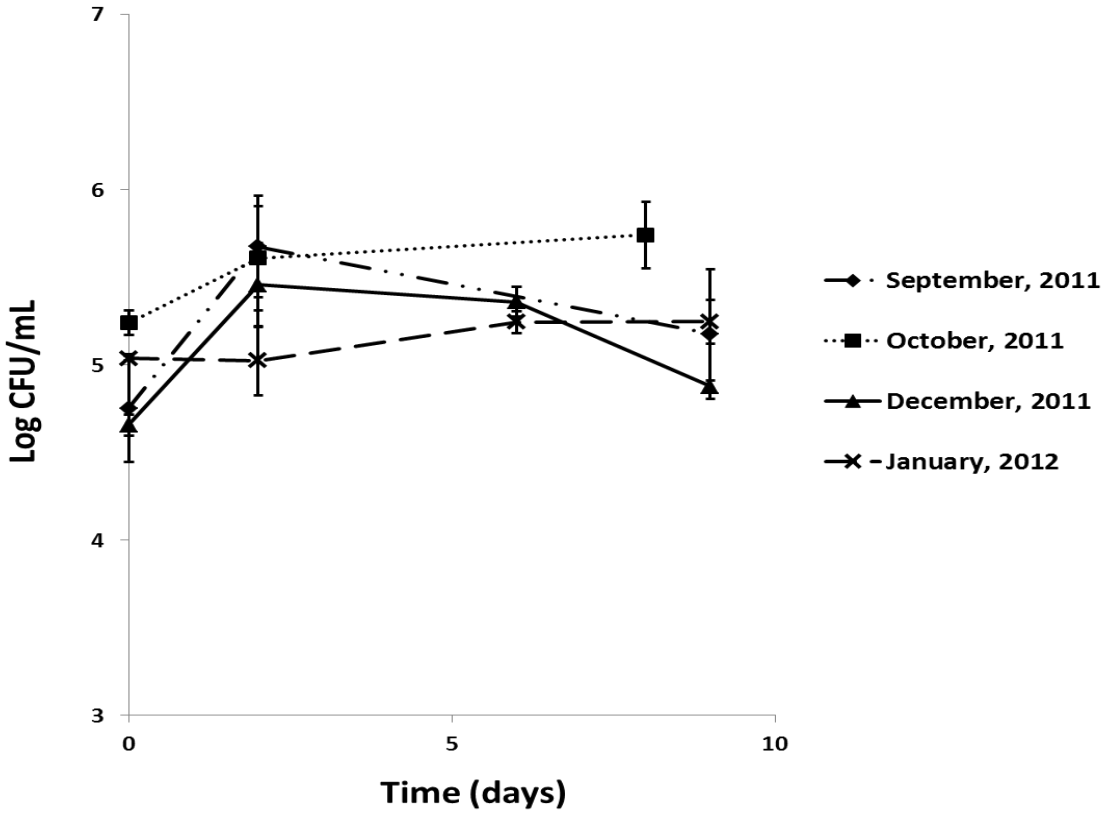


Figure 4-21: Anaerobic bacterial counts (CFU/mL) as a function of time (days) of central water plant samples (1-WP) obtained from September 2011 to January, 2012. The data shown are average counts for 2-3 dilutions; standard error bars are shown where these exceeded the size of the symbols.

4.3.3 Bioinformatics analyses of communities in SWs, PWs, 1-WP and 14-IW

As indicated in the previous sections oil is produced by reinjection of PW, which is mixed with SW in a water plant. SW is needed because not all water that is injected is recovered as PW. A question in microbiology of oil fields is whether the community present downhole is native or was introduced by injecting SW. This question can be answered by comparing community compositions of SW and PW.

Community compositions were analyzed by pyrosequencing of PCR-amplified 16S rRNA genes. The number of reads obtained is shown in Table 4-9. A total of 71,777 good reads (i.e. following chimera removal) was obtained for the 11 samples. Of these 21,392 reads were for 6 SW samples collected in September and October, coded as 22-SW-U-Sep, 22-SW-F-Sep, 22-SW-T-Sep, 22-SW-U-Oct, 22-SW-F-Oct and 22-SW-T-Oct, whereas 26,484 were for samples from producing wells 10-PW-Oct, 12-PW-Sep and 13-PW-Sep. Samples from injection well 14-IW-Aug and from the water plant 1-WP-Aug gave 13,376 and 10,525 good reads, respectively (Table 4-9). The entire set of the raw reads for the 11 samples is available from Sequence Read Archive (SRA) at NCBI under accession number SRP010235.

Community compositions of 1-WP and 14-IW samples are expected to be intermediate between SW and PW waters because they are mixtures of SW and PW waters. Therefore comparison of microbial community composition will be done primarily for just SW and PW samples. The amplicon sequence libraries obtained for SW samples were more diverse than those for PW samples, as indicated by rarefaction curves (Figure 4-22), which indicated more OTUs for a given number of sequence reads for SW

than for PW samples. The Chao estimated maximum number of OTUs was, likewise, higher for SW samples (227-933) than for PW samples (140-318). Shannon's H indices, which also measure diversity, were 2.36-4.05 and 1.49-2.16 for SW and PW samples, respectively (Table 4-9). SW OTUs were mostly affiliated with the *Bacteria*, whereas PW OTUs were mostly affiliated with the *Archaea* (Table 4-9).

The sequences obtained for all samples are compared by NMDS analysis in Figure 4-23 and by a relational tree in Figure 4-24. The NMDS plot indicates that sequences for SW samples separated clearly from the PW and WP-IW samples (Figure 4-23). Sequences for 22-SW-U-Oct, 22-SW-F-Oct and 22-SW-T-Oct formed a sub-cluster, which was distinct from those for 22-SW-U-Sep, 22-SW-F-Sep and 22-SW-T-Sep. Likewise, communities from PW samples clustered distinctly from those of WP and IW samples (Figure 4-24). Similar results were obtained in a relational tree, which indicated that SW samples clustered distinctly from PW and WP and IW samples (Figure 4-24). The data in Figure 4-23 and 4-24 indicate that the libraries for 22-SW-F-Oct and 22-SW-T-Oct were most similar.

This is also evident from a comparison of microbial community compositions in supplementary Table C-1, Appendix C. Both have a high fraction of *Lachnospiraceae*, lacking from most other samples except 22-SW-U-Oct. Average fractions for the six SW samples (F_{SW}) and the three PW samples (F_{PW}) are listed in Table 4-10 for 44 taxa with a combined representation ($F_{SW}+F_{PW}$) in excess of 0.5%. The ratio R indicating the average prevalence of a taxon in SW as compared to PW amplicon libraries has also been tabulated. The data indicate that, whereas all 44 taxa are represented in the SW samples,

19 are missing from the PW samples (Table 4-10: bold). This includes 9 taxa affiliated with the *Betaproteobacteria* (*Comamonadaceae, Zoogloea, Neisseriaceae/*uncultured and *Neisseriaceae, Azospira, Methylophilus, Nitrosomonas, Pelomonas* and *Malikia*). Amplicon libraries of PW were dominated by anaerobic microbes such as the SRB *Desulfomicrobium* and *Desulfovibrio*, the methanogenic archaea *Methanolinea*, *Methanosaeta, Methanoculleus* and others, like the syntroph *Smithella* and the nitrate-reducing genus *Thauera*. However, all of these anaerobic taxa were also present in SW samples (Table 4-10).

The overall conclusion is thus that all taxa found in PW are present in SW, but not all taxa found in SW are present in PW. The subsurface microbial community may well have originated from SW, but not all taxa present in SW can grow in the subsurface. In relation to CCS, it is clear that methanogens are a large fraction of the community in produced waters. Acetogens like *Acetobacterium* which belong to the phylum *Firmicutes* were not a major community component.

Table 4-9: Statistics and bioinformatics analysis of pyrotag sequences obtained for the amplicon libraries from 6 SW samples, 3 PW samples, and from an IW and WP sample.

Sample	ID	Reads			5% cutoff		
		Chimeras removed	*Archaea*	*Bacteria*	OTUs	Estimated OTUs (Chao)	Shannon's H index
12-PW-Sep	589	8571	6312	2259	172	318	1.75
13-PW-Sep	590	8428	7658	770	119	211	2.16
22-SW-F-Sep	598	8520	5	8324	219	394	3.25
22-SW-T-Sep	599	5211	94	5071	447	933	4.05
22-SW-U-Sep	600	1893	2	1891	144	231	2.77
22-SW-F-Oct	629	2865	227	2636	204	315	2.81
22-SW-T-Oct	630	2297	208	2087	139	227	2.36
22-SW-U-Oct	631	606	3	593	124	250	3.22
10-PW-Oct	653	9485	758	8727	101	140	1.49
1-WP-Aug	516	13376	2543	10833	405	686	2.79
14-IW-Aug	524	10525	2944	7581	394	682	3.67

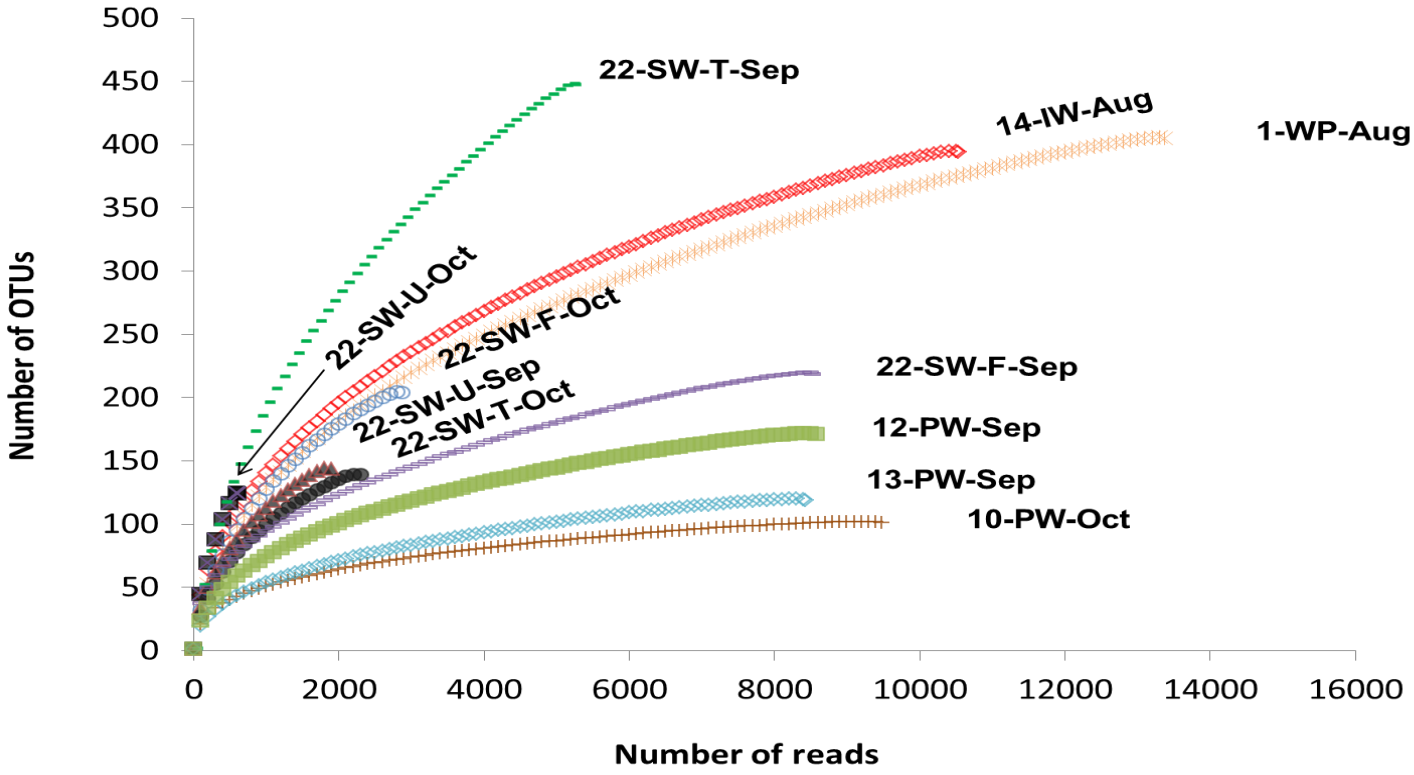


Figure 4-22: Rarefaction curves. Graphical representation of the diversity between each of the 11 amplicon libraries indicated in Table 4-10. The number of OTUs (Y-axis) is plotted against the number of sequence reads analyzed (X-axis).

