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Acetic acid formation by acetogens from hydrogen and CO₂:dissolution of carbonates and competition by methanogens

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Acetic acid formation by acetogens from hydrogen and CO₂: dissolution of
carbonates and competition with methanogens

by

Lindsay S Rollick

A THESIS

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Abstract

Methane-producing archaea (methanogens) and bacteria that produce acetic acid from H_2 and CO_2 (acetogens) exist in many low nutrient environments where other electron acceptors are absent. Methanogens usually outcompete acetogens because of a more powerful energy production but recent evidence suggests that acetogens may be able to compete through greater substrate diversity and energy efficiency (Lever 2012). Acetogenesis could be adapted as a biotechnology to induce carbonate dissolution in carbonate oil reservoirs.

Enrichments were conducted on the subsurface production waters from a conventional oil field. Variations in medium composition were tested to promote acetogenesis over methanogenesis. High levels of acetic acid of up to 2400 μmol or 28 mM were produced and the consumed along with various levels of methane. Analysis of 16S rRNA gene sequences show a progression from dominance of acetogenesis to methanogenesis and then a shift to a group of microbes that consume acetate to create biopolymers like polyhydroxybutyrate for storage of carbon in nutrient-limited environments. Factors that increase acetic acid production include ultra-low nutrient environments, sufficient pH buffering, not adding bicarbonate, and possibly increased surface area. Dissolution via microbial acetic acid was tested for powdered $CaCO_3$, crushed and solid carbon rock but results are inconclusive.

Acetogens can be competitive with methanogens under the lowest nutrient conditions. The excess of the H_2/CO_2 energy and carbon substrates used in these experiments along with differences seen in nutrient variation suggest that conditions other than substrate availability can influence this competition. Sampling loss, re-precipitation and other experimental factors make carbonate dissolution mediated by microbial acetic acid difficult to track.

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Dedication

I dedicate this thesis to my parents Gordon and Carol Rollick and my sister Natalie Rollick. I never would have made it without you.

Table of Contents

Approval Page	i
Abstract.....	ii
Acknowledgements	iii
Dedication	iv
Table of Contents	v
List of Tables	ix
List of Figures and Illustrations	xii
List of Symbols Abbreviations and Nomenclature	xv
 CHAPTER 1 INTRODUCTION	 1
1.1 Acetogens: functional definition and history	1
1.2 Metabolic diversity of acetogens.....	2
1.3 The Wood-Ljungdahl/Acetyl-CoA pathway for producing acetic acid	7
1.4 Potential for microbially enhanced oil recovery (MEOR)	12
1.5 The Grosmont.....	15
1.6 Chemistry of carbonate dissolution.....	21
1.7 Research challenges	24
1.8 Conclusions	27
CHAPTER 2 OBJECTIVES.....	28
CHAPTER 3 MATERIALS AND METHODS.....	31
3.1 Source inoculum and media	31
3.2 Enrichments	34
3.3 Experiment #1: Growth of hydrogenotrophic acetogens and methanogens	37
3.4 Experiment #2: Growth of acetotrophic methanogens	37
3.5 Experiment #3: Growth of acetogens and hydrogenotrophic methanogens in the presence of CaCO ₃ and NaHCO ₃	37
3.6 Experiment #4: Variation with no NaHCO ₃	38
3.7 Experiment #5: Variation with H ₂ or CO ₂ only headspace	38
3.8 Experiment #6: Variation with crushed Grosmont core	38
3.9 Experiment #7: Variations with nutrients	41

3.10 Experiment #8: Variation with 20 mM CaCO ₃	41
3.11 Experiment #9: Variation with Grosmont solid carbonate core	42
3.12 Sampling	46
3.13 Chemical analysis	46
3.14 Statistical analysis and comparison between experiments	54
3.15 Analysis of microbial community composition	54
3.16 Brightfield and epifluorescent microscopy	57
3.17 Porosity and permeability analysis	57
CHAPTER 4 RESULTS	58
4.1 Experiment #1: For growth of hydrogenotrophs	58
4.1.1 Primary enrichment	58
4.1.2 Secondary enrichment	63
4.1.3 16S rRNA microbial community analysis: Secondary enrichment.....	67
4.2 Experiment #2: For growth of acetotrophic methanogens	69
4.2.1 Primary enrichment	69
4.2.2 Secondary enrichment	73
4.2.3 16S rRNA microbial community analysis: Both enrichments	76
4.3 Experiment #3: For growth with CaCO ₃ and NaHCO ₃	78
4.3.1 Primary enrichment	82
4.3.2 16S rRNA microbial community analysis: Primary enrichment.....	76
4.3.1 Secondary enrichment	85
4.3.2 16S rRNA microbial community analysis: Primary enrichment.....	89
4.4 Experiment #4: for growth without NaHCO ₃	92
4.4.1 Primary enrichment	92
4.4.2 16S rRNA microbial community analysis: Primary enrichment	96
4.4.3 CaCO ₃ mass recovery: Primary enrichment.....	100
4.4.4 Secondary enrichment	102
4.4.5 16S rRNA microbial community analysis: Secondary enrichment.....	105
4.4.6 CaCO ₃ mass recovery: Secondary enrichment.....	109
4.4.7 Primary enrichment bright-field and epifluorescent microscopy with Nile red staining for polyhydroxybutyrate (PHB) granules	111

4.4.8 Primary enrichment ammonium assay	111
4.5 Experiment #5: Incubations with either H ₂ or CO ₂ only	114
4.5.1 Primary enrichment	114
4.5.2 CaCO ₃ mass recovery	118
4.6 Experiment #6: With crushed MgCa carbonate core	120
4.6.1 Primary enrichment	120
4.6.2 16S rRNA microbial community analysis: Primary enrichment	124
4.6.3 Crushed carbonate core mass recovery: Primary enrichment	129
4.6.4 Secondary enrichment	131
4.6.5 16S rRNA microbial community analysis: Secondary enrichment.....	134
4.6.6 Crushed carbonate core mass recovery: Secondary enrichment	138
4.7 Experiment #7: Effects of varying the concentrations of nutrients	140
4.7.1 Main enrichment.....	140
4.7.2 16S rRNA microbial community analysis	145
4.7.3 CaCO ₃ mass recovery.....	150
4.8 Experiment #8: 20 mM CaCO ₃	153
4.8.1 Primary and secondary enrichments.....	153
4.8.2 16S rRNA microbial community analysis.....	157
4.8.3 CaCO ₃ mass recovery.....	160
4.9 Experiment #9: Using solid MgCa carbonate core	162
4.9.1 Main enrichment.....	162
4.9.2 16S rRNA microbial community analysis	167
4.9.3 CaCO ₃ mass recovery.....	169
4.9.4 Porosity and permeability.....	171
4.10 Summary	174
CHAPTER 5 DISCUSSION.....	177
5.1 Acetogenesis optimization	177
5.1.1 pH limitation	177
5.1.2 Differences between primary and secondary enrichments	179
5.1.3 Acetic acid loss	182
5.1.4 16S rRNA microbial community analysis: Community succession	185

5.1.5 16S rRNA microbial community analysis: Discrepancies with activity data and actual production of acetic acid and methane	190
5.1.6 16S rRNA community analysis: Biofilms and late sampling	192
5.1.7 16S rRNA microbial community analysis: Alternative metabolic cycles	194
5.1.8 Variations in headspace gas	198
5.1.9 Carbon limitation	198
5.1.10 Nutrient variations	199
5.2 Evaluation as a method for microbially enhanced oil recovery (MEOR)	201
5.2.1 Carbonate dissolution: Absolute mass loss	201
5.2.2 Chemical loss	203
5.2.3 Sampling loss	204
5.2.4 Re-precipitation	205
5.2.5 Carbonate core dissolution	206
CHAPTER 6 CONCLUSIONS.....	208
6.1 Acetogenesis optimization	208
6.2 Evaluation as a method for microbially enhanced oil recovery (MEOR)	209
6.3 Future Work	210
CHAPTER 7 REFERENCES	213

List of Tables

Chapter 3 Tables

Table 1: Composition of low and high nutrient versions of CSB-K medium	33
Table 2: Composition of trace element solution used in CSB-K medium	33
Table 3: Composition of tungstate-selenite solution used in CSB-K medium	33
Table 4: Experimental set up of experiment #1	39
Table 5: Experimental set up of experiment #2	39
Table 6: Experimental set up of experiment #3	39
Table 7: Experimental set up of experiment #4	40
Table 8: Experimental set up of experiment #5	40
Table 9: Experimental set up of experiment #6	40
Table 10: Experimental set up of experiment #7	43
Table 11: Experimental set up for experiment #8	43
Table 12: Experimental set up for experiment #9	43
Table 13: Summary of parameter variations between experiments	49
Table 14: Calculated volumes of methane used to create standards	50
Table 15: Calculated masses of sodium acetate added to water used to create standards	53
Table 16: Calculated volumes of 1M ammonium chloride stock solution used to create standards	53

Chapter 4 Tables

Experiment #1

Table 17: DNA pyrosequencing data for experiment #1 secondary enrichment	68
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Experiment #2

Table 18: DNA pyrosequencing data for experiment #2 both enrichments	77
--	----

Experiment #3

Table 19: DNA pyrosequencing data for experiment #3 primary enrichment	84
--	----

Table 20: DNA pyrosequencing data for experiment #3 secondary enrichment	91
--	----

Experiment #4

Table 21: DNA pyrosequencing data for experiment #4 primary enrichment	99
--	----

Table 22: CaCO ₃ mass recovered from experiment #4 primary enrichment	101
--	-----

Table 23: Comparison of CaCO ₃ recovery to acetic acid for experiment #4 primary enrichment	101
Table 24: DNA pyrosequencing data for experiment #4 secondary enrichment	108
Table 25: CaCO ₃ mass recovered from experiment #4 secondary enrichment	110
Table 26: Comparison of CaCO ₃ recovery to acetic acid for experiment #4 secondary enrichment	110
Experiment #5	
Table 27: CaCO ₃ mass recovered from experiment #5	119
Experiment #6	
Table 28: DNA pyrosequencing data for experiment #6 primary enrichment	128
Table 29: Carbonate mass recovered from experiment #6 primary enrichment	130
Table 30: Comparison of carbonate recovery to acetic acid for experiment #6 primary enrichment	130
Table 31: DNA pyrosequencing data for experiment #6 secondary enrichment	137
Table 32: Carbonate mass recovered from experiment #6 secondary enrichment	139
Table 33: Comparison of carbonate recovery to acetic acid for experiment #6 secondary enrichment	139
Experiment #7	
Table 34: DNA pyrosequencing data for experiment #7 on day 21	148
Table 35: DNA pyrosequencing data for experiment #7 2xTM comparing days 7 and 21 .	149
Table 36: CaCO ₃ mass recovered from experiment #7	151
Table 37: Comparison of CaCO ₃ recovery to acetic acid for experiment #7	151
Experiment #8	
Table 38: DNA pyrosequencing data for experiment #8	159
Table 39: CaCO ₃ Mass recovered from experiment #8	161
Table 40: Comparison of CaCO ₃ recovery to acetic acid for experiment #8	161
Experiment #9	
Table 41: DNA pyrosequencing data for experiment #9	168
Table 42: Free mass recovered from experiment #9	170
Table 43: CaCO ₃ Mass lost in experiment #9	170
Table 44: Comparison of CaCO ₃ mass loss to acetic acid for experiment #9.....	170

Table 45: Analysis for porosity and permeability on carbonate core before treatment for experiment #9	173
Table 46: Analysis for porosity and permeability on carbonate core after treatment for experiment #9	173
Table 47: Analysis for standard change in porosity and standardized change in permeability on carbonate core after treatment for experiment #9	173
Summary	
Table 48: Comparison between experiments based on comparable parameters for acetic acid and methane	175
Table 49: Comparison between experiments based on comparable parameters for acetic acid and methane continued	176

List of Figures and Illustrations

Chapter 1 Figures

Figure 1: A sampling of identified acetogenic species	5
Figure 2: Phylogenetic diversity of known acetogenic species	6
Figure 3: Model of the Wood-Ljungdahl biochemical pathway	11
Figure 4: 16S rRNA pyrosequencing for the Medicine Hat Glauconitic C (MHGC) oil field ..	14
Figure 5: Acetic acid and gas use for MHGC waters in DSMZ medium	14
Figure 6: Location of Alberta's bitumen carbonate reservoirs	16
Figure 7: Sedimentary stratigraphic map contain Grosmont formation in Athabasca region ..	16
Figure 8: Model of steam assisted gravity drainage (SAGD)	20
Figure 9: Model of cyclic steam stimulation (CCS)	20
Figure 10: Forward and backward rates of calcite dissolution as a function of pH	26
Figure 11: Mechanism for 2-bromoethane sulfonate action	26

Chapter 3 Figures

Figure 12: Oil production by produced water re-injection	32
Figure 13: Example of a serum bottle	36
Figure 14: Example of a serum bottle adapted from a mason jar	44
Figure 15: Schematic of gas consumption measurement by syringe addition	48
Figure 16: Example of a standard line used for methane measurement for GC-FID	50
Figure 17: Example of a standard line used for acetic acid measurement for HPLC	53

Chapter 4 Figures

Experiment #1

Figure 18: Acetic acid and methane production for experiment #1 primary enrichment	61
Figure 19: Gas consumption and pH for experiment #1 primary enrichment	62
Figure 20: Acetic acid and methane production for experiment #1 secondary enrichment...	65
Figure 21: Gas consumption and pH for experiment #1 secondary enrichment	66
Figure 22: DNA pyrosequencing data for experiment #1 secondary enrichment	68

Experiment #2

Figure 23: Acetic acid and methane production for experiment #2 primary enrichment	71
Figure 24: Gas consumption and pH for experiment #2 primary enrichment	72
Figure 25: Acetic acid and methane production for experiment #2 secondary enrichment...	74

Figure 26: Gas consumption and pH for experiment #2 secondary enrichment	75
Figure 27: DNA pyrosequencing data for experiment #2 both enrichments	77
Experiment #3	
Figure 28: Acetic acid and methane production for experiment #3 primary enrichment	80
Figure 29: Gas consumption and pH for experiment #3 primary enrichment	81
Figure 30: DNA pyrosequencing data for experiment #3 primary enrichment	84
Figure 31: Acetic acid and methane production for experiment #3 secondary enrichment...	87
Figure 32: Gas consumption and pH for experiment #3 secondary enrichment	88
Figure 33: DNA pyrosequencing data for experiment #3 secondary enrichment	90
Experiment #4	
Figure 34: Acetic acid and methane production for experiment #4 primary enrichment	94
Figure 35: Gas consumption and pH for experiment #4 primary enrichment	95
Figure 36: DNA pyrosequencing data for experiment #4 primary enrichment	98
Figure 37: Acetic acid and methane production for experiment #4 secondary enrichment.	103
Figure 38: Gas consumption and pH for experiment #4 secondary enrichment	104
Figure 39: DNA pyrosequencing data for experiment #4 secondary enrichment	107
Figure 40: Bright-field and epifluorescent microscopy images from experiment #4 primary enrichment	113
Figure 41: Ammonium concentrations for experiment #4 primary enrichment	113
Experiment #5	
Figure 42: Acetic acid and methane production for experiment #5	116
Figure 43: Gas consumption and pH for experiment #5	117
Experiment #6	
Figure 44: Acetic acid and methane production for experiment #6 primary enrichment	122
Figure 45: Gas consumption and pH for experiment #6 primary enrichment	123
Figure 46: DNA pyrosequencing data for experiment #6 primary enrichment	127
Figure 47: Acetic acid and methane production for experiment #6 secondary enrichment.	132
Figure 48: Gas consumption and pH for experiment #6 secondary enrichment	133
Figure 49: DNA pyrosequencing data for experiment #6 secondary enrichment	136

Experiment #7	
Figure 50: Acetic acid and methane production for experiment #7	143
Figure 51: Gas consumption and pH for experiment #7	144
Figure 52: DNA pyrosequencing data for experiment #7 on day 21	147
Figure 53: DNA pyrosequencing data for experiment #7 2xTM comparing days 7 and 21	147
Figure 54: Two identical replicates from experiment #7, one normal and one with precipitate	152
Experiment #8	
Figure 55: Acetic acid and methane production for experiment #8	155
Figure 56: Gas consumption and pH for experiment #8	156
Figure 57: DNA pyrosequencing data for experiment #8.....	158
Experiment #9	
Figure 58: Acetic acid and methane production for experiment #9	164
Figure 59: Gas consumption and pH for experiment #9.....	165
Figure 60: Examples of turbidity in high nutrients, cores at the end of incubation and precipitate.....	166
Figure 61: DNA pyrosequencing data for experiment #9.....	168
Figure 62: Carbonate cores before treatment in experiment #9	172
Figure 63: Carbonate cores after treatment in experiment #9	172
Chapter 5 Figures	
Figure 64: A model for changes in the definition of nutrient levels	181
Figure 65: A representation of growth ranges and zones of competitiveness	181
Figure 66: A model of microbial community succession for experiment #3.....	187
Figure 67: A model of microbial community succession for experiment #4.....	188
Figure 68: A model of microbial community succession for experiment #6.....	189
Figure 69: A model for an alternative metabolic cycle	197

List of Symbols, Abbreviations and Nomenclature

<u>Symbol</u>	<u>Definition</u>
mya	million years ago
TDS	Total Dissolved Solids
GC-MS	Gas Chromatography Mass Spectrometry
SAGD	Steam Assisted Gravity Drainage
CSS	Cyclic Steam Cyclization
CoA	Coenzyme A
ACS	Acetyl-CoA Synthase
ATP	Adenosine Triphosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
CODH	Carbon Monoxide Dehydrogenase
CFeSP	Iron-Sulfur Protein
MeTr	Methyltransferase
PMF	Proton Motive Force
BESA	2-bromoethane sulfonic acid
MEOR	Microbially Enhanced Oil Recovery
MHGC	Medicine Hat Glauconitic C
CSB-K	Coleville Synthetic Brine K
NaCl	Experiment #7 Variation increasing concentration of NaCl
MgCa	Experiment #7 Variation decreasing concentration of MgCa
No TM	Experiment #7 Variation with no added trace metals
2xTM	Experiment #7 Variation with doubled added trace metals
No PO ₄	Experiment #7 Variation with no added phosphate

2xPO ₄	Experiment #7 Variation with doubled added phosphate
GC-FID	Gas Chromatography Flame Ionization Detector
HPLC	High Performance Liquid Chromatography
PHB	Poly-3-hydroxybutyrate

CHAPTER 1 INTRODUCTION

1.1. Acetogens: functional definition and history

Acetogens are bacteria that gain their carbon and energy from acetogenesis chemical reactions 1-4. They are obligate anaerobes, but not strictly so as they have been found to be able to exist in symbiosis with aerotolerant fermenting syntrophs. The acetogens consume excess CO₂ and H₂ from the syntroph and get protection via oxygen scavenging properties (Göbner *et al.* 1999). Acetogens are defined by their use of the acetyl-CoA (Wood-Ljungdahl) biochemical pathway for three purposes:

- 1) The reduction of CO₂ to the acetyl moiety of acetyl-CoA
- 2) Production/conservation of energy
- 3) Assimilation of CO₂ into biomass (Drake *et al.* 2008)

It should be noted that there are organisms that produce acetic acid that aren't considered acetogens, which are strictly defined by the production of acetic acid from CO₂, so if the microbe doesn't consume CO₂ it can't be called an acetogen even if it generates acetic acid. There are, however, acetogens that can ferment carbon compounds to CO₂ which is then used for acetogenesis. Many other organisms use the acetyl-CoA pathway for biomass fixation, not gaining energy by producing acetic acid from acetyl-CoA and are also no acetogens. Homoacetogens produce acetic acid as the sole end product, often growing autotrophically from H₂ and CO₂. Heteroacetogens sometimes they only use one CO₂ and source the other carbon as a carbonyl or methyl group from other molecules.

The microbial formation of acetic acid from H₂ and CO₂ was first demonstrated in 1932 by F. Fischer and associates using a culture enriched from sewage sludge. The first isolate came

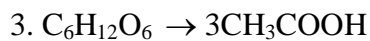
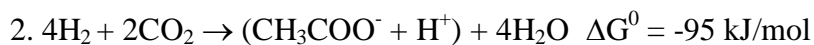
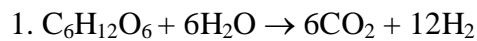
four years later from mesophilic soil by K.T. Wieringa (1936) and was named *Clostridium aceticum*. Unfortunately, this microbe was later lost and was then, ironically, rediscovered by chance in an old test tube during a tour of Wieringa's laboratory in 1980 just before his death (Gottschalk and Braun 1980).

Francis Fontaine and associates isolated a second acetogen in 1942, *Clostridium thermoaceticum* which has remained the model organism for studying acetogenesis since its discovery; 26 years would pass before a third acetogen was isolated. This organism could convert glucose into 3 moles of acetic acid almost stoichiometrically (Fontaine *et al.* 1942). Due to a review of the taxonomy of *Clostridium* in 1994, *C. thermoaceticum* was reclassified as *Moorella thermoacetica*. The third isolated acetogen, *Clostridium formicoaceticum* (El Ghazzawi 1967), was significant in that it helped to initiate the process of the elucidation of the biochemical pathway used for acetogenesis with its ability to produce both acetic and formic acids, hinting at the first step of CO₂ capture (Wood and Ljungdahl 1991).

1.2. Metabolism of acetogens

Acetic acid, depending on pH, can be represented as CH₃COOH or (CH₃COO⁻ + H⁺) is a product of the metabolism of acetogens. These produce acetic acid by fermenting carbohydrates first to CO₂ (equation 1) and then reacting the H₂ and CO₂ to acetic acid by the chemolithotrophic conversion of H₂ and CO₂ (reaction 2). Acetogens capable of catalyzing reaction 1 can convert carbohydrates, like glucose to three acetic acids (reaction 3). Acetogens are thought to be responsible for up to 10% of annual global production of acetic acid (Drake *et al.* 1997). Due to its consumption of a substantial amount of hydrogen, the acetogenesis reaction acts as an important hydrogen sink in environments where substantial amounts of hydrogen are

produced. If the level of hydrogen becomes too high in these environments fermentation reactions like reaction 1 can become thermodynamically unfavorable (Ragsdale and Pierce 2008). It also acts to recycle the hydrogen produced by acetate consumption in other heterotrophic reactions similar to reaction 1. Note that acetogenesis uses gaseous CO₂ dissolved in water, not the hydrated form bicarbonate (HCO₃⁻). So the amount of CO₂ available to acetogens to consume is controlled more by the partial pressure of CO₂ whereas the methanogens



Acetogens are environmentally beneficial in that they consume CO₂, our model greenhouse gas (GHG), and can also act as direct competitors to hydrogenotrophic methanogens which can aid in suppressing the level of methane production, an even more potent GHG. However, hydrogenotrophic methanogenesis is more thermodynamically favourable since it has a lower Gibb's free energy (Equation 4, Lapado and Whitman 1990). Hydrogenotrophic methanogens are therefore thought to outcompete acetogens.

Over 200 different types of acetogens have been isolated from many diverse environments with different levels moisture, temperature, pH, geography and salinity (Figure 1). Some species act as symbiotic organisms while others are completely independent. Acetogens

have a large genetic diversity that can be mono or polyphyletic (Figure 2). This means that there is currently no reliable way to identify an acetogen only through 16S rRNA sequencing.

Part of the acetogenesis pathway is considered to have very ancient evolutionary origins. Sections that deal with the production and consumption of acetyl-CoA are utilized by many microorganisms as a way to connect various other metabolic pathways. This means that many of the genes in the acetogenesis pathway are shared with non-acetogens. However, a recent study of the complete *M. thermoacetica* genome has led to the development of a molecular signature for acetogens comprising of the genes for acetyl-CoA synthase (*acsB*), carbon monoxide dehydrogenase (*acsA*), and both subunits of the corrinoid iron-sulfur protein (*acsC*, *acsD*); the key gene is the *acsA* gene which is thought to be most specific to acetogenic bacteria (Pierce *et al.* 2008).

TABLE 1. Acetogenic bacteria isolated to date^a

Acetogen	Source of isolate	Gram type ^b
<i>Acetitomaculum ruminis</i>	Rumen fluid, steer	+
<i>Acetoanaerobium noterae</i>	Sediment	–
“ <i>Acetoanaerobium romashkovii</i> ”	Oil field	+
<i>Acetobacterium bakii</i>	Wastewater sediment	+
<i>Acetobacterium carbinolicum</i>	Freshwater sediment	+
“ <i>Acetobacterium dehalogenans</i> ”	Sewage digester sludge	+
<i>Acetobacterium fmetarium</i>	Digested cattle manure	+
<i>Acetobacterium malicum</i>	Freshwater sediment	+
<i>Acetobacterium paludosum</i>	Fen sediment	+
“ <i>Acetobacterium psammolithicum</i> ”	Subsurface sandstone	–
<i>Acetobacterium tundrae</i>	Tundra soil	+
<i>Acetobacterium zweringae</i>	Sewage digester	+
<i>Acetobacterium woodii</i>	Marine sediment	+
<i>Acetobacterium</i> sp. AmMan 1	Freshwater sediment	+
<i>Acetobacterium</i> sp. B10	Wastewater pond	+
<i>Acetobacterium</i> sp. HA1	Sewage sludge	+
<i>Acetobacterium</i> sp. HP4	Lake sediment	+
<i>Acetobacterium</i> sp. KoB58	Sewage sludge	+
<i>Acetobacterium</i> sp. LuPhet1	Sewage sludge	+
<i>Acetobacterium</i> sp. LuTria3	Sewage sludge	+
<i>Acetobacterium</i> sp. MrTac1	Marine sediment	+
<i>Acetobacterium</i> sp. OyTac1	Freshwater sediment	+
<i>Acetobacterium</i> sp. RMMac1	Marine sediment	–
<i>Acetobacterium</i> sp. 69	Sea sediment	+
<i>Acetobacterium</i> sp.	Tundra wetland soil	+
<i>Acetohalobium arabaticum</i>	Saline lagoon	–
<i>Acetonema longum</i>	Wood-eating termite, gut	–
<i>Bryantella formatexigens</i>	Human feces	+
“ <i>Butyrubacterium methylotrophicum</i> ”	Sewage digester	+
<i>Caloramator fervidus</i> (?)	Hot spring	–
<i>Clostridium aceticum</i>	Soil	–
“ <i>Clostridium autoethanogenum</i> ” (?)	Rabbit feces	+
<i>Clostridium carboxidivorans</i>	Lagoon sediment	+
<i>Clostridium coccoides</i>	Mice feces, human feces	+
<i>Clostridium difficile</i> AA1	Rumen, newborn lamb	+
<i>Clostridium drakei</i>	Coal mine pond sediment	+
<i>Clostridium formicaceticum</i>	Sewage	–
<i>Clostridium glycolicum</i> 22	Sewage	+

Figure 1. An example of diversity of acetogens discovered to date. From Drake *et al.* 2008.

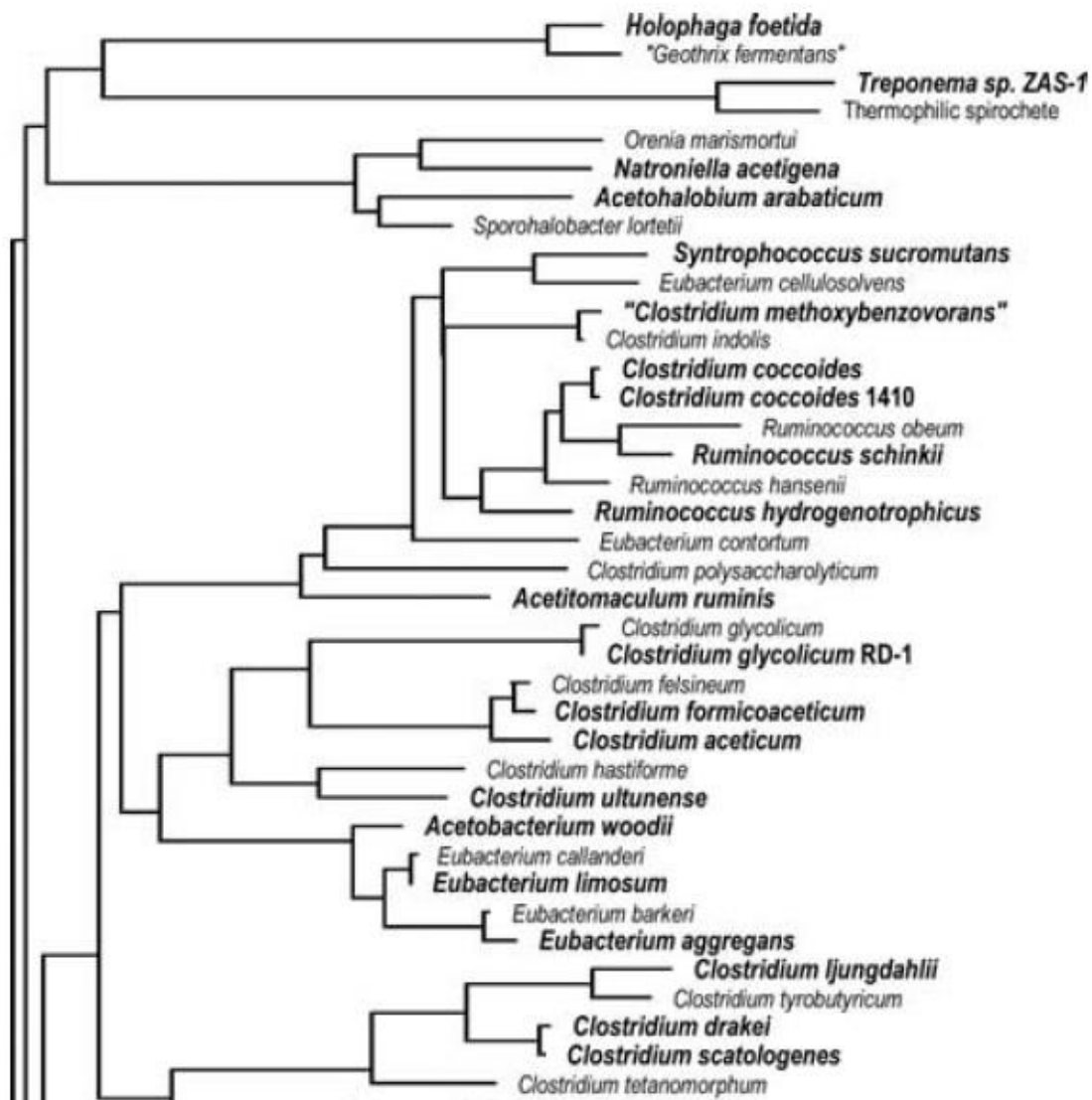


Figure 2. Phylogenetic diversity of known acetogenic (bold) and related non-acetogenic (not bold) bacteria as defined by 16S rRNA analysis. From Drake *et al.* 2008.

1.3. The Wood-Ljungdahl/Acetyl-CoA pathway: a mechanism for acetogenesis and carbon fixation with variations

The Wood-Ljungdahl pathway has been studied from the 1960's, when pathway components were first identified using C-14 radioisotope and C-13 stable isotope pulse-labelling experiments, to the 1980's, when enzymology studies were performed. Its study in the context of acetogenesis was integral in the discovery of acetyl-CoA and the enzymes involved in its metabolism. This occurred before it was found to act as an intermediate pathway connecting several important metabolic pathways in many other organisms including humans which connect sugar degradation reactions using acetyl-CoA as the intermediate (glycolysis and the tricarboxylic acid cycle) and link it with fatty acid breakdown (beta-oxidation). Plants also use it as an intermediate during sugar formation for the Calvin cycle.

The pathway is reversible and can be used in both the oxidative (acetate/acetyl-CoA to CO₂) or reductive (CO₂ to acetyl-CoA/acetate) directions (Ragsdale 1997). Many organisms, such as hydrogenotrophic methanogens, use it primarily as a carbon fixation pathway taking acetyl-CoA and transferring it to other metabolic pathways for biomass formation. These then use other reactions for energy production. Acetogens use this pathway for both carbon fixation and energy production. Acetate-utilizing sulphate-reducing bacteria and acetotrophic methanogens reverse this pathway to convert acetate to acetyl-CoA and then to CO₂ (Ferry 1999).

In wider perspective, the Wood-Ljungdahl pathway converts 2 one-carbon compounds (2 CO₂) along with 4 H₂ (which can be sourced from other reduced molecules) into one two-carbon compound (CH₃COOH). Due to this basic building block concept of creating more complex

compounds from CO₂, this form of autotrophy is thought to be among the first that evolved in the emergence of life 3.8 billion years ago. This is supported by C-isotope fractionation patterns in fossil records, which are similar to those produced by the Wood-Ljungdahl pathway (Schidlowski *et al.* 1983). This is also supported by the widespread distribution of the Wood-Ljungdahl pathway among microorganisms, especially anaerobes which likely predominated before the evolution of an oxygen atmosphere.

The pathway consists of two branches (Figure 3), called the “Eastern” and “Western” branches, each reacting with one CO₂ molecule. The Eastern branch is the part commonly shared among organisms and involved the reduction of CO₂ to formate and eventually to a methyl (-CH₃) group carried on a tetrahydrofolate carrier (referred to as H₄-folate). Three H₂ molecules are used in this process. Several of the enzymes found in the Eastern branch are extremely sensitive to oxygen and are likely the reason why acetogens are anaerobes (Park *et al.* 1991). The methyl group is then transferred from methyl-H₄folate onto the cobalt ion of corrinoid iron-sulfur protein (CFeSP) using the enzyme methyltransferase (MeTr) (Ljungdahl *et al.* 1966). The CFeSP connects with the Western branch when it transfers the methyl group to acetyl-CoA synthase where it becomes the methyl component of the acetyl group on acetyl-CoA.

The methyltransferase reaction is an important step in this process because there is substantial variation in this enzyme which acts as an important interface with other heterotrophic reactions. Different versions of methyltransferase can move the methyl group from several types of compounds onto CFeSP including from methanol, methylamines, methyl thiols and aromatic methyl ethers (Ragsdale 2008). There can be intense competition in an oil field microbial community with not only hydrogenotrophic methanogens, but also with heterotrophic sulphate-reducing bacteria where they are present (Mayumi *et al.* 2011). Acetogens survive by being very

metabolically diverse and having alternate pathways for energy production when other more energetically favourable electron acceptors are available. Heteroacetogens can have multiple end products along with acetic acid and can metabolize carbon monoxide, formate, alcohols, sugars, glyoxylate, glycolate, oxalate, lactate, pyruvate, fumarate, succinate, short chain fatty acids and several sugars by lifting off a methyl and carboxyl groups with methyltransferase. Another group of microbes, found in the same environments as acetogens that can have an impact on its growth, are the fermenters, which can be inhibitory through the production of acetic acid (incomplete fermentation) or commensal through the production of CO₂ and H₂ from sugar degradation (Drake et al. 2008).

In the Eastern branch one molecule of CO₂ is essentially reduced to –CH₃. In the Western branch, the other CO₂ is converted to carbon monoxide (CO) using the nickel-containing enzyme carbon monoxide dehydrogenase (which uses the fourth H₂), which then becomes the carbonyl group of the acetate (Ragsdale 2006).

In homoacetogenesis, the entire eastern branch of the pathway is ATP neutral (ATP neither consumed nor produced) but ATP is generated through the consumption of a chemiosmotic proton gradient (Ljungdahl 1994) created by the Wood-Ljungdahl pathway or through the consumption of a sodium gradient created by a bifunctional methyltransferase (Müller 2003). After the two intermediates produced by the branches are combined to form the acetyl on acetyl-CoA with the enzyme acetyl-CoA synthase. Acetyl-CoA is then either directed into other metabolic pathways to produce biomass or is used for the production of further energy using the enzymes phosphotransacetylase to convert acetyl-CoA to acetyl-phosphate and acetate kinase to produce free acetate and an ATP via substrate level phosphorylation (Drake *et al.* 1981).