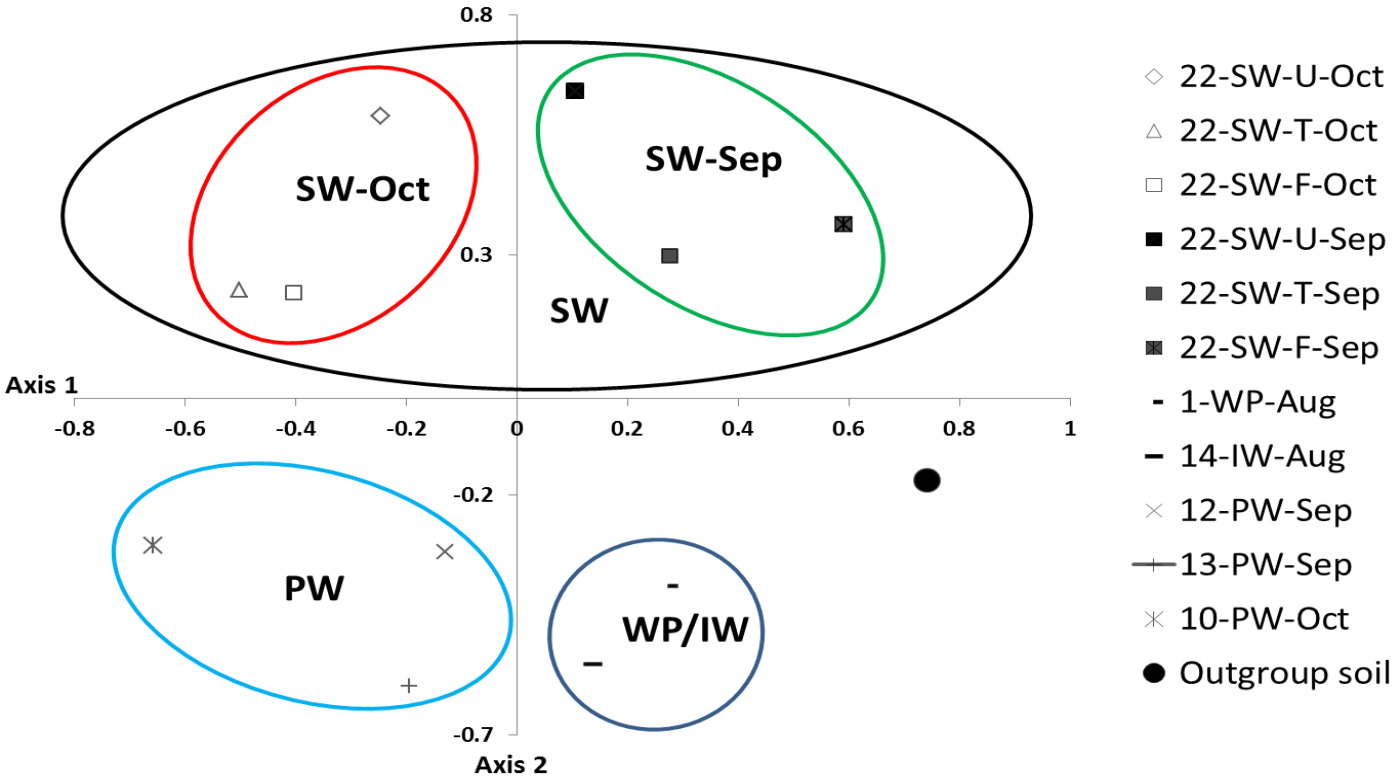


Figure 4-23: Non-metric multidimensional scaling (NMDS) ordination. Each point in the plot represents an amplicon library, as indicated in Table 4-10. Separation of libraries for SW samples from those for PW and WP/IW samples is indicated by the ellipses drawn. The amplicon library from soil (NCBI SRA accession number SRX1147758) served as the outgroup.

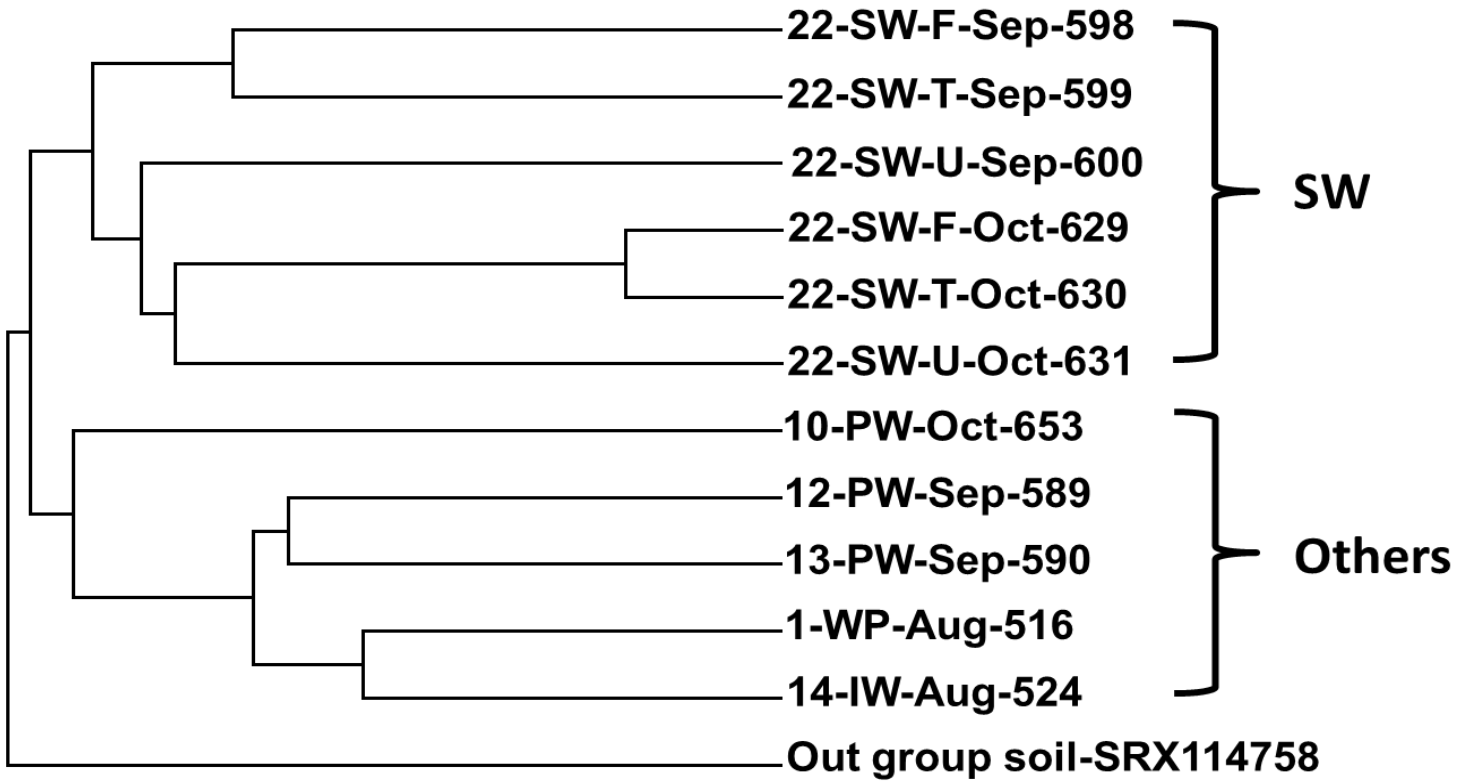


Figure 4-24: Bray Curtis sample relation tree generated using the UPGMA algorithm and visualized with Dendroscope software. The sequences for SW sampled clustered distinct from those for other (PW and WP/IW) samples. The amplicon library from soil (NCBI SRA accession number SRX1147758) served as the outgroup.

Table 4-10: Average distribution of reads (%) over taxa for 6 SW samples (F_{SW}) and for 3 PW samples (F_{PW}). The distribution of reads (%) obtained from 454 sequencing of 16S rRNA genes over taxa are shown.

Number of reads	21392	26484		
Taxon (Phylum; class; order; family; genus)	**F_{SW}**	**F_{PW}**	**$F_{SW}+F_{PW}$**	**Ratio R**
Firmicutes_Clostridia_Clostridiales_Clostridiaceae_Clostridium	7.894	**0.000**	7.894	7894
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae	5.898	**0.000**	5.898	5898
Bacteroidetes_Flavobacteria_Flavobacteriales_Flavobacteriaceae_Flavobacterium	4.602	**0.000**	4.602	4602
Bacteroidetes_Sphingobacteria_Sphingobacteriales_Chitinophagaceae_Niabella	2.249	**0.000**	2.249	2249
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Zoogloea	2.087	**0.000**	2.087	2087
Proteobacteria_Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Sphingopyxis	1.912	**0.000**	1.912	1912
Proteobacteria_Gammaproteobacteria_Xanthomonadales_Sinobacteraceae_Nevskia	1.396	**0.000**	1.396	1396
Proteobacteria_Betaproteobacteria_Neisseriales_Neisseriaceae_uncultured	1.395	**0.000**	1.395	1395
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Azospira	1.208	**0.000**	1.208	1208
Proteobacteria_Betaproteobacteria_Methylophilales_Methylophilaceae_Methylophilus	1.134	**0.000**	1.134	1134
Proteobacteria_Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae	1.070	**0.000**	1.070	1070
Proteobacteria_Betaproteobacteria_Nitrosomonadales_Nitrosomonadaceae_Nitrosomonas	0.937	**0.000**	0.937	936.8
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Pelomonas	0.923	**0.000**	0.923	923.3
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae	10.253	0.012	10.265	809.4
Planctomycetes_Planctomycetacia_Planctomycetales_Planctomycetaceae_Planctomyces	0.771	**0.000**	0.771	771.3
Proteobacteria_Epsilonproteobacteria_Campylobacterales_Helicobacteraceae_Sulfuricurvum	0.771	**0.000**	0.771	770.8
Bacteroidetes_Flavobacteria_Flavobacteriales_Flavobacteriaceae	0.650	**0.000**	0.650	650.3
Proteobacteria_Betaproteobacteria_Neisseriales_Neisseriaceae	0.623	**0.000**	0.623	622.7
Proteobacteria_Gammaproteobacteria_Aeromonadales_Aeromonadaceae_Aeromonas	0.615	**0.000**	0.615	614.7
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Malikia	0.597	**0.000**	0.597	597.2
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Moraxellaceae	6.619	0.019	6.638	325.5
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Aquabacterium	0.961	0.004	0.965	192.3
Proteobacteria_Betaproteobacteria_Burkholderiales_Oxalobacteraceae	2.140	0.012	2.152	164.6
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Moraxellaceae_Acinetobacter	0.773	0.004	0.777	154.5
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Rhodoferax	1.943	0.027	1.970	68.56
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Methylibium	0.501	0.007	0.508	62.67
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae	0.610	0.023	0.633	25.05
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Pseudomonadaceae	13.748	2.246	15.993	6.119
Proteobacteria_Epsilonproteobacteria_Campylobacterales_Helicobacteraceae_Sulfurimonas	0.465	0.230	0.695	2.017
Proteobacteria_Deltaproteobacteria_Desulfuromonadales_Geobacteraceae_Geobacter	0.233	0.516	0.748	0.450
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Candidatus_Methanoregula	0.140	0.378	0.518	0.369
Proteobacteria_Epsilonproteobacteria_Campylobacterales_Campylobacteraceae_Arcobacter	0.131	0.523	0.654	0.250
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Thauera	0.288	1.737	2.025	0.166
Proteobacteria_Deltaproteobacteria_Desulfovibrionales_Desulfovibrionaceae_Desulfovibrio	0.316	1.996	2.312	0.158
Proteobacteria_Deltaproteobacteria_Syntrophobacterales_Syntrophaceae_Smithella	0.085	0.807	0.892	0.105
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanoculleus	2.033	31.372	33.405	0.065
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosaetaceae_Methanosaeta	0.147	2.992	3.139	0.049
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanocalculus	0.165	3.520	3.685	0.047
Spirochaetes_Spirochaetes_Spirochaetales_Spirochaetaceae_uncultured	0.028	0.621	0.649	0.045
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanofollis	0.071	1.673	1.744	0.042
Proteobacteria_Alphaproteobacteria_Rhodospirillales_Rhodospirillaceae_uncultured	0.023	0.559	0.583	0.042
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanolinea	0.293	7.684	7.978	0.038
Euryarchaeota_Methanomicrobia_Methanosarcinales	0.250	8.970	9.221	0.028
Proteobacteria_Deltaproteobacteria_Desulfovibrionales_Desulfomicrobiaceae_Desulfomicrobium	0.022	1.429	1.451	0.015

Values for individual samples are shown in Appendix C Table C3. Only entries with (F_{SW} +F_{PW})>0.5% and entries with a taxon description beyond the phylum level are shown.