Acetogens are thought to survive by being very metabolically diverse and having alternate pathways for energy production when other more energetically favourable electron acceptors are available. Besides using variations in methyltransferase can use electron acceptors other than CO₂, including nitrate, nitrite, sulfoxide and dimethylsulfoxide (Ragsdale and Pierce 2008), and can even fix N₂ (Bogdahn *et al.* 1983). Still even in situations where acetogenesis isn't the best option for energy production, the mechanism used for acetogenesis, referred to as either the Wood-Ljungdahl or acetyl-CoA pathway, can still be used separately for carbon fixation. As such the acetogenesis pathway isn't always constitutive with some organisms able to switch between a nitrate-reducing and acetogenic lifestyles, gaining a competitive edge and improved growth efficiency for doing so (Seifritz *et al.* 1993).

The Wood-Ljungdahl Pathway

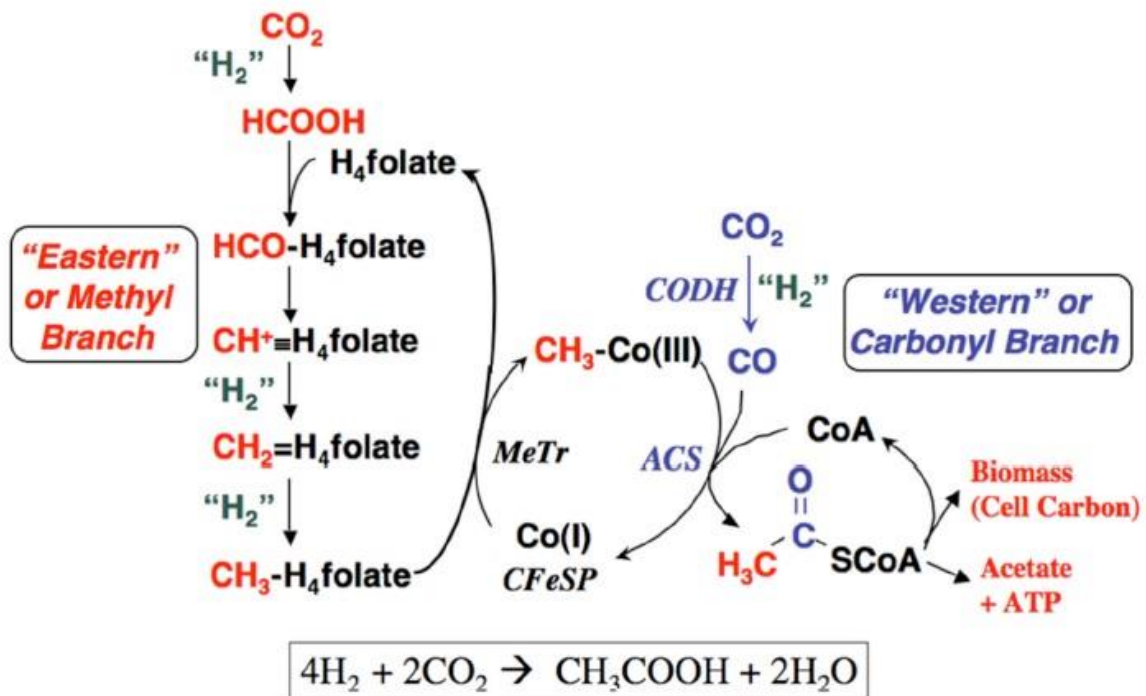


Figure 3. The Wood-Ljungdahl pathway. The left side highlighted in red is the common Eastern branch and the right side highlighted in blue is the Western branch unique to acetogenesis. From Ragsdale and Pierce 2008.

1.4. Potential for microbially enhanced oil recovery (MEOR)

Acetogens are being examined for potential use in microbially enhanced oil recovery (MEOR). This involves stimulating the growth of native acetogens in oil reservoirs to produce acetic acid to dissolve the surrounding rock matrix. Traditionally biogenic acid MEOR has been done by injecting exogenous microbes known to produce acid with their favored substrate glucose in the form of aqueous molasses (Youssef *et al.* 2009). Analysis of this process has indicated that too little acetic acid was being produced with the observed increased oil recovery being due to plugging from excess biomass production rather than to carbonate rock dissolution. The effect was also considered to be quite transient due to the inability of the injected microbes to compete with native ones (Gray *et al.* 2008).

In 2012, a metagenomic 16S rRNA pyrosequencing survey was conducted on the oil and produced water of the Medicine Hat Glauconitic C oil field which is mesothermic (30⁰C) and methanogenic. The 16S rRNA reads had 3% *Acetobacterium*, a common acetogen (Kryachko *et al.* 2012, Figure 4). This was unusual, in that *Acetobacterium* appeared to be co-existing in an environment with many methanogenic archaea present. When the produced waters were enriched in a medium designed to grow *Acetobacterium* with a 80% H₂/20% CO₂ headspace, over 100 mM of acetic acid was produced (Nathoo *et al.* 2012, Figure 5). This effect still occurred when the headspace and the culture were separated by a layer of heavy oil.

Further study of these oil field acetogens is warranted for the understanding the scientific questions around acetogen and methanogen competition and to study the potential applications for microbially enhanced oil recovery. If *Acetobacterium* growth could be promoted by the injection of H₂ and CO₂ gas into an oil reservoir this would solve some of the problems

associated with adding aqueous nutrient packages and penetration beyond the well-bore region. It would also be using native microbes, which would avoid the transience issues associated with injecting foreign microbes. Other methods besides biogenic acid production have been examined for MEOR application to carbonate oil reservoirs. Some have found some success in reducing interfacial tension wettability in carbonate rock using the microbial production of biosurfactants (Kowalewski *et al.* 2006, Sarafzadeh *et al.* 2013) which could also be used to improve bitumen production from carbonate rocks.

There is substantial evidence that the oil of the Grosmont reservoir has been heavily biodegraded. Using gas chromatography-mass spectrometry (GC-MS) to look for biomarkers, Zhao and Machel (2011) found that even the compounds most resistant to biodegradation, hopanes, rearranged steranes and tricyclic terpanes, were depleted or completely missing. This likely accounts at least in part for the very high viscosity of the bitumen (Wo. *et al.* 2011), which makes current activity of heterotrophic hydrocarbon-degrading bacteria unlikely.

#	Phylum;Class;Order;Genus	OCP: number of reads	OCP: fraction of total reads (%)
1	Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanosarcina	68	0.154
2	Proteobacteria;Alphaproteobacteria;Sphingomonadales;Sphingomonas	80	0.181
3	Proteobacteria;Gammaproteobacteria;Oceanospirillales;Thalassolituus	1,863	4.224
4	Proteobacteria;Betaproteobacteria;Rhodocyclales;Azovibrio	203	0.460
5	Actinobacteria;Actinobacteria;Actinomycetales;Propioniceella	1,151	2.610
6	Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanomethylovorans	252	0.571
7	Firmicutes;Clostridia;Clostridiales;Acetobacterium	1,458	3.306
8	Euryarchaeota;Methanomicrobia;Methanosarcinales;Methanolobus	5,878	13.328
9	Tenericutes;Mollicutes;Acholeplasmatales;Acholeplasma	305	0.692
10	Actinobacteria;Actinobacteria;Actinomycetales;Rhodococcus	63	0.143
11	Proteobacteria;Alphaproteobacteria;Rhodobacterales;Rhodovulum	2,990	6.780
12	Euryarchaeota;Methanobacteria;Methanobacteriales;Methanobacterium	6,106	13.845
13	Deferribacteres;Deferribacterales;Deferribacteraceae;Calditerrivibrio	92	0.209
14	Proteobacteria;Alphaproteobacteria;Caulobacteriales;Brevundimonas	532	1.206
15	Proteobacteria;Alphaproteobacteria;Rhodobacterales;Stappia	2,265	5.136

Figure 4. 16S rRNA gene amplified DNA pyrosequencing results from DNA extracted from oil of Medicine Hat Glauconitic C oil field. Percentage of reads found for *Acetobacterium* highlighted in orange. From Kryachko *et al.* 2012. OCP is the pooled microbial community associated with oil phase.

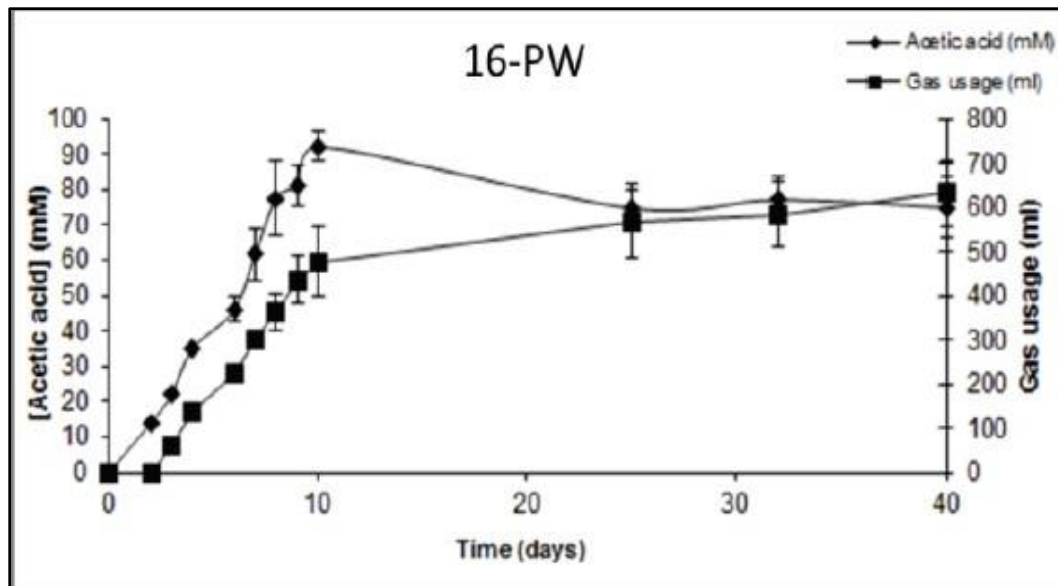


Figure 5. Mean acetic acid in millimolar and mean gas usage in mL for MHGC produced waters enriched in DSMZ medium for *Acetobacterium woodii*. From Nathoo *et al.* 2012.

1.5. The Grosmont

The Grosmont is a formation of four layers of carbonate rock in north-central Alberta. It is estimated to contain up to 500 billion barrels of oil (Wo *et al.* 2011) with 85-95% oil saturation (Machel *et al.* 2012). This formation and three smaller carbonate oil reservoirs underlie the same region as the McMurray-based Athabasca oil sands within a geographical area loosely referred to as the “carbonate triangle” (Figure 6). These exist in two separate sedimentary layers delineated by their deposition in different periods of geological time with the oil sands being deposited in the Cretaceous period and the Grosmont carbonates in the Early Devonian period (419-393 million years ago [mya], Figure 7). The Grosmont formation is considered to be part of a larger group of connecting rock layers called the Woodbend group formed within the Devonian period over a larger geographical spread of Alberta. The reservoir is relatively shallow with the eastern, shallower edge just under 200 m below surface (mbs) and the deeper western part more than 1000 mbs.



Figure 6. Location of Alberta’s bitumen carbonate reservoirs within the “Carbonate Triangle”.
 4From the Government of Alberta.

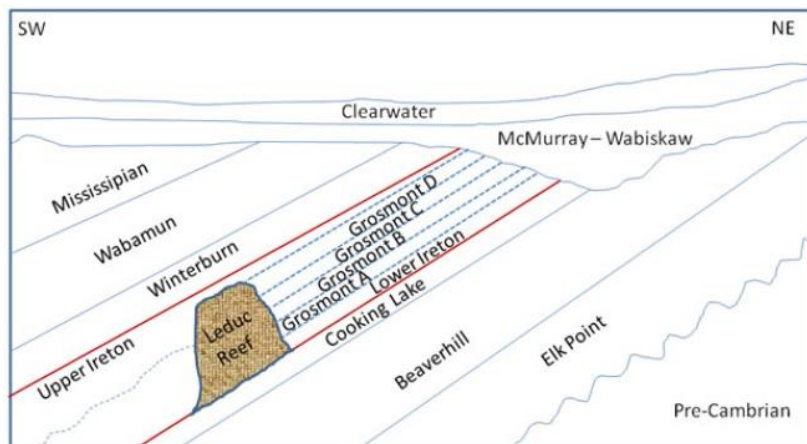


Figure 7. Stratigraphic map of the sedimentary layers in the Athabasca region that contain the Grosmont formation. The larger Woodbend group of formation consists of the Grosmont layers plus the upper and lower Ireton layers and the Leduc reef.. From Ezeuko *et al.* 2013.

The Grosmont was originally formed as marine-sourced calcium carbonate (CaCO_3). Starting relatively soon after deposition, most of the CaCO_3 was converted to dolomite ($\text{CaMg}[\text{CO}_3]_2$), to the point that nearly 98% of the reservoir is now dolomite. Two main dolomitization events had significant impact on the crystal structure of the formation. The first event occurred and consisted of evaporite dolomitization reflux (interaction with evaporating dense brines) just before its first burial in the late Devonian (370 mya) to early Mississippian (358 mya). The second event consisted of burial dolomitization (interaction with groundwaters in the reservoir) during the Grosmont's initial burial phase from the early Mississippian to late Pennsylvanian period (346 to 303 mya). The intragranular porosity of rocks changed in the earlier event and resulted in finer crystal structures, whereas the later event caused more coarse crystal structures. Unfortunately, most of the bitumen is located in areas consisting of rock with finer structures and lower porosity (Machel *et al* 2012).

Another significant source of geological change was the uplifting of the Grosmont formation over a long period of time. Based on data presented in Mossop and Shetsen (1994) the formation was completely buried by the late Devonian (370 mya) and resurfaced in the Late Jurassic (161-145 mya). Based on more recent data (Machel *et al.* 2012), the period of exposure may have been for much longer than previously reported with possible sub-aerial exposure as soon as the late Mississippian (330-323 mya). It was then exposed to surface waters, weathering and erosion until the early Cretaceous (146 to 100 mya). It was after the reburial of the rock that the period of hydrocarbon formation occurred from the middle Cretaceous (100 mya) to well into the Tertiary period (66 to 2.5 mya).

When the Grosmont was at or near the surface, the formation was exposed to low salinity, slightly acidic meteoric waters (precipitation) that caused the exposed rock to undergo

karstification, the partial dissolution of the carbonate rock. This is supported by the isotopic signatures of the water, which also suggest that at some point the reservoir was exposed to glacial melt water (Grasby and Chen 2005). This created large, uneven pores (vugs) and even caves in the rock based on where the meteoric water flowed. This made the porosity of the rock much more complex and heterogeneous (anywhere from 7 to 40% [Wo *et al.* 2011]). This reduced the permeability by having mixed sections very low discontinuous porosity that are difficult to push bitumen out of combined with sections of very high porosity that is very difficult to get steam through for thermal technologies. Today, the formation has a distinct aquifer that has been found to be saline to hypersaline, with total dissolved salts (TDS) ranging from 10 to 100 g/L. There is an average TDS of 70 g/L in the western part of the reservoir which also contains up to 5 g/L of sulfate (SO_4^{2-}). The eastern Grosmont has a lower salinity and a higher concentration of HCO_3^- (Bachu *et al.* 1993).

The geological features of the Grosmont present a challenge for the creation of flow for oil extraction by presenting a combination a tight non-porous crystal matrix and a heterogeneous, discontinuous mix of fractures and vugs. Another important challenge is the heavy nature of the bitumen. The bitumen in the Grosmont has the highest viscosity of any of the known comparable carbonate oil reservoirs with a viscosity of 1 to 10 million cP and API gravity of 5 to 9 degrees. The high viscosity precludes production of this material using conventional water flooding re-pressurization methods. The reservoir also happens to be oil-wet, which further increases the difficulty of extraction (McDougall *et al.* 2008), which can be improved by the addition of surfactants to carbonate rocks (Jarrahain *et al.* 2012).

Several forms of thermal extraction technology have been tested with limited success on the reservoir with the main two methods being steam-assisted gravity drainage (SAGD) and

cyclic steam stimulation (CSS). Both techniques involve generating steam and injecting it into an oil well to melt bitumen to allow it to be pumped to the surface. SAGD uses horizontal wells with steam being injected into an upper well where the melted oil drains via gravity into a lower extraction well (Figure 8). CSS uses vertical wells with a central steam injection well and several peripheral extraction wells, as can be seen in Figure 9. An issue with both of these schemes is the premature escape of the steam because large pockets of vugs act as alternate pathways to reroute the steam back to the surface before it can act at depth to melt bitumen. Most of the attempts with CSS and SAGD occurred in the mid 70's to early 80's. Work was largely stopped until 2007, when new attempts were made using cold solvents to attempt to dilute the bitumen (Jiang *et al.* 2009). Other technologies that have been tested include using additives with steam injection or water flooding (ineffective), CO₂ injection (same issues as steam), and *in situ* combustion (too difficult to control) (Dougall *et al.* 2008).

Given our knowledge of the geology and bitumen properties of the Grosmont, it is clear that novel technologies need to be explored for effective production of this resource. The microbial production of acids (e.g. formic or acetic acid) from small diffusible substrates (e.g. H₂ and CO₂) could potentially increase reservoir porosity and is considered here.

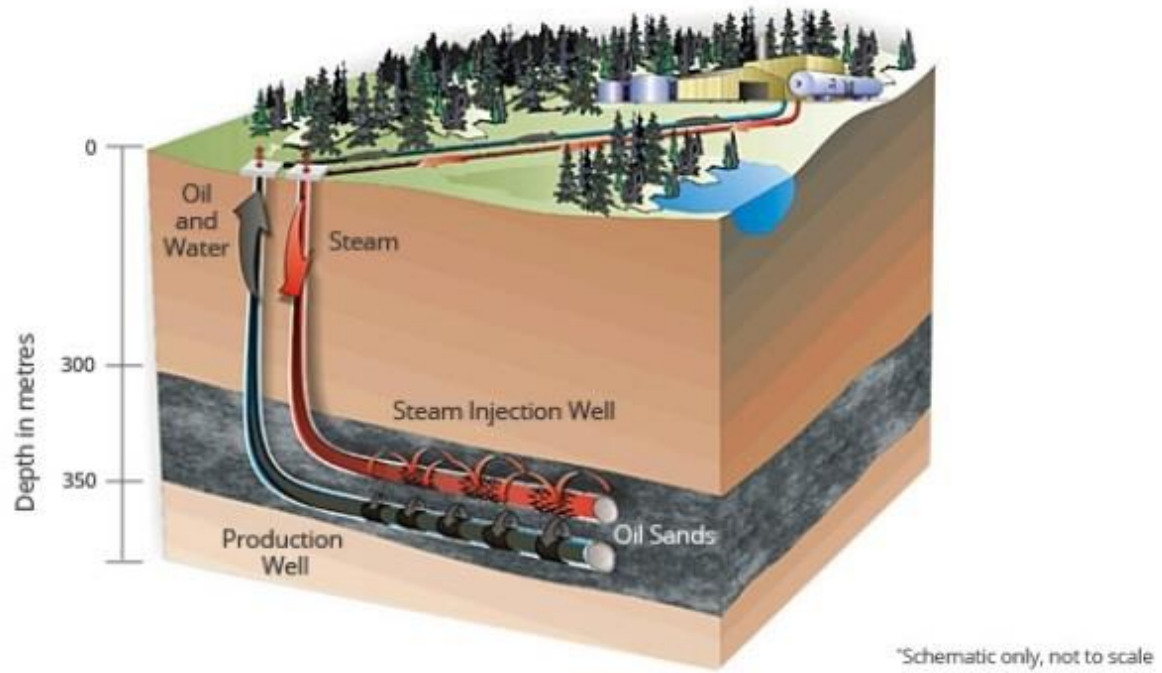


Figure 8. Steam assisted gravity drainage (SAGD). From www.megenergy.com.

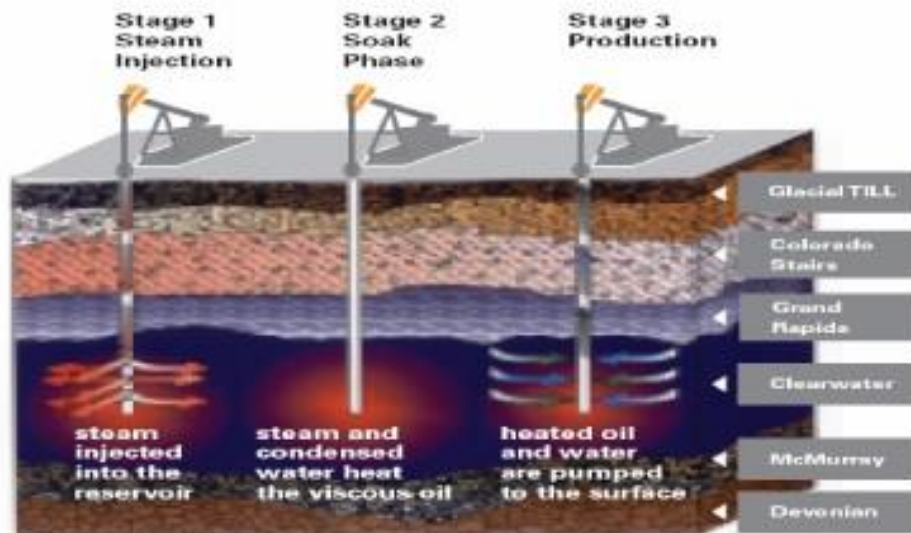
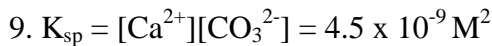
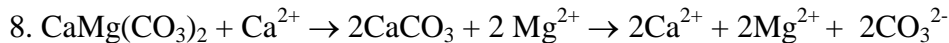
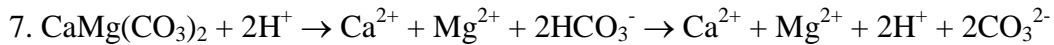
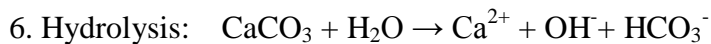
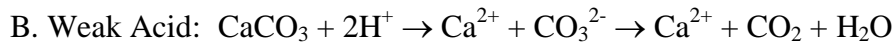
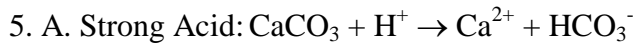


Figure 9. Cyclic steam stimulation (CSS). From www.odec.ca.

1.6. Chemistry of carbonate dissolution

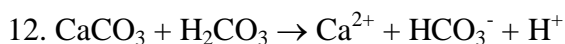
The Grosmont was originally deposited as calcite/aragonite (hence referred to as calcite), two different crystal structures but with the same chemical formula CaCO_3 . Soon after, this rock was altered by environmental processes to dolomite, $\text{CaMg}(\text{CO}_3)_2$ also written as $(\text{CaMg})(\text{CO}_3)_2$. Since the rock is permeated with groundwater it exists in aqueous phase equilibria, both with acid in the water (represented by protons, Equation 5) and the water itself (hydrolysis [Equation 6]). Acidity based on the concentration of protons (H^+) has a strong effect on the dissolution of carbonates. Dolomite has similar (but not the same) dissolution kinetics, but because of the magnesium ion it can be represented by multiple equilibria as seen in reactions 7 and 8.



The hydrolysis reaction has a relatively minor effect in the dissolution at groundwater pH since it requires a high level of acidity to have a significant effect. Both solubility equilibria are quantified by their solubility products (equation 9 for calcite, equation 10 for dolomite). The solubility product of dolomite is difficult to analyze with accuracy because though it is a very common sedimentary rock, there isn't any dolomite found forming in nature today under "normal" environmental conditions and it has only been prepared under relatively extreme conditions in the lab (very high temperatures, pH above 9.5, high SO_4^{2-} and NO_3^- concentrations). This geological mystery is commonly referred to as the "Dolomite Problem",

though due to its regular and highly ordered crystal structure, long periods of time are probably required for dolomite formation (Langmuir 1997). Both calcite and dolomite solubility are affected by the concentration of calcium ions with dolomite also being affected by the concentration of magnesium ions.

Due to involvement of the carbonate ion, the acid equilibrium of CaCO_3 is strongly influenced by CO_2 and the carbonic acid (H_2CO_3) equilibrium in water (Equation 11). It is also a source of acidity, so that Equation 11 can also be represented by Equation 12.



From this it can be noted that the concentration of H_2CO_3 in the aqueous phase is dependent on the concentration of CO_2 dissolved in solution, which is in turn defined by the partial pressure of CO_2 in an adjacent gas phase. Carbonic acid doesn't have to be the only acid present in the water or even be the acid that controls the overall pH of the solution. Interconnection between CO_2 , the CO_3^{2-} ion and $\text{CaCO}_3/\text{CaMg}(\text{CO}_3)_2$ has an important influence on the solubility of both types of rock in water, but the acidity of carbonic acid has an even greater effect. This is very important both in consideration of technologies for injecting high concentrations of CO_2 into the ground (carbon capture) and in how this relates to CO_2 producing and consuming microbes.

Other chemical species that are present in water, many due to microbial activity, may also affect these equilibria via their effect on the pH. Under anaerobic conditions these can include H_2S and CH_3COOH , which increase the concentration of protons ($[\text{H}^+]$) and thus increases solubility, and NH_3 which due to its basic nature decreases $[\text{H}^+]$ and also solubility. The hydrolysis reaction produces an OH^- ion, decreasing solubility. Under oxic conditions, the

predominant aerobic respiration produces CO_2 , again increasing solubility (Langmuir 1997) and decreasing dissolution.

High partial pressure of CO_2 increases carbonate solubility by increasing the amount of aqueous carbonic acid, which would be important for *in situ* experiments but not for laboratory work. While high temperature does affect the carbonate solubility equilibria, it decreases the solubility of CO_2 (and thus the $[\text{CO}_2]_{\text{aq}}$) more than it increases the solubility of the carbonate with a net effect of a decrease in the solubility. Other ions may have an inhibitory effect by forming a complex with the calcium ion, preventing it from forming solid CaCO_3 , instead creating CaSO_4 , CaHCO_3 or CaCl_2 , with CaSO_4 having the most stability and impact.

The factors that have the largest effect on dissolving carbonate rock are acidity $[\text{H}^+]$ and undersaturation of calcium, magnesium and/or carbonate ion. Most other factors are related to how they influence these four main effects. Calcium and magnesium levels may be affected by microbial activity through their influence on sulfate levels, which can complex with calcium. Carbonate ion concentration is controlled by CO_2 levels in water and carbonate rock can be dissolved by increasing aqueous CO_2 concentrations. This increases dissolution by increasing acidity through carbonic acid more than it increases solubility by increasing carbonate ion levels. Decreasing pressure and temperature could also affect this, but these would be stable and difficult to change for an *in situ* treatment. Acidity and CO_2 levels are the most amenable factor to influence by microbial activity including the production of acetic acid to increase protons and microbial CO_2 consumption. As stated above, acidity can also be affected by other microbial products that have an acid-base activity. They may also be affected by exposure to meteoric surface water to dilute the water of the reservoir and circulate these ions away from the carbonate rock.

1.7. Research Challenges

In terms of applications of acetogens for biotechnology, a significant challenge is that most acetogens are not able to grow below pH 5.5, i.e. in the presence of high concentrations of CH_3COOH , dissociated to CH_3COO^- and H^+ . (Note that CH_3COOH may well be truly toxic agent, as it is membrane permeable and inside the cell, decreasing the proton motive force [PMF]). They are in part dependent on proton motive force to generate ATP. This requires a relatively higher concentration of protons on the inside of the bacteria relative to outside the membrane to create a transmembrane potential. At low pH the concentration of protons outside the bacterial cell membrane is very high and disrupts transmembrane potential. Though some acid tolerant acetogens have been found (Traunecker *et al.* 1991), the sensitivity of acetogens to high concentrations of end-product poses a potential challenge for attempts at their *in situ* biostimulation.

A low pH is important for carbonate dissolution, but it can also be limiting further acetogenic growth. A balance must be found between the range of limitation of acetogenesis (<5.5) and the rate of calcite dissolution, which according to Chou *et al.* 1989, increases in rate in ranges of 5.5 to 6.5 though re-precipitation does not start to become significant until pH 7.2 (Figure 10).

Another challenge for acetogenic growth is the direct competition of acetogens with methanogens. These can be either hydrogenotrophic methanogens which compete for substrate with acetogens or they can be acetotrophic methanogens which consume the acetic acid formed causing re-precipitation of carbonates. How acetogens interact and compete with these methanogens needs to be further studied. One way to do this is by adding a methanogen inhibitor

such as sodium 2-bromoethane sulfonate (BESA) to compare how acetogens grow when methanogens are not active. BESA inhibits the final enzymatic step of methanogenesis by competing with methyl-coenzyme M for the active site of methyl-coenzyme M methylreductase (Figure 11). Adding BESA is useful as a method for understand the effects of an acetogens dominated culture versus a mixed acetogen-methanogen culture, but it is not a viable option for an *in situ* treatment as BESA is highly ionic and would not dissolve in oil.

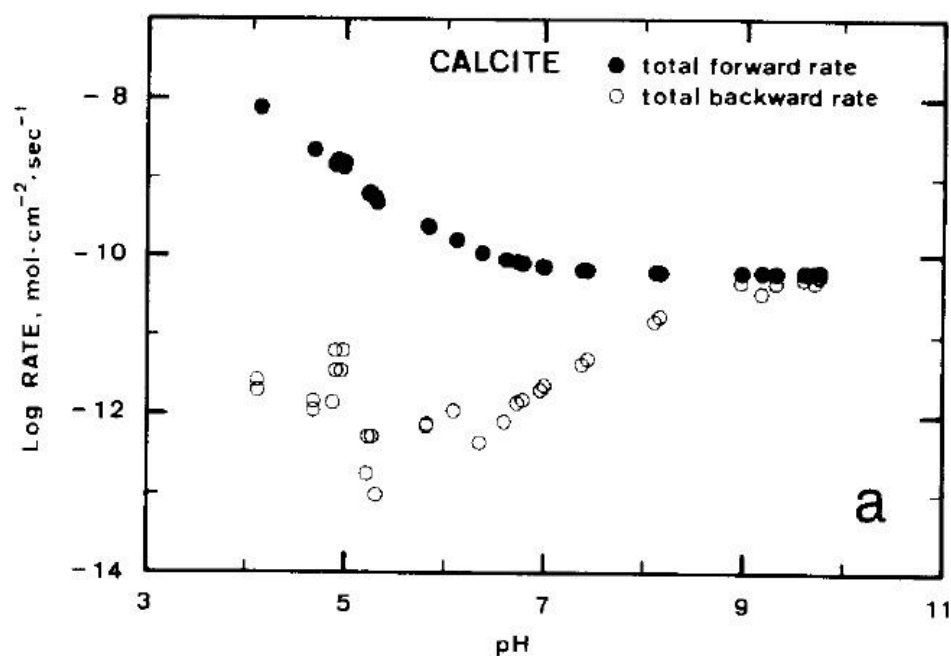


Figure 10. The total forward and backward rates of calcite dissolution as a function of pH. From Chou *et al.* 1989.

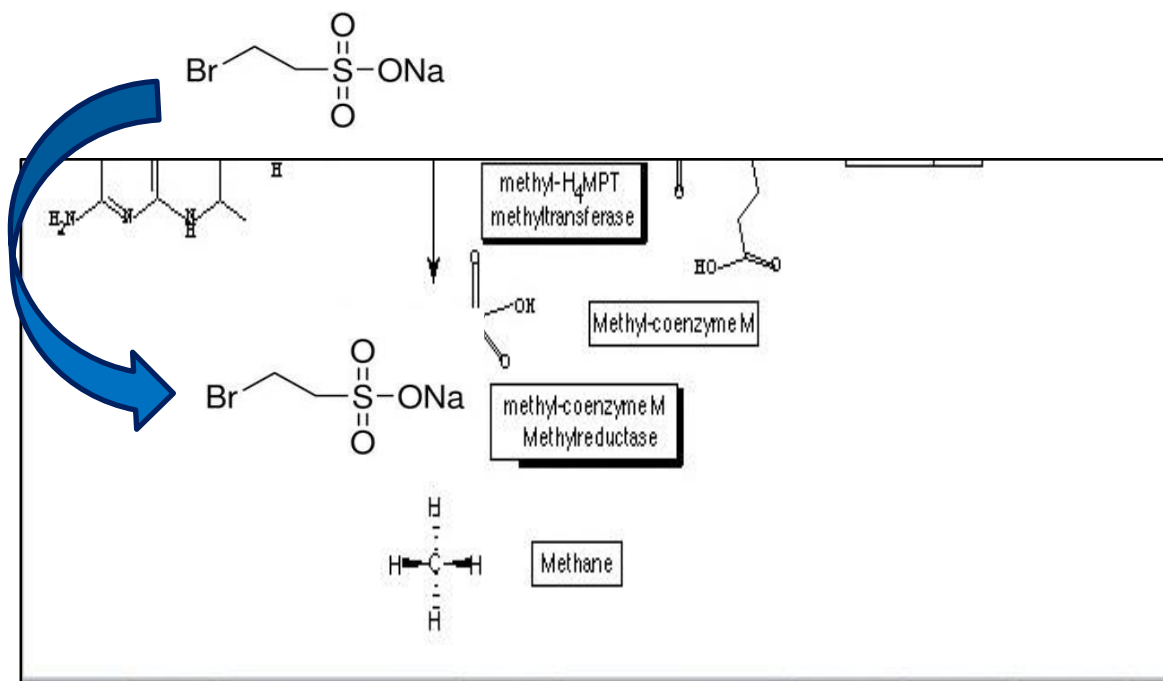


Figure 11. Mechanism of BESA action to inhibit methanogenesis. BESA interferes with the methanogenic biochemical pathway by inhibiting the final enzyme in the pathway to receive a methyl group that would otherwise be converted to methane. Figure from Ma 2011.

1.8. Conclusions

There are many factors to consider in evaluating the potential value and utility of acetogen-based MEOR technologies, including the geology and chemistry of the Grosmont bitumen reservoir, the resident microbial community and the mechanisms of acetogenesis itself. The *in situ* use of native microbes may solve some of the problems associated with previous attempts at using biogenic acid production for MEOR, but there are other issues that still need to be addressed including the challenge in balancing the importance of pH levels in carbonate dissolution with their inhibitory effect on acetogenic activity.

CHAPTER 2 OBJECTIVES

Acetogens in the context of oil reservoirs have not been widely studied with most work associating acetogens and oil field focusing on identification (Youssef *et al* 2009, Davydova-Charakhch'yan *et al.* 1993, Herman *et al.* 1992) with only a few papers further studying their metabolic capacities and competitiveness (Grabowski *et al.* 2005). Some potential biotechnological applications have been explored but these have used acetogens in supportive roles for technologies focusing on biocorrosion (Mori *et al.* 2010) and bioremediation (Zhao *et al.* 2011). The goals of this thesis work are as follows:

1) Promote acetogenic microbial growth over methanogenic growth under methanogenic mesophilic conditions.

Acetogens have been found to be competitive with methanogens under conditions that are not considered to be favourable to methanogenic growth such as cold environments of less than 20°C. For acetogen biostimulation to be viable as a biotechnology, their growth should be promotable under mesophilic (20-30°C) temperatures as well. Also it would just as useful to further the understanding of conditions affecting and suppressing methanogenic growth in an environment in which methanogens would otherwise be viable. Also important is to characterize the effect of acetotrophic methanogenesis and whether it has the potential to completely reverse the effects of acetogenesis and if there is anything that can be done to prevent this.

2) Optimize culture conditions of oil-field sourced acetogenic microbes.

Acetogens have been enriched successfully from oil field produced waters before, but the basal medium included yeast extract and added tryptone, which are undefined and as a biostimulation nutrient package would be expensive. Other potentially factors that influence acetogenic growth have not been examined and may be useful in understanding how to increase competitiveness of acetogens. Factors tested here include: nutrient levels, culture time, gas composition, and presence or absence of solids.