The ratio R was used to rank the list, $R = F_{SW}/(F_{PW} + 0.001)$; 0.001 was added to be able to represent entries with F_{PW}=0.

Chapter 5 DISCUSSION

The reduction in the emission of greenhouse gases such as CO_2 has become of great concern amongst industrialized countries (e.g. Canada) around the world. Carbon capture and storage (CCS) is one of the solutions to this problem. Although research on this topic has been done in other areas of science (such as geology and chemistry) and engineering, very little understanding on the effect of microorganisms in subsurface locations such as oil fields with regards to CCS has been obtained. Research on the microbiology of oil fields has shown that microbes in the subsurface are able to convert CO_2 to products such as acetic acid and methane. We have therefore studied the microbial community in oil field waters as well as the activity of microorganisms capable of utilizing CO_2 with the hope to better understand how the process of CO_2 storage in depleted oil fields may affect subsurface microbial communities.

5.1 Microbial community in oil field produced water

The microbial community present in 3-PW (Figure 4-1 and 4-2) provided us with knowledge of a microbial community present in a specific subsurface oil reservoir. The top microbial genera present were mostly methanogens regardless of when the 3-PW sample was obtained. The microbial genera present in 3-PW obtained in October, 2010 (Figure 4-1) included hydrogenotrophic methanogens such as *Methanoculleus* (66.5%), *Methanobacterium* (1.4%), *Methanocalculus* (1.2%) and *Methanofollis* (0.9%) which are capable of converting H_2/CO_2 to methane (Barret et al., 2012; Sakai et al., 2012; Zhu et al., 2011; Mori et al., 2000 and Lai & Chen, 2001). The acetotrophic methanogen *Methanosaeta* was also present at 22.9% and is capable of converting acetic acid to

methane (Zhu et al., 2012). Other microbial genera present in 3-PW from October 2010 were nitrate reducing bacteria *Thauera* (0.7%) and *Rhodobacter* (0.5%) (Scholten et al., 1999 and Pino et al., 2006). The top microbial genera present in the 3-PW sample obtained in September 2011 (Figure 4-2) also included hydrogenotrophic methanogen *Methanoculleus* (58.8%), acetotrophic methanogen *Methanoseata* (9.3%) and higher percentage of the nitrate reducing bacterium *Thauera* (5.4%). Other microbial genera present in the 3-PW September 2011 sample were hydrogenotrophic methanogen *Methanolinea* (4.6%) and *Pseudomonas* (4.2%) (Imachi et al., 2008). *P. putida*, a species of the microbial genera *Pseudomonas* is a common hydrocarbon degrader (Nelson et al., 2002). In the paper by Magot et al., (2000) on the microbiology of petroleum reservoirs, hydrogenotrophic methanogens such as *Methanobacterium* and *Methanofollis* and acetotrophic methanogen such as *Methanosarcina* were found to be important components of oil reservoirs. The temperature and salinity of an oil reservoir was also proposed to affect its microbial community composition (Magot et al., 2000). Therefore microorganisms which convert CO_2 and/ acetic acid to methane were found to be present in high abundance in the oil field tested (MHGC) and in those tested by others.

5.2 Enrichment of 3-PW in media X, X5 and A

The results from the microbial activity measurements of methanogens and acetogens with X, X5 and A media always showed initial high acetogenic activity followed much later by methane production except for experiments with inhibitor (20 mM BESA in Figure 4-2B and 4-4A; and 10 mg/L rifampicin in Figure 4-4B). BESA has been used to inhibit methanogenesis in experiments where high concentrations of acetate were

observed (Horn et al., 2003). Although acetogens of the phylum *Firmicutes* such as *Acetobacterium* were not found by pyrosequencing in the 3-PW sample used for inoculation (Table 4-1), concentrations of acetic acid at the end of the enrichment period were between 22-54 mM.

Methanogenic activity was always observed after 20 days of incubation in some cases, even in the presence of the antibiotic rifampicin (Figure 4-4B). These results were not expected as the community composition of 3-PW was dominated by different genera of methanogens (92.9%). Rifampicin is able to inhibit *Bacteria* (such as acetogens) and not *Archaea* (such as methanogens) because their metabolic processes and cell wall compositions are different (Dridi et al., 2011). Rifampicin inhibits the bacterial RNA polymerase, an enzyme responsible for DNA replication and transcription in *Bacteria* (Dridi et al., 2011). There was no literature found on the time it takes for rifampicin to breakdown and become active.

The conversion of acetate to methane by acetotrophic methanogens also did not occur (Figure 4-3B), even though *Methanosaeta* was present at a relatively high abundance in 3-PW (22.9%). *Methanosaeta* is known to grow in conditions of lower acetate conditions in comparison to other acetotrophic methanogens such as *Methanosarcina* which can grow when acetate concentration is between 0.2 mM and 1.2 mM (Jetten et al., 1992, Conrad, 1999 and Zinder, 1993). The threshold concentrations of acetate required to culture some species of *Methanosaeta* is 7-70 µM, much lower than 20 mM of acetate used in our experiments (Conrad, 1999 and Janssen, 2003). *Methanosaeta* have also been known to be sensitive to agitation (or shaking) which was

also required in our experimental procedure to keep the culture homogenous (Dannenberg et al., 1997).

Research on the activity of homoacetogens has shown that they compete for H_2 with hydrogenotrophic methanogens. Competition has also been observed under various temperature conditions including at 30°C in which our experiments were incubated (Stams, 1994; Schink, 1997; Fey and Conrad, 2000; Kotsyurbenko et al., 2001; Grabowski et al., 2005). In anaerobic environments, there is a stiff competition by different microbial groups for H_2, which is an intermediate produced from the anaerobic degradation of organic matter and thus has a high turnover. The main consumers of H_2 are usually methanogens and homoacetogens and the results of their competition is production of acetate followed by production of methane or, production of methane directly from H_2 and CO_2 (Fey & Conrad, 2000; Westermann, 1994 and Conrad, 1999).

It seems that the methanogens thrive at low pH because methane production was observed when pH of some enrichments was as low as 5.2 (Table 4-3). This was also observed in a study where the methanogen *Methanosarcina barkeri* was able to grow in conditions where the pH was between 6.25 and 5.5 (Staley et al., 2011). The genus *Methanosarcina* was not present in the community compositions of 3-PW but some members of the order *Methanosarcinales* under which it is classified, such as the genera *Methanosaeta* and *Methanolobus* were present (Tables 4-1 and 4-2). The pH of enrichments as a function of time was measured in subsequent experiments in order to determine the effect of pH on acetogenesis and methanogenesis.

The microbial community composition results from enrichment derived from 3-PW with media X, X5 and A with a headspace of H_2/CO_2 (80:20) showed a high

percentage of methanogens (Table 4-5). The hydrogenotrophic methanogen *Methanobacterium* was mostly present in enrichments with defined nutrients content (media X and X5) at pH 5.5 while the hydrogenotrophic methanogen *Methanoculleus* was mostly present in enrichments with high nutrient content (A media) (Table 4-5). *Acetobacterium* was also present in all three media, but at low percentage between 0.5 – 2.5% even though high concentrations of acetic acid was observed (Figure 4-6 and Table 4-5). This may be due to the fact that isolation of DNA was not done until the end of enrichment when production of methane was at its peak. Also, because we do not have the community composition of the methanogens capable of growing when rifampicin was added to enrichments with X5 medium, we are not able to determine which genus or genera of methanogen is responsible for the activity we observed. This is because *Methanobacterium* which was the most active methanogens in X5 medium with H_2/CO_2 (80:20) has been reported to be sensitive to antibiotics such as kanamycin (Patel et al., 1990). Although rifampicin has not been reported to inhibit methanogenesis (Brauer et al., 2004; Youngster et al., 2008 and Figure 4-5B), it would be interesting to have determined what type of methanogens thrive when it is used to inhibit acetogenesis. The community composition however contained a low percentage of *Methanocalculus* (0.02% in X5 and 0.004% in A media), *Methanofollis* (0.01% in X and A media) and no *Methanosaeta* which were present in 3-PW. Other microbial taxa which were present included sulfate reducing bacteria *Desulfovibrio* (Zhang et al., 2007) as well as anaerobic fermentative bacteria *Spirochaeta* (Breznak & Warnecke, 2008) and *Petrimonas* (Grabowski et al., 2005). A higher diversity was obtained in medium A (1.09) in comparison to media X and X5, 0.59 and 0.40 respectively, as shown in Shannon index

column of Table 4-4. This is possibly because yeast extract which is present in medium A (2 g/L) provided additional nutrient for the growth of wide range of microorganisms.

5.3 Effect of hydrogen and pH on microbial activity

The production of high concentrations of acetic acid by acetogens reduced the pH of enrichment media (Table 4-2). It was also observed that the methanogens seemed to be more active after the pH of enrichment medium is lowered by the acetogens (sections 4.1.3, 4.1.4 and 4.15). It was therefore necessary to monitor the pH of subsequent experiment in order to validate our observations.

Although the energy yield required for H_2-dependent methanogenesis (- 131 kJ) is higher than that for H_2-dependent acetogenesis (- 95 kJ), the results we obtained in our experiments show that acetogenesis starts before methanogenesis (equations 2 and 4; Figure 4-6). Although the acetogens out compete the methanogens for available H_2 at high concentrations (Fey and Conrad, 2000; Westermann, 1994, Conrad et al., 1986, and Conrad, 1999), in our case it was because the methanogens were mostly inactive in 3-PW samples which were stored for a long time.

We also decided to determine if a lower percentage of headspace H_2 in enrichments bottles will favour either acetogenesis or methanogenesis. Results obtained were from X5 enrichments inoculated with 3-PW obtained from the field in September, 2011. The primary enrichment experiments showed the usual trend of high acetogenic activity early into incubations followed by methanogenic activity. In experiments with H_2/CO_2 (80:20), methanogenic activity started on day 18 when the acetate concentration was 26.3 mM and enrichment pH was 6 (Figure 4-7). The gas calculations from the

volume of H_2/CO_2 (80:20) used showed that methanogenic activity was strictly by hydrogenotrophic methanogens. In the experiments with $H_2/CO_2/N_2$ (5:10:85), methanogenic activity started on day 8 when acetate concentration was 0.4 mM and no drop in pH was observed (Figure 4-8). Because the percentage of H_2 and CO_2 were quite low, the amount of gas use was not easily observed (except in calculations) and could therefore not be measured. It was thus evident from our results that when high concentrations of hydrogen were present (80%), methanogenic activity continued while acetogenic activity stopped. It was also evident that when low concentrations of hydrogen was present (5%), both methanogenic and acetogenic activity continued to occur. In experiments carried out to constrain H_2 concentration in subsea floor sediments, the authors suggest that in the upper methanogenic zone of the ocean the low concentrations of H_2 available is suitable for methanogens to compete favourable against the acetogens (Lin et al., 2012). This is because methanogens are capable of survival in the presence of low amounts of energy and at low H_2 concentration (Kotsyurbenko et al., 2001; Hoehler et al., 2001 and Lin et al., 2012).

A study on the effect on acetic acid concentrations on acetogenesis (Baronofsky et al, 1984) showed that the growth of the acetogen *Clostridium thermoaceticum*, is hindered when the growth medium reaches a pH of about 5. This is because the acetic acid produced acts as an uncoupling agent and causes the cytoplasm of the cells of *C. thermoaceticum* to become acidified to an untolerable level (Baronofsky et al., 1984). The effect of pH on the activity of acetogens and methanogens was tested by transferring 1 mL sample of primary enrichment with H_2/CO_2 (80:20) into X5 medium with starting pH of 5.6, 6.2, 7.6 and 8.5 by amending the medium with different concentration of

acetate (Figure 4-9A). Overall only methanogenic activity was observed in all enrichments immediately after incubation (Figure 4-9B). It seemed that the conditions in this experiments favoured methanogenesis because transfer from primary to secondary enrichment occurred during the peak of methanogenic activity and no acetogenic activity. Acetogenic activity did not recover from low pH conditions from high concentrations of acetic acid produced in the primary enrichment (Figure 4-7). Therefore the theory postulated by Baronfsky et al., 1984 was validated. It also seems that the hydrogenotrophic methanogen(s) present in our produced water (3-PW) needed longer period of time to grow regardless of pH or the percentage of H_2 present in the headspace of our enrichments. This was probably because the methanogens present in the 3-PW samples (October 2010 and September, 2011) were exposed to oxygen during sampling. The methanogens are very strictly anaerobic *Archaea* and exposure to oxygen causes their metabolic activity to stop (Peters and Conrad, 1995; and Brauer et al., 2004).

The microbial community composition analysis done on selected samples of primary and secondary enrichments enabled us to determine which types of acetogens and methanogens where stimulated at different stages of microbial activity. A change in community composition from day 8 to 40 was observed in primary enrichments with H_2/CO_2 (80:20) (Table 4-7 and Figure 4-11), as the microbial community went from being composed mostly of the acetogenic genus *Acetobacterium* to one mostly composed of methanogens (*Methanofollis*, *Methanobacterium* and *Methanospirillum*). Comparison of this with communities present in early enrichments of the 3-PW sample obtained in October, 2010 in defined media X and X5, show similarity in the presence but not percentage of *Acetobacterium* and *Methanobacterium* (Table 4-5). The microbial

community composition analysis of 3-PW showed that *Methanospirillum* was present at 0.01% in the sample received in October, 2010 but absent in the sample received in September, 2011 (Tables 4-1 and 4-2). It is thus possible for *Methanospirillum* to be present in this enrichment.

The methanogenic genera *Methanospirillum* and *Methanofollis* were present in high percentages in the secondary enrichment with pH 7.6 while genus *Methanobacterium* was found in high percentage at pH 5.5. It was also interesting to see that a high percentage of *Methanobacterium* was detected when the pH of primary enrichment went as low as 5.2. Studies done on the methanogenic community in peat soils (Yavitt et al., 2012; Horn et al., 2003; Williams & Crawford, 1985) have identified members of the orders *Methanosarcinales*, *Methanonomicrobiales*, *Methanobacteriales* and *Methanocellales* as being active under low pH conditions. All the hydrogenotrophic methanogenic genera (*Methanoculleus*, *Methanobacterium*, *Methanofollis* and *Methanospirillum*) which have been present in enrichments with 3-PW belong to the order *Methanomicrobiales* (Tables 4-4, 4-6 and 4-7). Although we do not have results for community composition for primary enrichments with 5% H_2, one may expect that either *Methanospirillum* or *Methanofollis* would be mostly present at pH 7.6 while *Methanobacterium* would be mostly present at pH 5.5.

Although methanogenesis has been found to occur at pH 4.5 in some studies, it was suggested that the presence of acetate might inhibit acetotrophic methanogenic activity (Horn et al., 2003; Lansdown et al., 1992; Williams and Crawford, 1984; Chasar et al., 2000 and Popp et al., 1999). Even though the formation of methane from acetate has been reported to occur in the presence of 100 mM volatile fatty acids (van den Berg

et al., 1976; Williams & Crawford, 1984; Wang et al., 1997; Kotsyurbenko et al., 1996; and Lansdown et al., 1992), the acetotrophic methanogen *Methanosaeta* found in our produced water sample 3-PW requires a maximum acetate threshold of 70 µM (Conrad, 1999). The conditions in our secondary enrichments were therefore still not suitable for the conversion of acetate to methane. Enrichments with acetate concentrations below 70 µM will have to be done to observe acetotrophic methanogensis in our produced water sample 3-PW.

5.4 Hydrogen producing reactions in the subsurface

H_2 is essential for the success of subsurface conversion of CO_2 to acetic acid and then to methane, with the latter being catalyzed by acid tolerant methanogens (Horn et al., 2003). The question that arises is where the H_2 needed along with stored CO_2 for acetic acid and/or methane production will be obtained from in the subsurface. This is because H_2 has to be readily available to the anaerobic microorganisms present in the subsurface ecosystem (Nealson et al., 2005). H_2 is an important electron donor in the subsurface as it is also used in the microbial reduction of sulfur and sulfate (Stevens, 1997).

It is believed that H_2 is formed by the action of hydrogen-producing thermophilic bacteria (HPTB) in subsurface environments (Sugai et al., 2008; Sugai et al., 2010). Although this has not been tested under high temperatures, HPTBs were suspected to be present in oil reservoirs since they can make use of sugars or oil organics in the presence of water to produce acetate, hydrogen and carbon dioxide (equation 6; Sugai et al., 2008; Sugai et al., 2010). These can then be used by either the hydrogenotrophic or the acetotrophic methanogens to produce methane and the acetogenic bacteria to produce

acetate (Fujiwara et al., 2006). Some other types of bacteria are also capable of directly degrading oil organics or sugar to produce butyrate, hydrogen and carbon dioxide (equation 7; Fujiwara et al., 2006).

$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2$ $\Delta G'^\circ = -136.06$ kJ **equation 6**

$C_6H_{12}O_6 \rightarrow C_3H_7COOH + 2H_2 + 2CO_2$ $\Delta G'^\circ = -224.21$ kJ **equation 7**

This also corroborates the hypothesis of Chapelle et al., 2002; where it was predicted that carbon dioxide might be the most abundant electron acceptor for hydrogen-utilizing bacteria in the subsurface of the earth. However, growth of hydrogenotrophic methanogens and subsequent production of methane in the subsurface requires a regular supply of hydrogen (Sugai et al., 2010). Zengler also stated that bacteria can degrade alkanes such as hexadecane to acetate and hydrogen (equation 8; Zengler et al., 1999):

$C_{16}H_{34} + 16H_2O \rightarrow 8CH_3COOH + 17H_2$ $\Delta G^\circ' = -929$ kJ **equation 8**

The syntrophic reaction carried out by certain anaerobic microorganisms facilitates the transfer of H_2 to hydrogen utilizing partners such as acetogens and methanogens (Schink & Stams, 2006 and Horn et al., 2003). This transfer requires the continuous uptake of hydrogen by hydrogenotrophic methanogens or acetogens in order to prevent the partial pressure of hydrogen from increasing to levels where continued consumption of hexadecane is prevented (Sugai et al., 2008). The amount of extractable H_2 measured in relation to groundwater at depth > 700 m in the subsurface was found to be almost 100 µL/L which equals to 0.01% (Pedersen et al., 2008), therefore the headspace concentrations of 5% and 80% H_2 used in our experiments is very much unlikely to be available in the subsurface.

5.5 Contribution of SW to microbial composition of in PW

The question whether microorganisms in oil reservoirs are native to the reservoir or introduced by water injection has been addressed before (Magot, 2005). In cases where the physical or chemical properties of the source and the formation waters, which are introduced and native to the reservoir, respectively, are vastly different some conclusions may be reached. As an example, Magot (1996) postulated that thermophiles of the genera *Thermotoga*, *Thermoanaerobacter* and *Thermodesulfobacterium* are native to the reservoir because of the high resident temperature (Magot 1996, 2000, 2005), which is very different from the low surface temperature. The more recent discoveries of thermophiles in arctic sediments, that are at a constant low temperature of about 5^{o}C (Hubert et al. 2009), and also in surface soil (Quaiser et al., 2002) indicates that this conclusion is not necessarily straightforward. In the MHGC field there is no significant temperature difference between conditions above-ground and downhole. However, a strong effort is made to kill microorganisms in the source water through continuous chlorination and periodic treatment with biocide with the goal of keeping microbes originating from the sewage treatment plant out of the reservoir.

We determined if these treatments were effective by performing microbial counts on mixtures of SW and PW were mixed with and without nutrient addition (Figure 4-13 and 4-14) under aerobic and anaerobic conditions. In general microbial counts with nutrient addition were higher than those without nutrient addition irrespective of whether incubations were done aerobically or anaerobically. Evidence of additional microbial growth was also observed as mixtures without either of the SWs added gave lower

microbial growth expect for experiments without nutrient addition where incubations were done anaerobically (Figure 4-14B).

In order to further investigate the effect of mixing SW with PW, experiments without nutrient addition under anaerobic conditions were done (Figures 4-15, 4-16, 4-17 and 4-18). Microbial counts obtained for untreated SW (22-SW-U) and treated SW (22-SW-T) gave low number of culturable anaerobic heterotrophs except in samples obtained in September, 2011 and January, 2012. The log CFU/mL obtained in 22-SW-F were mostly high except in samples obtained in January, 2012. This could be the result of backwashing the filter bed every 1-2 hours to prevent accumulation of organic matter. Hence, the SW-F samples may contain bacteria filtered from a larger volume, explaining the higher numbers. Also treatment with acrolein reduces the organic matter present and is done quarterly. Therefore bacterial numbers in SW samples can fluctuate, depending on length of time since the acrolein treatment. The mixture of 22-SW with 2-PW sometimes resulted in additional growth of at most one log unit except for experiments with samples obtained in September, 2011. Our results therefore suggest that most of the viable bacteria injected originate from the PW, not from the SW. Irrespective of the fact the inappropriate dilutions were sometimes done, it seems that the eventual number of anaerobic heterotrophs present in 22-SW-T was influenced by the numbers obtained in 22-SW-U which is obtained from the effluent of the municipal sewage treatment plant. The water plant sample is a combination of several PW in the field with 22-SW-T (Figure 1-7). It was interesting to observe that its anaerobic heterotrophic bacteria number between $10^5 – 10^6$ log CFU/mL was similar to that obtained in mixture of 22-SW-T with 2-PW from September 2011 to January 2012.

Nevertheless, results from community composition analysis of SW and PW show that all microbial taxa that predominate in the PW are found in the SW community (Table 4-10 and supplementary Table C-1), suggesting that many of the microbes that now thrive in the reservoir originated from the sewage treatment plant. Microorganisms lacking in PW samples, i.e. the top entries in Table 4-10, are mostly strictly aerobic or facultative bacteria. This includes the genera *Niabella* and *Nevskia*, which are strictly aerobic bacteria isolated from soil (Kim et al., 2011) and *Nitrosomonas*, which derives energy for growth from aerobic ammonium oxidation. *Neisseriaceae* and *Zooglea* have been described as components of sewage sludge (Thomsen et al., 2004). In contrast anaerobic microorganisms such as the methanogens *Methanoculleus*, *Methanosaeta* and *Methanolinea*, the nitrate-reducing *Thauera* and the syntrophic bacterium *Smithella* predominated in PW samples (Table 4-10). Of these *Thauera* has been described both as a major component of oil field waters (Agrawal et al., 2012) and of waste waters (Cabezas et al., 2006). However, although these anaerobic microbes dominated in PW, they were found in low fractions in the SW samples, indicating that these could have been the source for their proliferation in the field. Also acetogens such as *Acetobacterium* were not detected in either the SW or PW water samples (Table 4-10 and supplementary Table C-1). *Clostridium* was found to be present in SW only at 7.9%. This is interesting because some species of the genus *Clostridium* are known acetogens such as *Clostridium mayombei* (Kane et al., 1991).

5.6 Effect of injecting supercritical CO_2 into the subsurface

Although microorganisms may not be in direct contact with CO_2 after supercritical fluid injection, neighbouring areas in the subsurface are expected to have increased levels of CO_2. Experiments done in this study were with gaseous CO_2. However sequestration of CO_2 involves the injection of CO_2 in the supercritical state (section 1.1.3). Storage in this state requires an average depth of 1 km, temperature greater than 31.5°C and pressure not less than 73 atm (Figure 1-5; Riekkola & Manninen, 1993 and Mitchell et al., 2008). Exposing microbes to liquid supercritical CO_2 (Sc-CO_2) is expected to disrupt their physical structure (White et al., 2006). This is because Sc-CO_2 is a known disinfectant inhibiting the growth of microorganisms (White et al., 2006; Mitchell et al., 2008; Mendiola et al., 2008 and Ivanovic et al., 2010). Sc-CO_2 has been used to inactivate a wide range of bacteria and yeast (Spilimbergo & Bertucco, 2003). The unique phase property of Sc-CO_2 and its ability to deeply penetrate in porous media makes it a good sterilizing agent (Ge et al., 2002 and Vandervelde et al., 1992). Theories such as cell rupture, acidification, lipid modification, and inactivation of essential enzymes have been proposed as the mechanism of inactivating bacteria by Sc-CO_2 (Dillow et al., 1999 and Spilimbergo & Bertucco, 2003). However after exposure to Sc-CO_2, bacterial cells remain intact, therefore the theory of cell rupture can be ruled out (White et al., 2006 and Mitchell et al., 2008). Anaesthesia effect caused by disruption of the lipid bilayer in bacteria cells due to mass transfer by Sc-CO_2 may also lead to the inactivation of bacteria (Isenschmid et al., 1995). The process of CO_2 mass transfer (from gas to liquid) is proposed to increase CO_2's ability to penetrate the phospholipid bilayer and prevents it from repair (White et al., 2006). Injection of Sc-CO_2 may cause microorganisms in the

subsurface to become inactive. This may also cause methanogens and acetogens present in subsurface oil reservoirs to become inactive. Ongoing related studies (Bordenave, unpublished work), is addressing the effects of high pressure CO_2 on microbial communities in subsurface oil reservoirs.