3) Test for potential biotechnology applications.

Since oil fields can be considered desirable targets for biotechnology applications, the potential benefits (or undesirable side effects) of using biotechnology involving acetogenic growth should be explored. Acetogens can produce an organic acid (CH_3COOH) from the gaseous substrates of H_2 and CO_2 , gases which also happen to be oil-soluble and have the ability to diffuse much further into a reservoir than the aqueous solutions that are typically used. The production of an acid may be useful in separating oil from tightly bound rock through rock dissolution which is especially problematic in carbonate oil reservoirs and may benefit particularly from having some of the rock dissolved. To that effect the potential of acetogenic enrichment to effect carbonate rock dissolution, permeability and porosity was also tested.

A specific hypothesis or hypotheses for each experiment are as follows:

Experiment #1: If aqueous carbon (bicarbonate) is removed, acetogens grow from dissolved CO_2 gas from the headspace and produce acetic acid.

Experiment #2: If all sources of hydrogen are removed, acetotrophic methanogens consume acetate and produce methane.

Experiment #3: If 100 mM CaCO_3 and 30 mM NaHCO_3 are added to the medium, pH is sufficiently buffered during enrichment to prevent acid-limitation and increase acetic acid levels.

Experiment #4: a) If the NaHCO_3 is removed, CaCO_3 alone is sufficient to pH buffer the medium, prevent acid limitation and increase acetic acid.

b) If microbial acetic acid is produced in the presence of CaCO_3 , dissolution of CaCO_3 results in a mass loss is detected that is statistically different from sterile controls.

Experiment #5: If a 100% CO_2 headspace is used CaCO_3 will dissolve chemically and significantly less CaCO_3 will be recovered than from an environment with a 100% H_2 headspace.

Experiment #6: If powdered CaCO_3 is replaced with crushed carbonate core, then sampling loss will be reduced and result in a significant loss of mass where the highest amount of microbial acetic acid is produced. Is acetic acid or methane affected?

Experiment #7: If a single nutrient (NaCl , MgCl_2 , CaCl_2 , trace metals or PO_4^{2-}) is increased then acetic acid will increase and if that nutrient is decreased then acetic acid will also decrease.

Experiment #8: If the concentration of CaCO_3 is decreased from 100 mM to 20 mM then a significant mass loss of CaCO_3 will be observed in bottles with more microbial acetic acid. Is acetic acid or methane affected?

Experiment #9: If a solid carbonate core is incubated in medium that produces a significant amount of microbial acetic acid then a proportional mass of carbonate rock dissolves and increases porosity and/or permeability.

CHAPTER 3 MATERIALS AND METHODS

3.1. Source inoculum and media

Produced water samples were collected along with associated heavy oil (API gravity of 16 degrees) from the Medicine Hat Glauconitic C (MHGC) field near Medicine Hat, Alberta in sterile 1-L Nalgene bottles, filled to the brim to exclude as much air (and oxygen) as possible and transported back to the lab in Calgary within 5 hours (Figure 12). They were then stored in the anaerobic hood containing an atmosphere of 90% (v/v) N₂ and 10% CO₂. Samples from production wells 13-PW and 5-PW were used.

The medium used in all enrichments was a modified Coleville Synthetic Brine variant K (CSB-K) medium (Tables 1,2 and 3). It is representative of the chemical environment found in the MHGC field. Low and high nutrient versions of this medium were used. Sodium bromoethane sulfonate (BESA) was added to 20 mM from a 1 M stock solution to inhibit methanogenic activity.

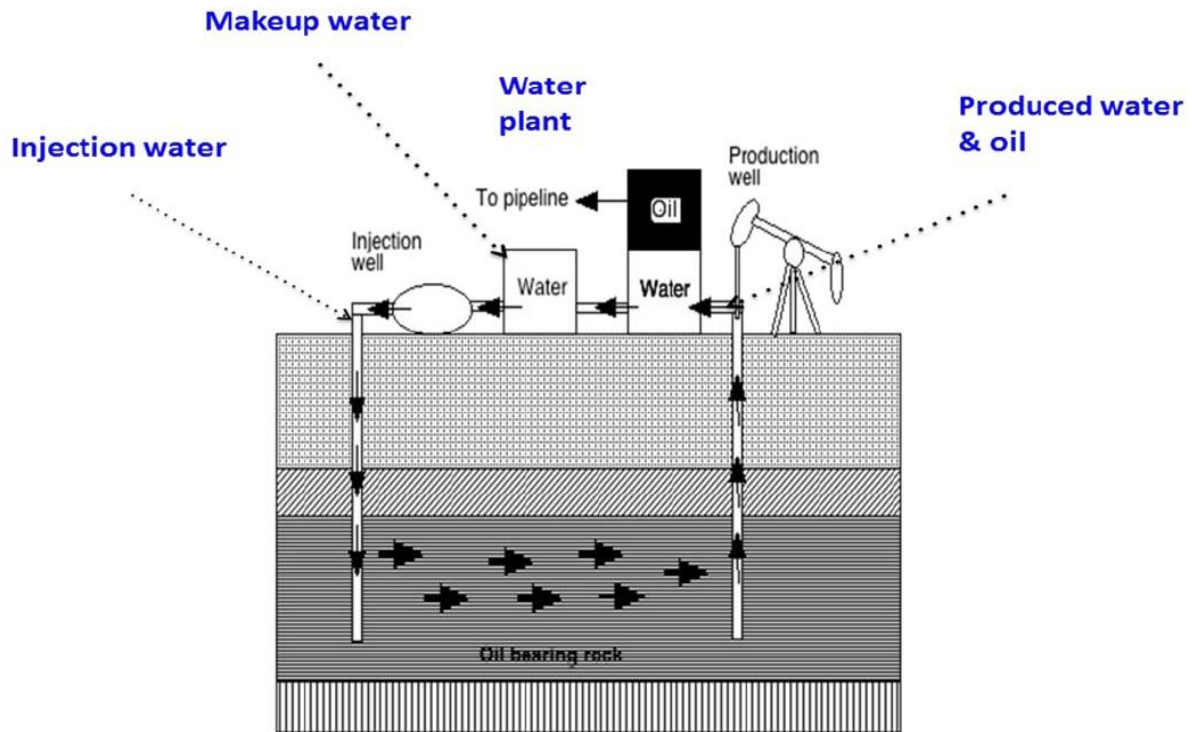


Figure 12. Oil production by produced water re-injection. Water is injected into an oil reservoir to re-pressurize the reservoir and push more oil out of the rock towards the production well. A water and oil mixture is recovered at the production well, the oil is separated out and sent to market and the water is reused and re-injected into the well. The water is considered to contain a microbial community representative of that found in the oil reservoir (From Folarin 2013).

Table 1. Composition of high and low nutrient CSBK media.

Nutrient	Concentration (g/L)	
Medium Type	High	Low
NaCl	1.5	1.5
CaCl ₂ .H ₂ O	0.21	0.21
MgCl ₂ .H ₂ O	0.54	0.54
NH ₄ Cl	0.3	0
KCl	0.1	0.1
KH ₂ PO ₄	0.05	0
Reseazurin	1-2 drops	1-2 drops
Trace Elements	1 mL	0 mL
Tungstate Selenite	1 mL	0 mL
NaHCO ₃ Solution	0 mL	0 mL
Adjust pH to 7.2-7.5 with 2N HCl.		

Table 2. Composition trace element solution used in CSBK media (Table 1).

	Concentration (mg/L)
Na ₂ EDTA	5200
FeSO ₄ .7H ₂ O	2100
H ₃ BO ₃	30
MnCl ₂	100
CoCl ₂ .6H ₂ O	190
NiCl ₂ .6H ₂ O	24
CuSO ₄ .5H ₂ O	3
ZnCl ₂	68
Na ₂ MoO ₄ .2H ₂ O	36
Adjust pH to 6.5 with 5N NaOH.	

Table 3. Composition tungstate-selenite solution used in CSBK media (Table 1).

	Concentration (mg/L)
Na ₂ SeO ₃ (anhydrous)	4
or	
NaOH + Na ₂ SeO ₃ .5H ₂ O	400 + 6
Na ₂ WO ₄ .2H ₂ O	8

3.2. Enrichments

Enrichments of the microbial community, for the purpose of increasing the growth and activity of acetogens and in some cases methanogens, were performed in closed serum bottles.

These were set up by:

- 1) Prepare either low nutrient or high nutrient medium, as indicated in Table 1 without heat sensitive trace elements and tungstate-selenite.

- 2) Transfer to a 1 or 2 L Widdel flask depending on the size of the experiment and autoclaving at 120⁰C along with empty serum bottles, stoppers stored in deionized water and forceps.

- 3) Cool to room temperature and bubbling with 90% N₂, 10% CO₂ (N₂-CO₂) to an anoxic (oxygen-free) state which takes approximately 30 minutes. Resazurin dye will turn pink.

- 4) Add filter-sterilized trace elements solution and tungstate-selenite solution to high nutrient medium and bubble with N₂/CO₂ until anaerobic.

- 5) Adjust the pH of the medium to 7.2 to 7.5 with 2 M HCl and 5 M NaOH. When correctly adjusted and anoxic, the solution will be light blue due to the presence of resazurin dye.

- 6) Transfer medium to sterile serum bottles anoxically and using a gas syringe to flush the bottle head space. Cap the bottles with sterile rubber stoppers. If powdered CaCO₃ was added, it will be milky white in suspension.

- 7) Crimp serum bottles with aluminum rings to maintain seal (Figure 13).

- 8) Inject 1 ml of 1 M BESA stock per 50 mL of medium through stopper, if needed.

9) Inoculate with 10% (v/v) of produced waters wells (13-PW or 5-PW depending on availability) from the MHGC field. Inoculate the serum bottles at a proportion of 10% which involves adding 10 mL of produced water to 90 mL of medium or 5 mL to 45 mL of medium depending on the final desired volume. The solution turns clear after inoculation or stays light blue due to the reduction of the resazurin dye.

10) Flush the headspace of the serum bottles with the desired gas, either 80% H₂ and 20% CO₂ (H₂-CO₂) or 90% N₂/ 10% CO₂ (N₂-CO₂). If the resazurin in the medium turns pink over the course of the experiment, then the redox potential is high and the medium has likely been exposed to oxygen. Inject drops of 0.2 mM sodium sulfide into the bottles using a 1 mL syringe to act as an oxygen scavenger. When the bottles become clear again this indicates an anaerobic state with the desired redox potential.

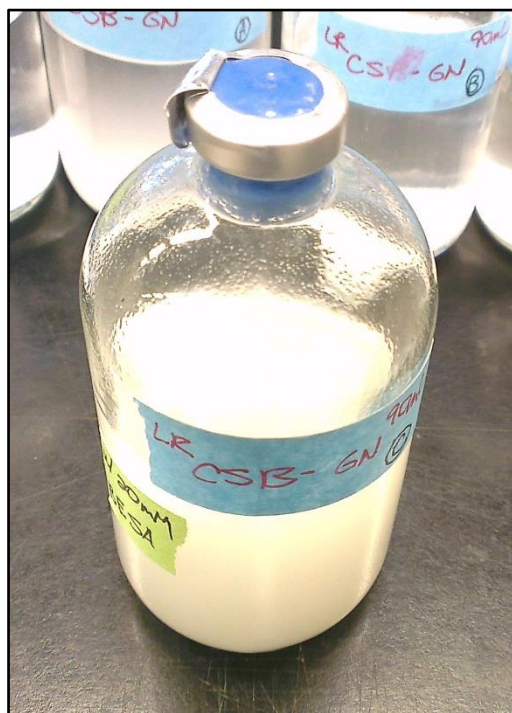


Figure 13. An example of a serum bottle batch test after inoculation from experiment #3. 100 mM CaCO_3 was added and is in suspension here.

3.3. Experiment #1: Growth of hydrogenotrophic acetogens and methanogens

Enrichments were prepared as detailed in section 2.1 with 45 mL of medium and 5 mL of inoculum in 120 mL bottles. Of six bottles four contained high nutrient medium and two contained low nutrient medium. Two bottles of high nutrient medium were amended with BESA. Hence the experiment consisted of two replicates each of the low nutrient medium, high nutrient medium and high nutrient medium with BESA (Table 4). After inoculation, the headspace inside these bottles was flushed with $\text{H}_2\text{-CO}_2$. The first set of six bottles (the “primary” enrichments) were incubated with oil field produced water inoculum (13-PW, September 2012) and incubated for 28 days at 30°C . On day 21, 5 mL of each bottle was removed via 5 mL syringe and injected into a second set of six identical serum bottles (the “secondary” enrichments), which was then incubated for 30 days at 30°C .

3.4. Experiment #2: Growth of acetotrophic methanogens

Duplicate bottles with media as in experiment #1 were amended with either 0, 1 or 5 mM sodium acetate (pH 7.1) from a 1 M stock (Table 5). The headspace in the bottles was $\text{N}_2\text{-CO}_2$. The primary enrichment of experiment #1 on day 28 was used as the inoculum. Because this is the first acetate amended experiment it should be considered a primary enrichment. A secondary enrichment used this primary enrichment as an inoculum following 21 days of growth. Both enrichments were incubated at 30°C for 99 days. All bottles were in duplicate.

3.5. Experiment #3: Growth of acetogens and hydrogenotrophic methanogens in the presence of CaCO_3 and NaHCO_3

The third set of experiments was run under the same parameters as experiment #1 (Table 6) except that 1000 ± 10 mg of powdered CaCO_3 was added to the serum bottles. The

CaCO₃ was suspended in 2 mL of deionized water before autoclaving. 30 mM of NaHCO₃ was added using a 1 M stock. The medium volume was increased to 100 mL (90 mL medium, 10 mL inoculum) in 150 mL serum bottles (headspace is 50 mL). The primary enrichment was run for 60 days and the secondary enrichment was run for 62 days. All bottles were in duplicate.

3.6. Experiment #4: Variation with no NaHCO₃

The fourth experiment contained 100 mM of CaCO₃ but no NaHCO₃ was added (Table 7). At the end of the experiment, the remaining CaCO₃ solid was collected by vacuum filtration on 0.2 µm nylon GNWP Millipore filters and weighed on an analytical balance. To minimize CaCO₃ loss in sampling, suspended solid was allowed to settle out, inverted with the sampling needle inserted before sampling. The number of replicates for each experimental state was increased to 3 for a total of 18 bottles. Both enrichments were incubated for 97 days at 30⁰C.

3.7. Experiment #5 Variation with H₂ or CO₂ only headspace

Experiment #5 was conducted similar parameters to experiment #4 but instead of using 80% H₂/20% CO₂ headspace gas, either 100% H₂ or 100% CO₂ was using in the two sets of bottles and sterile controls were not used (Table 8). The CaCO₃ was again recovered at the end of the experiment. Both enrichments were incubated for 58 days at 30⁰C.

3.8. Experiment #6: Variation with crushed Grosmont core

The sixth experiment was performed under the same parameters as experiment #4 but using crushed carbonate core from the Grosmont formation sorted to a size fraction of 0.06 to 0.19 inches (Table 9). The primary enrichment was incubated for 97 days, the secondary enrichment was incubated for 91 days. Both enrichments were incubated at 30⁰C.

Table 4. Experimental set up of experiment #1.

	# of Bottles
Low Nutrient Medium	2
High Nutrient Medium	2
High nutrient + BESA	2
Total	6

Table 5. Experimental set up of experiment #2.

Medium	Acetate Added (mM)
Low Nutrient Medium	0
	1
	5
High Nutrient Medium	0
	1
	5
High Nutrient + BESA	0
	1
	5

Table 6. Experimental set up of experiment #3.

Changes: CaCO ₃ + NaHCO ₃ Added	# of Bottles
Low Nutrient Medium	2
Low Nutrient Sterile Control	2
High Nutrient Medium	2
High Nutrient Sterile Control	2
BESA Medium	2
BESA Sterile Control	2
Total	12

Table 7. Experimental set up of experiment #4.

Changes: no NaHCO₃ added	# of Bottles
Low Nutrient Medium	3
Low Nutrient Sterile Control	3
High Nutrient Medium	3
High Nutrient Sterile Control	3
BESA Medium	3
BESA Sterile Control	3
Total	18

Table 8. Experimental set up of experiment #5.

Changes: 100 % H₂ or 100% CO₂ headspace	# of Bottles
Low Nutrient Medium	
H₂ Only	3
CO₂ Only	3
High Nutrient Medium	
H₂ Only	3
CO₂ Only	3
BESA Medium	
H₂ Only	3
CO₂ Only	3
Total	18

Table 9. Experimental set up of experiment #6

Changes: crushed carbonate rock instead of CaCO₃	# of Bottles
Low Nutrient Medium	3
Low Nutrient Sterile Control	3
High Nutrient Medium	3
High Nutrient Sterile Control	3
BESA Medium	3
BESA Sterile Control	3
Total	18

3.9. Experiment #7: Variations with nutrients

Multiple sets of medium were tested with single nutrient variations in the medium (Table 10). The amount of NH_4Cl added (nitrogen source) was doubled from 0.35 g/L to 0.7 g/L for all nutrient bottles. The size of serum bottle used was changed to 50 mL with 25 mL of medium.

In the “NaCl” variation, the concentration of NaCl was increased from 1.5 g/L to 5.0 g/L. The “ MgCl_2 ” variation had the amount of CaCl_2 decreased from 0.21 g/L to 0.14 g/L and MgCl_2 was decreased from 0.54 g/L to 0.14 g/L. The final 6 variations had salts adjusted to these experimental levels with NaCl at 5.0 g/L and CaCl_2 and MgCl_2 at 0.14 g/L. The “No TM” variation had no added trace metal solution, with the regular amounts of added phosphate. The “2xTM” variation had doubled trace metals. The “No PO_4 ” had normal amount of added trace metals and no added KH_2PO_4 . The “2x PO_4 ” variation had doubled the normal amount of phosphate. Two live controls of the high nutrient medium and the low nutrient medium were also included to control for the change in medium volume and the increase in nitrogen instead of sterile controls. Powdered CaCO_3 (100 mM) was still added so the amount added was scaled down by a quarter to 250 ± 10 mg. The enrichment was run for 58 days at 30°C .

3.10. Experiment #8: Variation with 20 mM CaCO_3

The concentration of added CaCO_3 was decreased to 20 mM for this experiment. Medium variations were for both a primary and secondary enrichment in low nutrient medium, no trace metals and doubled trace metals (Table 11). A sterile control was also included.

3.11. Experiment #9: Variation with Grosmont solid carbonate core

The final set of experiments was the enrichment in media containing carbonate cores sourced from the Grosmont that had been stripped of its oil. Since a carbonate core could not fit inside a typical commercial serum bottle, a serum bottle was created from Mason canning jars. With the assistance of the University of Calgary Glassblowing Workshop, the lids were altered with the top part of a serum bottle and stopper to create a sampling port to take samples during the experiment without having to open the jars (Figure 14). The seal generated wax inlay on the inside of canning lid maintained an anaerobic air-tight seal for 30 days (incubation temp 30⁰C). Both an autoclaved sterile control and an unamended control were performed. Only 8 cores were available, so one of each control was performed along with three replicates each of low nutrient and high nutrient medium (Table 12). A summary of experiments and their main parametric difference are summarized in Table 13.

Table 10. Experimental set up of experiment #7.

Changes: ↓ medium volume, nutrient variations	# of Bottles
NaCl	3
MgCa	3
No TM	3
2xTM	3
No PO₄	3
2XPO₄	3
High Nutrient Control	3
Low Nutrient Control	3
Total	24

Table 11. Experimental set up of experiment #8.

Changes: ↓ CaCO₃ to 20 mM	# of Bottles
Low Primary	3
Low Secondary	3
No TM	3
2xTM	3
Sterile	3
Total	15

Table 12. Experimental set up of experiment #9.

Changes: Grosmont solid carbonate core instead of CaCO₃	# of Bottles
Sterile Control	1
Unamended Control	1
Low Nutrients	3
High Nutrients	3
Total	8

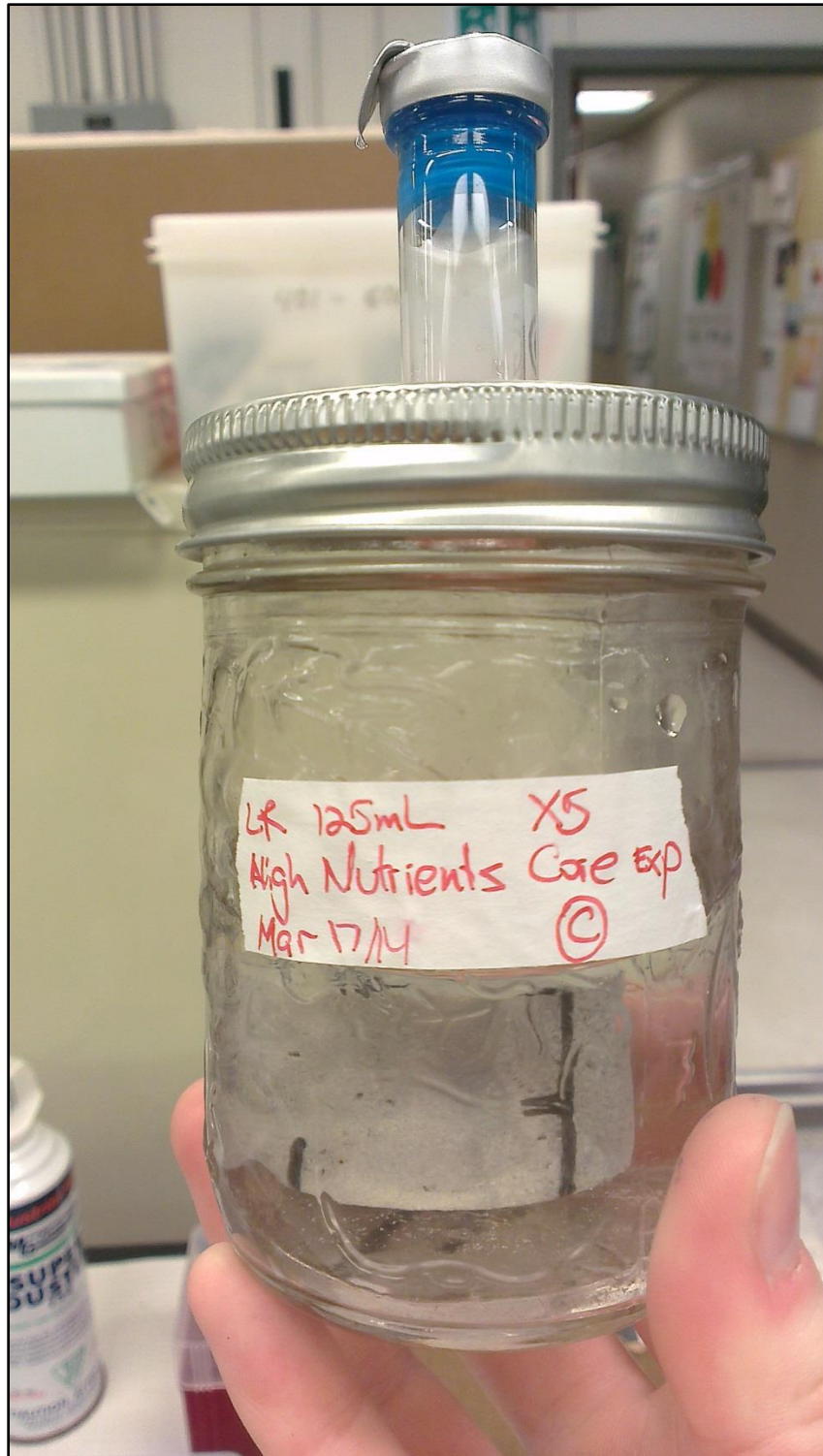


Figure 14. An example of a serum bottle adapted from a mason jar used to test the dissolution of solid pieces of carbonate core in experiment #9.

Table 13. Summary of parameter variations between experiments.

Parameter Comparison	Headspace Gas	Solid	30 mM Bicarbonate	Nutrient Variation	Change
Experiment #1	80% H ₂ /20% CO ₂	☒	☒	☒	Base Line
Experiment #2	90% N ₂ /10% CO ₂	☒	☒	☒	N ₂ /CO ₂
Experiment #3	80% H ₂ /20% CO ₂	100 mM CaCO ₃	✓	☒	Added CaCO ₃ and NaHCO ₃
Experiment #4	80% H ₂ /20% CO ₂	100 mM CaCO ₃	☒	☒	Removed NaHCO ₃
Experiment #5	100% H ₂ or 100% CO ₂	100 mM CaCO ₃	☒	☒	100% H ₂ or CO ₂
Experiment #6	80% H ₂ /20% CO ₂	100 mM crushed carbonate rock	☒	☒	Crushed carbonate rock
Experiment #7	80% H ₂ /20% CO ₂	100 mM CaCO ₃	☒	Variations	Nutrient Variations
Experiment #8	80% H ₂ /20% CO ₂	20 mM CaCO ₃	☒	2 x nitrogen and trace metals	Reduced [CaCO ₃]
Experiment #9	80% H ₂ /20% CO ₂	Solid carbonate cores	☒	2 x nitrogen and trace metals	Solid carbonate core

Gas consumption, methane production, pH and acetic acid levels and DNA analysis were measured as detailed in sections 3.13-3.14. “Sampling and Analysis”. Sampling methods are detailed in the “Sampling and Analysis” section 3.12.

3.12. Sampling

Sampling was performed three times a week for experiments #1 through #3 and once a week for experiments #4 through #9. After gas use analysis, a 0.2 mL sample of headspace was taken from the bottles using a 1 mL sterile syringe with a 24 gauge sterile needle and used for methane analysis. A 1 mL liquid sample was then taken from medium of each bottle and then stored in a labelled 1.5 mL microcentrifuge tube. This sample was then used for pH measurement, sealed with parafilm, and then frozen at -20°C until DNA extraction and analysis. The bottles had to be inverted to sample the liquid medium which in experiments using powdered CaCO_3 resulted in re-suspension of the CaCO_3 which could then be removed during sampling and increase error in the mass recovery analysis. To minimize this, after the needle was inserted, the bottles were rested upside down until the CaCO_3 had settled out of the medium. A sample was then taken.

3.13. Chemical analysis

Sampling and analysis occurred over two days. On the first day gas sampling and related measurements involving the first two analyses were conducted. The day after, liquid sampling and related measurements were conducting involving next two analyses. Samples were then frozen until DNA analysis (the fifth analysis) could be performed. The ammonium assay was performed for the primary enrichment of experiment#4.

1) Headspace gas consumption was measured by inserting a 30 mL syringe flushed with the headspace gas of the experiment (either H₂/10% CO₂, 90% N₂/10% CO₂, 100% H₂ or 100% CO₂) into the top of the serum bottle (Figure 15). Gas consumption causes the pressure of the headspace in the bottle to become negative which pulls the gas from the syringe into the bottle until the pressures equalizes to atmospheric pressure. The amount of gas pulled into the bottle (ml) was quantified.

Determining gas usage

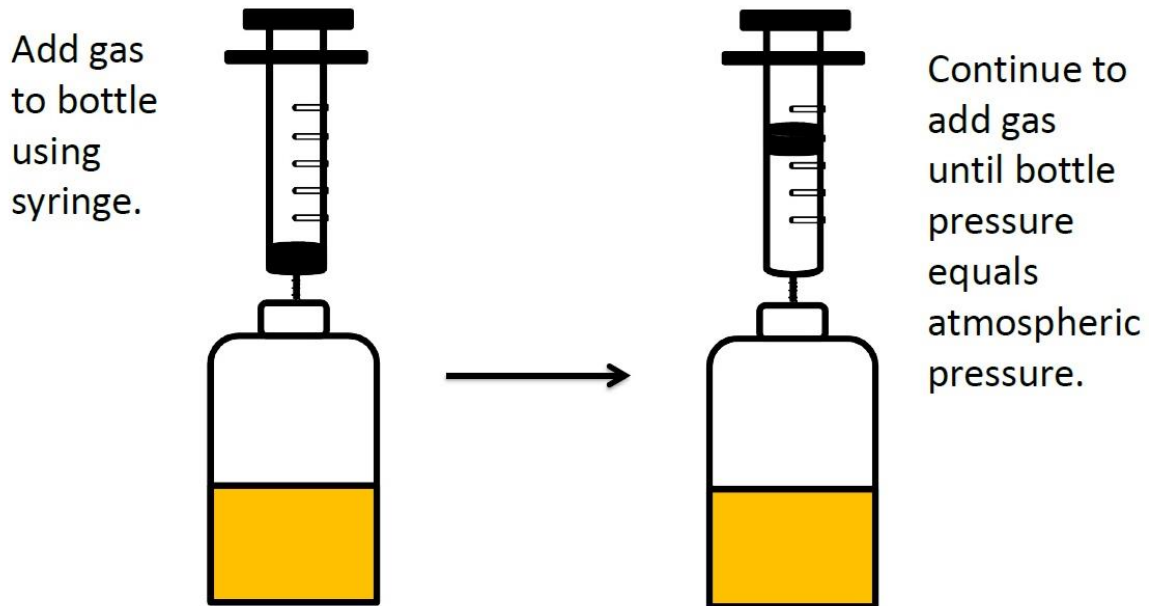


Figure 15. Schematic of gas consumption measurement by syringe addition. A syringe was filled with the requisite headspace gas and injected through the bottle stopper. Negative pressure created inside the serum bottle headspace due to gas consumption pulled the gas inside the syringe into the bottle until the headspace pressure equalized back to atmospheric pressure. The consumed volume was recorded.

2) Methane measurement: a 0.2 mL sample of headspace gas was injected into a Hewlett Packard Model 5890 gas chromatograph equipped with a flame ionization detector (GC-FID). The column used was packed stainless steel of the Poropak R model (80/100, 0.0049 cm by 5.49 m). Injector temperature was 150⁰C, detector temperature was 200⁰C and oven temperature was 100⁰C. An example of a calibration curve is shown in Figure 16. Serum bottles with defined methane concentrations were created as follows:

- a) Closed, stoppered and crimped 150 mL serum bottles were flushed with 100% N₂ gas.
- b) A volume of 100% CH₄ gas was added to each bottle to adjust them to a certain percentage, which was then converted to micromolar (μM) methane concentrations by considering that 1 L of gas is 24.52 L/mol at 22⁰C and 1 atmosphere. (Table 14).

Table 14. Calculated volume that needs to be added to a 100% N₂ serum bottle to create a certain percentage of CH₄ and conversion of % CH₄ to concentration of CH₄ (μM).

CH ₄ % (v/v)	Volume Added CH ₄ (mL)	Concentration CH ₄ (μM)
0.1	0.155	41.64
0.2	0.31	83.28
0.5	0.775	208.19
1	1.55	416.39
2	3.1	832.77
5	7.75	2081.94
10	15.5	4163.87

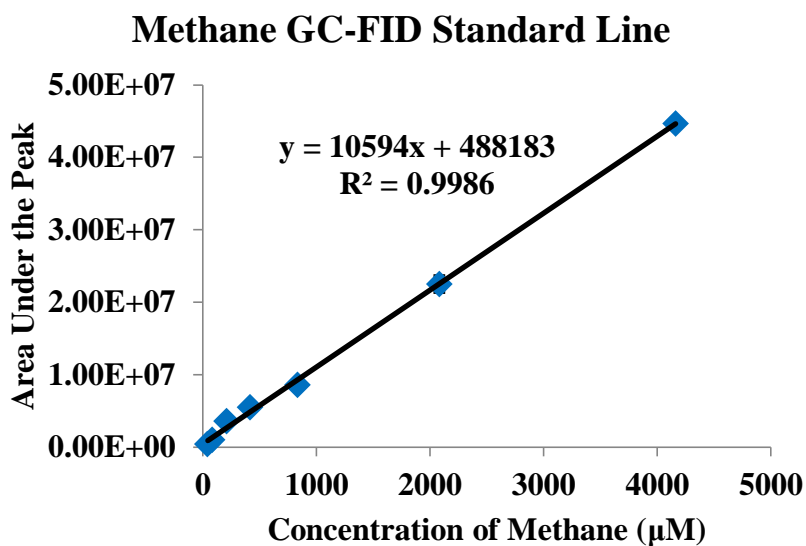


Figure 16. An example of a standard curve for methane measurements by gas chromatography with flame ionization detection (GC-FID).

3) Acetic acid concentration measurements: the concentration of acetate in the medium was measured with high-performance liquid chromatography (HPLC) using an organic acids column (Alltech, 250 by 4.6 mm) with an ultraviolet detector (Waters 2487 detector, $\lambda = 210\text{nm}$). The column was swept with a 25 mM KH_2PO_4 mobile phase with a pH of 2.5 at a flow rate of 0.8 mL/min for determination of acetate. Samples were centrifuged at 13,300 g for 5 minutes on a Fisher Scientific Accuspin Micro 17 centrifuge to pellet solids and 300 μL of supernatant was run on the HPLC after acidizing with 20 μL of 1 M phosphoric acid (H_3PO_4). Run time for each sample was 10 minutes. Standard solutions (mM) were created in 25 mL of deionized water and were preserved in closed serum bottles kept at 4 $^{\circ}\text{C}$ (Table 15). An example of a calibration curve is shown in Figure 17. BESA interfered with the detection of acetic acid so a different HPLC (Dionex ICS 5000 had to be used with an IonPac AS18 column (250 mm by 4mm). The mobile phase was Milli-Q water (neutral pH) at a flow rate of 1.0 mL/min.

4) pH Measurement: The pH of liquid samples was measured before freezing using an Orion pH meter (model 370) with 3 point calibration.

5) Ammonium Measurement: The indophenol blue colorimetric assay was used as follows:

Reagent A

Mix 2.9 grams of phenol in 91 mL of Milli-Q water then add 6 mL of 0.5 g/L sodium nitroprusside solution. Store in the dark at 4 $^{\circ}\text{C}$. Solution turns green when expired.

Reagent B

Mix 2.0 grams of sodium hydroxide and 1 mL of sodium hypochlorite (10-15%) in MilliQ water and dilute to 100 mL in a volumetric flask. Store in the dark at 4⁰C. Prepare new standards after 2 weeks of storage.

1M ammonium chloride (NH₄Cl) stock solution

Dry ammonium chloride at 100⁰C for 1 hour. Dissolve 5.35 grams in 100 mL MilliQ water using a volumetric flask. Store in the dark at 4⁰C for up to six months. Create standards for concentrations 0.1 to 2 mM (Table 16).

a) Add 950 µL of Milli-Q water at pH 3 to 1.5 mL a microcentrifuge tube. Add 30 µL aqueous sample, and then 100 µL Reagent A and then 100 µL of Reagent B in that order. Vortex and incubate at room temperature in the dark for 1 hour. Create a duplicate for every sample and standard. Use MilliQ water as a blank to calibrate the spectrophotometer.

b) Measure absorbance in spectrophotometer at 635 nm wavelength. An Iron (III) concentration of higher than 2 mg/L can interfere with assay.

Table 15. Calculated mass of sodium acetate added to 25 mL of deionized water to create a specific concentration of acetate for standards. Based on a molar mass of 82.03 g/mol.

Concentration of Acetic Acid (mM)	Mass of sodium acetate added to 25 mL H ₂ O (mg)
1	2.05
2	4.10
5	10.25
10	20.51
25	51.27

Table 16. Calculated volumes of 1M ammonium chloride stock solution and MilliQ water mixed to created standards. Based on a molar mass of 53.49 g/mol.

Concentration of Ammonium (mM)	Volume of 1M Ammonium Stock Solution (μL)	Volume of MilliQ H ₂ O (μL)
0.1	10	990
0.2	20	980
0.5	50	950
1	100	900
1.5	150	850
2.0	200	800

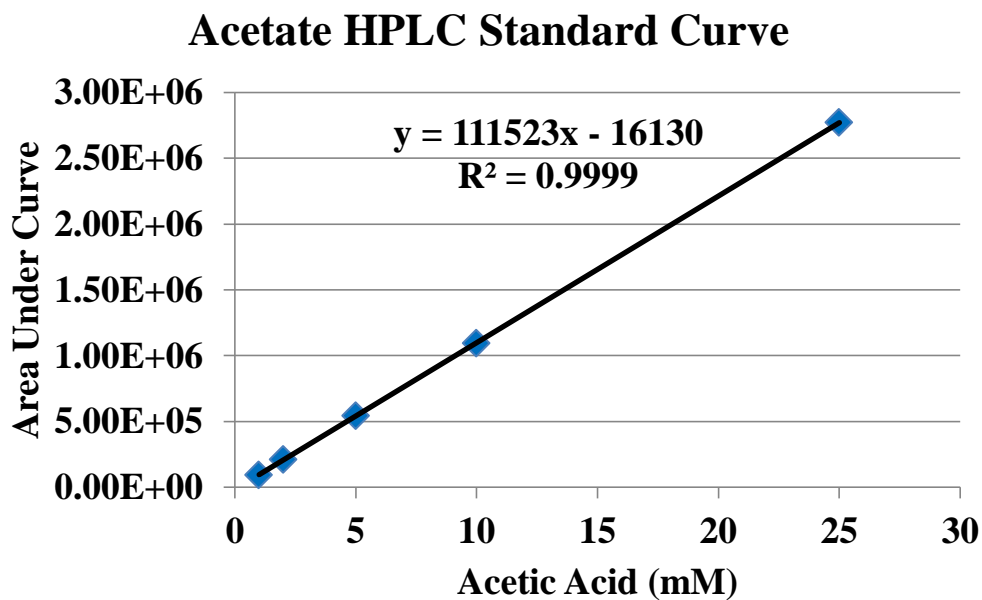


Figure 17. An example of a standard curve for acetic acid measurement by HPLC.

3.14. Statistical analysis and comparison between experiments

Patterns and trends in gas consumption, methane and acetic acid were analyzed and compared for statistical significance using one-factor analysis of variance (ANOVA) which is represented as a p value in the text. Significantly different groups have a p-value of less than 0.05 and values that are the same have a p-value of greater than 0.05.

3.15. Analysis of microbial community composition

DNA was extracted from pelleted cells in the liquid sample and processing for sequencing as follows:

1) Sample combination: cells in the liquid sample were pelleted by centrifuging at 12,300 rpm for 5 minutes. Pellets from replicates were combined together by transfer with sterile MQ water and re-pelleted.

2) DNA extraction: samples were processed with the Fast DNA Spin Kit for soil (500 mg) using the FastPrep Instrument (MP Biomedicals, Santa Ana, CA) for bead beating.

a) The cell pellet was transferred to the Lysing Matrix tube A using deionized water to a volume of 500 μ L to which 978 μ L sodium phosphate buffer and 122 μ L MT buffer were added. The contents of Lysing Matrix tube A were homogenized in the FastPrep Instrument for 40 seconds at a speed of 6.0 m/s. The Lysing Matrix tube A was cooled on ice for 7 minutes then centrifuged at 12,300 rpm for 7 minutes.

b) The supernatant from Lysing Matrix tube A was transferred to a 2.0 mL microcentrifuge tube and 250 μ L of protein-precipitating solution (PPS) were added. The tube was inverted by hand 10 times. The tube was centrifuged at 12,300 rpm for 5 minutes.

c) The supernatant was transferred to a Fisher Scientific 15 mL centrifuge tube and 500 μ L of Binding Matrix and 500 μ L of GTC solution were added. The centrifuge tube was gently inverted for 2 minutes on a rotating table. Afterward the Binding Matrix in the tube was allowed to settle out upright on the bench for 5 minutes.

d) Once the Binding Matrix had settled out, the supernatant was removed and discarded to create a final volume of 1.5 mL in the tube. The Binding Matrix was re-suspended in solution and 600 μ L were transferred to a two part SPINTM filter tube with a top filter inset into a lower catchment tube. The tube was centrifuged at 12,300 rpm for 1 minute. Flow through in the catchment tube was discarded. This process was repeated until all of the Binding Matrix had been passed through the filter.

e) SEWS-M ethanol solution (500 μ L) was added to the SPINTM filter and the Binding Matrix was gently re-suspended with a pipette tip. The filter tube was allowed to rest on the desktop for 2 minutes. The filter tube was then centrifuged at 12,300 rpm for 1 minute and the flow through was discarded. The filter tube was centrifuged again for 2 minutes.

f) The top of the filter tube containing the dried Binding Matrix was transferred to a new 1.5 mL microcentrifuge tube. The DNA was eluted by adding 75 μ L of DES solution and incubated for 5 minutes in a water bath at 55⁰C and then centrifuged for 1 minute at 12,300 rpm. The filter was discarded.

3) DNA Concentration Measurement: DNA concentration was determined using a Qubit Fluorometer (Invitrogen) and a Quanti-itTM dsDNA HS Assay Kit (Invitrogen). This method of quantification was also used to determine the concentration of PCR products.

4) PCR Amplification: DNA was amplified using a two-step procedure. In the first step, the 16S rRNA gene was amplified from the genomic DNA using primers 926F (AAACTYAAAKGAATTGRCGG) and 1392R (ACGGGCGGTGTGTRC). The PCR thermocycle was 3 minutes at 95⁰C followed by 25 repeated cycles of 30 seconds at 95⁰C, 30 seconds at 55⁰C and 1 minute at 72⁰C with a final step of 72⁰C for 5 minutes and storage at 4⁰C. The PCR product was purified from leftover primers using a QIAquick PCR Purification Kit (Qiagen) and the quality of the PCR product was confirmed by running on a 1.5% agarose gel, which was imaged under UV light using DNA Safe Stain C138 (Lambda Biotech). The second round of PCR used FLX titanium amplicon primers 454T_RA_X which has a 25 nucleotide A-adaptor (CGTATCGCCTCCCTCGCGCCATCAG) and 10 nucleotide multiplex identifier barcode sequence X as a reverse primer. The forward primer 454T_FwB had a 25 nucleotide B-adaptor sequence (CTATGCGCCTTGCCAGCCCGCTCAG).

5) Final Processing: the 2nd PCR product was purified again using the same purification kit as indicated above and purity was confirmed by running on a 1.5% agarose gel. The final DNA concentration was determined with the above assay kit. Once purity was confirmed, the final sample was diluted to a volume of 20 µL with a DNA concentration of 20 ng/µL. The final PCR product was sent for pyrosequencing at the Genome Quebec and McGill University Innovation Centre in Montreal, Quebec. Pyrosequencing was performed with a Genome Sequencer FLX Instrument, using a GS FLX Titanium Series Kit XLR70 (Roche Diagnostics Corporation).

6) Data Processing: the metagenomic sequencing data were analyzed using the Phoenix 2 software package (Soh *et al.* 2013). Data were subjected to stringent checks to remove low quality reads and minimize sequencing errors that can be introduced during the pyrosequencing

process (Huse *et al.*, 2007). The remaining high quality sequences were compared against SILVA102 (Pruesse *et al.*, 2007) database using the Tera-BLAST algorithm on a TimeLogicDecypher system (Active Motif, Inc.). The filtered sequences that passed the quality control, problematic, chimerical, and eukaryotic sequence removal stages were clustered into OTUs (Operational Taxonomic Units) at 5% distance by using the complete linkage algorithm in Mothur (Schloss *et al.*, 2009). A taxonomic consensus of representative sequences from each OTU was derived from the recurring species within 5% of the best bitscore from a BLAST search against the SILVA database.

3.16. Brightfield and epifluorescent microscopy

To examine for potential polyhydroxybutyrate granules in the cells of experiment #4, liquid samples were examined using a Olympus BX51 microscope. Images were taken with Retiga 2000R 12-bit monochrome camera and processed with Image Pro Express 6.0 software. After cell clusters were identified under bright field settings, the cells were dyed with Nile Red fluorescent stain (250 µg/mL) for 45 minutes in the dark and re-examined under epifluorescence setting of the same microscope using a BX2 fluorescence light (BX-RFA) with CY5 brightline fluorescence filter set (EX 628/40, 660 DM, EM 692/40, ZPIXEL).

3.17. Porosity and permeability analysis

Porosity and permeability values were determined for the solid carbonate core by the lab of Dr. Apostolos Kantzas before and after microbial enrichment. Porosity was analyzed by injecting helium inside the solid carbonate cores to a pressure of 100 psig. The volume of gas required to achieve this pressure was compared to a standard volume related to porosity.

Permeability was also measured by injecting helium gas, but in relation to the rate of gas flow as

pressure is applied to one side of the core in a core-flow apparatus. The differential of pressure required to achieve a specific flow rate is also incorporated into the calculation.

CHAPTER 4 RESULTS

4.1. Experiment #1: For growth of hydrogenotrophs

4.1.1 Primary Enrichment

The first experiment was performed to enrich both acetogens and methanogens autotrophically (solely from headspace CO₂) and as such CSB-K medium was used with the sodium bicarbonate removed. In the primary enrichment, a peak of 198.7 µmol of acetic acid and minimal methane were produced in the low nutrient medium (Figure 18A). A similar amount of acetic acid was produced in the high nutrient medium ($p>0.05$). A maximum of 564 µmol of methane was produced which was higher than in the low nutrient medium ($p<0.05$, Figure 18B). Adding BESA to high nutrient medium caused a peak of 475 µmol of acetic acid, higher than both high and low nutrient media ($p<0.05$), with minimal methane (Figure 18C) which is consistent with expected effects of adding a methanogen inhibitor. Peak values for acetic acid and methane are represented in Figure 18D. These values are substantially lower than the values found in Nathoo *et al.* 2012 which found concentrations of 100 mM which translate to 5000 µmol of acetic acid in 50 mL of medium in a 120 mL serum bottle.

A total of 179 mL of headspace gas (80% H₂/20% CO₂) was consumed in the high nutrient medium (Figure 19B). Gas consumption plateaued early on day 5 at 37 mL of gas, which was associated with acetic acid production. Gas consumption resumed after day 14 to the final total and was associated with methane production. Minimal gas was consumed with low nutrient medium (Figure 19A) and 40 mL of gas was consumed in the BESA medium which stopped by day 5 (Figure 19C). The gas consumption in the low nutrient and BESA media was associated with acetic acid production. All media started in the pH range of 6.6 to 6.7. Low nutrient medium dropped to pH of 5.9 (Figure 19A) and high nutrient medium dropped to pH 5.7

(Figure 19B). BESA medium dropped to pH 5.2, significantly lower than that of low and high nutrient medium ($p < 0.05$, Figure 19C). The extent of the pH drop was associated with acetic acid production in all media and was greater than seen in other CSB-K enrichments which had added bicarbonate (Nathoo *et al.* 2012).

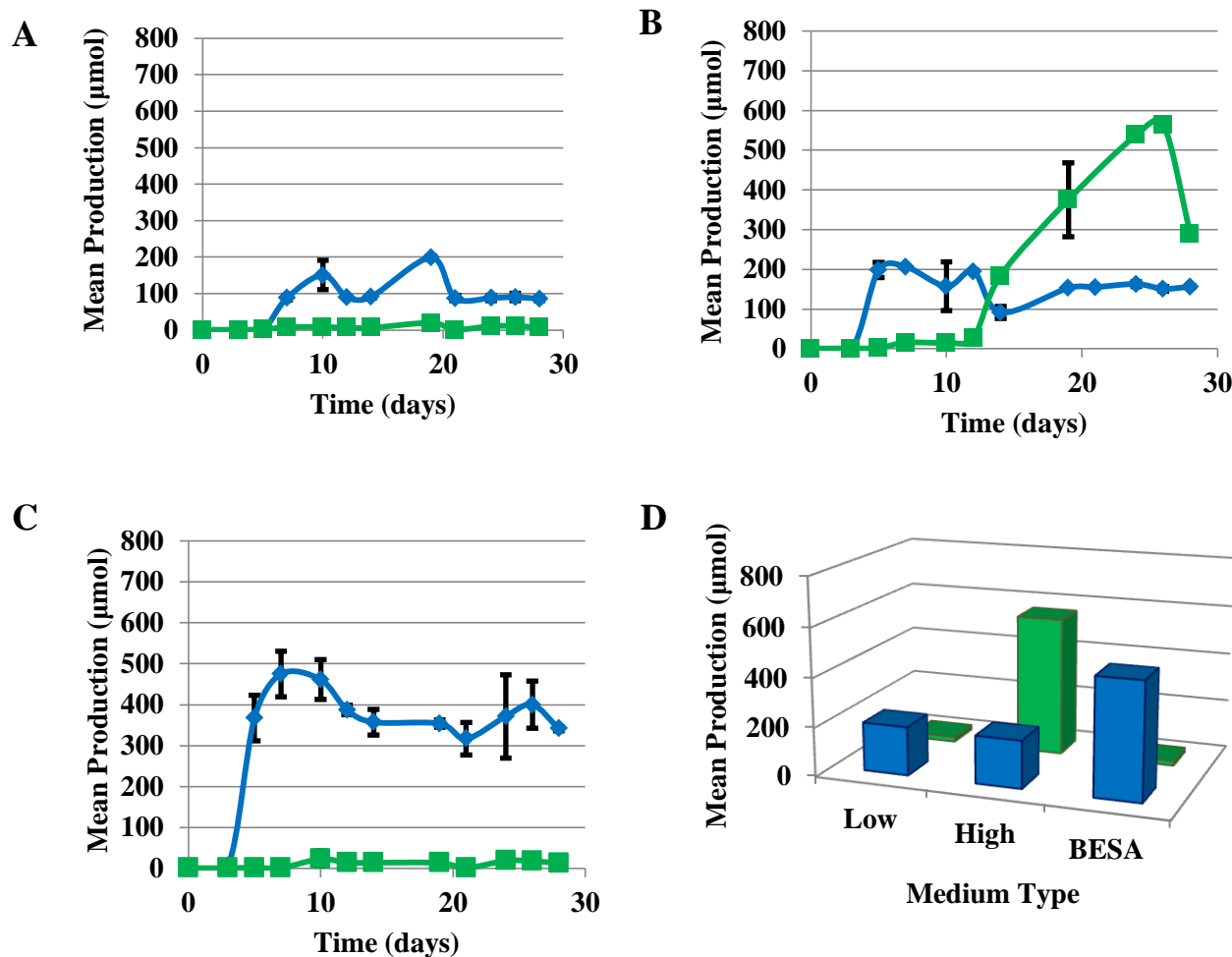


Figure 18. Mean acetic acid (blue) and mean methane (green) production as a function of time for experiment #1 primary enrichment. CSB-K medium was used with no added bicarbonate with H_2/CO_2 headspace gas (80:20). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BES and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates.

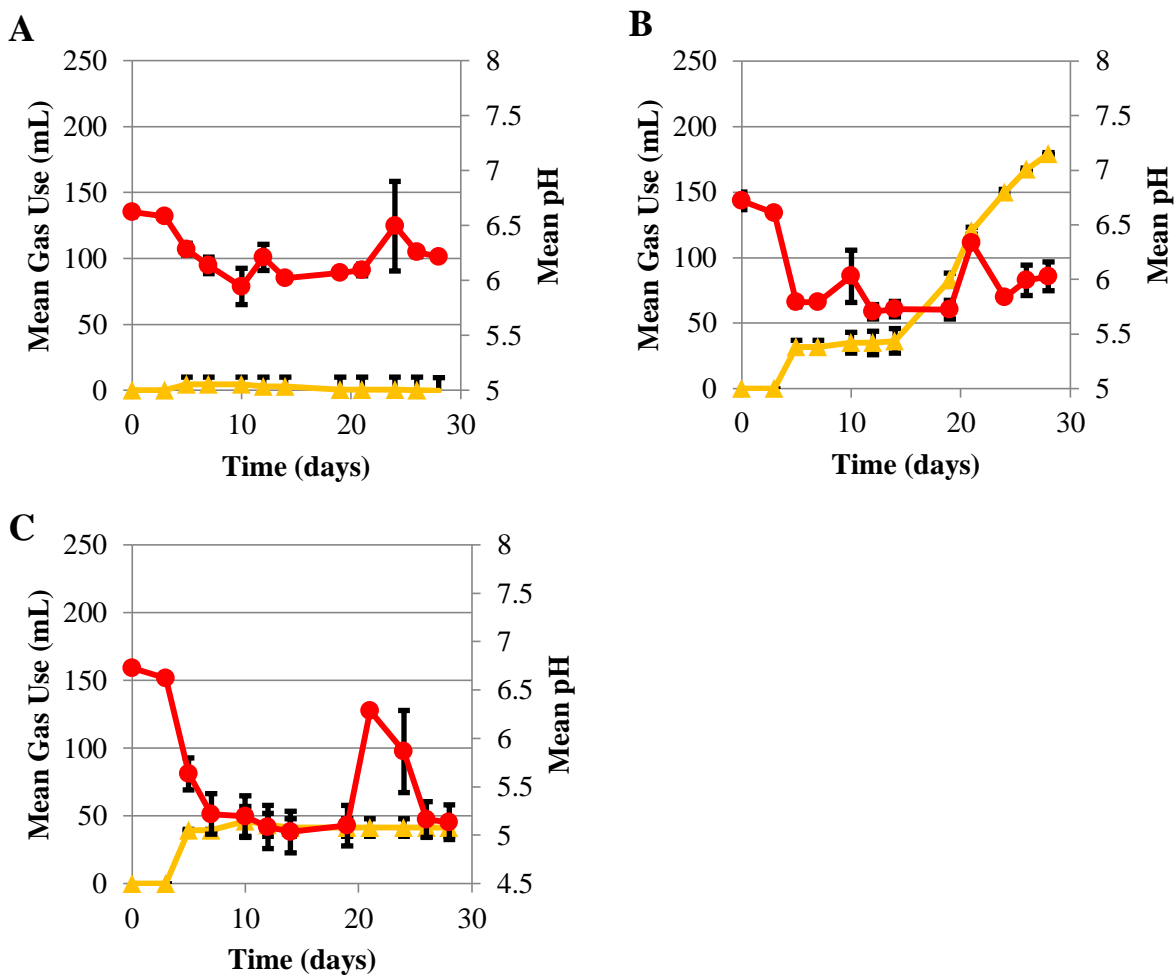


Figure 19. Mean pH (red) and mean gas consumption (yellow) production as a function of time for experiment #1 primary enrichment. CSB-K medium was used with no added bicarbonate and H_2/CO_2 headspace gas (80:20). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BES. The error bars represent an average of 3 replicates.

4.1.2 Secondary Enrichment

Medium was taken from the original culture (primary enrichment) on day 21 to create a subculture (secondary enrichment) to account for the effect of oxygen-exposed transport from the field to the lab on sensitive anaerobic microbes especially methanogens. This also accounts for effects of any unknown nutrients in the inoculum water not associated with medium which also suggests that the secondary enrichment will have a somewhat lower overall level of nutrients and thus growth.

The low nutrient medium produced minimal acetic acid (less than 15 μmol) and 276 μmol of methane (Figure 20A). Both of these were lower than in the primary enrichment ($p < 0.05$). High nutrient medium produced same amount of acetic acid (127 μmol), similar to the primary enrichment but it then decreased to almost zero by the end of the experiment ($p > 0.05$, Figure 20B). Less methane was produced (659 μmol , $p < 0.05$). In the BESA medium, acetic acid peaked at 365 μmol and had negligible methane which was not statistically different from the primary enrichment ($p > 0.05$, Figure 20C). Peak values for acetic acid and methane are represented in Figure 20D. Acetic acid was generally lower in the secondary enrichment and was again much lower than as seen previously in Nathoo *et al.* 2012.

pH trends were similar to the primary enrichment though the initial pH drop was not observed due to a lack of day 0 data point. There was a small pH rise in BESA medium from 5.2 to 5.6, but this was not statistically significant ($p > 0.05$, Figure 21). Gas use trends were the same as for the primary enrichment for low nutrient and BESA medium ($p > 0.05$) and were associated with acetic acid production. Gas consumption was higher in the high nutrient medium, increasing

to 219 mL (Figure 21C). The majority of the gas consumption was associated with methane production.

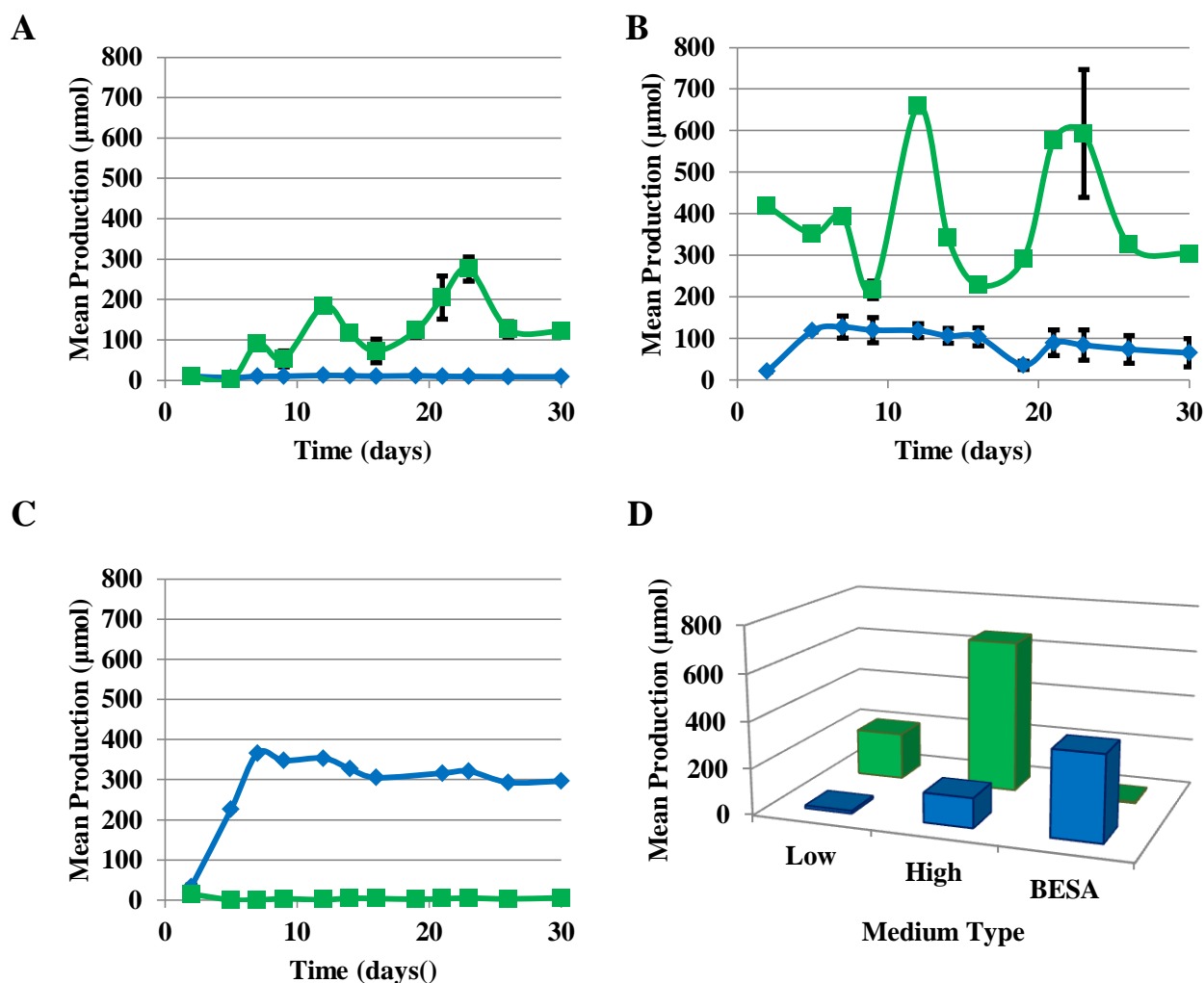


Figure 20. Mean acetic acid (blue) and mean methane (green) production as a function of time for experiment #1 secondary enrichment (subculture). CSB-K medium is used with no added bicarbonate with H_2/CO_2 (80:20). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BESA and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates.

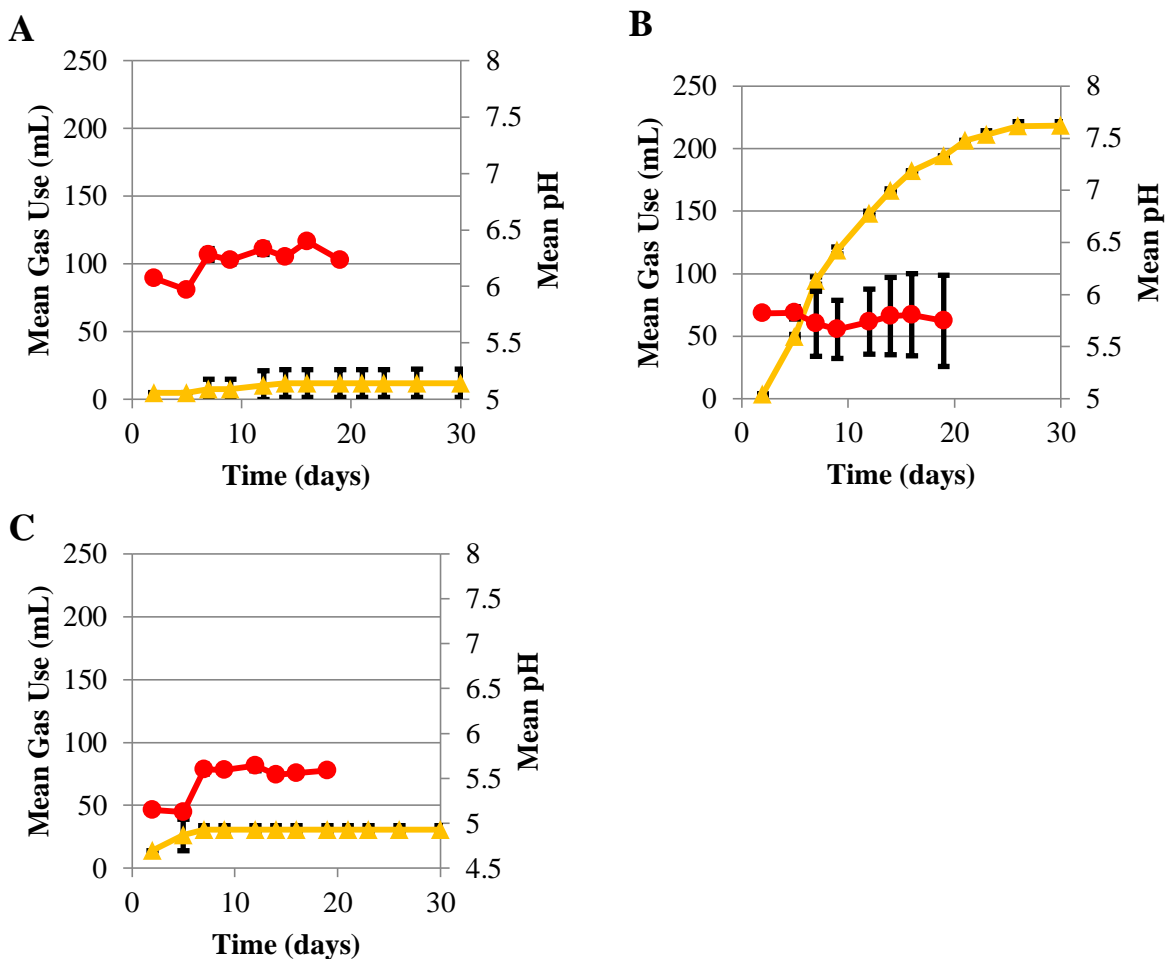


Figure 21. Mean pH (red) and mean gas consumption (yellow) production as a function of time for experiment #1 secondary enrichment. CSB-K medium was used with no added bicarbonate with H₂/CO₂ headspace gas (80:20). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates.

4.2.2 16S rRNA microbial community analysis: Secondary enrichment

DNA community analysis was performed by sequencing the 16S rRNA genes in the secondary enrichment (Figure 22 and Table 17). Samples were a mix of multiple days to achieve sufficient DNA extraction. A 16S rRNA gene product could not be amplified from the low nutrient medium. Genera were considered significant members if they constituted over 1% of the overall community.

Over 95% of the detectable reads were matched to two methanogenic archaea, 69.5% from the *Methanobacterium* genus and 26.2% from an unidentified member of the *Methanobacteriaceae* family. The only other two genera present were 2.5% *Roseibacterium* and just over 1% *Acetobacterium*. These same two methanogens were also found in the medium with BESA in slightly smaller proportions, 57.9% *Methanobacterium* and 15.9% *Methanobacteriaceae*, whereas the proportion of *Roseibacterium* increased (25.7%). It appears the detected microbial community at the times sampled was largely methanogenic which partially agrees with the lower than expected acetogenesis although 365 μmol of acetic acid was produced.

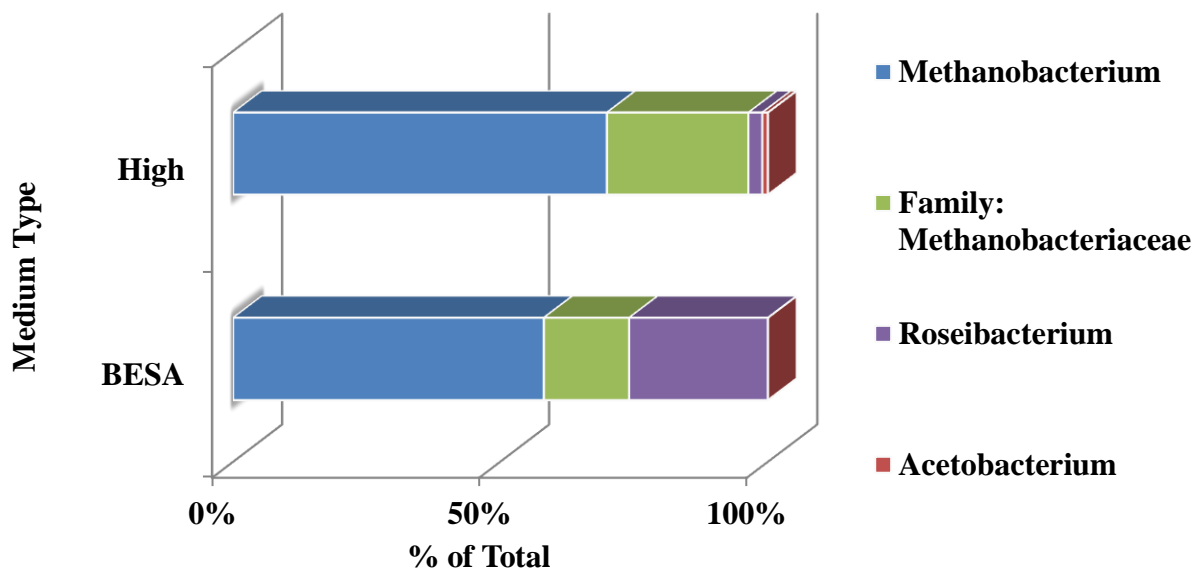


Figure 22. 16S rRNA pyrosequencing data for experiment #1 secondary enrichment represented at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 2 combined replicates.

Table 17. Experiment #1 16S rRNA pyrosequencing data for the secondary enrichment at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	High	BESA
<i>Methanobacterium</i>	69.52	57.85
<i>Methanobacteriaceae</i>	26.21	15.88
<i>Roseibacterium</i>	2.57	25.69
<i>Acetobacterium</i>	1.02	0

4.2. Experiment #2: For growth of acetotrophic methanogens

4.2.1 Primary Enrichment

Previous work has found acetic acid to peak and then decrease back to zero. A potential microbial reason for this acetic acid loss is acetotrophic methanogens converting acetic acid to methane. To test for this the headspace gas was switched from H_2/CO_2 to N_2/CO_2 which prevents any hydrogenotrophs such as acetogens and hydrogenotrophic methanogens so the effects of acetotrophic methanogenesis are isolated. To allow greater recovery of oxygen-sensitive methanogens, the primary enrichment inoculum of this experiment was taken from the primary enrichment of experiment #1 instead of directly inoculating with produced waters from the MHGC field. Activity was only observed in the high medium with 5 mM sodium acetate added, not in control media with only 1 mM or with 0 mM of acetate added or in low nutrient or medium with BESA added.

There was minimal methane production in the low nutrient and BESA media. Acetic acid levels only declined slightly from 270 to 219 μmol in low nutrients and from 482 to 332 μmol with BESA (Figure 23A and 23C) which is likely due to abiotic loss. Minimal to no acetotrophic methanogenesis occurred. The high nutrient medium had the highest level of activity with 347.5 μmol of acetic acid that was consumed to less than 10 μmol with a production of 254 μmol of methane starting day 70 (Figure 23B). Only high nutrient medium with added acetate showed acetotrophic methanogenesis. Final values for acetic acid and methane are represented in Figure 23D.

All pH values remained in a range of 6.0 to 6.6 (Figure 24) for low nutrients and BESA. Starting on day 56, pH increased from 6.2 to 7.0 in the high nutrient medium and this was

associated with acetic acid loss (Figure 24B). All gas consumption was less than 2 mL (Figure 23). Statistical analysis of experiment #2 was not possible due to missing raw data.

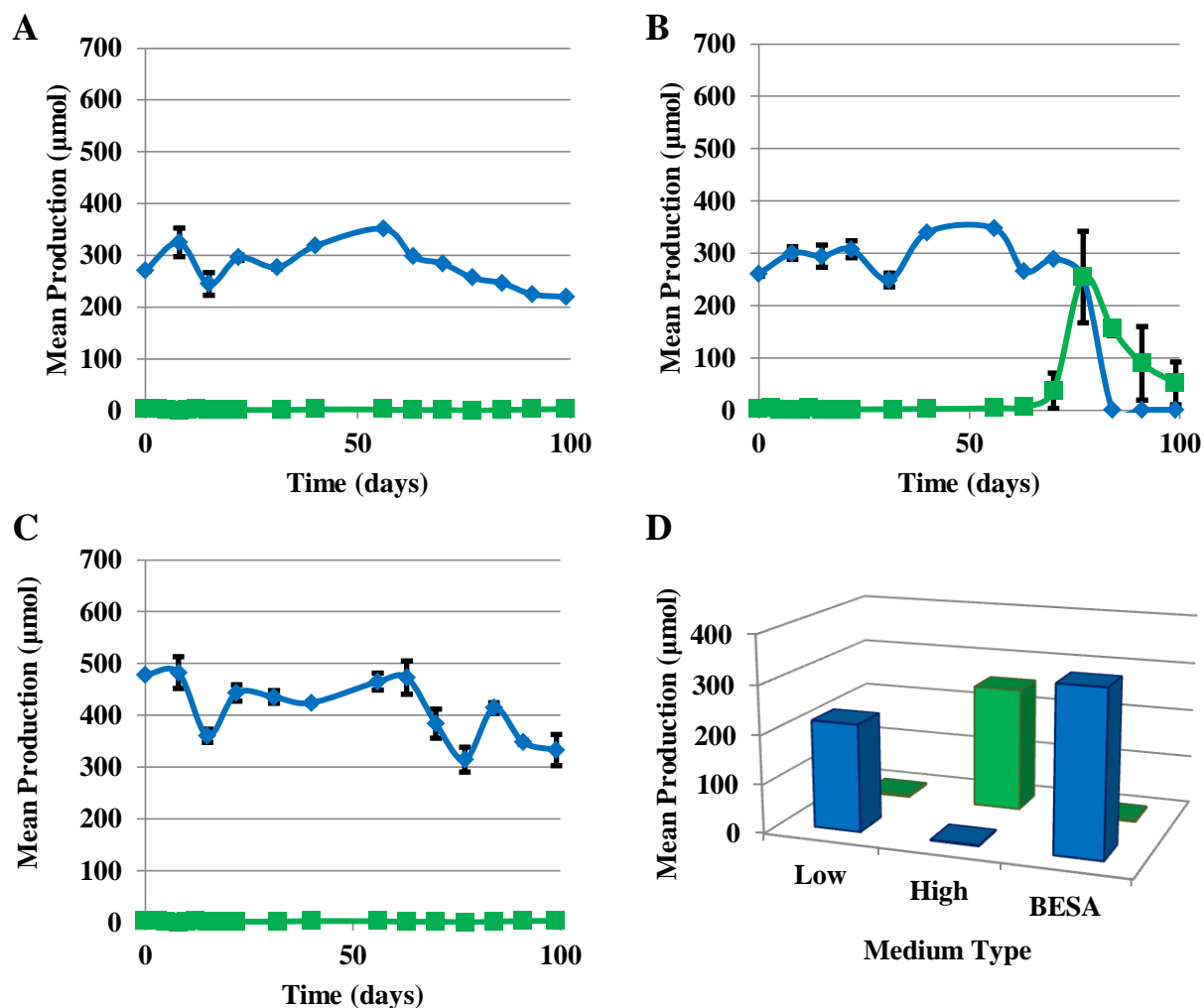


Figure 23. Mean acetic acid (blue) and mean methane (green) production as a function of time for experiment #2 primary enrichment. The headspace was switched from H_2/CO_2 (80:20) to N_2/CO_2 (90:10). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BESA and (D) final values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates.