5.7 Conclusions

The microbial community in our oil field produced water (3-PW) samples were mostly composed of methanogens. High percentage of the hydrogenotrophic methanogen *Methanoculleus* and the acetotrophic methanogen *Methanosaeta* were present. Microorganisms which convert H_2/CO_2 and/ acetic acid to methane were therefore present in the produced water samples. Hydrogenotrophic methanogenesis always occurred after acetogenesis regardless of media composition. The microbial community in the defined media was mostly composed of the hydrogenotrophic methanogen *Methanobacterium* while that in rich medium was mostly composed of hydrogenotrophic methanogen *Methanoculleus*. Primary enrichments with medium X5 which contained salt concentration similar to our produced water sample (5 g/L NaCl) produced the best activity of acetogenesis and methanogenesis in our experiments.

Hydrogenotrophic methanogenesis also occurred after acetogenesis in primary enrichments where 80% or 5% headspace H_2 was used. In both cases, methane production was observed after 10 days of incubation. We can therefore not conclude from our experiments that the percentage of headspace H_2 had an effect on the activity of acetogens or methanogens as similar trends were observed in both cases but at different concentrations. In the secondary enrichment at different pH, microbial activity was by

hydrogenotrophic methanogenesis. Methane concentrations increased at all pH conditions; therefore enrichment pH had no effect on the activity of methanogenesis. The activity of acetogens however ceased to occur when enrichment pH was about 5.5 due to accumulation of acetic acid in the enrichments bottles. This was evident in all the results where microbial community compositions were analyzed. The percentage of the acetogen *Acetobacterium*, decreased as enrichment days progressed. The acetotrophic acetogen *Methanoseata*, was also not active in our enrichment bottles due to the high concentration of acetic acid in all our enrichment experiments. Acetogenesis therefore requires the continuous uptake of acetate by acetotrophic methanogens for it to continue to occur in the subsurface.

Despite significant efforts to lower the numbers of viable bacteria in source waters through chlorination and biocide treatment, it appears that these have served as inoculum for the microbial community that is currently present in the MHGC field. As a result there is considerable overlap between communities in PWs from the MHGC field and from SWs. Strict aerobes, e.g. the genus *Nitrosomonas*, are present in SWs but not in PWs, whereas anaerobic microorganisms (e.g. syntrophs and methanogens which may catalyze the anaerobic hydrolysis of oil) predominate in PWs, but are also found in the SWs. Acetogens such as the genus *Acetobacterium* were not detected in either the SW or PW water samples but high percentage of methanogens were present in the PW samples.

Also, the H_2 concentrations in the subsurface (0.01%) is much lower than that which we have tested. We can therefore not conclude which microbial activity (acetogenesis or methanogenesis) will most likely occur in the subsurface. We can however predict that acetogenesis is mostly likely to occur first followed by continuous

production of methane in subsurface oil reservoirs since they both compete favourably for H_2 in the subsurface.

5.8 Future directions

In this study, the activity of methanogenesis always occurred after acetogenic activity possibly because oxygen exposure during sampling inhibited the methanogens. It would be beneficial to obtain new samples of produced water with conditions of minimal or zero exposure to oxygen. This can be done by injecting produced water into enrichments bottles with headspace H_2/CO_2 immediately during sampling. Procedures used in the paper by Caffrey et al., 2007 and Caffrey et al., 2008 may also be used to prevent inhibition of methanogenesis by oxygen contamination in headspace of enrichment while it is being transported to the lab.

The effect of H_2 in this study was done with 5% and 80% H_2 concentrations. Since we know that subsurface hydrogen is most like 0.01% experiments with H_2 concentrations 0.05% should be done as 0.01% might not be obtainable in the lab. Headpace gas can be should be filled up with CO_2 and/or N_2 with enrichment in defined medium. The salt concentrations of produced water sample should be adjusted if need be, since we observed optimum activity when salt concentrations in medium were similar to PW sample.

Studying the activity of acetogens and methanogens in primary enrichments with different pH will also help us understand the relationship of *Acetobacterium* and *Methanobacterium* since they were present at high percentages in our experiments. We may also find different methanogens and/or acetogens are active at certain pH conditions.

The activity of acetotrophic methanogen *Methanosaeta* should also be studied but at acetate concentrations of 70 µM and below. The effect of agitation on their growth should also be tested since other studies found this to be problematic (Dannenberg et al., 1997).

The effect of producing high concentrations of acetic acid on the geology of oil reservoirs is also important. This might be problematic because consumption of high concentrations acetic acid by *Methanosaeta* does not occur in our study. Production of excess acetic acid may dissolve oil bearing rock formation in the subsurface. How this impacts the process of CCS should be tested as it may hinder safe sequestration.

REFERENCES

Agrawal, A., Park, H. S., Nathoo, S., Gieg, L. M., Jack, T. R., Miner, K., Ertmoed, R., Benko, A., and Voordouw, G., 2012. Toluene depletion in produced oil contributes to souring control in a field subjected to nitrate injection. Environmental Science & Technology 46, 1285-1292.

Bachu, S., and Stewart, S., 2002. Geological sequestration of anthropogenic carbon dioxide in the western Canada sedimentary basin: Suitability analysis. Journal of Canadian Petroleum Technology 41, 32-40.

Baronofsky, J. J., Schreurs, W. J. A., and Kashket, E. R., 1984. Uncoupling by acetic-acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. Applied and Environmental Microbiology 6, 1134-1139.

Barret, M., Gagnon, N., Morissette, B., Topp, E., Kalmokoff, M., Brooks, S. P., Matias, F., Masse, D. I., Masse, L., and Talbot, G., 2012. *Methanoculleus* spp. as a biomarker of methanogenic activity in swine manure storage tanks. FEMS Microbiology Ecology 80, 427-440.

Basso, O., Lascourreges, J. F., Le Borgne, F., Le Goff, C., and Magot, M., 2009. Characterization by culture and molecular analysis of the microbial diversity of a deep subsurface gas storage aquifer. Research in Microbiology 160, 107–116.

Borg, I., and Groenen, P., 2010. Modern multidimensional scaling: Theory and applications. Springer, New York, NY.

Brauer, S. L., Yavitt, J. B., and Zinder, S. H., 2004. Methanogenesis in McLean Bog, an acidic peat bog in upstate New York: stimulation by H_2/CO_2 in the presence of rifampicin, or by low concentration of acetate. Geomicrobiology Journal 21, 433-443.

Braun, K., and Gottchalk, G., 1981. Effect of molecular hydrogen and carbon dioxide on chemo-organotrophic growth of *Acetobacterium woodii* and *Clostridium aceticum*. Archives of Microbiology 128, 294-298.

Bray J. R., and Curtis J. T., 1957. An ordination of the upland forest communities of Southern Wisconsin. Ecological Monographies 27, 325-349.

Breznak, J. A., and Warnecke, F., 2008. *Spirochaeta cellobiosiphila* sp nov., a facultatively anaerobic, marine spirochaete. International Journal of Systematic and Evolutionary Microbiology 58, 2762-2768.

Cabezas, A., Draper, P., Muxí, L., and Etchebehere, C., 2006. Post-treatment of a slaughterhouse wastewater: stability of the microbial community of a sequencing batch

reactor operated under oxygen limited conditions. Water Science & Technology 54, 215-221.

Caffrey, M., S., Park, H., Voordouw, J. K., He, Z., Zhou, J., and Voordouw, G., 2007. Function of periplasmic hydrogenases in the sulphate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. Journal of Bacteriology 189, 6159-6167.

Caffrey, S. M., Park, H., Been, J., Gordon, P., Sensen, C. W., and Voordouw, G., 2008. Gene expression by the sulphate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough grown on an iron electrode under cathodic protection conditions. Applied and Environmental Microbiology 74, 2404-2413.

Chao, A., and Shen, T. J., 2003. Nonparametric estimation of Shannon's index of diversity when there are unseen species in sample. Environmental and Ecological Statistics, 10, 429-443.

Chapelle, F. H., O'Neil, K., Bradley, P. M., Methe, B. A., Clufo, S. A., Knobel, L. L., and Lovely, D. R., 2002. A hydrogen-based subsurface microbial community dominated by methanogens. Nature 415, 312-315.

Chasar, L. S., Chanton, J. P., Glaser, P. H., Siegel, D. I., and Rivers, J. S., 2000. Radiocarbon and stable carbon isotopic evidence for transport and transformation of dissolved organic carbon, dissolved inorganic carbon, and CH_4 in a northern Minnesota peatland. Global Biogeochemical Cycles 14, 1095-1108.

Conrad, R., 1999. Contribution of hydrogen to methane production and control of hydrogen concentrations in methanogenic soils and sediments. FEMS Microbiology Ecology 28, 193-202.

Conrad, R., Schink, B., and Phelps, T. J., 1986. Thermodynamics of H_2-consuming and H_2-producing metabolic reactions in diverse methanogenic environments under in situ conditions. FEMS Microbiology Ecology 38, 353-360.

Conrad, R., Bak, F., Seitz, H. J., Thebrath, B., Mayer, H. P., and Schutz, H., 1989. Hydrogen turnover by psychrotrophic homoacetogenic and mesophilic methanogenic *Bacteria* in anoxic paddy soil and lake sediment. FEMS Microbiology Letters 62, 285-293.

Dandge, D. K., Heller, J. P., and Wilson, K. V., 1985. Structure solubility correlations: Organic compounds and dense carbon dioxide binary systems. Industrial and Engineering Chemistry Product Research and Development 24, 162-166.

Dannenberg, S., Wudler, J., and Conrad, R., 1997. Agitation of anoxic paddy soil slurries affects the performance of the methanogenic microbial community. FEMS Microbiology Ecology 22, 257–263.

Diekert, G., 1990. CO_2 reduction to acetate in anaerobic *Bacteria*. FEMS Microbiology Letters 87, 391-395.

Dikert, G., and Wohlfarth, G., 1994. Metabolism of homoacetogens. Antonie Van Leeuwenhoek 66, 209-221.

Dillow, A. K., Dehghani, F., Hrkach, J. S., Foster, N. R., and Langer, R., 1999. Bacterial inactivation by using near- and supercritical carbon dioxide. Proceedings of the National Academy of Sciences 96, 10344-10348.

Drake, H. L., Daniel, S. L., Küsel, K., Matthies, C., Kuhner, C., and Braus-Stromeyer, S., 1997. Acetogenic *Bacteria*: What are the in situ consequences of their diverse metabolic versatilities? BioFactors 6, 13-24.

Dridi, B., Fardeau, M. L., Ollivier, B., Raoult, D., and Drancourt, M., 2011. The antimicrobial resistance pattern of cultured human methanogens reflects the unique phylogenetic position of *Archaea*. Journal of Antimicrobial Chemotherapy 66, 2038–2044.

Emberley, S., Hutcheon, I., Shevalier, M., Durocher, K., Gunter, W. D., and Perkins, E. H., 2004. Geochemical monitoring of fluid-rock interaction and CO_2 storage at the Weyburn CO_2-injection enhanced oil recovery site, Saskatchewan, Canada. Energy 29, 1393-1401.

Fazal, M. A., Haseeb, A. S. M. A., and Masjuki, H. H., 2011. Effect of different corrosion inhibitors on the corrosion of cast iron in palm biodiesel. Fuel Processing Technology 92, 2154-2159.

Fey, A., and Conrad, R., 2000. Effect of temperature on carbon and electron flow and on the archaeal community in methanogenic rice field soil. Applied and Environmental Microbiology 66, 4790-4797.

Fuchs, G., 1986. CO_2 fixation in acetogenic bacteria: Variations on a theme. FEMS Microbiology Letters 39, 181-213.

Fujiwara, K., Mukaidani, T., Kano, S., Hattori, Y., Maeda, H., Miyagawa, Y., and Tkabayashi, K., 2006. Research study for microbial restoration of methane deposit with subsurface CO_2 sequestration depleted gas/oil fields. Society of Petroleum Engineers, 101248.

Ge, Y. Q., Yan, H., Hui, B. D., Ni, Y. Y., Wang, S. X., and Cai, T. Y., 2002. Extraction of natural vitamin E from wheat germ by supercritical carbon dioxide. Journal of Agricultural and Food Chemistry 50, 685-689.

Gieg, L. M., Jack, T. R., and Foght, J. M. 2011. Biological souring and mitigation in oil reservoirs. Applied Microbiology and Biotechnology 92, 263-282.

Goodwin, R., Conrad, R., and Zeikus, J. G., 1988. Influence of pH on microbial hydrogen metabolism in diverse sedimentary ecosystems. Applied Environmental Microbiology 54, 590-593.