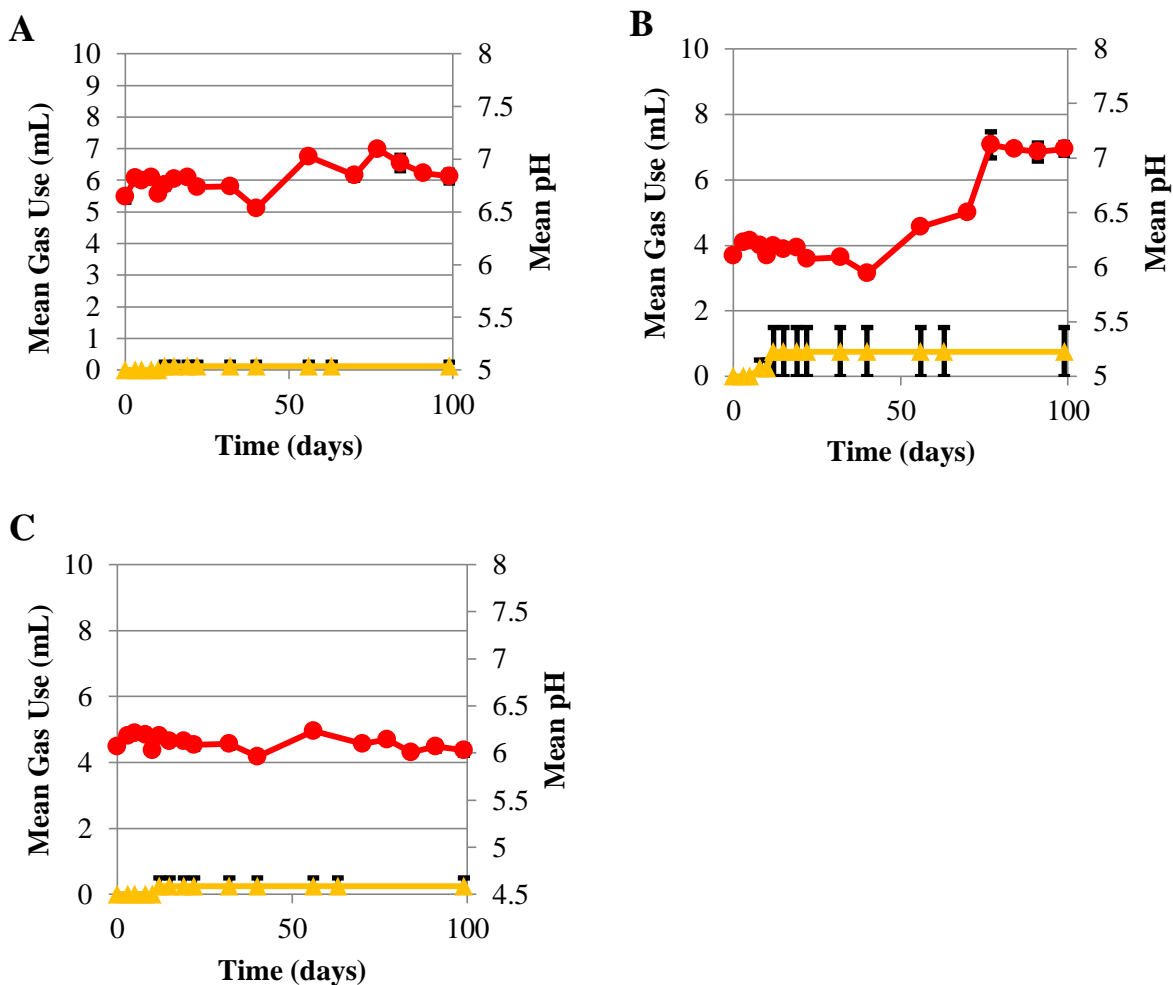


Figure 24. Mean pH (red) and mean gas consumption (yellow) production as a function of time for experiment #2 primary enrichment. The headspace gas was switched from H_2/CO_2 (80:20) to N_2/CO_2 (90:10). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates.

4.2.2 Secondary enrichment

A secondary enrichment was performed to allow for even further methanogen recovery. Activity was again only seen in high nutrient medium with 5 mM added acetate which is consistent with acetotrophic methanogenesis. The acetic acid loss started sooner on day 26 with 319 μmol of acetic acid decreasing to less than 5 μmol . Methane increased to 598 μmol (Figure 25B). Trends for the low nutrient and BESA media were similar to the primary enrichment and showed minimal evidence of microbial activity (Figures 25 and 26). Final values for acetic acid and methane are represented in Figure 25D. Acetotrophic methanogenesis in these experiments started at the earliest on day 40 which is too late for the acetic acid loss seen in experiment #1 and subsequent experiments.

The pH in all medium types started at 6.9, higher than the starting pH in the primary enrichment though the amount of acetic acid initially present was similar (347 μmol in the primary enrichment and 319 μmol in the secondary enrichment, Figure 26B). There was probably trace acetogenic activity in the inoculum used in the primary enrichment which came from the acetogenic experiment #1 culture. The secondary enrichment would not have the effects of this extra acetic acid production and this may account for the higher starting pH than in the primary enrichment ($p < 0.05$). The rise in pH from 7.0 to 7.2 in the secondary enrichment was much smaller than the primary enrichment which is associated with higher initial pH. Gas consumption was again less than 2 mL (Figure 26).

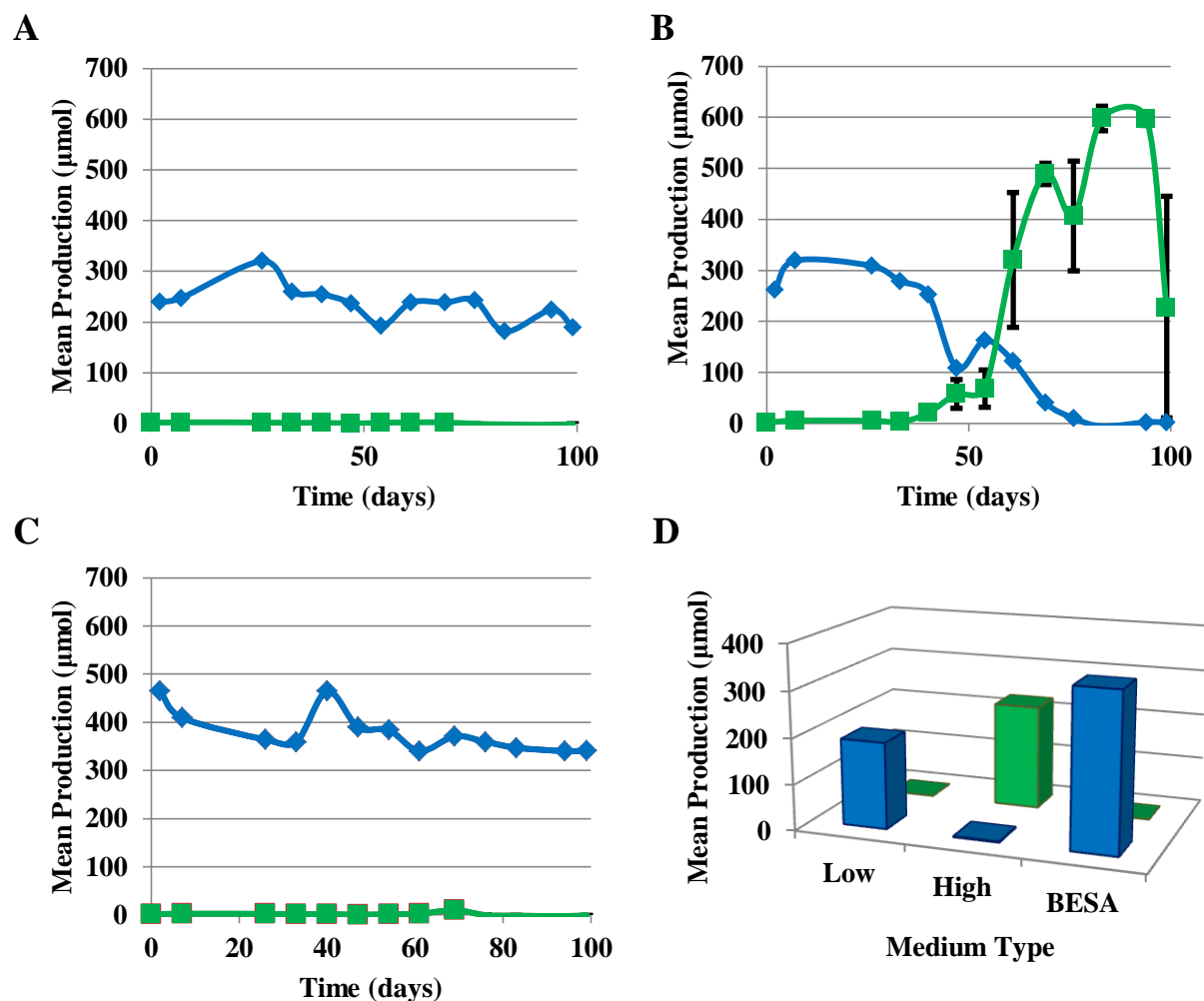


Figure 25. Mean acetic acid (blue) and mean methane (green) production as a function of time for experiment #2 secondary enrichment. The headspace was switched from H_2/CO_2 (80:20) to N_2/CO_2 (90:10). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BESA and (D) final values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates.

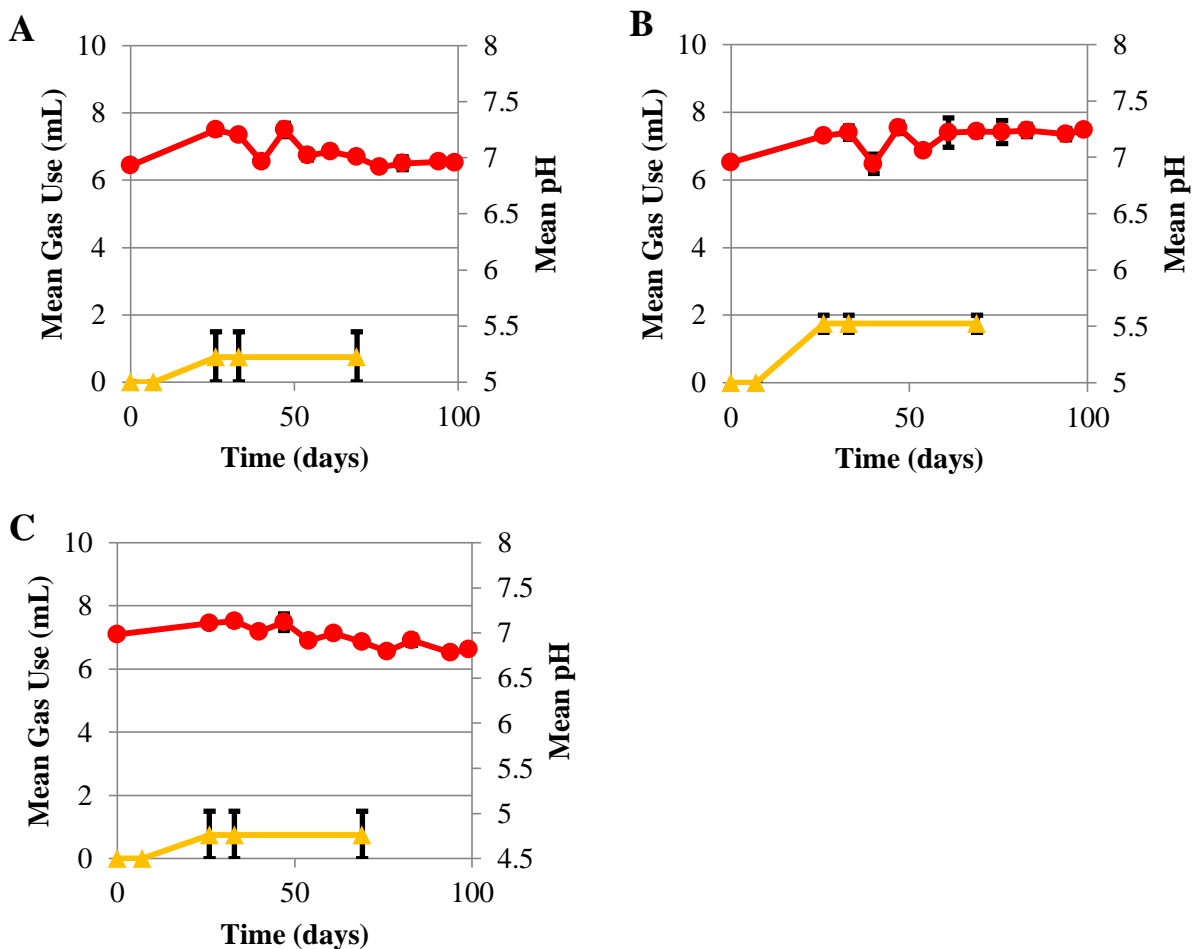


Figure 26. Mean pH (red) and mean gas consumption (yellow) production as a function of time for experiment #2 secondary enrichment. The headspace gas was switched from H_2/CO_2 (80:20) to N_2/CO_2 (90:10). The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates.

4.2.3 16S rRNA microbial community analysis: Both enrichments

DNA community analysis was only performed for the high nutrient medium with added acetate for both the primary and secondary enrichments. Both enrichments had high proportions of methanogenic archaea but of different genera along with a substantial portion of *Roseibacterium*. The primary enrichment had 49% *Methanobacteriaceae* and 34% *Methanobacterium* and 12% *Roseibacterium*. The secondary enrichment sequencing reads contained less *Methanobacterium* (19%) and more 31% *Roseibacterium* (Figure 27 and Table 18). *Methanosaeta* (46%), a known acetotrophic methanogen largely replaced the *Methanobacteriaceae* in the secondary enrichment but this appears to be an insignificant change.

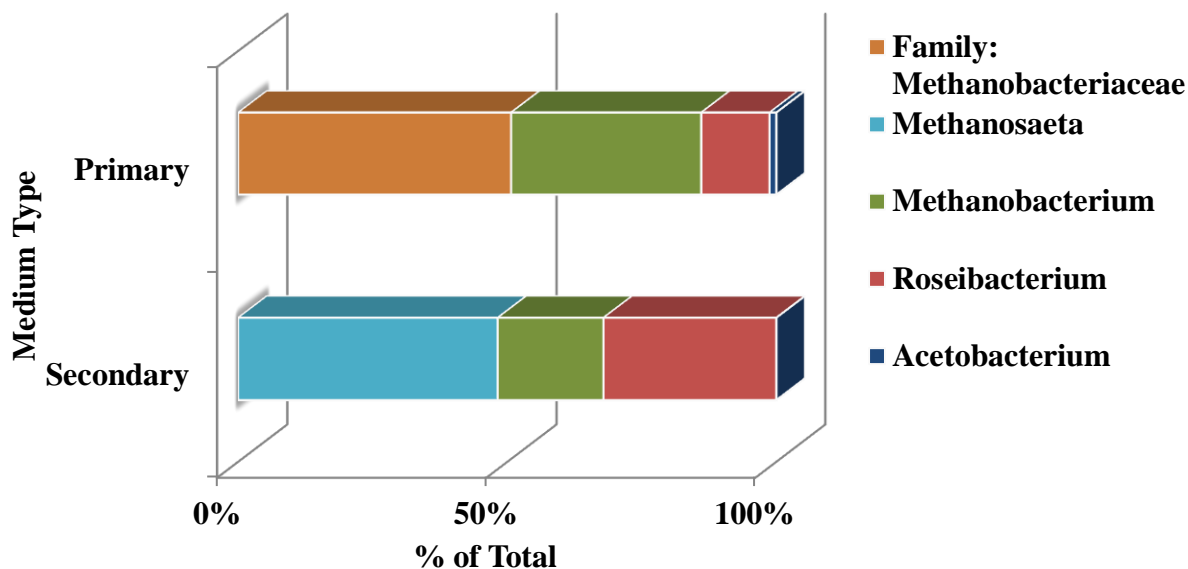


Figure 27. Experiment #2 16S rRNA pyrosequencing data for both enrichments at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 18. Experiment #2 16S rRNA pyrosequencing data for both enrichments at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	Primary	Secondary
<i>Methanobacteriaceae</i>	49.35	0
<i>Methanosaeta</i>	0	45.76
<i>Methanobacterium</i>	34.39	18.64
<i>Roseibacterium</i>	12.38	30.51
<i>Acetobacterium</i>	1.21	0

4.3. Experiment #3: For growth with CaCO_3 and NaHCO_3

4.3.1 Primary Enrichment

The first experiment had a substantial pH drop that likely resulted in acid-limitation of acetogens and methanogens. In an attempt to prevent this, the third experiment had an added carbonate pH buffering system consisting of 1000 ± 10 milligrams of laboratory grade powdered CaCO_3 (a concentration of 100 mM) and 30 mM of sodium bicarbonate. The volume of medium was increased to 100 mL in a 150 mL serum bottle to increase number of samplings possible, which was thought to be a benign change.

In the primary enrichment, 373 μmol of methane and 194 μmol of acetic acid were produced in the low nutrient medium (Figure 28A). This is statistically the same as experiment #1 ($p > 0.05$). Acetic acid (777 μmol) and methane (917 μmol) were both higher in the high nutrient medium ($p < 0.05$, Figure 28B). The acetic acid reached a peak in the high nutrient medium and then decreased to zero whereas the low nutrient and BESA media reached a plateau of acetic acid with no loss. The medium with BESA produced more methane than in experiment #1 (647 μmol , $p < 0.05$) and had much higher acetic acid (2579 μmol , $p < 0.05$, Figure 28C) which is likely due to the prevention of acid-limitation.

The pH for all three media was 6.7 to 7.3 (Figure 29) and did not drop as in experiment #1. The carbonate buffering system was effective and also acted as an early proxy of a carbonate reservoir. The pH for BESA medium still dropped but to a lesser extent, from 7.0 to 6.7 ($p < 0.05$). This occurred just as acetic acid production peaked. At the end of the experiment, the pH of low nutrient medium was the same as its sterile control. The pH of high nutrient medium increased relative to the sterile control from 7.0 to 7.35 ($p < 0.05$). This was associated with the

loss of the acetic acid. The pH for BESA medium dropped from 7.0 to 6.7 ($p < 0.05$), which occurred just as acetic acid production peaked. Gas use increased to 401 mL for BESA medium and was associated with greater acetic acid production ($p < 0.05$, Figure 29). Gas consumption for low and high nutrient medium had higher averages (68 mL and 269 mL respectively) than experiment #1 but this increase was not statistically significant ($p > 0.05$).

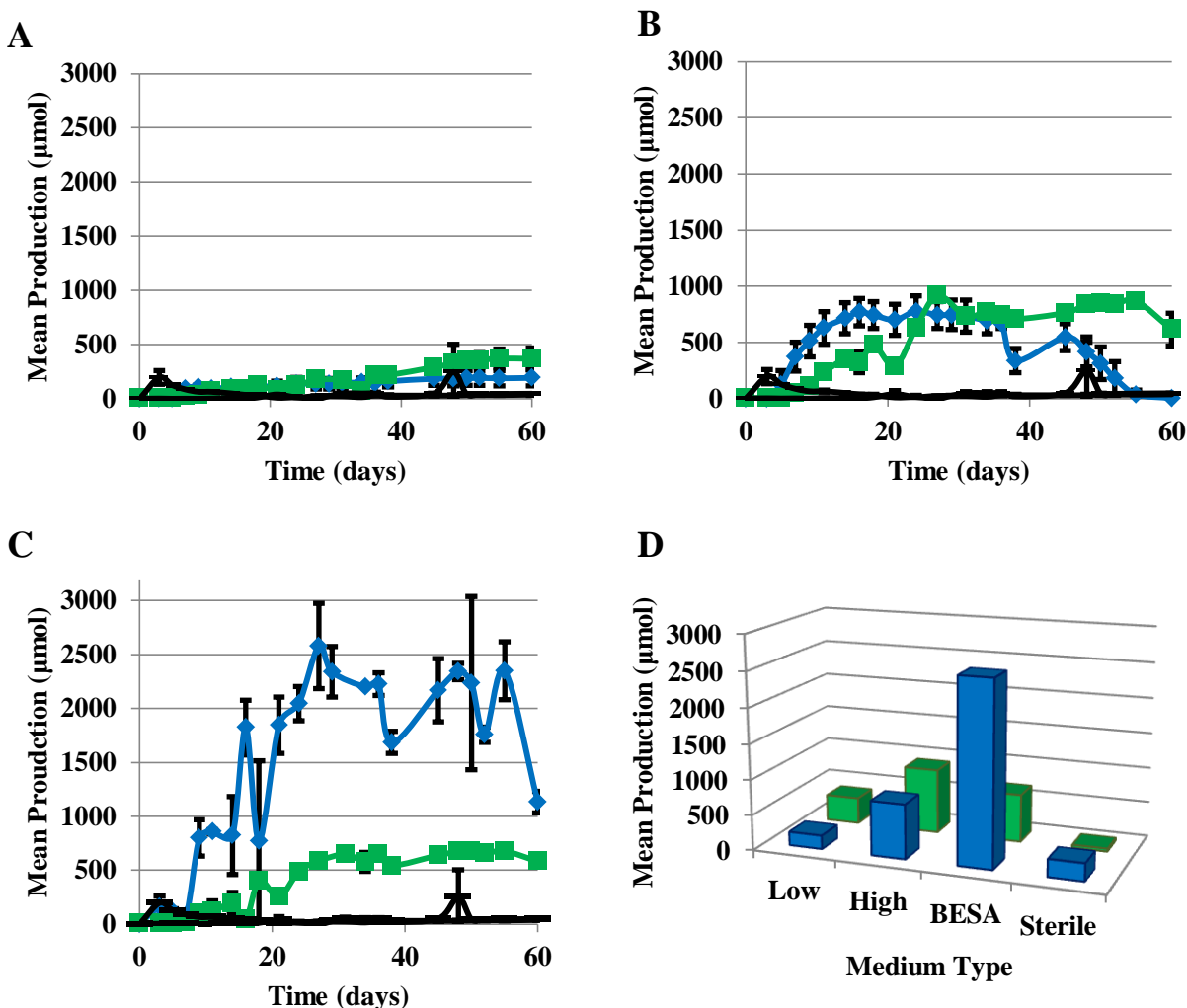


Figure 28. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #3 primary enrichment. 100 mM of CaCO_3 and 30 mM of NaHCO_3 were added with an H_2/CO_2 (80:20) headspace. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BESA and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

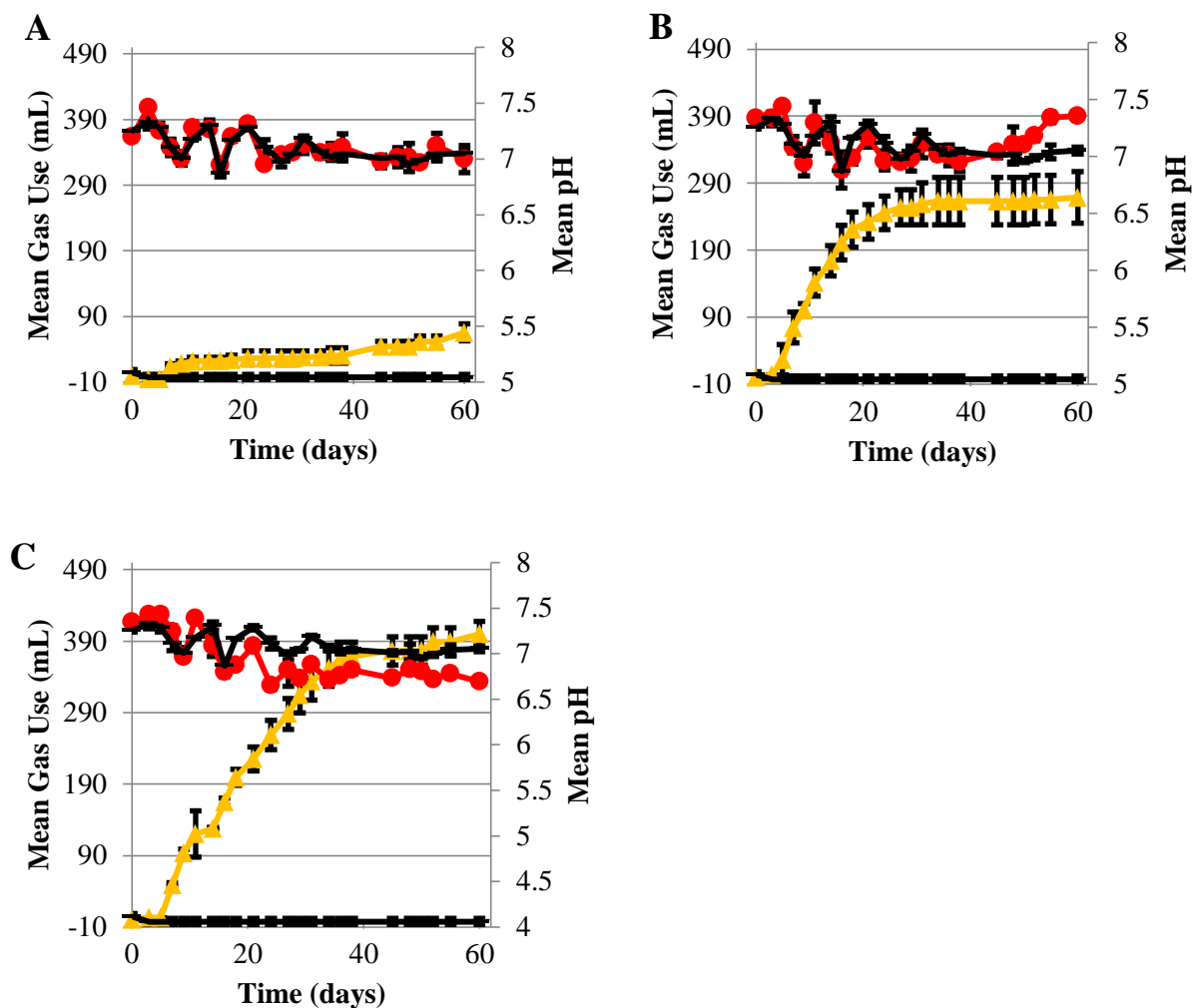


Figure 29. Mean pH (red) and mean gas consumption (yellow) as a function of time for experiment #3 primary enrichment. 100 mM of CaCO_3 and 30 mM of NaHCO_3 were added with an H_2/CO_2 (80:20) headspace. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

4.3.2 16S rRNA microbial community analysis: Primary enrichment

DNA community analysis was performed on the primary enrichment (Figure 30 and Table 19). One sample each was taken from the low and BESA media and two samples taken from the high nutrient medium at the peak of acetic acid production (day 27) and at the end of the experiment (day 60). The other two medium types used samples from the end of the experiment (day 55 for low nutrients and day 60 for BESA).

The low nutrient medium was dominated by a single unidentified member of the *Methanobacteriales* order (93.8%) with a small proportion of the sequencing reads being identified as *Acetobacterium* (3.2%). This microbial community is methanogenic in nature.

When at peak activity the high nutrient medium contained a more diverse assortment of methanogens, *Methanobacteriales* (37%), *Methanospirillum* (51.6%), *Methanofollis* (5.2%) and *Methanobacterium* (2.1%), but no *Acetobacterium*. Towards the end of the experiment, the microbial community shifted to 85.2% *Ralstonia* genus with 3% *Pelomonas*, 2.5% *Massilia*, and 2.4% *Aquabacterium*. *Ralstonia*, *Pelomonas*, *Massilia* and *Aquabacterium* are identified in the literature as capable of converting CO₂ and acetate to carbon storage forms like polyhydroxybutyrate (PHB) under nutrient-limited conditions (Volova *et al.* 2002, Bernard *et al.* 2007 and Xie and Yokota 2005). This microbial community is a mix between methanogenic and microbes that could be storing carbon as PHB. These microbes are only seen in the high nutrient medium which correlates with acetic acid loss only seen in high nutrient medium.

The community in BESA medium resembled that in high nutrient medium at peak activity by being methanogen dominant. It contained more *Methanobacterium* (12.1%), *Methanosaeta* (32.6%) and *Methanosarcina* (14%) and less *Methanobacteriales* (7.2%),

Methanofollis (3%), and *Methanospirillum* (26.2%). This increase in methanogens appears to be associated with the higher amount of methane seen in BESA medium in experiment #3.

The communities in BESA medium and in low nutrient medium appear to have ended in a “methanogen dominant” stage while that in the high nutrient appears to have shifted from methanogen dominant to “carbon storage dominant”. Acetogenic bacteria are only detected in the low nutrient medium which was the slowest growing of the media suggesting that the samples from the other medium types were taken from parts of the growth cycle after acetogens had died off.

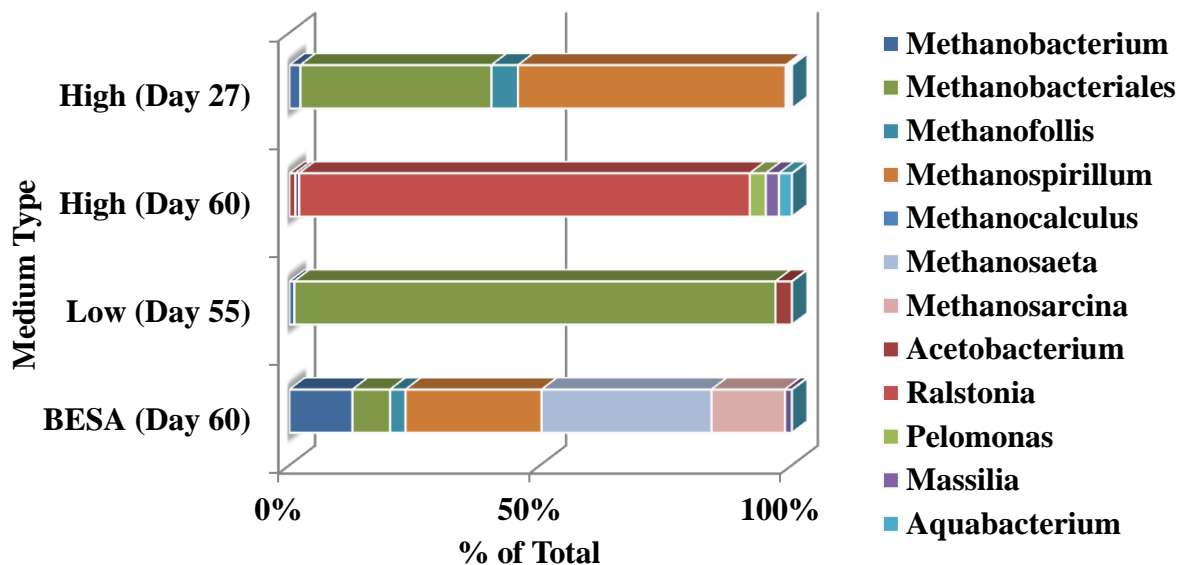


Figure 30. Experiment #3 primary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 19. Experiment #3 primary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	High (Day 27)	High (Day 60)	Low (Day 55)	BESA (Day 60)
<i>Methanobacterium</i>	2.12	0	0.97	12.13
<i>Methanobacteriales</i>	36.97	0	93.83	7.24
<i>Methanofollis</i>	5.15	0	0	2.98
<i>Methanospirillum</i>	51.65	0	0	26.18
<i>Methanocalculus</i>	0.43	0	0	0
<i>Methanosaeta</i>	0	0	0	32.58
<i>Methanosarcina</i>	0	0	0	14.04
<i>Acetobacterium</i>	0.40	1.12	3.17	0
<i>Ralstonia</i>	0	85.29	0	0
<i>Pelomonas</i>	0	2.99	0	0
<i>Massilia</i>	0	2.49	0	0
<i>Aquabacterium</i>	0	2.43	0	0

4.3.3 Secondary enrichment

The only difference between a primary and secondary enrichment of the same experiment was the source of the inoculum, all other parameters were kept the same. Inoculum taken directly from the MHGC field was measured for nitrogen and sulfur species, but the presence and level of other nutrients was unknown. As the inoculum is 10% of the overall medium, these unknowns could have had an impact on the overall nutrient levels. The secondary enrichment is designed to mitigate that effect while allowing for greater recovery from the shock of oxygen in transport. Since the secondary enrichment would have the unknown additional nutrients of the inoculum removed, the overall level of growth was expected to be a little different from the primary enrichment but not majorly.

The secondary enrichment was significantly different from the primary enrichment for experiment #3 (Figure 31A). The low nutrient medium produced a higher average of acetic acid (508 μmol) and less methane (172 μmol) than the high nutrient medium, although this was not statistically significant ($p>0.05$). However the low nutrient medium still produced at least *the same* amount of acetic acid. This was higher than anything seen in previous secondary enrichments. The acetic acid production in high nutrient medium peaked at 215 μmol on day 8 and then slowly decreased and since methane did not increase substantially after methane peaked, this loss is not due to acetotrophic methanogenesis. As compared to the primary enrichment, the acetic acid mean was lower but not significantly different. Methane was the same at 798 μmol ($p>0.05$, Figure 31B).

The secondary enrichment in BESA medium produced similar acetic acid (2077 μmol , $p>0.05$) and less methane (436 μmol) than the primary enrichment ($p<0.05$, Figure 31C). Much

more methane was produced in the presence of BESA than previous work. As compared to experiment #1, the acetic acid production was higher in low nutrient and in BESA medium ($p < 0.05$) and the same for the high nutrient medium ($p > 0.05$). Methane was the same for low nutrient medium ($p > 0.05$), but higher for high nutrient medium and BESA ($p < 0.05$).

The pH of the secondary enrichment was slightly lower for the low nutrient medium and decreased from 7.2 to 7.0, relative to the pH of the sterile control ($p < 0.05$, Figure 32A). These values were still similar to those of the primary enrichment ($p > 0.05$) and much more buffered than experiment #1. The pH rose from 7.3 to 8.0 by day 10 in the high nutrient medium ($p < 0.05$, Figure 32B), which was different from both the sterile control and the primary enrichment ($p < 0.05$). The difference from the sterile control may be associated with acetic acid loss. This is somewhat ambiguous as less acetic acid was consumed in the secondary enrichment, but a larger rise in pH was observed (0.7 units rather than 0.3 in the primary enrichment). The pH in BESA medium was not different from its control and dropped from 7.3 to 7.0, less than in experiment #1 ($p < 0.05$, Figure 32C).

Where there were some significant pH changes, pH buffering overall was still in control and not in the acid-limiting ranges. Gas consumption did not have a strong association in the secondary enrichment. It was similar to the primary enrichment for low nutrient and high nutrient medium and greater for BESA medium and was associated with acetic acid (Figure 32).

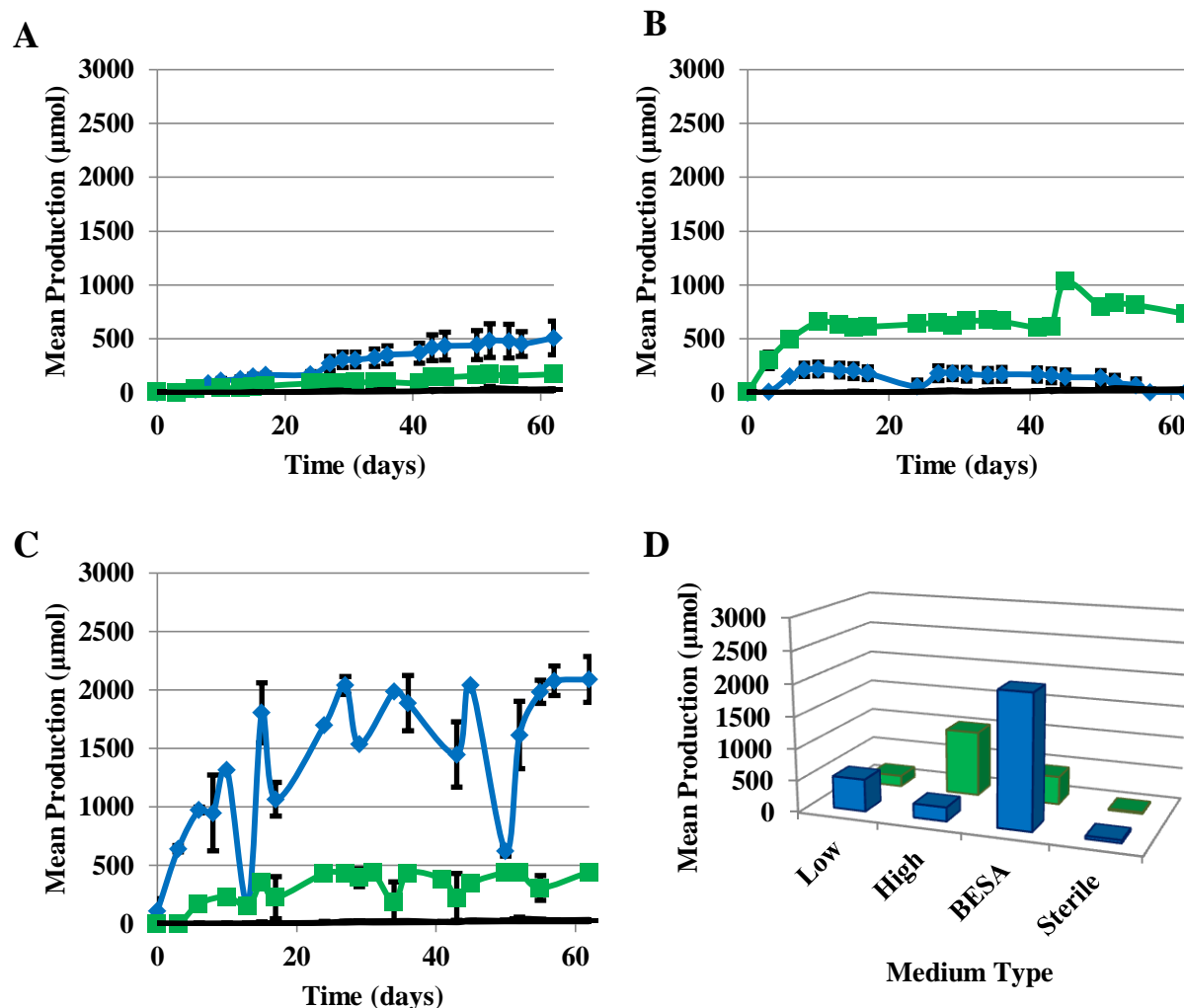


Figure 31. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #3 secondary enrichment. 100 mM of CaCO_3 and 30 mM of NaHCO_3 were added with an H_2/CO_2 (80:20) headspace. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BES and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

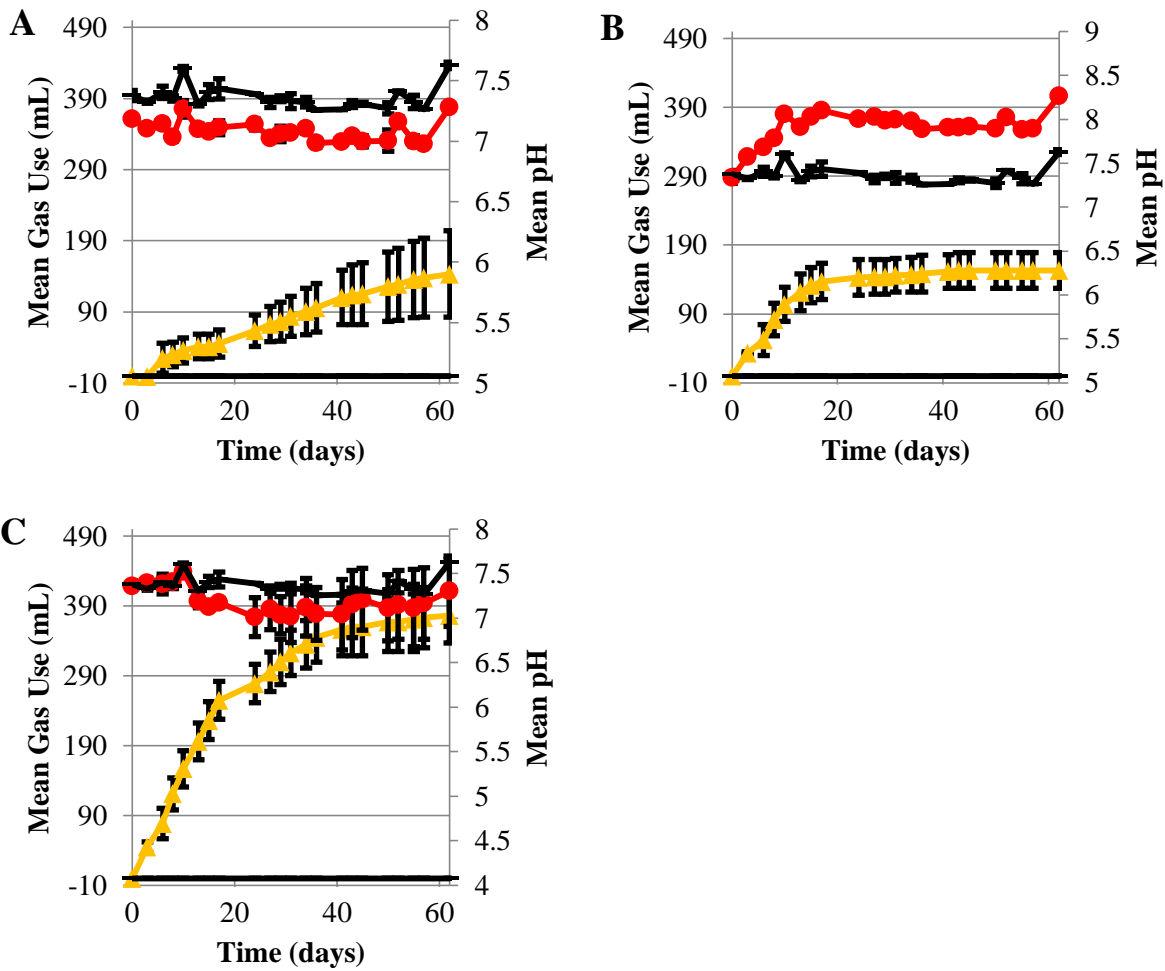


Figure 32. Mean pH (red) and mean gas consumption (yellow) as a function of time for experiment #3 secondary enrichment. 100 mM of CaCO_3 and 30 mM of NaHCO_3 were added with an H_2/CO_2 (80:20) headspace. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

4.3.4 16S rRNA microbial community analysis: Secondary enrichment

DNA community analysis was performed for samples at the beginning, peak and end of the incubation in high nutrient medium and at the end in low nutrient and BESA media. Low nutrient medium had 51% *Ralstonia*, 26.5% *Methanospirillum*, 3.3% *Methanofollis* and 4.4% *Pelomonas* (Figure 33 and Table 20). This appears to show a transition from a methanogenic to a carbon storing community, but no acetic acid loss was observed. *Ralstonia* is known to start forming PHB due to a specific nutrient limitation usually nitrogen. High nutrient medium grows faster than low nutrient medium which may cause it to reach a specific nutrient limitation to stimulate acetic acid consumption.

High nutrient medium started with approximately equal proportions of *Ralstonia* (40%) and *Methanospirillum* (47%) which shifted to progressively more *Ralstonia* (84.3% at peak, 84.8% at the end) and less methanogens. Other microbes present were identified as *Pelomonas* (9.3% at peak, 4.2% at the end) and *Mesorhizobium* (3.2% at peak, 3.3% at the end). *Mesorhizobium* is also capable of carbon storage (Lodwig *et al.* 2005). At the end of the incubation in high nutrient medium, 2.7% *Acetobacterium* was found which appears to only be a remnant of previous growth. The microbial community appears to have shifted sooner to a stage completely dominated by carbon storing microbes than in the primary enrichment, probably due to sooner nutrient limitation from the removal nutrients in the inoculum.

The incubation in BESA medium gave mostly of *Methanospirillum* (76.9%), *Methanobacterium* (12.1%) and *Methanofollis* (4.2%). The BESA medium incubation appears not to have progressed past the methanogenic stage and this may be associated with the greater methane production observed than in previous experiments.

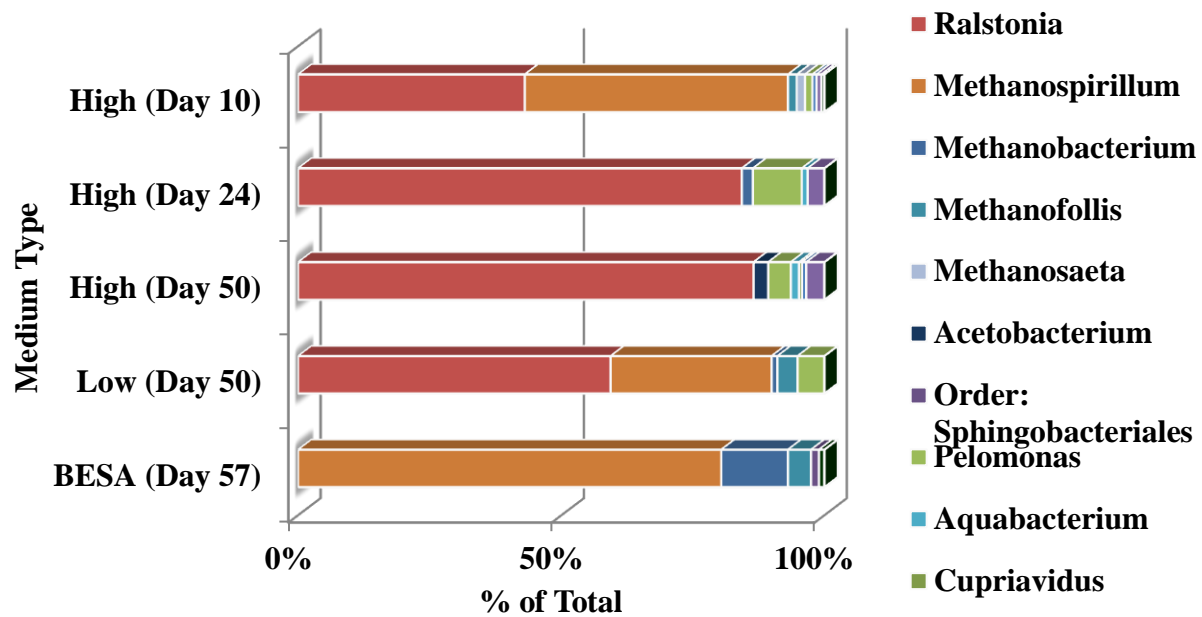


Figure 33. Experiment #3 secondary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 20. Experiment #3 secondary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	High (Day 10)	High (Day 24)	High (Day 57)	Low (Day 50)	BESA (Day 57)
<i>Ralstonia</i>	40.40	84.32	84.83	51.34	0
<i>Methanospirillum</i>	46.94	0	0	26.46	76.86
<i>Methanobacterium</i>	1.56	0	0	3.31	4.22
<i>Methanofollis</i>	3.25	0	0	8.35	12.20
<i>Methanosaeta</i>	1.48	0	0	0	0
<i>Acetobacterium</i>	0	1.13	2.74	0	0
<i>Sphingobacteriales</i>	0	0	0	0	1.44
<i>Pelomonas</i>	1.32	9.302	4.22	4.41	0
<i>Aquabacterium</i>	0	1.13	1.53	0	0
<i>Cupriavidus</i>	0	0	0.58	0	0

4.4 Experiment #4: For growth without NaHCO₃

4.4.1 Primary Enrichment

Experiment #3 had bicarbonate added to further buffer the pH of the medium. However, this added bicarbonate would further load the carbonic acid equilibrium towards higher concentrations of carbonate ion (CO_3^{2-}) in the medium and prevent dissolution of the CaCO_3 . Experiment #4 had the same experimental setup as experiment #3 but without added bicarbonate, just added CaCO_3 . The headspace of the bottles was flushed after 30 days of incubation in attempt to stimulate later acetogenesis. However plateaus or peaks in acetic acid occurred within the same time frame as previous experiments (20-40 days depending on the medium type). Methanogens appear to have been the main beneficiaries as methane plateaued later than previous work (day 40-60 as opposed to day 30-40 in experiment #3). This extra hydrogenotrophic methanogenesis makes it difficult to observe any acetotrophic methanogenesis that may or may not have occurred.

In the primary enrichment, the low nutrient medium had a higher level of acetic acid (832.6 μmol) and methane (1180 μmol) than observed in experiment #3 ($p < 0.05$, Figure 34A). In the high nutrient medium, levels of acetic acid were similar to the previous experiment (702 μmol , $p > 0.05$) which was again consumed starting on day 27. Unlike in experiment #3, acetic acid in the low nutrient and high nutrient medium was statistically the same in the primary enrichment in the secondary enrichment. Methane production was higher than in both the low nutrient and the high nutrient medium in experiment #3 at 1054 and 1546 μmol respectively ($p < 0.05$, Figure 34B). BESA medium produced a peak of 2281 μmol of acetic acid, similar to experiment #3 ($p > 0.05$) with less methane (less than 100 μmol , $p > 0.05$, Figure 34C). Peak values for methane and acetic acid are represented in Figure 34D. Removing bicarbonate had either a

positive or neutral effect on acetic acid production. It may have also increased methane production but this is inconclusive because flushing the headspace also appears to have increased methane levels.

The pH was within the range of 6.7 to 7.1 for low nutrient and BESA medium similar to sterile controls ($p > 0.05$, Figure 35A and 35C). This suggests that though bicarbonate was removed, CaCO_3 provides sufficient pH buffering on its own. The pH rose from 6.7 to 7.4 in the high nutrient medium by day 55 ($p < 0.05$, Figure 35B), which was again associated with either acetic acid or CO_2 loss. Compared to experiment #3 gas consumption was higher for the low nutrient medium (335 mL, $p < 0.05$) and the same for high nutrient and BESA medium at 318 and 442 mL respectively ($p > 0.05$, Figure 35). Gas consumption was mostly associated with methanogenesis for high nutrient medium. It was associated with acetogenesis for BESA medium and with both acetogenesis and methanogenesis in low nutrient medium.

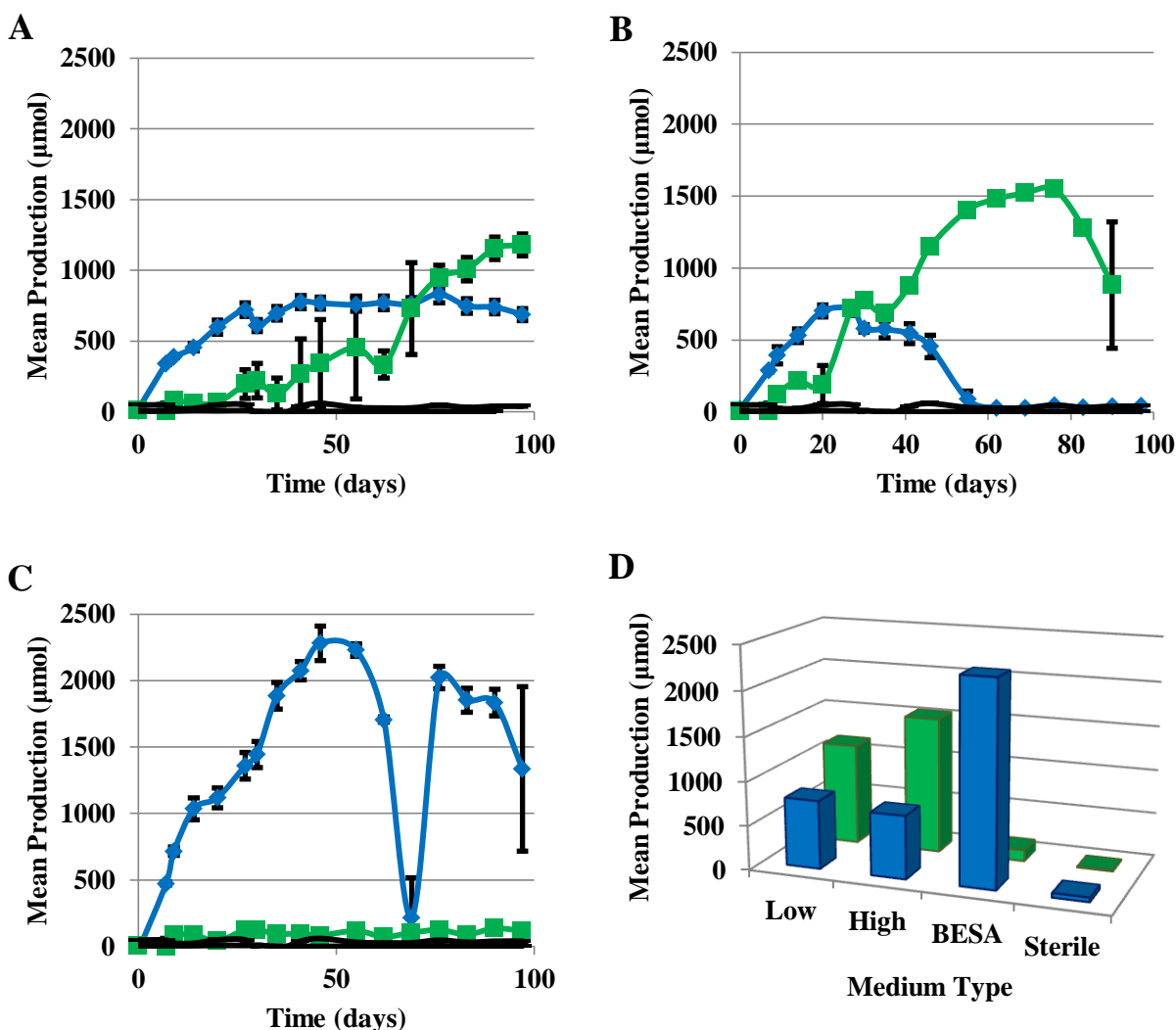


Figure 34. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #4 primary enrichment. Bicarbonate was removed from the medium. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BESA and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

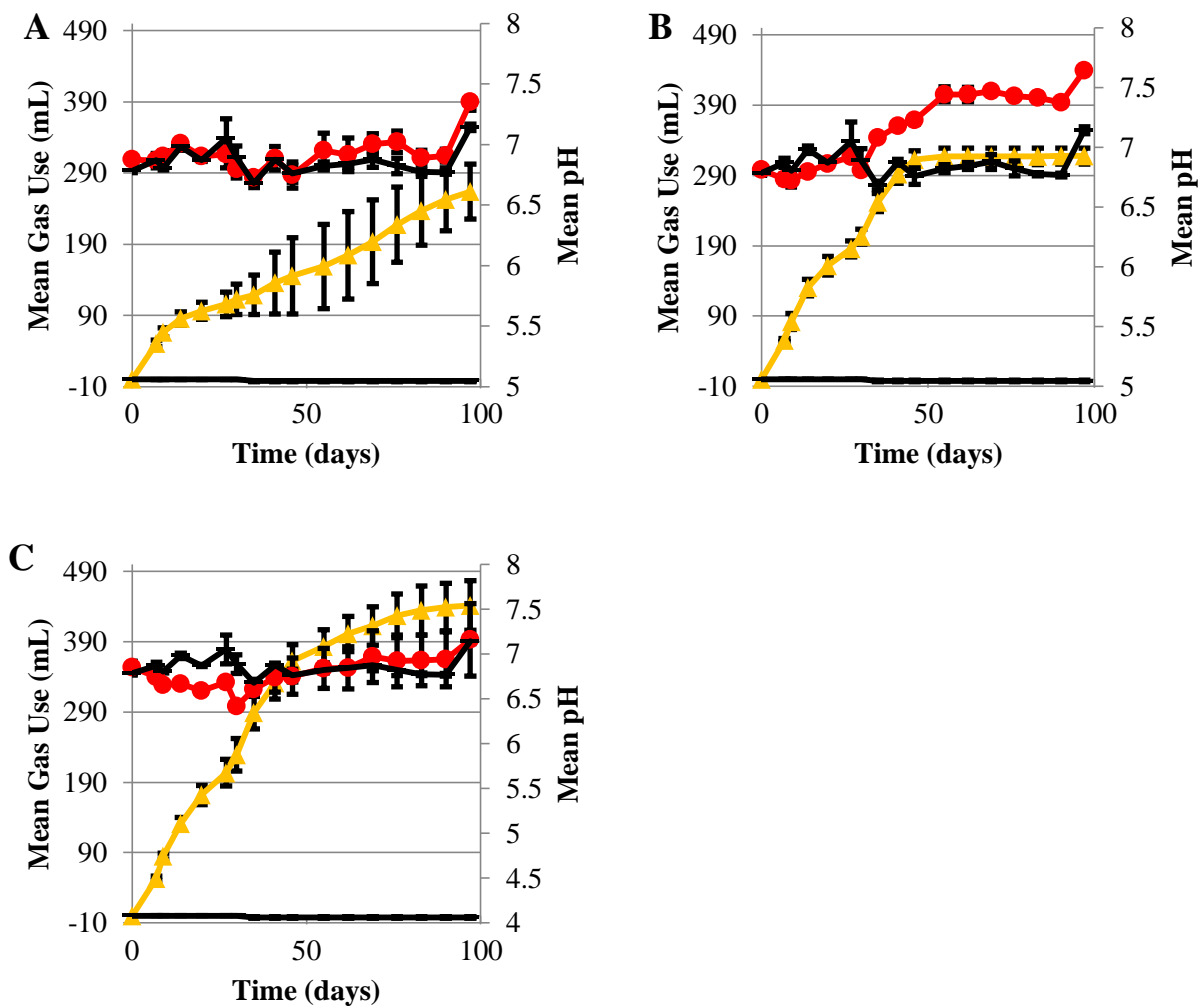


Figure 35. Mean pH (red) and mean gas consumption (yellow) as a function of time for experiment #4 primary enrichment. Bicarbonate was removed from the medium. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

4.4.2 16S rRNA microbial community analysis: Primary enrichment

Sampling days used in experiment #3 captured the microbial community in later stages of incubation during the transition from methanogenic growth to end stage carbon storage. While this is valuable for study, the analysis only captured tiny amounts of *Acetobacterium* growth and no other known acetogens. Earlier days were used in an attempt detect more acetogenic growth which tends to occur before day 20 (Figure 36 and Table 21).

Low nutrient medium had lower biomass with less extractable genomic DNA, so samples were taken on day 7 and day 35 and combined to increase levels of DNA. 22% of detectable reads were *Acetobacterium* which is supported by the acetic acid produced in the medium. The other amplified sequences represented a defined group of methanogens, with *Methanofollis* as the most abundant (22 %), followed by *Methanospirillum* (1.34%), *Methanocorpusculum* (9.7%) and *Methanobacterium* (4.5%). Mixing samples from days that were 3 weeks apart may have combined acetogen-dominant and methanogen-dominant communities.

A small proportion of detectable DNA read involves microbes active in sulfur cycle metabolism. 5.9% of reads match the nitrate or oxygen reducing sulfide-oxidizer *Sulfurospirillum* (Hubert and Voordouw 2007). Small fractions of sulfate reducers were also detected (0.5% *Desulfomicrobium*, Theveniaeu *et al.* 2007) which may be using the sulfate produced by *Sulfurospirillum* and recycle it back to sulfide. Acetogenic and methanogenic microbes were the largest community components and represented about half of the sequencing reads.

A few other minor contributors were 2.2% *Proteiniphilum* and 4.4% *Sphingobacteriales*, which are indentified as syntrophs capable of degrading fatty acids and polyaromatic compounds

(respectively) in association with methanogens (Mao *et al.* 2012, Chen and Dong 2005). These microbes may survive off of trace amounts of oils from the inoculum. A substantial number of aerobes and microaerophiles, including the aerobic pathogen *Shigella* (13.7%) were detected (Zaika *et al.* 1994), which may be getting oxygen from *Cyanobacteria* (2.4%) or oxygen leakage through the stoppers. However the *Cyanobacteria* detected is only identified at the phylum level, some non-photosynthetic members of the phylum exists and this microbes could be one of them (Kulik 1995).

The high nutrient medium was sampled a week before acetic acid loss started to occur. 56% of sequencing reads were from *Acetobacterium*, making this community acetogen-dominant. The next most abundant community group were sulfur cyclers with 23.8% *Sulfurospirillum* and 2.4% *Arcobacter* (another sulfide-oxidizer, Wirsén *et al.* 2002), which were more abundant than in the low nutrient medium. The methanogenic community was starting to form, (190 µmol was methane present) with 1.8% *Methanofollis* and 1.6% *Methanobacterium*. Once acetic acid loss started to occur, the community became more methanogenic in composition with 39.7% *Methanofollis*, 19.7% *Methanospirillum* and 3.7% *Methanocorpusculum*. Syntrophic members *Sphingobacteriales* (11.1%) and *Proteiniphilum* (5.0%) were then also present in higher proportion. The microbial community shifted from acetogen-dominant to methanogen-dominant between day 20 and day 46. While sufficient PCR product was obtained, an issue at Genome Quebec prevented access to the sequences for the sample from late days in high nutrient medium.

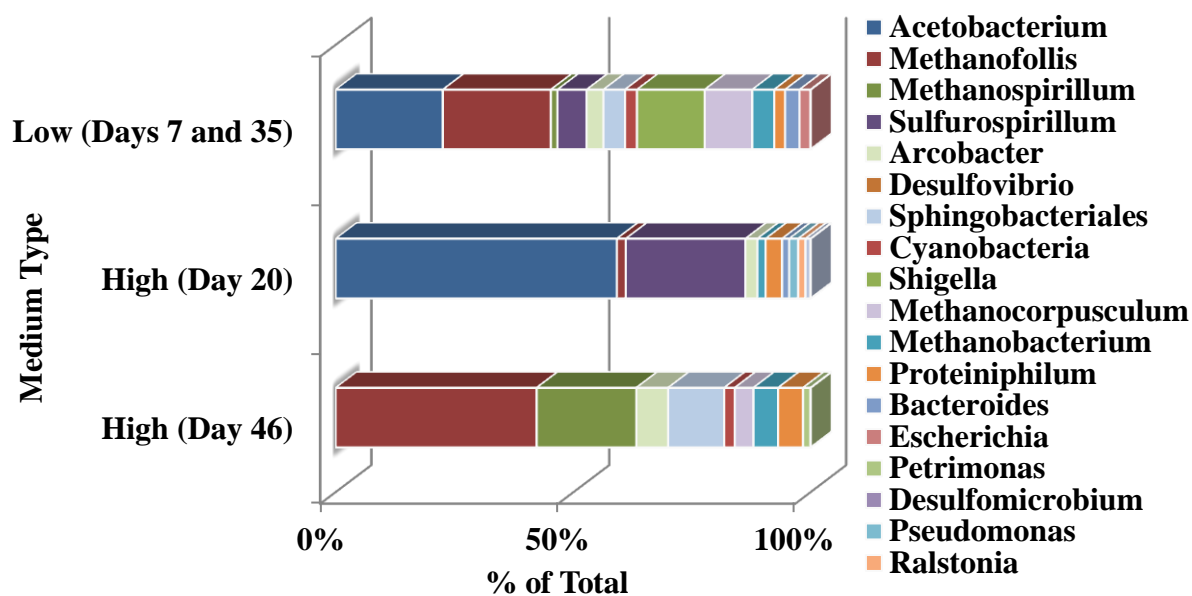


Figure 36. Experiment #4 primary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 21. Experiment #4 primary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	Low (Days 7 and 35)	High (Day 20)	High (Day 46)
<i>Bacteroides</i>	2.93	1.44	0.55
<i>Sphingobacteriales</i>	4.36	0	11.12
<i>Proteiniphilum</i>	2.22	3.29	4.95
<i>Methanofollis</i>	21.82	1.80	39.70
<i>Methanobacterium</i>	4.45	1.64	4.89
<i>Desulfovibrio</i>	0.009	0.15	0
<i>Ruminococcaceae</i>	0.11	1.08	0
<i>Methanospirillum</i>	1.34	0	19.67
<i>Acetobacterium</i>	21.69	56.01	0.26
<i>Escherichia</i>	2.27	0.051	0
<i>Arcobacter</i>	3.40	2.41	6.28
<i>Sulfurospirillum</i>	5.89	23.87	0.38
<i>Cyanobacteria</i>	2.42	0	2.09
<i>Petrimonas</i>	0.082	0	1.49
<i>Pseudomonas</i>	0.95	1.80	0
<i>Ralstonia</i>	0.055	1.44	0.033
<i>Methanocorpusculum</i>	9.68	0	3.72
<i>Methanosaeta</i>	0	0	0.93
<i>Shigella</i>	13.71	0.15	0.011
<i>Desulfomicrobium</i>	0.49	0	0.31

4.4.3 CaCO₃ mass recovery: Primary enrichment

1000 milligrams of CaCO₃ was originally added to the bottles at the beginning of the experiment. The remaining mass was recovered at the end of the incubation by vacuum filtration and drying. The amount of mass recovered ranged from 837 mg in the BESA medium to 953 mg in the low nutrient medium. However, there was also a substantial amount of mass lost in the sterile controls, with an average of 903 mg recovered (Table 22). The amount of mass recovered in the microbially active bottles was not different from the sterile controls ($p>0.05$).

When this amount was subtracted from the experimental measurements, the net change was positive in the low nutrient medium and near zero in the high nutrient medium. 0.65 mmol of CaCO₃ was lost in the BESA medium, though 2.28 mmol of acetic acid was produced suggesting at best only partial dissolution that did not result in a loss of mass significantly different from the BESA control ($p<0.05$, Table 23).

Table 22. CaCO₃ mass recovered from experiment #4 primary enrichment. 1000 mg \pm 10 mg of ACS grade powdered CaCO₃ was originally added to bottles to create a concentration of 100 mM in suspension. Standard error and mean were calculated based on 3 replicates.

Sample ID	Mean CaCO ₃ Recovered (mg)	Mean Std Error (mg)	Mean % Recovered	Mean % Recovered Std Error	Ratio Active/Sterile
Sterile Low	896.47	17.58	89.65	0.176	N/A
Sterile High	952.70	16.65	95.27	0.167	N/A
Sterile BESA	859.30	29.93	85.93	0.299	N/A
Low	915.07	24.40	91.51	0.244	1.02
High	899.70	35.70	89.97	0.357	0.94
BESA	837.47	4.41	83.75	0.044	0.93

Table 23. Comparison of CaCO₃ mass change to peak acetic acid produced for experiment #4 primary enrichment after removal of abiotic loss (as represented by loss in the sterile control), precipitation occurred if the net value was positive. Dissolution occurred if mass recovery was negative. The sterile control is calculated as an average of sample loss found in the sterile controls for the three types of medium.

Sample ID	Mean Acetic Acid (mmol)	Mean CaCO ₃ Change (mg)	Mean CaCO ₃ change (mmol)	Mean CaCO ₃ change – mean sterile loss (mmol)	Precipitation or Dissolution
Sterile	0.050	-97.18	-0.97	N/A	N/A
Low	0.78	-84.93	-0.85	+0.12	Precipitation
High	0.71	-100.30	-1.00	-0.03	Dissolution
BESA	2.28	-162.53	-1.63	-0.65	Dissolution

4.4.4 Secondary enrichment

In the secondary enrichment, the low nutrient medium acetic acid plateaued at 688 μmol and methane peaked at 589 μmol (Figure 37A). In the high nutrient medium methane peaked at 1546 μmol and acetic acid peaked at 545 μmol at day 7 and then decreased (Figure 37B). The acetic acid in the high nutrient medium was significantly lower than in the low nutrient medium ($p < 0.05$) and the methane was the same ($p > 0.05$). Compared to the primary enrichment, high nutrient medium had the same acetic acid ($p > 0.05$) and lower methane ($p < 0.05$). Acetic acid in BESA medium peaked at 2394 μmol and methane was less than 100 μmol . This was the same as the primary enrichment and experiment #3 (Figure 37C, $p > 0.05$). Peak values for methane and acetic acid are represented in Figure 37D. As compared to experiment #3, acetic acid was higher or the same and methane was higher. Removing bicarbonate appears to promote acetogenesis somewhat and at least it doesn't hurt it. Methane also increased and this may have been due to the removal of bicarbonate, but this could also be due to the flushing of the headspace gas.

The pH ranged from 6.7 to 7.4 for low nutrient medium which is similar to that of sterile controls ($p > 0.05$, Figure 38A). The high nutrient enrichment pH rose from 6.7 to 7.5 in association with acetic acid or CO_2 loss (Figure 38B, $p < 0.05$). The pH of the BESA medium was slightly above the control ($p < 0.05$) and even though pH appears to have increased from 6.9 to 7.4, the sterile control rose from 6.7 to 7.3 too (Figure 38C) so this was due to instrumental fluctuation. Gas consumption was the same as for the primary enrichment experiment #3 with similar associations with acetic acid and methane production (Figure 38).