Grabowski, A., Nercessian, O., Fayolle, F., Blanchet, D., and Jeanthon, C., 2005. Microbial diversity in production waters of a low-temperature biodegraded oil reservoir. FEMS Microbiology Ecology 54, 427-443.

Grabowski, A., Tindall, B. J., Bardin, V., Blanchet, D., and Jeanthon, C., 2005. *Petrimonas sulfuriphila* gen. nov., sp nov., a mesophilic fermentative bacterium isolated from a biodegraded oil reservoir. International Journal of Systematic and Evolutionary Microbiology 55, 1113-1121.

Heise, R., Muller, V., and Gottschalk, G., 1993. Acetogenesis and ATP synthesis in *Acetobacterium woodii* are coupled via a transmembrane primary sodium ion gradient. FEMS Microbiology 112, 261-268.

Hoehler, T. M., Alperin, M. J., Albert, D. B., and Martens, C. S., 2001. Apparent minimum free energy requirements for methanogenic *Archaea* and sulfate-reducing *Bacteria* in an anoxic marine sediment. FEMS Microbiology Ecology 38, 33-41.

Holloway, S., 1997. An overview of the underground disposal of carbon dioxide. Energy Conversion and Management 38, 193-198.

Horn, M. A., Matthies, C., Kusel, K., Schramm, A., and Drake, H. L., 2003. Hydrogenotrophic methanogenesis by moderately acid-tolerant methanogens of a methane-emitting acidic peat. Applied and Environmental Microbiology 69, 74-83.

Hubert, C., Loy, A., Nickel, M., Arnosti, C., Baranyi, C., Brüchert, V., Ferdelman, T., Finster, K., Christensen, F. M., Rosa de Rezende, J., Vandieken, V., and Jørgensen, B. B., 2009. A constant flux of diverse thermophilic *Bacteria* into the cold Arctic seabed. Science 325, 1541-1544.

Huson, D., Richter, D., Rausch, C., Dezulian, T., Franz, M., and Rupp, R., 2007. Dendroscope: An interactive viewer for large phylogenetic trees. BMC Bioinformatics 8, 460.

Imachi, H., Sakai, S., Sekiguchi, Y., Hanada, S., Kamagata, Y., Ohashi, A., and Harada, H., 2008. *Methanolinea tarda* gen. nov., sp. nov., a methane-producing archaeon isolated from a methanogenic digester sludge. Internationl Journal Systematic and Evolutionary Microbiology 58, 294-301.

Isenschmid, A., Marison, I. W., and Vonstockar, U., 1995. The influence of pressure and temperature of compressed CO_2 on the survival of yeast-cells. Journal of Biotechnology 39, 229-237.

Ivanovic, J., Misic, D., Ristic, M., Pesic, O., and Zizovic, I., 2010. Supercritical CO_2 extract and essential oil of bay (*Laurus nobilis* L.) - chemical composition and antibacterial activity. Journal of the Serbian Chemical Society 75, 395-404.

Janssen, P. H., 2003. Selective enrichment and purification of cultures of *Methanosaeta spp*. Journal of Microbiological Methods 52, 239-244.

Jetten, M. S. M., Stams, A. J. M., and Zehnder, A. J. B., 1992. Methanogenesis from acetate: comparison of the acetate metabolism in *Methanothrix soehngenii* and *Methanosarcina spp.* FEMS Microbiology Reviews 88, 181–198.

Kane, M. D., Brauman, A., and Breznak, J. A., 1991. *Clostridium mayombei* sp-nov, an H_2/CO_2 acetogenic bacterium from the gut of the african soil-feeding termite, *Cubitermes speciosus*. Archives of Microbiology 156, 99-104.

Karl, T. R., and Trenberth, K. E., 2003. Modern global climate change. Science 302, 1719-1723.

Kim, S. J., Weon, H. Y., Kim, Y. S., Park, I. C., Son, J. A., and Kwon, S. W., 2011. *Nevskia terrae* sp. nov., isolated from soil. International Journal of Systematic and Evolutionary Microbiology 61, 1226-1229.

Korenblum, E., Valoni, E., Penna, M., and Seldin L., 2010. Bacterial diversity in water injection systems of Brazilian offshore oil platforms. Applied Microbiology and Biotechnology 85, 791-800.

Kotsyurbenko, O. R., Glagolev, M. V., Nozhevnikova, A. N., and Conrad, R., 2001. Competition between homoacetogenic *Bacteria* and methanogenic *Archaea* for hydrogen at low temperature. FEMS Microbiology Ecology 38, 153-159.

Kotsyurbenko, O. R., Nozhevnikova, A. N., Soloviova, T. I., and Zavarzin, G. A., 1996. Methanogenesis at low temperatures by microflora of tundra wetland soil. Antonie Van Leeuwenhoek 69, 75-86.

Kuijvenhoven, C., Noirot, J., Bostock, A. M., Chappell, D., and Khan, A., 2006. Use of nitrate to mitigate reservoir souring in Bonga deepwater development offshore Nigeria. SPE Production & Operations 21, 467–474.

Lacis, A. A., Schmidt, G. A., Rind, D., and Ruedy, A. A., 2010. Atmospheric CO_2: Principal control knob governing Earth's temperature. Science 330, 356-359.

Lai, M. C., and Chen, S. C., 2001. *Methanofollis aquaemaris* sp nov., a methanogen isolated from an aquaculture fish pond. International Journal of Systematic and Evolutionary Microbiology 51, 1873-1880.

Lansdown, J. M., Quay, P. D., and King, S. L., 1992. CH_4 production via CO_2 reduction in a temperate bog - A source of C-13-depleted CH_4. Geochimica Et Cosmochimica Acta 56, 3493-3503.

Leybo, A. I., Netrusov, A. I., and Conrad, R., 2006. Effect of hydrogen concentration on the community structure of hydrogenotrophic methanogens studied by T-RELP analysis of 16S rRNA gene amplicons. Microbiology 75, 786-791.

Li, H., Wang, L., and Shen, L., 2005. Potential CO_2 emission reduction by development of non-grain-based bioethanol in China. Environmental Management 46, 555-564.

Liamleam, W., and Annachhatre, A. P., 2007. Electron donors for biological sulfate reduction. Biotechnology Advances 25, 452–463.

Lin, Y. S., Heuer, V. B., Goldhammer, T., Kellermann, M. Y., Zabel, M., and Hinrichs, K. U., 2012. Towards constraining H_2 concentration in subseafloor sediment: A proposal for combined analysis by two distinct approaches. Geochimica et Cosmochimica Acta 77, 186-201.

Magot, M., 1996. Similar *Bacteria* in remote oil fields. Nature 379, 681.

Magot, M., 2005. Indigenous microbial communities in oil fields. In: Ollivier, B., Magot, M. (eds) Petroleum Microbiology. ASM Press, Washington, pp 21–33.

Magot, M., Ollivier, B., and Patel, B. K. C., 2000. Microbiology of petroleum reservoirs. Antonie Van Leeuwenhoek 77, 103-116.

Mendiola, J. A., Santoyo, S., Cifuentes, A., Reglero, G., Ibanez, E., and Senorans, F. J., 2008. Antimicrobial activity of sub- and supercritical CO_2 extracts of the green alga *Dunalielia salina*. Journal of Food Protection 71, 2138-2143.

Mitchell, A. C., Dideriksen, K., Spangler, L. H., Cunningham, A. B., and Gerlach, R., 2010. Microbially enhanced carbon capture and storage by mineral-trapping and solubility-trapping. Environment Science and Technology 44, 5270-5276.

Mitchell, A. C., Philips, A. J., Hamilton, M. A., Gerlach, R., Kirk Hollis, W., Kaszuba, J. P., and Cunningham, A. B., 2008. Resilience of planktonic and biofilm cultures to supercritical CO_2. Journal of Supercritical Fluids 47, 318-325.

Mitchell, A. C., Phillips, A. J., Hiebert, R., Gerlach, R., Spangler, L. H., and Cunningham, A. B., 2008. Biofilm enhanced geologic sequestration of supercritical CO_2. International Journal of Greenhouse Gas Control 3, 90-99.

Mladek, B. M., Charbonneau, P., and Frenkel, D., 2007. Phase Coexistence of Cluster Crystals: Beyond the Gibbs Phase Rule. Physical Review Letters, 99.

Mori, K., Yamamoto, H., Kamagata, Y., Hatsu, M., and Takamizawa, K., 2000. *Methanocalculus pumilus* sp nov., a heavy-metal-tolerant methanogen isolated from a waste-disposal site. International Journal of Systematic and Evolutionary Microbiology 50, 1723-1729.

Nealson, K. H., Inagaki, F., and Takai, K., 2005. Hydrogen-driven subsurface lithoautotrophic microbial ecosystems (SLiMEs): Do they exist and why should we care? Trends in Microbiology 13, 405-410.

Neelis, M. L., Patel, M., and Blok, K., 2005. CO_2 emissions and carbon storage resulting from non-energy use of fossil fuels in the Netherlands, NEAT results for 1993-1999. Resources, Conservation and Recycling 45, 251-274.

Nelson, K. E., Weinel, C., Paulsen, I. T., Dodson, R. J. Hilbert, H., dos Santos, V. A. P. M., Fouts, D. E., Gill, S. R., Pop, M., Holmes, M., Brinkac, L., Beanan, M., DeBoy, R. T., Daugherty, S., Kolonay, J., Madupu, R., Nelson, W., White, O., Peterson, J., Khouri, H., Hance, I., Lee, P. C., Holtzapple, E., Scanlan, D., Tran, K., Moazzez, A., Utterback, T., Rizzo, M., Lee, K., Kosack, D., Moestl, D., Wedler, H., Lauber, J., Stjepandic, D., Hoheisel, J., Straetz, M., Heim, S., Kiewitz, C., Eisen, J., Timmis, K. N., Dusterhoft, A., Tummler, B., and Fraser, C. M., 2002.
Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environmental Microbiology 12, 799 -808.

Noble, R. R. P., Stalker, L., Wakelin, S. A., Pejcic, B., Leybourne, M. I., Hortle, A. L., and Michael, K., 2012. Biological monitoring for carbon capture and storage – A review and potential future developments. International Journal of Greenhouse Gas Control 10, 520-535.

Park, H. S., Chatterjee, I., Dong, X., Wang, S. H., Sensen, C. W., Caffrey, S. M., Jack, T. R., Boivin, J., and Voordouw, G., 2011. Effect of sodium bisulfite injection on the microbial community composition in failed pipe sections of a brackish-water-transporting pipeline. Applied and Environmental Microbiology 77, 6908-6917.

Patel, G. B., Sprott, G. D., and Fein, J. E., 1990. Isolation and characterization of *Methanobacterium espanolae* sp-nov, a mesophilic, moderately acidiphilic methanogen. International Journal of Systematic Bacteriology 40, 12-18.

Patton, C. C., 1990. Injection-water quality. SPE Journal of Petroleum Technology 42, 1238-1240.

Pedersen, K., 2000. Exploration of deep intraterrestrial microbial life: Current perspectives. FEMS Microbiology Letters 185, 9-16.

Pedersen, K., Arlinger, J., Eriksson, S., Hallbeck, A., Hallbeck, L., and Johansson, J., 2008. Numbers, biomass and cultivable diversity of microbial populations relate to depth and borehole-specific conditions in groundwater from depths of 4-450 m in Olkiluoto, Finland. ISME Journal 7, 760-775.

Peters, V., and Conrad, R., 1995. Methanogenic and other strictly anaerobic *Bacteria* in desert soil and other oxic soils. Applied and Environmental Microbiology 61, 1673-1676.

Pino, C., Olmo-Mira, F., Cabello, P., Castillo, F., Roldan, M. D., and Moreno-Vivian, C., 2006. The assimilatory nitrate reduction system of the phototrophic bacterium *Rhodobacter capsulatus* E1F1. Biochemical Society Transactions 34, 127-129.

Planckaert, M., 2005. Oil reservoirs and oil production. In: Ollivier, B., Magot, M. (eds) Petroleum Microbiology. ASM Press, Washington, pp 3-19.

Popp, T. J., Chanton, J. P., Whiting, G. J., and Grant, N., 1999. Methane stable isotope distribution at a Carex dominated fen in north central Alberta. Global Biogeochemical Cycles 13, 1063-1077.

Princiotta, F., 2009. Global climate change and the mitigation challenge. Journal of the Air & Waste Management Association 59, 1194-1211.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B., Ludwig, W., Peplies, J., and Glöckner, F. O., 2007. SILVA: A comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Research 35, 7188-7196.