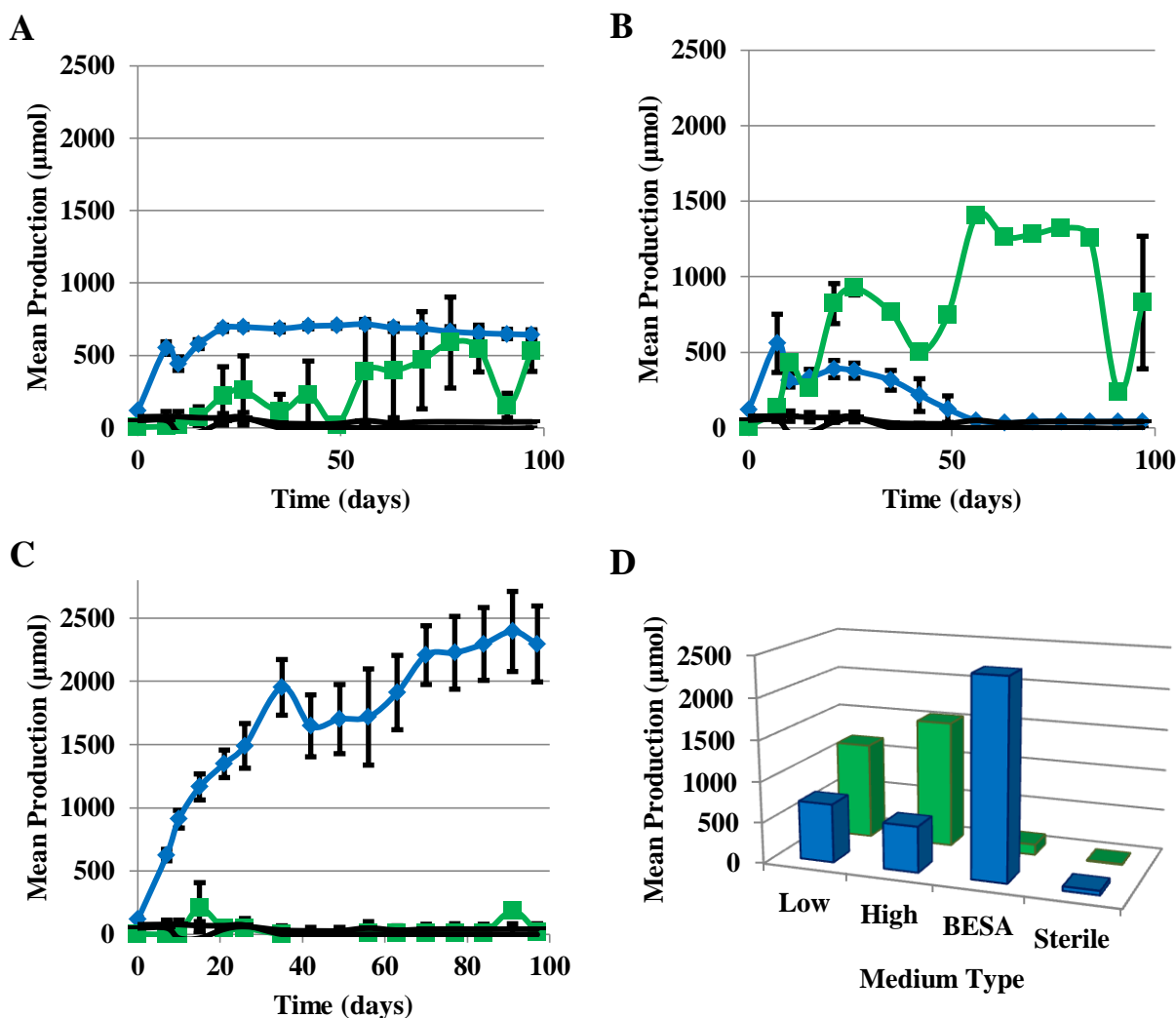


Figure 37. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #4 secondary enrichment. Bicarbonate was removed from the medium. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BESA and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

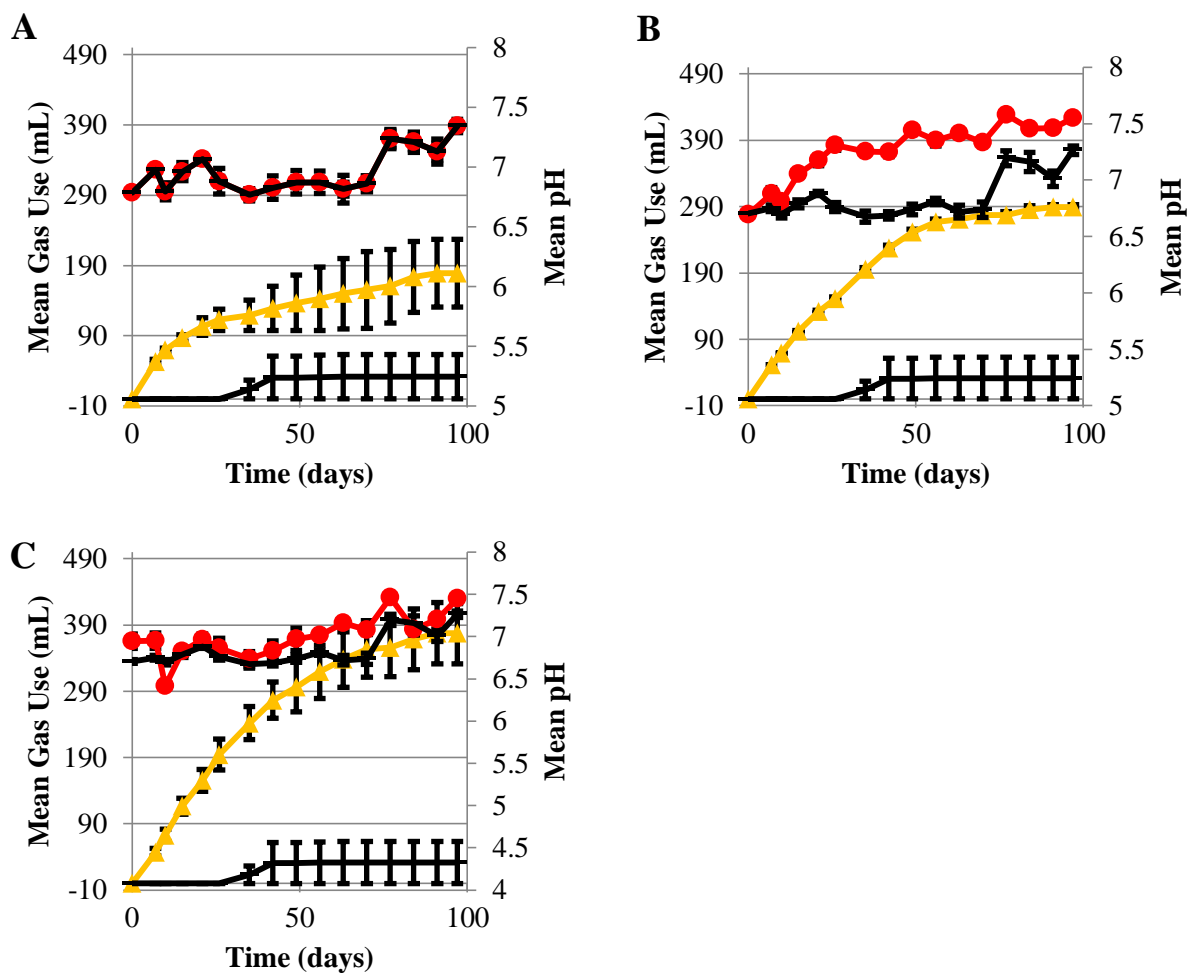


Figure 38. Mean pH (red) and mean gas consumption (yellow) as a function of time for experiment #4 secondary enrichment. Bicarbonate was removed from the medium. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

4.4.5 16S rRNA microbial community analysis: Secondary enrichment

A PCR product could not be obtained for the low nutrient medium in the secondary enrichment. Samples were sequenced for high nutrient medium from time points before and after peak acetic acid production. A sample from the incubation with BESA medium at day 21 was also analyzed (Figure 39 and Table 24).

The sequencing reads in high nutrient medium at peak acetic acid production (day 15) were 64.8% *Acetobacterium*, which appears to be main acetogen present in all of these experiments. The remainder of the sequencing reads are associated with sulfide metabolism with sulfide-oxidizers *Arcobacter* (2.8%) and *Sulfurospirillum* (4.2%) and sulfate-reducers *Desulfovibrio* (12.4%) and *Desulfomicrobium* (2.2%). Syntrophs *Proteiniphilum* (1.1%) and *Sphingobacteriales* (0.6%) are still present. Less than 0.05% of any methanogenic archaea were detected even though 434 μmol of methane had already been produced and was still actively being produced. There appears to be a lag in changes in microbial activity as compared to what is seen in the DNA data. The microbial community was acetogen-dominant in earlier days of the high nutrient medium. The sulfur cycling community was a greater than in the primary enrichment but does not appear to have affected the activity of acetogens or methanogens which remains equal or higher.

At the later time point in the high nutrient enrichment, 64.8% of reads were *Acetobacterium*, essentially the same as the earlier time point even though the acetic acid had been reduced by from 560 to 219 μmol , Sulfide oxidizers *Sulfurospirillum* (5.9%) and *Arcobacter* (14.1%) were detected and the sulfate they produced may have supported sulfate reducers *Desulfovibrio* (5.2%) and *Desulfomicrobium* (2.2%). Sulfide oxidation may use oxygen

made by *Cyanobacteria* (10.7%) or perhaps through leakage of atmosphere through the stopper. Almost no methanogenic archaea are detected with less than 0.1% of *Methanofollis*, *Methanobacterium* and *Methanospirillum* though 498 μmol of methane had been produced and was still increasing. At this time point the headspace had been flushed a week earlier to replenish CO_2 . This change is not reflected in the molecular microbial community data. The secondary enrichment appears to have remained acetogen-dominant longer than experiment #3.

Though BESA medium produced the most acetic acid, the 16S RNA sequencing reads were mostly of methanogens with 46% *Methanofollis*, 1.6% *Methanobacterium*, 2.6% *Methanocorpusculum* and 23.2% *Methanospirillum*. Syntrophs (2.1% *Proteiniphilum* and 3.4% *Shingobacteriales*) and sulfide oxidizer *Arcobacter* (9.5%) still present. The BESA medium appears to have progressed to the methanogenic stage by day 21 (*Acetobacterium* constituted only 2.2% of the sequencing reads) even though 1348 μmol of acetic acid was produced after that sampling time point with almost no methane. There a defined discrepancy between the activity data and the molecular 16S rRNA data for BESA medium.

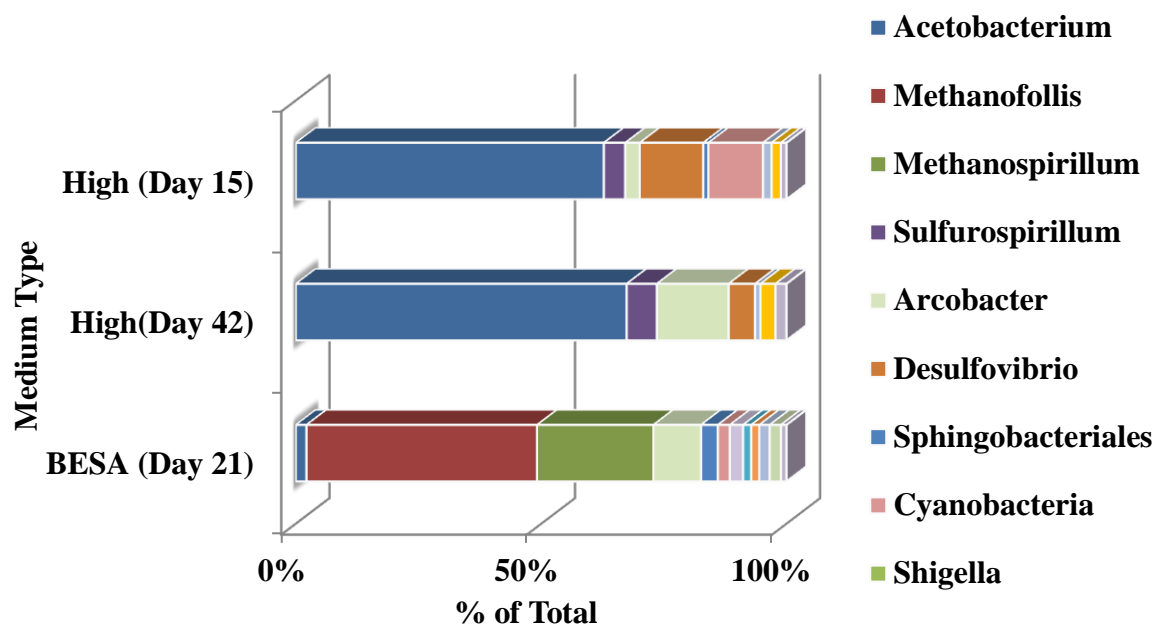


Figure 39. Experiment #4 secondary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 24. Experiment #4 secondary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	High (Day 15)	High (Day 42)	BESA (Day 21)
<i>Bacteroides</i>	2.94	1.80	0.94
<i>Sphingobacteriales</i>	0.59	1.01	3.36
<i>Proteiniphilum</i>	1.06	1.68	2.08
<i>Methanofollis</i>	0.040	0.025	45.97
<i>Methanobacterium</i>	0.01	0	1.62
<i>Desulfovibrio</i>	5.17	12.41	0
<i>Ruminococcaceae</i>	0.59	0.17	0
<i>Methanospirillum</i>	0.01	0	23.21
<i>Acetobacterium</i>	64.77	60.14	2.16
<i>Escherichia</i>	0	0.025	0
<i>Arcobacter</i>	14.09	2.84	9.51
<i>Sulfurospirillum</i>	5.92	4.18	0.90
<i>Cyanobacteria</i>	0.23	10.68	2.41
<i>Petrimonas</i>	0	0	2.24
<i>Pseudomonas</i>	0.03	0.074	0.028
<i>Ralstonia</i>	0	0	0
<i>Methanocorpusculum</i>	0	0.025	2.64
<i>Methanosaeta</i>	0	0	0
<i>Shigella</i>	0.02	0.12	0
<i>Desulfomicrobium</i>	2.15	1.09	0.21

4.4.6 CaCO₃ mass recovery: Secondary enrichment

For mass recovery of CaCO₃ from the secondary enrichment, the amount of mass recovered in the sterile controls was again less than in the primary enrichment (Table 25). The amount of mass recovered in microbially active low nutrient medium was significantly *more* than in the sterile control ($p < 0.05$). Re-precipitation may also have occurred. The amount of CaCO₃ recovered in the high nutrient and BESA media were not significantly different from their controls.

Accounting for sterile loss resulted in net gains in mass for all three medium types ranging from +0.19 to +1.47 mmol (Table 26). BESA medium, which produced the most acetic acid, had the lowest gain but this was not statistically significant ($p > 0.05$).

Table 25. CaCO₃ mass recovered from experiment #4 secondary enrichment. 1000 mg ± 10 mg of ACS grade powdered CaCO₃ was originally added to bottles to create a concentration of 100 mM in suspension. Standard error and mean were calculated based on 3 replicates.

Sample ID	Mean CaCO ₃ Recovered (mg)	Mean Std Error (mg)	Mean % Recovered	Mean % Recovered Std Error	Ratio Active/Sterile
Sterile Low	811.20	1.20	81.12	0.12	N/A
Sterile High	874.70	32.86	87.47	3.29	N/A
Sterile BESA	919.43	40.68	91.94	4.07	N/A
Low	929.33	2.71	92.93	0.27	1.15
High	1015.57	84.95	101.56	8.49	1.16
BESA	887.80	13.17	88.78	1.32	0.97

Table 26. Comparison of CaCO₃ mass recovery to peak acetic acid produced for experiment #4 secondary enrichment. After removal of abiotic loss (as represented by loss in the sterile control), precipitation occurred if the mass recovery was positive and dissolution occurred if mass recovery was negative. The sterile control is calculated as the mean of sample loss found in the sterile controls for the three types of medium.

Sample ID	Mean Acetic Acid (mmol)	Mean CaCO ₃ Change (mg)	Mean CaCO ₃ change (mmol)	Mean CaCO ₃ change – sterile loss (mmol)	Precipitation or Dissolution
Sterile	0.052	-131.6	-1.32	N/A	N/A
Low	0.72	-70.67	-0.71	+0.61	Precipitation
High	0.56	+15.57	+0.16	+1.47	Precipitation
BESA	2.39	-112.2	-1.12	+0.19	Precipitation

4.4.7 Primary enrichment bright-field and epifluorescent microscopy with Nile red staining for polyhydroxybutyrate (PHB) granules

In high nutrient medium for experiment #3 and #4, the acetic acid decreased to almost zero. 16S rRNA sequencing data suggests that this may be due to formation of carbon-storage bodies that store CO₂ and acetate in the form of polyhydroxyalkanoates like polyhydroxybutyrate. To investigate this, samples were taken and dyed with Nile Red fluorescent dye and examined under microscopy with both bright-field and epifluorescent settings (Figure 40). These samples were taken from days 21 (A and B) and 33 (C and D) of the primary enrichment of experiment #4 which is when acetic acid was actively decreasing. Under bright field microscopy, several cell clusters appeared to contain lipid-like inclusions which were red under epifluorescence and which covered most of the cells. This is consistent with the presence of polyhydroxybutyrate (PHB) and other polyhydroxyalkanoates but they may also be artifacts. Further investigation and chemical confirmation is warranted.

4.4.8 Primary enrichment ammonium assay

It appears the nutrient difference in the inoculum between the primary and secondary enrichment is important. It allows the nutrient differences between the low and high nutrient medium to have a significant effect on the competition between acetogens and methanogens. But the low nutrient medium has four of the added nutrients removed and needed to be further defined. Ammonium (NH₄⁺) was assayed using the indophenol blue method to look for active loss during the primary enrichment (Figure 41). If loss was occurring then ammonium may be the nutrient causing the differential effects seen between the nutrient levels. Ammonium was at the expected levels in the different medium types, 0.01 mM in low nutrient medium and 4.0 and

3.2 mM in high nutrient and BESA medium, respectively. There was a slight decline in ammonium levels in the latter two media to 3.8 mM and 3.0 mM but this decrease is small. It is unlikely that the differences between primary and secondary enrichments are due to different ammonium (nitrogen) levels.

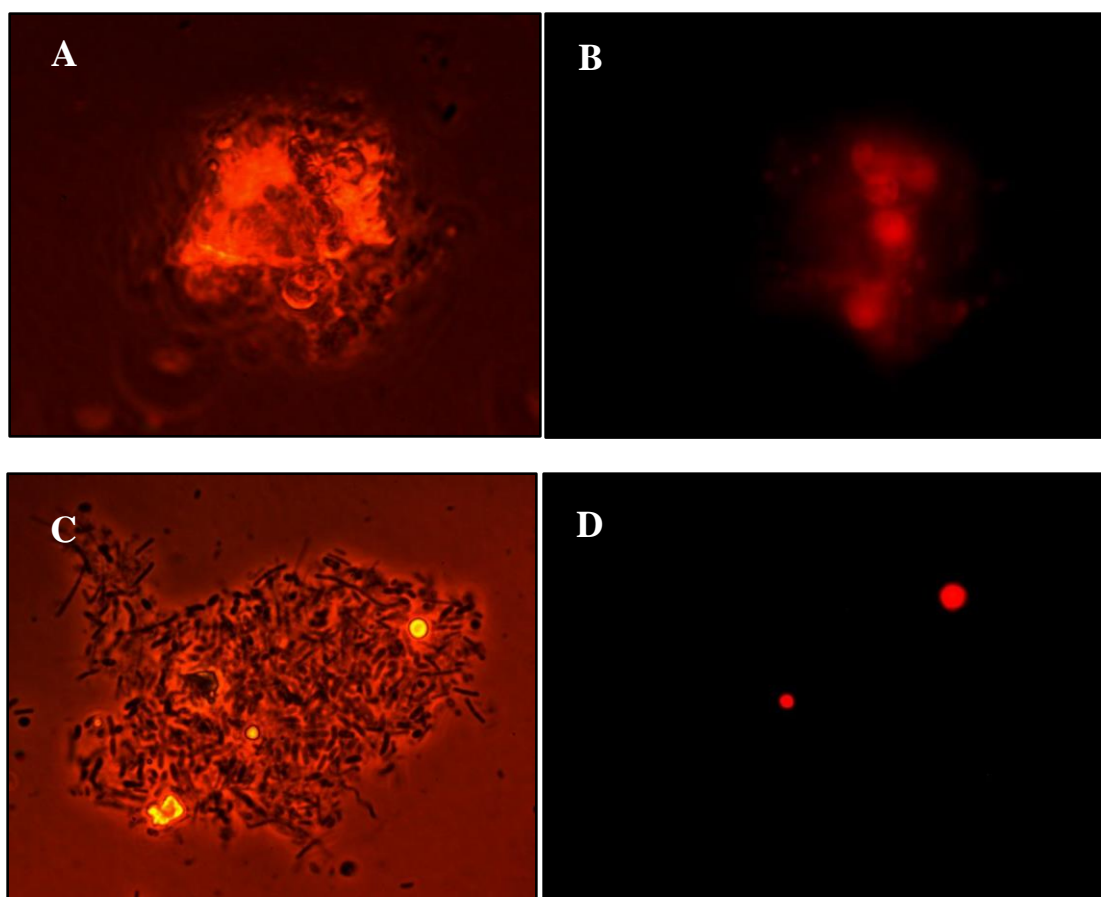


Figure 40. Bright field (A and C) and epifluorescent (B and D) microscopy images of samples from experiment #4 high nutrient medium primary enrichment. Image B was the epifluorescent image of A from day 21 and image D was the epifluorescent image of C from day 33. Fluorescent images were obtained by dying with Nile Red fluorescent dye and viewing under a CY5 brightline fluorescence filter.

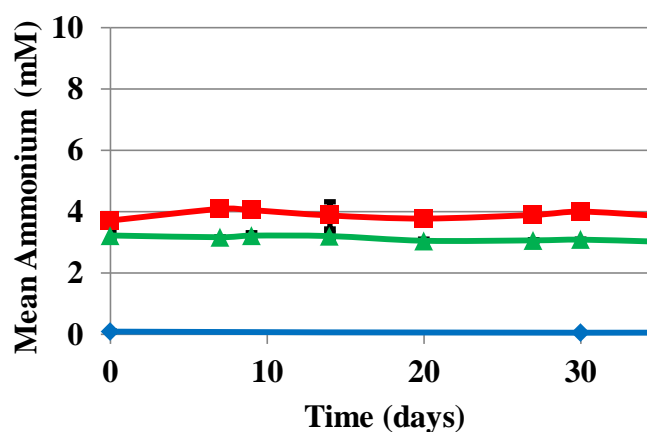


Figure 41. Mean ammonium concentrations for low nutrients (blue), high nutrients (red) and BESA medium (green) for the primary enrichment of experiment #4 primary enrichment. Error bars are standard error for 3 replicates.

4.5. Experiment #5: Incubations with either H₂ or CO₂ only

Sterile loss had a significant effect in the mass recovery of CaCO₃ in experiment #4. This could be due to loss of CaCO₃ into the needle during sample or this could be due to chemical loss. Chemical loss would involve dissolution of CaCO₃ due to CO₂ acidification. The concentration of 20% CO₂ in the headspace of previous experiments is much higher than the CaCO₃ was exposed to in the atmosphere. To test whether a higher concentration of CO₂ caused dissolution, the experimental set up from experiment #4 was repeated using two sets of replicates with either 100% H₂ headspace gas or 100% CO₂ headspace gas. Only a primary enrichment was performed.

For the 100% H₂ headspace, the low and high nutrient media produced similar amounts of acetic acid (380 µmol and 400 µmol respectively, $p>0.05$), which were much lower than in experiment #4 ($p<0.05$, Figure 42A). This is not surprising as CO₂ is much more limited in these conditions and CO₂ would have to come from trace amounts or the carbonate and carbonic acid equilibria. Less than 20 µmol of acetic acid was produced in the BESA medium and methane for all media was less than 100 µmol ($p>0.05$). In all media, the pH rose from 7.5 to a range of 8.7 to 8.9. In BESA medium it did not rise as much (due BESA's chemical acidity) and it was lower than low and high nutrient medium ($p<0.05$, Figure 42ABC). This increase in pH is due to removal of the acidifying effect of CO₂. Gas consumption was approximately 30 mL for all medium types (Figure 43ABC) which is also very low. Peak values for acetic acid and methane are shown in Figure 41B.

In the 100% CO₂ headspace, relatively low acetic acid and methane production was observed for most media. Less than 2 µmol of methane was detected in all three media which

were not different from each other in any way ($p>0.05$, Figure 41C). Acetic acid was only detected in the low and high nutrient media (58 μmol), higher than in the BESA medium ($p<0.05$, Figure 42C). The pH declined over time in all media from 6.7 to 6.4, but this was not significantly different from the sterile controls ($p>0.05$, Figure 42DEF). The low pH levels were likely due to with CO_2 acidification. Gas consumption was about 30 mL for high nutrient medium and 20 mL for low nutrient and BESA medium and was not associated with a particular microbial activity (Figure 43DEF). Peak values for acetic acid and methane are shown in Figure 42D.

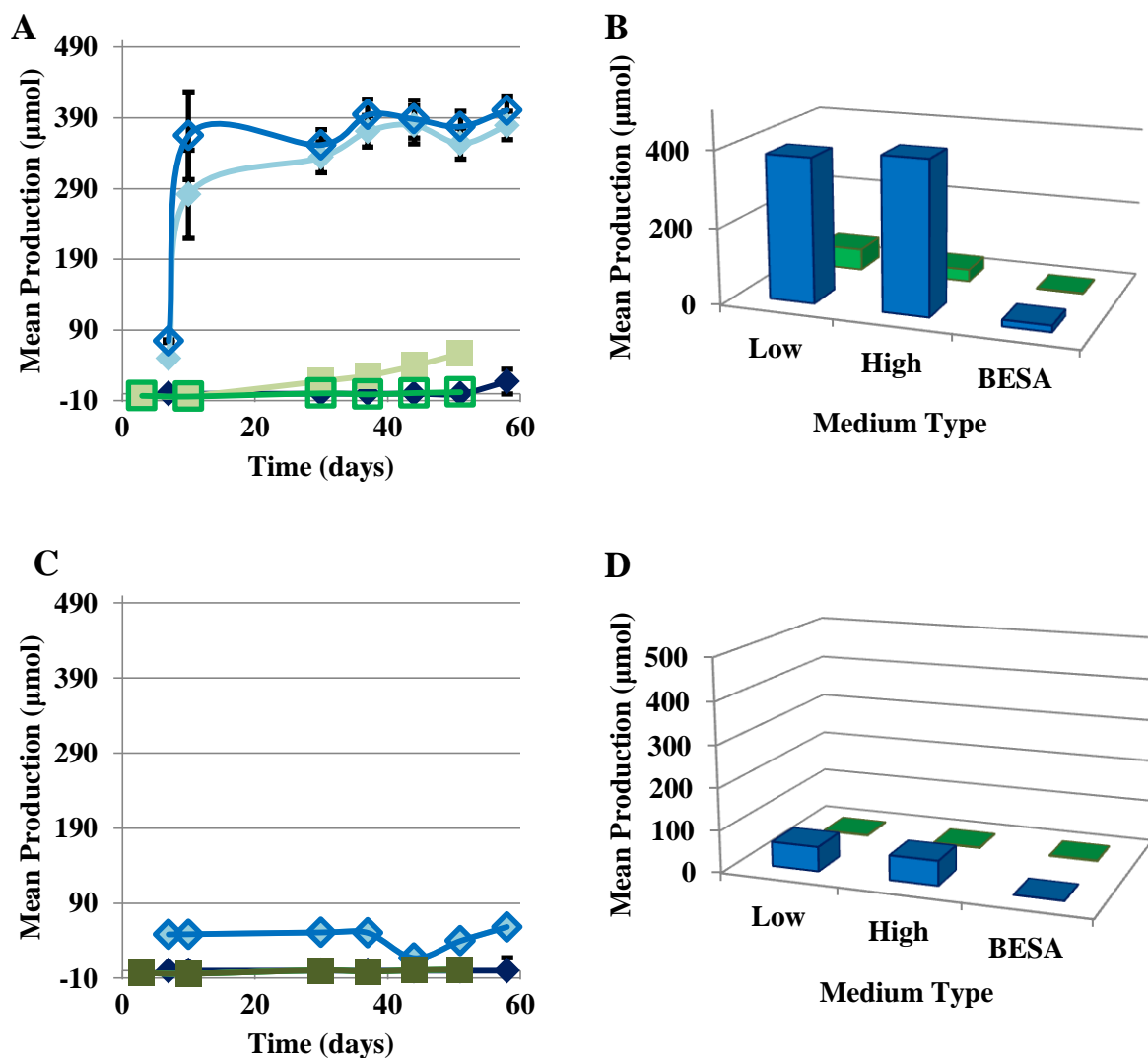


Figure 42. Mean acetic acid (blue) and mean methane (green) production for experiment #5. The H₂/CO₂ headspace was replaced with either 100% H₂ or 100% CO₂. Hollow points represent low nutrient medium, and solid points represent high nutrient medium. The top graphs show (A) 100% H₂ as a function of time and (B) peak values for 100% H₂ in all three medium types. The bottom graphs show (C) 100% CO₂ as a function over time and (D) peak values for 100% CO₂ in all three medium types. The error bars represent an average of 3 replicates.

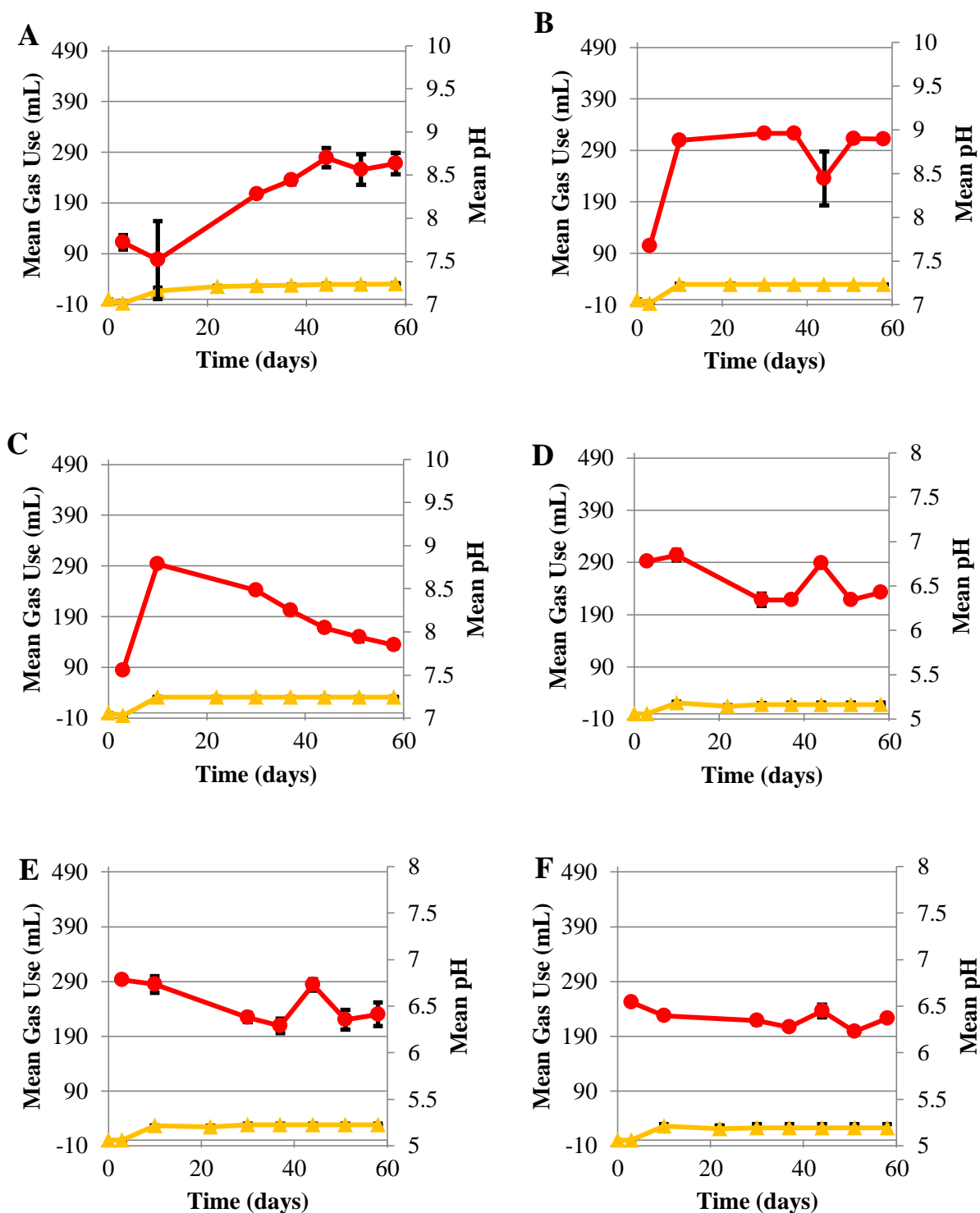


Figure 43. Mean pH (red) and mean gas consumption (yellow) for experiment #5. The H₂/CO₂ headspace was replaced with either 100% H₂ or 100% CO₂. The top graphs show 100% H₂ as a function of time for (A) low nutrient medium and (B) high nutrient medium. The middle graphs show (C) 100% H₂ as a function of time for BESA medium and (D) 100% CO₂ as a function of time for low nutrient medium. The bottom graphs show 100% CO₂ as a function over time for (E) high nutrient medium and (F) BESA medium. The error bars represent an average of 3 replicates.

5.1.2 CaCO₃ mass recovery

When CaCO₃ mass recovery was compared between 100% H₂ and 100% CO₂, there was significantly less mass recovered with the 100% CO₂ headspace for low nutrient and BESA medium (Table 27, $p < 0.05$). There is no difference in mass recovered for high nutrient medium ($p > 0.05$). This is due to chemical loss from greater acidity in 100% CO₂ which resulted in dissolution of 7.15% in low nutrient medium and 3.45% in BESA medium. Sampling loss is not accounted for and may have influenced the results especially since the more acidic BESA medium had less dissolution. Abiotic dissolution is present, but it would occur for both sterile and microbially active bottles and is relatively small.

Table 27. CaCO₃ mass recovered from experiment #5. 1000 mg \pm 10 mg of ACS grade powdered CaCO₃ was originally added to bottles to create a concentration of 100 mM in suspension. Standard error and mean were calculated based on 3 replicates.

Sample ID	Mean CaCO ₃ Recovered (mg)	Mean Std Error (mg)	Mean % Recovered	Mean % Recovered Std Error
H ₂ Low	978.50	10.15	97.85	1.02
H ₂ High	952.25	13.95	95.23	1.39
H ₂ BESA	959.63	3.68	95.96	0.37
CO ₂ Low	907.03	4.36	90.70	0.44
CO ₂ High	927.63	11.44	92.76	1.14
CO ₂ BESA	925.07	5.46	92.51	0.55

4.6. Experiment #6: For growth with MgCa crushed carbonate core

4.6 Primary Enrichment

The set-up of experiment #4 was repeated using crushed MgCa-carbonate core, which has an overall lower surface area than the previously used powdered CaCO_3 . It was provided by Suncor from the bitumen-containing Grosmont reservoir in Northern Alberta, though the bitumen had been stripped from the rock. This rock was more representative of the intended dissolution environment. It also had a larger particle size which reduced sampling loss as it did not fit into the sampling needle like the powdered CaCO_3 did.

In the primary enrichment, the low nutrient medium had less acetic acid (428 μmol , $p < 0.05$) and similar methane than experiment #4 (1176 μmol , $p > 0.05$, Figure 44A). The acetic acid plateau occurred earlier and methane production started sooner. High nutrient medium produced similar amounts of acetic acid (643 μmol , $p > 0.05$), which again peaked and disappeared with a little less methane (1287 μmol , $p < 0.05$, Figure 44B). BESA medium had less acetic acid (1791 μmol , $p < 0.05$) with the same minimal methane (<125 μmol , $p > 0.05$, Figure 44C). Peak values for acetic acid and methane production are shown in Figure 43D. There is less growth overall in this experiment which suggests that available surface area is associated with acetogenic and methanogenic growth. This experiment appears to have favoured methanogenesis more than experiment #4.

The pH remained similar to the sterile control at around 7.0 in the low nutrient medium ($p > 0.05$, Figure 45A). In high nutrient medium the pH dropped slightly and then rose from 7.0 to 7.3 ($p < 0.05$, Figure 45B). The rise in pH was not associated with acetic acid loss as it did not start until after loss had finished but it could be associated with CO_2 loss. The pH in BESA

medium dropped from 6.7 to 5.6 and then slowly rose to 6.0 ($p < 0.05$, Figure 45C). Gas consumption was the same for the low nutrient medium (227 mL, $p > 0.05$) and lower for high nutrient (235 mL) and BESA medium as compared to experiment #4 (330 mL, $p < 0.05$). It was associated with methane production for low and high nutrient medium and with acetic acid production for BESA medium.