Quaiser, A., Ochsenreiter, T., Klenk, H. P., Kletzin, A., Treusch, A. H., Meurer, G., Eck, J., Sensen, C., and Schleper, C., 2002. First insight into the genome of an uncultivated *Crenarchaeote* from soil. Environmental Microbiology 4, 603-611.

Raval, A., and Ramanathan, V., 1989. Observational determination of the greenhouse effect. Nature 342, 758-761.

Riekkola, M. L., and Manninen, P., 1993. Supercritical-fluid extraction as an alternative sample preparation method. Trac-Trends in Analytical Chemistry 12, 108-114.

Sakai, S., Ehara, M., Tseng, I. C., Yamaguchi, T., Brauer, S. L., Cadillo-Quiroz, H., Zinder, S. H., and Imachi, H., 2012. *Methanolinea mesophila* sp nov., a hydrogenotrophic

methanogen isolated from rice field soil, and proposal of the archaeal family *Methanoregulaceae* fam. nov within the order *Methanomicrobiales*. International Journal of Systematic and Evolutionary Microbiology 62, 1389-1395.

Schink, B., and Stams, A. J. M., 2006. Syntrophism among prokaryotes. The prokaryotes, Dworkin, M., et al., New York: Springer, pp 309-335.

Schink, B., 1997. Energetics of syntrophic cooperation in methanogenic degradation. Microbiology and Molecular Biology Reviews 61, 262-280.

Schloss, P. D., Westcott, S. L., Thomas, R., Hall, J. R., Hartmann, M., Hollister, E. B., Lesniewski, R. A., Oakley, B. B., Parks, D. H., Robinson, C. J., Sahl, J. W., Stres, B., Thallinger, G. G., Van Horn, D. J., and Weber, C. F., 2009. Introducing mothur: Open-source, platform independent, community-supported software for describing and comparing microbial communities. Applied Microbiology and Biotechnology 75, 7537–7541.

Schloss, P. D., and Westcott, S. L., 2011. Assessing and improving methods used in OTU-based approaches for 16S rRNA gene sequence analysis. Applied Microbiology and Biotechnology 77, 219-3226.

Scholten, E., Lukow, T., Auling, G., Kroppenstedt, R. M., Rainey, F. A., and Diekmann, H., 1999. *Thauera mechernichensis* sp nov., an aerobic denitrifier from a leachate treatment plant. International Journal of Systematic Bacteriology 49, 1045-1051.

Sen, R., 2008. Biotechnology in petroleum recovery: The microbial EOR. Progress in Energy and Combustion Science 34, 714-724.

Shannon, C. E., 1948. A mathematical theory of communication. Bell System Technical Journal 27, 379-423 and 623-656.

Spilimbergo, S., and Bertucco, A., 2003. Non-thermal bacteria inactivation with dense CO_2. Biotechnology and Bioengineering 84, 627-638.

Spilimbergo, S., Dehghani, F., Bertucco, A., and Foster, N. R., 2003. Inactivation of *Bacteria* and spores by pulse electric field and high pressure CO_2 at low temperature. Biotechnology and Bioengineering 82, 118-125.

Stams, A. J. M., 1994. Metabolic interactions between anaerobic *Bacteria* in methanogenic environments. Antonie Van Leeuwenhoek 66, 271-294.

Sugai, Y., Niimi, T., Sasaki, K., Hattori, Y., Kano, S., Mukaidani, T., Fujiwara, K., and Okatsu, K., 2008. Screening of oil-degrading and hydrogen producing microorganisms for microbial conversion of CO_2 into CH_4 in oil reservoir. Society of Petroleum Engineers, 115009.

Sugai, Y., Purwasena, I. A., Sasaki, K., Fujiwara, K., Ji, C., and Okatsu, K., 2010. Evaluation of the potential of microbial conversion process of CO_2 into CH_4 by investigating the microorganisms in high CO_2 content oilfield. Canadian Society for Unconventional Gas Society of Petroleum Engineers, 137568.

Taconi, K. A., Zappi, M. E., French., W. T., and Brown, L. R., 2008. Methanogenesis under acidic pH conditions in a semi-continuous reactor system. Bioresource Technology 99, 8075-8081.

Thomsen, T. R., Nielsen, J. L., Ramsing, N. B., and Nielsen, P. H., 2004. Micromanipulation and further identification of FISH labelled microcolonies of a dominant denitrifying bacterium in activated sludge. Environmental Microbiology 6, 470-479.

Tischler, A., Woodworth, T. R., Burton, S. D., and Richards, R. D., 2010. Controlling bacteria in recycled production water for completion and workover operations. SPE Production & Operations 25, 232-240.

van den Berg, L., Patel, G. B., Clark, D. S., and Lentz, C. P., 1976. Factors affecting rate of methane formation from acetic acid by enriched methanogenic cultures. NRC Research Press 22, 1312-1319.

Vandervelde, E. G., Dehaan, W., and Liem, A. K. D., 1992. Supercritical fluid extraction of polychlorinated-biphenyls and pesticides from soil - comparison with other extraction methods. Journal of Chromatography 626, 135-143.

Voordouw, G., Grigoryan, A. A., Lambo, A., Lin, S., Park, H. S., Jack, T. R., Coombe, D., Clay, B., Zhang, F., Ertmoed, R., Miner, K., and Arensdorf, J. J., 2009. Sulfide remediation by pulsed injection of nitrate into low temperature Canadian heavy oil reserviour. Environmental Science and Technology 43, 9512–9518.

Wang, Y. S., Odle, W. S., Eleazer, W. E., and Barlaz, M. A., 1997. Methane potential of food waste and anaerobic toxicity of leachate produced during food waste decomposition. Waste Management & Research 15, 149-167.

Westermann, P., 1994. The effect of incubation-temperature on steady-state concentrations of hydrogen and volatile fatty-acids during anaerobic degradation in slurries from wetland sediments. FEMS Microbiology Ecology 13, 295-302.

White, A., Burns, D., and Christensen, T. W., 2006. Effective terminal sterilization using supercritical carbon dioxide. Journal of Biotechnology 123, 504-515.

Williams, R. T., and Crawford, R. L., 1984. Methane production in Minnesota peatlands. Applied and Environmental Microbiology 47, 1266-1271.

Williams, R. T., and Crawford, R. L., 1985. Methanogenic *Bacteria*, including an acid-tolerant strain, from peatlands. Applied and Environmental Microbiology 50, 1542-1544.

Yavitt, J. B., Yashiro, E., Cadillo-Quiroz, H., and Zinder, S. H., 2012. Methanogen diversity and community composition in peatlands of the central to northern Appalachian Mountain region, North America. Biogeochemistry 109, 117-131.

Youngster, L. K. G., Somsamak, P., and Haggblom, M. M., 2008. Effects of co-substrates and inhibitors on the anaerobic O-demethylation of methyl tert-butyl ether (MTBE). Applied Microbiology and Biotechnology 80, 1113-1120.

Zengler, K., Richnow, H. H., Rossello-Mora, R., Michaelis, W., and Widdel, F., 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401, 266-269.

Zhang, W. W., Culley, D. E., Nie, L., and Scholten, J. C. M., 2007. Comparative transcriptome analysis of *Desulfovibrio vulgaris* grown in planktonic culture and mature biofilm on a steel surface. Applied Microbiology and Biotechnology 76, 447-457.

Zhu, J. X., Liu, X. L., and Dong, X. Z., 2011. *Methanobacterium movens* sp nov and *Methanobacterium flexile* sp nov., isolated from lake sediment. International Journal of Systematic and Evolutionary Microbiology 61, 2974-2978.

Zhu, J. X., Zheng, H. J., Ai, G. M., Zhang, G. S., Liu, D., Liu, X. L., and Dong, X. Z., 2012. The genome characteristics and predicted function of methyl-group oxidation pathway in the obligate aceticlastic methanogens, *Methanosaeta* spp. Plos One 7.

Zinder, S. H., 1993. Physiological ecology of methanogens J.G. Ferry (Ed.), Methanogenesis: Ecology, Physiology, Biochemistry and Genetics. Chapman & Hall, New York, pp 128–206.

Appendix A

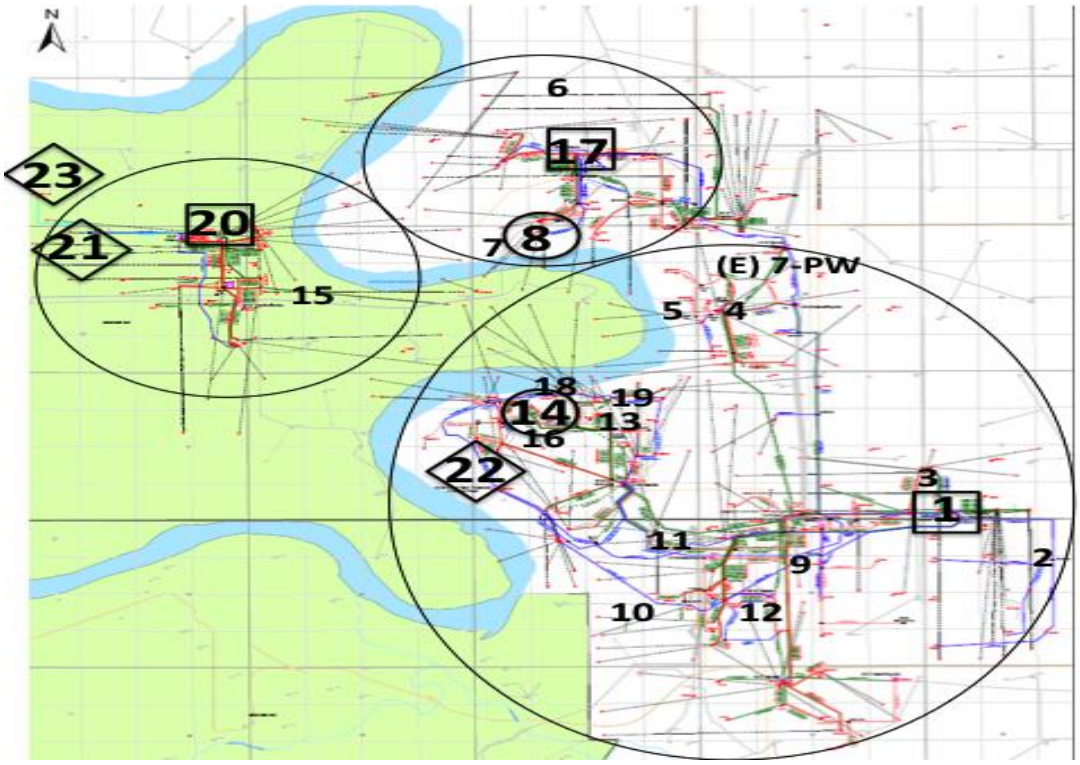


Figure A-1: Survey of sampling sites in the MHGC field. The map shows the location of sampling points for source water (e.g. 22-SW), injection waters (e.g. 14-IW), produced waters (e.g 2-PW, 3-PW, 10-PW, 12-PW and 13-PW) and central water plant (1-WP) used in this study.

Figure A-2: The make–up water treatment plant at the MHGC field. Make-up water is referred to as source water (SW) in the text.

Figure A-3: Sample collection of the untreated source water (22-SW-U) by a technician in the field.

Figure A-4: The four filter beds that filter 22-SW-U to 22-SW-F.

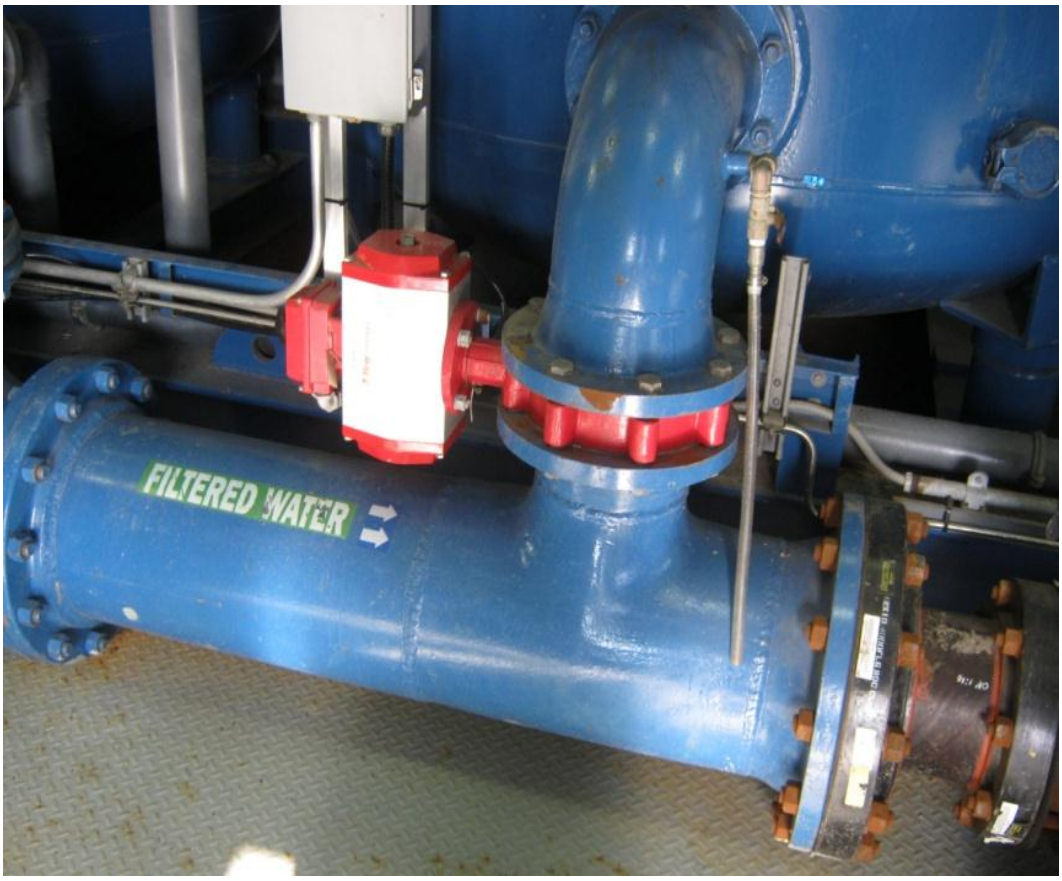


Figure A-5: The sampling point for filtered source water (22-SW-F).

Figure A-6: Sample collection of the ammonium bisulfite treated source water (22-SW-T) by a technician in the field and a technician from the Voordouw labs.

Appendix B

Table B-1: Composition of the trace element solution.

Ingredients	Concentration (g/Liter)
EDTA	0.5
$MgSO_4.H_2O$	3.0
NaCl	0.5
$CaCl_2.\ 2H_2O$	1.0
$ZnSO_4.7H_2O$	0.1
$FeSO_4.7H_2O$	0.1
$CuSO_4.7H_2O$	0.01
$Na_2MnO_4.2H_2O$	0.01
H_3BO_3	0.01
Na_2SeO_4	0.005
$NiCl_2.6H_2O$	0.003

Table B-2: Composition of the batch vitamins solution.

Ingredients	Concentration (g/Liter)
Biotin	2.0
Folic acid	2.0
Pyridoxine - HCl	10.0
Thiamine – HCl	5.0
Riboflavin	5.0
Nicotinic acid	5.0
DL - Calcium pantothenate	0.1
Vitamin B12	5.0
PABA	5.0
Lipoic acid	5.0
Mercaptoethane – sulfonic acid (MESA)	5.0

Appendix C

Table C-1. Fractions of pyrosequencing reads for all 11 amplicon libraries generated for communities in SWs, PWs, IW and WP waters. The fraction of reads for 6 SW samples (F_{SW}) and for 3 samples from producing wells (F_{PW}), from an injection well (IW) and a water plant (WP) are given. The sum $F_{SW} + F_{PW}$ is indicated, as well as the ratio $R = F_{SW}/(F_{PW} + 0.001)$. Entries with ($F_{SW} + F_{PW}$) <0.5 and entries not described beyond the phylum level are not represented in the table.

Sample type	22-SW-F	22-SW-T	22-SW-U	22-SW-F	22-SW-T	22-SW-U		10-PW	12-PW	13-PW		1-WP	14-IW		
Sample ID	Sep-598	Sep-599	Sep-600	Oct-629	Oct-630	Oct-631		Oct-653	Sep-589	Sep-590		Aug-516	Aug-524		
Number of reads	8520	5211	1893	2865	2297	606	21392	9485	8571	8428	26484	13376	10525	47876	
Taxon (Phylum; class; order; family; genus)							**F_{SW}**				**F_{PW}**			**$F_{SW}+F_{PW}$**	**Ratio R**
Firmicutes_Clostridia_Clostridiales_Clostridiaceae_Clostridium	0.012	0.211	0	19.756	25.076	2.31	7.894	0	0	0	0	0.022	0	7.894	7894
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae	23.721	4.529	4.754	0.314	0.087	1.98	5.898	0	0	0	0	0.366	0.513	5.898	5898
Bacteroidetes_Flavobacteria_Flavobacteriales_Flavobacteriaceae_Flavobacterium	5.023	19.075	2.007	0.349	0	1.155	4.602	0	0	0	0	0.075	0	4.602	4602
Bacteroidetes_Sphingobacteria_Sphingobacteriales_Chitinophagaceae_Niabella	3.967	3.646	0.053	0.384	0	5.446	2.249	0	0	0	0	0	0.01	2.249	2249
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Zoogloea	0.528	0	11.992	0	0	0	2.087	0	0	0	0	0	0	2.087	2087
Proteobacteria_Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae_Sphingopyxis	3.768	7.407	0.211	0	0.087	0	1.912	0	0	0	0	0.03	0	1.912	1912
Proteobacteria_Gammaproteobacteria_Xanthomonadales_Sinobacteraceae_Nevskia	8.228	0.077	0	0.07	0	0	1.396	0	0	0	0	0	0	1.396	1396
Proteobacteria_Betaproteobacteria_Neisseriales_Neisseriaceae_uncultured	0.023	1.785	0.158	4.258	0	2.145	1.395	0	0	0	0	0	0	1.395	1395
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Azospira	2.054	0.518	4.015	0.035	0.131	0.495	1.208	0	0	0	0	0.007	0	1.208	1208
Proteobacteria_Betaproteobacteria_Methylophilales_Methylophilaceae_Methylophilus	5.704	0.96	0.106	0.035	0	0	1.134	0	0	0	0	0	0	1.134	1134
Proteobacteria_Alphaproteobacteria_Sphingomonadales_Sphingomonadaceae	4.953	1.363	0.106	0	0	0	1.070	0	0	0	0	0	0	1.070	1070
Proteobacteria_Betaproteobacteria_Nitrosomonadales_Nitrosomonadaceae_Nitrosomonas	2.195	3.339	0	0	0.087	0	0.937	0	0	0	0	0	0	0.937	936.8
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Pelomonas	1.937	0.902	1.849	0.105	0.087	0.66	0.923	0	0	0	0	0	0	0.923	923.3
Firmicutes_Clostridia_Clostridiales_Lachnospiraceae	0	0.173	0	24.782	34.088	2.475	10.253	0.011	0	0.024	0.012	0	0.01	10.265	809.4
Planctomycetes_Planctomycetacia_Planctomycetales_Planctomycetaceae_Planctomyces	4.249	0.23	0	0.105	0.044	0	0.771	0	0	0	0	0	0	0.771	771.3
Proteobacteria_Epsilonproteobacteria_Campylobacterales_Helicobacteraceae_Sulfuricurvum	0	4.625	0	0	0	0	0.771	0	0	0	0	0	0	0.771	770.8
Bacteroidetes_Flavobacteria_Flavobacteriales_Flavobacteriaceae	0.059	2.994	0.317	0.314	0.218	0	0.650	0	0	0	0	1.189	0	0.650	650.3
Proteobacteria_Betaproteobacteria_Neisseriales_Neisseriaceae	0	0.729	2.007	0.175	0	0.825	0.623	0	0	0	0	0.03	0	0.623	622.7
Proteobacteria_Gammaproteobacteria_Aeromonadales_Aeromonadaceae_Aeromonas	0.012	0.557	0	0.314	0	2.805	0.615	0	0	0	0	0	0	0.615	614.7
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Malikia	0.023	2.667	0.475	0.035	0.218	0.165	0.597	0	0	0	0	0.007	0	0.597	597.2
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Moraxellaceae	0.059	0.384	38.669	0.105	0	0.495	6.619	0.011	0.023	0.024	0.019	0	0	6.638	325.5
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Aquabacterium	0.329	2.188	0.211	0.07	0	2.97	0.961	0	0.012	0	0.004	0.007	0	0.965	192.3
Proteobacteria_Betaproteobacteria_Burkholderiales_Oxalobacteraceae	9.988	0.806	1.638	0.035	0.044	0.33	2.140	0	0.012	0.024	0.012	0.09	0	2.152	164.6
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Moraxellaceae_Acinetobacter	0.07	0.115	4.12	0	0	0.33	0.773	0	0	0.012	0.004	0	0	0.777	154.5
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Rhodoferax	0.704	4.759	2.219	0.628	0.871	2.475	1.943	0	0.082	0	0.027	0.075	0	1.970	68.56
Proteobacteria_Betaproteobacteria_Burkholderiales_Comamonadaceae_Methylibium	1.89	0.403	0.053	0.314	0.348	0	0.501	0.021	0	0	0.007	0	0	0.508	62.67
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae	0.035	0.633	1.902	0.209	0.218	0.66	0.610	0	0.07	0	0.023	0.164	0.2	0.633	25.05
Proteobacteria_Gammaproteobacteria_Pseudomonadales_Pseudomonadaceae	0.704	4.107	12.361	18.115	15.02	32.178	13.748	0.074	5.18	1.483	2.246	6.9	5.805	15.993	6.119
Proteobacteria_Epsilonproteobacteria_Campylobacterales_Helicobacteraceae_Sulfurimonas	0	0.038	0	1.501	1.088	0.165	0.465	0.327	0.362	0	0.230	0.12	0.152	0.695	2.017
Proteobacteria_Deltaproteobacteria_Desulfuromonadales_Geobacteraceae_Geobacter	0	0.019	0	0.593	0.784	0	0.233	1.465	0.082	0	0.516	0.09	0.513	0.748	0.450
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Candidatus_Methanoregula	0	0.019	0	0.349	0.305	0.165	0.140	0.496	0.152	0.486	0.378	0.045	0.057	0.518	0.369
Proteobacteria_Epsilonproteobacteria_Campylobacterales_Campylobacteraceae_Arcobacter	0	0.019	0	0.175	0.261	0.33	0.131	1.476	0.093	0	0.523	2.318	0.979	0.654	0.250
Proteobacteria_Betaproteobacteria_Rhodocyclales_Rhodocyclaceae_Thauera	0.035	1.247	0	0.14	0.305	0	0.288	0.274	4.76	0.178	1.737	42.748	14.556	2.025	0.166
Proteobacteria_Deltaproteobacteria_Desulfovibrionales_Desulfovibrionaceae_Desulfovibrio	0	0.038	0	0.908	0.784	0.165	0.316	2.636	2.415	0.937	1.996	0.12	0.067	2.312	0.158
Proteobacteria_Deltaproteobacteria_Syntrophobacterales_Syntrophaceae_Smithella	0.012	0	0	0.279	0.218	0	0.085	0.148	1.75	0.522	0.807	0.882	1.264	0.892	0.105
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanoculleus	0.047	0.998	0	5.026	5.964	0.165	2.033	2.636	67.25	24.229	####	14.182	10.679	33.405	0.065
Euryarchaeota_Methanomicrobia_Methanosarcinales_Methanosaetaceae_Methanosaeta	0	0.134	0	0.314	0.435	0	0.147	0.179	1.82	6.977	2.992	0.621	0.96	3.139	0.049
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanocalculus	0	0.173	0	0.384	0.435	0	0.165	2.035	0.35	8.175	3.520	0.441	3.116	3.685	0.047
Spirochaetes_Spirochaetes_Spirochaetales_Spirochaetaceae_uncultured	0	0.019	0	0.105	0.044	0	0.028	0.042	0.408	1.412	0.621	0.157	0.162	0.649	0.045
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanomicrobiaceae_Methanofollis	0.012	0.038	0.053	0.105	0.218	0	0.071	0.928	1.493	2.598	1.673	0.12	0.114	1.744	0.042
Proteobacteria_Alphaproteobacteria_Rhodospirillales_Rhodospirillaceae_uncultured	0	0	0	0.105	0	0	0.023	1.666	0.012	0	0.559	0	0.048	0.583	0.042
Euryarchaeota_Methanomicrobia_Methanomicrobiales_Methanolinea	0	0.173	0.053	0.105	0.871	0	0.293	0.063	1.027	21.963	7.684	1.338	8.979	7.978	0.038
Euryarchaeota_Methanomicrobia_Methanosarcinales	0	0.115	0	0.105	0.479	0	0.250	0.221	1.132	25.558	8.970	1.503	2.081	9.221	0.028
Proteobacteria_Deltaproteobacteria_Desulfovibrionales_Desulfomicrobiaceae_Desulfomicrobium	0	0	0	0.105	0.131	0	0.022	4.217	0.07	0	1.429	0	0.257	1.451	0.015