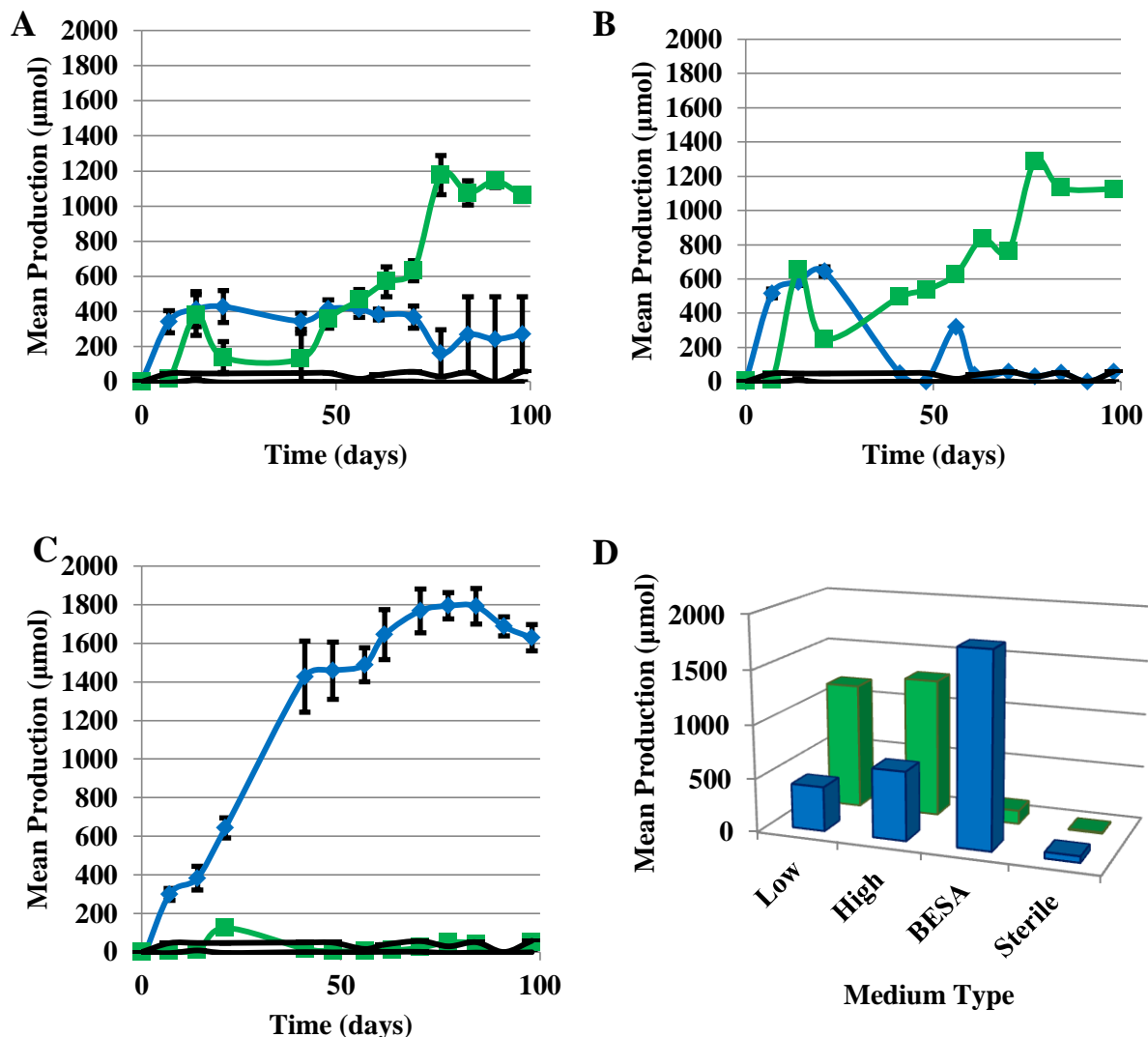


Figure 44. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #6 primary enrichment. Crushed carbonate rock was used instead of powdered CaCO_3 . The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BES and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

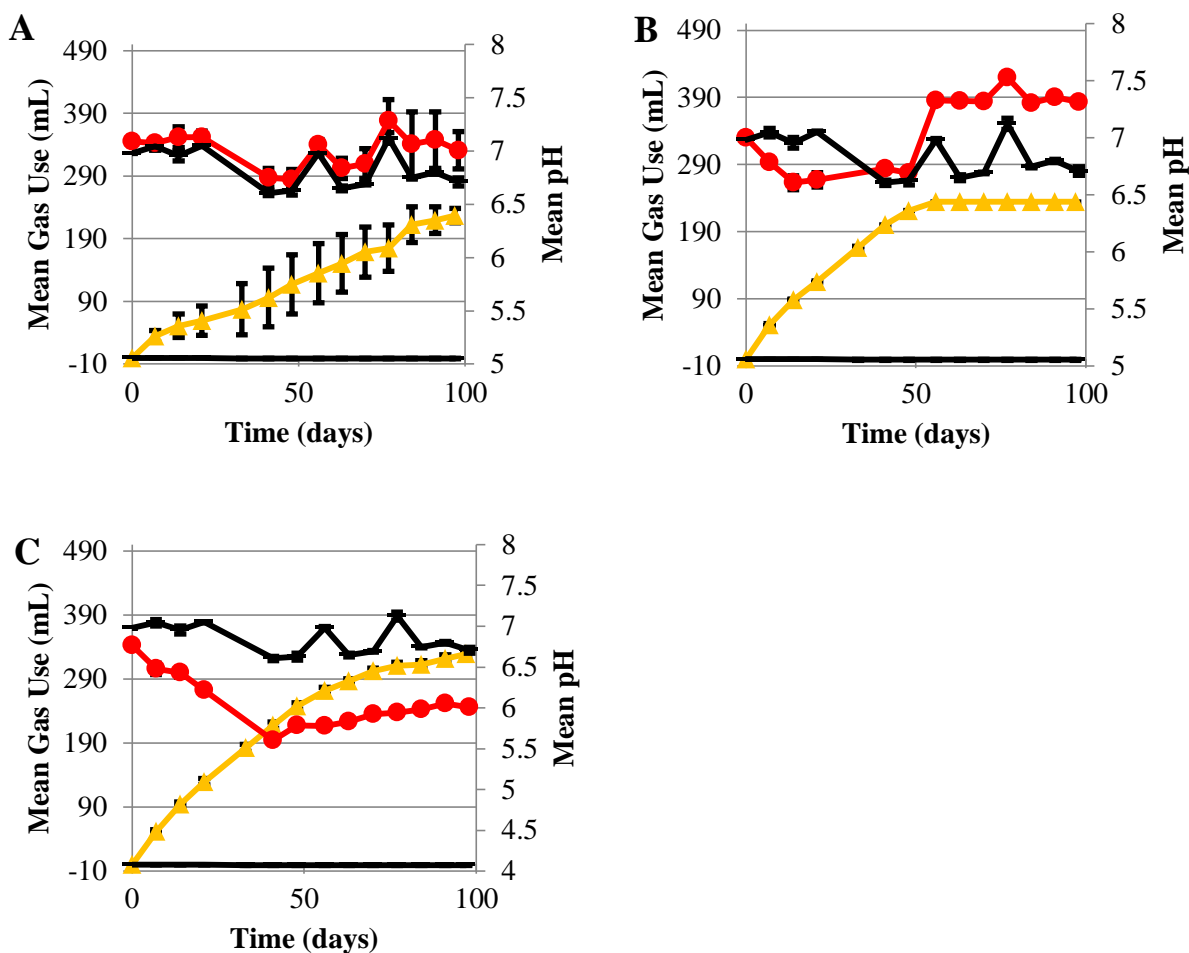


Figure 45. Mean pH (red) and mean gas consumption (yellow) as a function of time for experiment #6 primary enrichment. Crushed carbonate core was used instead of powdered CaCO_3 . The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

4.6.2 16S rRNA microbial community analysis: Primary enrichment

The main change in experiment #6 was switching from powdered CaCO_3 to crushed MgCa-carbonate core. This results in less available solid surface area and the crystal structure of the solid is dolomite instead of amorphous CaCO_3 and calcite.

Acetobacterium was again a major community component in low nutrient medium (37%, Figure 46 and Table 28) which is a higher proportion than in experiment #4. Even though the activity data was a little more methanogenic, the microbial community at the molecular levels had less methanogens. The only significant methanogen found was *Methanospirillum* (2.7%), but this could be due to using an earlier sampling date, which focuses on the acetogenic phase of growth. The sampling day used in experiment #6 (days 7 and 14) is earlier than those used in experiment #4 (days 7 and 35) to focus more on the acetogenic phase of growth. It could also just be due to semi-quantitative nature of DNA extraction and amplification.

The other major organisms were *Rhodobacter* (26.4%) and *Citrobacter* (13.3%), which are facultative anaerobic and aerobic respectively and typically ferment sugars but can also ferment organic acids (Yokoi *et al.* 1993, Fujita *et al.* 2000) and may be feeding on components produced by other microbial groups. Oxygen infiltration through microbial means or through the stopper could have supported these microbes. They did not consume acetic acid as this was not seen in the low nutrient medium. Other community members included the syntroph *Proteiniphilum* (5.1%) and sulfur cyclers *Sulfurospirillum* (6.1%), *Desulfovibrio* (1.4%) and *Arcobacter* (1.2%). Low nutrient medium was acetogen-dominant within the first two weeks of culture.

High nutrient medium from early in the enrichment (days 7 and 14) had a high proportion of *Acetobacterium* (64.2%) similar to experiment #4. Also, 12.0% of the reads were associated with the fermenter *Citrobacter*. *Proteiniphilum* was present again (2.8%) as well as the sulfide oxidizers *Sulfurospirillum* (4.5%) and *Arcobacter* (2.9%). 7.8% of reads were attributed to *Pseudomonas*, some strains of which are known to store carbon in the form of PHB but the genus is so diverse that conclusions cannot be made on identification alone. The high nutrient medium was also acetogen-dominant within the earlier growth phase. This was well before the transition to a methanogenic phase since less than 0.5% of reads represented methanogens.

At later time points (days 48 and 56) during acetic acid loss, potential carbon-storers *Pseudomonas* (34.5%), *Alishewanella* (7.7%), *Acinetobacter* (1.3%), *Rhizobium* (4.2%) and *Flavobacterium* were present (1.8%) (Hui *et al.* 2012, Lakshman *et al.* 2004, Majone *et al.* 2006). A different group of fermenters *Cellvibrio* (8.1%) and *Cyclobacteriaceae* (3.5%) were also detected (Khan 1977, Albuquerque *et al.* 2013). It appears that the shift to carbon storage phase was complete in the DNA data which is not in agreement with the activity data as no methanogens were detected though 660 μmol more of methane was produced after day 56.

For the first time BESA-containing high nutrient medium had *Acetobacterium* (27.8%) as a major community component. Less acetic acid was produced in experiment #6 than experiment #4 but BESA still produced the most acetic acid. There was a higher proportion of methanogenic sequencing reads in BESA medium than in the low and high nutrient media even though less than 10 μmol of methane had been produced by day 14. 7.0% of reads matched to *Methanofollis* along with 2.9% *Methanosarcina*, 1.0% *Methanosaeta*, 1.3% *Methanospirillum* and 9.7% *Methanobacterium*. Other community components include fermenter *Citrobacter* (7.6%) and *Pseudomonas* (5.9%). The syntrophs *Proteiniphilum* (10.6%) and *Sphingobacteriales* (2.6%)

were present in higher proportions than in the other media and the sulfur-cycling microbial community were still present with 2.9% *Sulfurospirillum* and 2.4% *Arcobacter*.

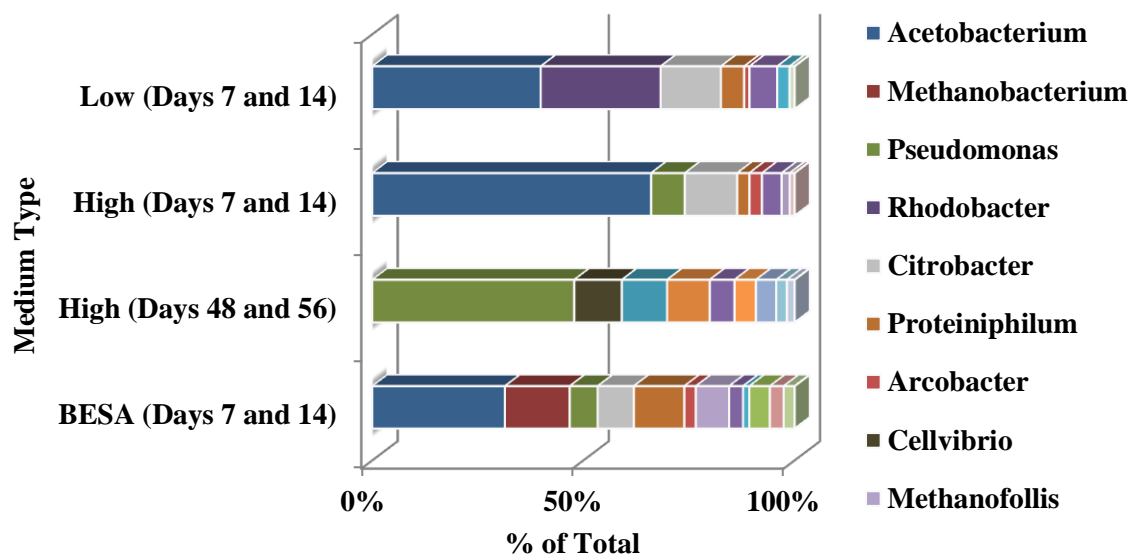


Figure 46. Experiment #6 primary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 28. Experiment #6 primary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	Low (Days 7 and 14)	High (Days 7 and 14)	High (Days 48 and 56)	BESA (Days 7 and 14)
<i>Bacteroides</i>	0.005	1.86	0	1.00
<i>Sphingobacteriales</i>	0.021	0.015	0	2.25
<i>Alishewanella</i>	0	0	7.72	0
<i>Proteiniphilum</i>	5.11	2.80	0	10.56
<i>Methanofollis</i>	0.64	0.092	0	6.95
<i>Methanospirillum</i>	2.71	0.17	0	1.31
<i>Acetobacterium</i>	37.06	64.22	0	27.76
<i>Citrobacter</i>	13.26	12.04	0.46	7.55
<i>Arcobacter</i>	1.15	2.91	0	2.41
<i>Sulfurospirillum</i>	6.10	4.51	0	2.91
<i>Cyanobacteria</i>	0.011	0	0	4.30
<i>Acinetobacter</i>	0	0	1.29	0
<i>Rhizobium</i>	0.63	0.031	4.22	0
<i>Cyclobacteriaceae</i>	0	0	3.46	0
<i>Desulfovibrio</i>	1.43	1.23	0	0.84
<i>Methanobacterium</i>	0.43	0.15	0	13.56
<i>Methanosaeta</i>	0.021	0	0	1.00
<i>Acinetobacter</i>	0	0	3.66	0
<i>Shigella</i>	0.011	0.015	7.26	0
<i>Methanosarcina</i>	0	0	0	2.88
<i>Flavobacterium</i>	0	0	1.81	0
<i>Pseudomonas</i>	0.68	7.77	34.46	5.85
<i>Rhodobacter</i>	26.37	0	0.66	0.026
<i>Shewanella</i>	1.09	0.031	0.02	0.079
<i>Cellvibrio</i>	0.005	0	8.11	0
<i>Enterobacteriaceae</i>	0.98	1.11	0	0.11

4.6.3 Crushed carbonate core mass recovery: Primary enrichment

Mass recovery for the primary enrichment was significantly lower than the sterile control for the BESA medium which had the highest acetic acid production ($p < 0.05$, Table 29). Low nutrient and high nutrient media were not significantly different from their controls ($p > 0.05$). This resulted in net gains in the low nutrient medium (+0.18 mmol) and a slight loss in the high nutrient medium (-0.18 mmol). There was a loss of 0.97 mmol of carbonate core in the BESA medium based on a CaCO_3 molar mass (Table 30).

Table 29. CaCO₃ mass recovered from experiment #6 primary enrichment. 1000 mg ± 10 mg of crushed Grosmont carbonate core provided by Suncor was originally added to bottles to create a concentration of 100 mM in suspension. Standard error and mean were calculated based on 3 replicates.

Sample ID	Mean CaCO ₃ Recovered (mg)	Mean Std Error (mg)	Mean % Recovered	Mean % Recovered Std Error	Ratio Active/Sterile
Sterile Low	979.40	5.44	97.94	0.54	N/A
Sterile High	984.83	4.03	98.48	0.40	N/A
Sterile BESA	929.03	1.79	92.90	0.18	N/A
Low	981.55	12.75	98.16	1.28	1.00
High	945.80	0.00	94.58	0.00	0.96
BESA	867.20	11.76	86.72	1.18	0.93

Table 30. Comparison of CaCO₃ mass change to peak acetic acid produced for experiment #6 primary enrichment after removal of abiotic loss as represented by loss in the sterile control. Precipitation occurred if the net change was positive and dissolution occurred if the net change was negative. The sterile control is calculated a mean of the sterile controls of the three medium types.

Sample ID	Mean Acetic Acid (mmol)	Mean CaCO ₃ Change (mg)	Mean CaCO ₃ change (mmol)	CaCO ₃ change – sterile loss (mmol)	Precipitation or Dissolution
Sterile	0.059	-35.58	-0.36	N/A	N/A
Low	0.42	-18.45	-0.18	+0.18	Precipitation
High	0.64	-54.2	-0.54	-0.18	Dissolution
BESA	1.79	-132.8	-1.33	-0.97	Dissolution

4.6.4 Secondary enrichment

In the secondary enrichment, acetic acid in the low nutrient medium (571 μmol) was the same as the primary enrichment and experiment #4 ($p>0.05$, Figure 47A). At 935 μmol , methane was also the same as the primary enrichment and experiment #4 ($p>0.05$). The high nutrient medium had a lower acetic acid peak than the primary enrichment (378 μmol , $p<0.05$), but it was the same as experiment #4 ($p>0.05$). Methane was similar to that of the primary enrichment at 1244 μmol ($p>0.05$) and less than experiment #4 ($p<0.05$, Figure 47B). BESA medium peaked at 1547 μmol acetic acid, the same as the primary enrichment and experiment #4 ($p>0.05$). Less than 100 μmol of methane was produced, less than the primary enrichment ($p<0.05$) and similar to experiment #4 ($p>0.05$, Figure 47C). Peak acetic acid and methane values are shown in Figure 47D. This experiment had lower overall levels of both acetogenesis and methanogenesis, but methanogenesis started earlier than experiment #4 it can be considered a little more methanogenic.

Though the pH rose from 6.7 to 7.2 it remained similar to sterile controls for low nutrient medium ($p<0.05$, Figure 48A). The pH rose from 6.7 to 7.5 in the high nutrient medium ($p<0.05$) and this was associated with acetic acid or CO_2 loss ($p<0.05$, Figure 48B). For BESA medium, the pH dropped from 6.5 to 5.6 and slowly rose back to 6.1 ($p<0.05$, Figure 48C). Gas use was the same as in experiment #4 for low nutrient medium ($p>0.05$), but less for the high nutrient and BESA medium ($p<0.05$, Figure 48). Gas use was more associated with methanogenesis in the low nutrient and high nutrient medium than in experiment #4. Gas use in BESA medium was associated with acetic acid production.

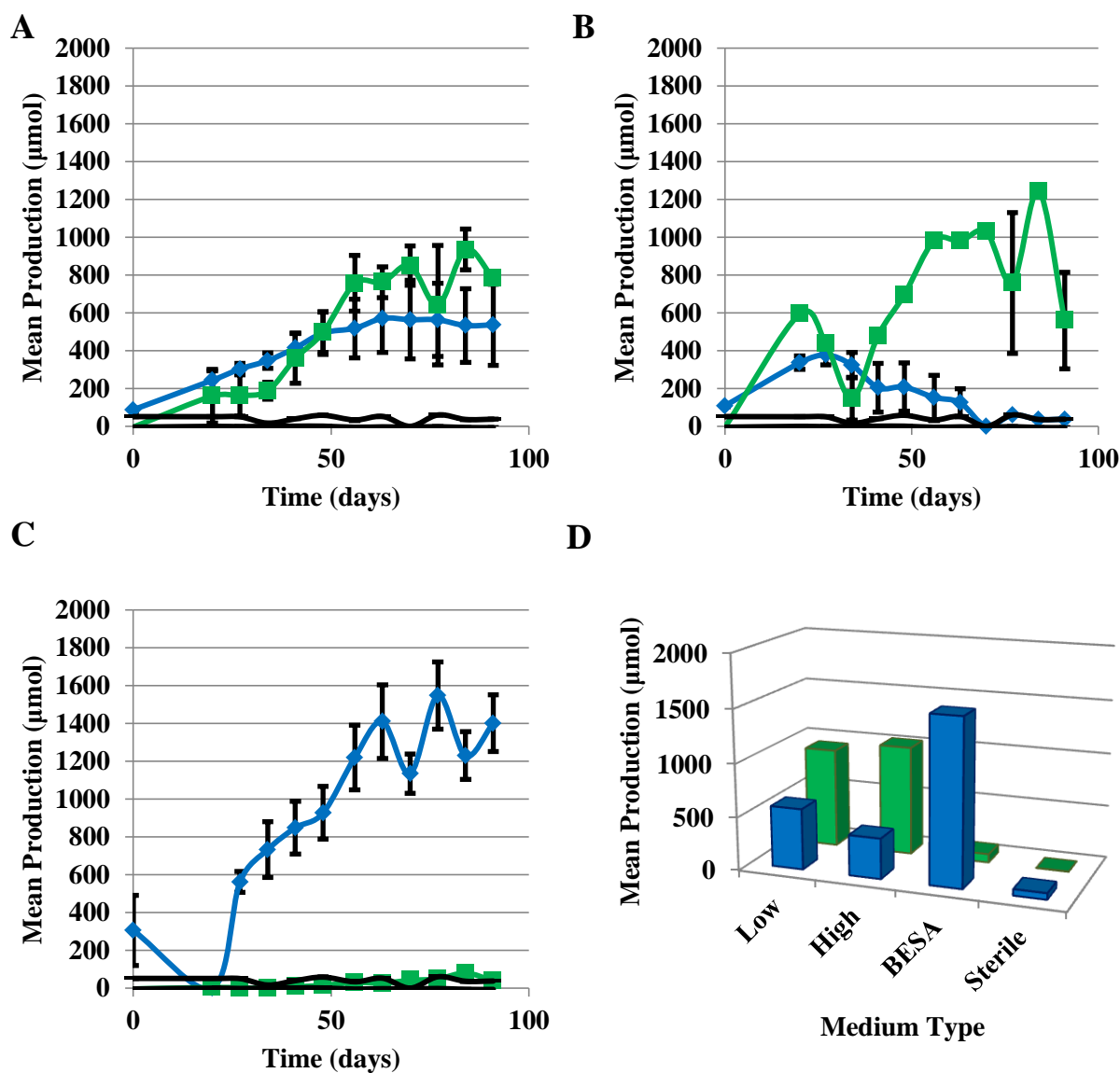


Figure 47. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #6 secondary enrichment. Crushed carbonate rock was used instead of powdered CaCO_3 . The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graphs show (C) high nutrient medium with 20 mM BES and (D) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

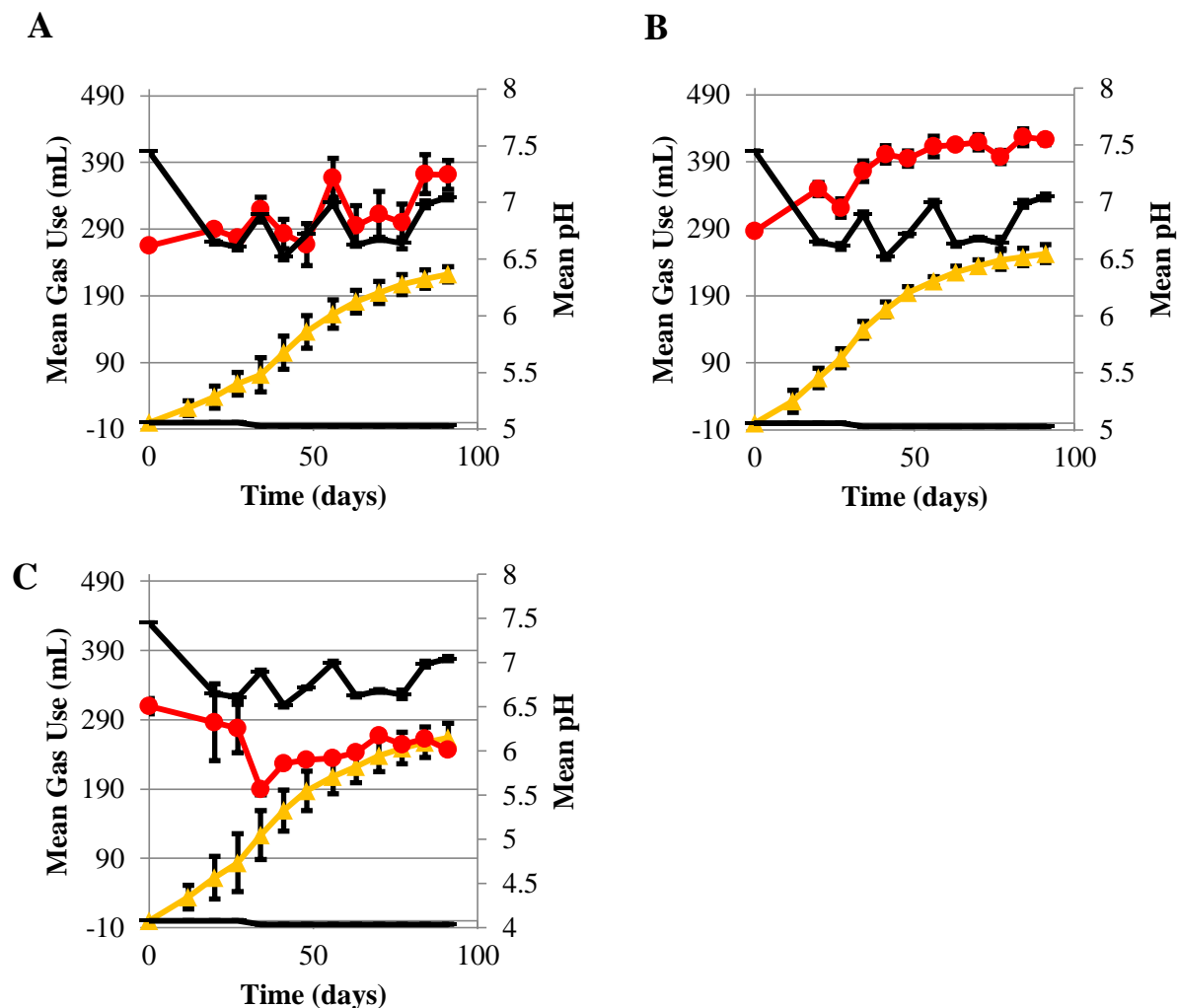


Figure 48. Mean pH (red) and mean gas consumption (yellow) as a function of time for experiment #6 secondary enrichment. Crushed carbonate core was used instead of powdered CaCO_3 . The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) high nutrient medium with 20 mM BESA. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

4.6.5 16S rRNA microbial community analysis: Secondary enrichment

Four metagenomic PCR amplification products were obtained from early in the low nutrient and BESA media and early and late in the high nutrient medium. Low nutrient medium in the secondary enrichment had substantially more *Acetobacterium* (51.2%) than the primary enrichment which replaced *Rhodobacter* (Figure 49 and Table 31). However the same acetic acid was produced in experiments #4 and #6 so this is probably due improved DNA extraction. Fermenter *Citrobacter* was about the same at 15.6% and there were more methanogenic reads than in the primary enrichment with 8.0% *Methanofollis*, 4.3% *Methanospirillum* and 1.0% *Methanobacterium*. The sulfur cycling (3.3% *Arcobacter*, 3.4% *Desulfovibrio*) and syntrophic (1.8% *Sphingobacteriales*, 2.3% *Proteiniphilum*) taxa were still present. The transition from acetogenic to methanogenic dominance appears to have occurred earlier than in the primary enrichment but it is difficult to tell if this was associated with earlier methane production due to the timing of the flushing the headspace. Using crushed core instead of CaCO₃ seems to take away some of the advantage seen in the low nutrient medium of the secondary enrichment. Having excess surface area available is important for the promotion of acetogenesis.

In the high nutrient medium early samples of the secondary enrichment had a much lower proportion of *Acetobacterium* (7.0%) than of the primary enrichment or of the enrichments in experiment #4, which is in agreement with the lower level of acetic acid produced. But the community was only starting to become methanogenic with 4.26% *Methanospirillum* and 2.52% *Methanobacterium*. This may have resulted in or was the result of greater growth by fermenters *Ketogulonigenium* (16.7%, Urbance *et al.* 2001) and *Rhodobacter* (39.5%) with syntrophs *Shingobacteriales* (1.0%) and *Proteiniphilum* (3.78%) that may have provided substrates for during their breakdown of trace oil components. While acetogenic and methanogenic groups

were present, this medium was dominated by fermentation. This is in accordance with the lower levels of acetic acid and methane seen. A small sulfur cycling community was present as well (2.74% *Sulfurospirillum*).

In the later part of the secondary enrichment in high nutrient medium, 18% of reads were associated with *Acetobacterium*. This is more than obtained from the sample on days 7 and 14 and supports acetic acid data that peaked at day 27. The acetogenic community appears to have grown less and slower, but persisted longer. The sulfur cycling community was more prominent than the earlier time point (8.41% *Arcobacter* and 1.34% *Sulfurospirillum*). The only (dubiously) potential PHB-forming microbe present is *Pseudomonas* (3.76%). Since the rest of the reads were from *Methanobacterium* (48.75%), the culture can be considered to have almost finished the transition to methanogen-dominance with early signs of transition to carbon storing. The cultures in the primary enrichment and experiment #4 had completely transitioned carbon storage by day 48 but the secondary enrichment appears to still be in acetogenic to methanogenic transition; microbial succession appears to have occurred at a slower pace.

Similar to previous experiments, BESA medium had high proportions of methanogens (62.9% *Methanobacterium* and 3.7% *Methanosaeta*) with lower levels of *Acetobacterium* (6.4%). Smaller fractions of sulfur cycling (1.80% *Arcobacter*), fermenting (9.50% *Rhodobacter*) and syntrophic (6.35% *Proteiniphilum* and 2.48% *Sphingobacteriales*) microbes were still present.

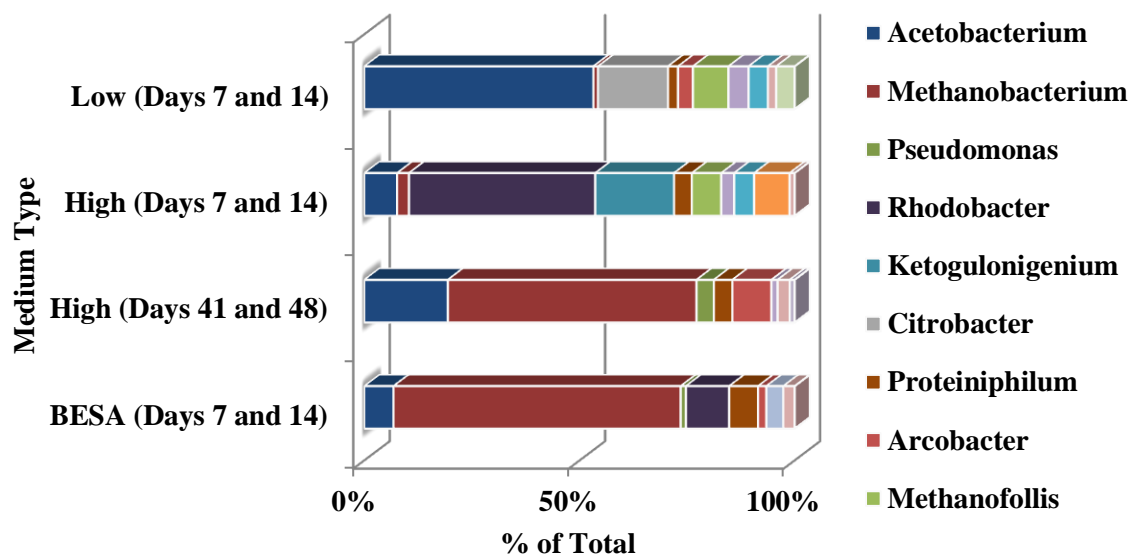


Figure 49. Experiment #6 secondary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 31. Experiment #6 secondary enrichment 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	Low (Days 7 and 14)	High (Days 7 and 14)	High (Days 41 and 48)	BESA (Days 7 and 14)
<i>Bacteroides</i>	0.95	0.043	1.07	0
<i>Sphingobacteriales</i>	1.84	1.04	2.61	2.48
<i>Proteiniphilum</i>	2.25	3.78	3.99	6.35
<i>Methanofollis</i>	7.95	6.25	0.74	0.90
<i>Desulfovibrio</i>	5.21	0.273	0.31	0.031
<i>Methanospirillum</i>	4.34	4.26	0.12	0.016
<i>Acetobacterium</i>	51.17	6.99	17.97	6.41
<i>Citrobacter</i>	15.64	0.17	0	0
<i>Arcobacter</i>	3.33	0.17	8.41	1.80
<i>Sulfurospirillum</i>	4.50	2.74	1.34	0.30
<i>Cyanobacteria</i>	0.40	0.35	1.73	0.88
<i>Acinetobacter</i>	0	0	0	0
<i>Ketogulonigenium</i>	0	16.72	0	0.078
<i>Rhizobium</i>	0	7.56	0	0.016
<i>Methanobacterium</i>	1.03	2.52	53.42	62.87
<i>Methanosaeta</i>	0.036	0	0.14	3.73
<i>Shigella</i>	0.018	0.043	0.019	0.016
<i>Pseudomonas</i>	0.054	0.13	3.76	1.16
<i>Rhodobacter</i>	0	39.52	0.019	9.50
<i>Shewanella</i>	0.09	0	0	0
<i>Enterobacteriaceae</i>	0.23	0	1.03	0.19

4.6.6 CaCO₃ mass recovery: Secondary enrichment

Mass recovery of the carbonate rock had net positive recoveries for low and high nutrient media which were not different from sterile controls ($p>0.05$). BESA however had a net loss of 0.58 mmol which was significantly less than its sterile control ($p<0.05$, Tables 32 and 33).

Table 32. CaCO₃ mass recovered from experiment #6 secondary enrichment. 1000 mg ± 10 mg of crushed Grosmont carbonate core provided by Suncor was originally added to bottles to create a concentration of 100 mM in suspension. Standard error and mean were calculated based on 3 replicates.

Sample ID	Mean CaCO ₃ Recovered (mg)	Mean Std Error (mg)	% Recovered	% Recovered Std Error	Ratio Active/Sterile
Sterile Low	907.77	16.02	90.78	1.60	N/A
Sterile High	962.67	4.68	96.27	0.468	N/A
Sterile BESA	962.40	5.89	96.24	0.589	N/A
Low	958.23	14.21	95.82	1.42	1.06
High	960.77	14.30	96.08	1.43	1.00
BESA	884.23	13.73	88.42	1.37	0.92

Table 33. Comparison of CaCO₃ mass change to peak acetic acid produced for experiment #6 secondary enrichment after removal of abiotic loss as represented by loss in the sterile control. Precipitation occurred if the net change was positive and dissolution occurred if the net change was negative. The sterile control is calculated a mean of the sterile controls of the three medium types.

Sample ID	Mean Acetic Acid (mmol)	Mean CaCO ₃ Change (mg)	Mean CaCO ₃ change (mmol)	Mean CaCO ₃ change – sterile loss (mmol)	Precipitation or Dissolution
Sterile	0.059	-55.75	-0.58	N/A	N/A
Low	0.57	-41.77	-0.42	+0.16	Precipitation
High	0.38	-39.23	-0.39	+0.19	Precipitation
BESA	1.55	-115.77	-1.16	-0.58	Dissolution

4.7. Experiment #7: Effect of varying the concentration of nutrients

4.7.1 Main enrichment

In an attempt to ascertain whether a difference in a specific nutrient could account for the differences between low and high nutrient medium types, an experiment was run testing variations in trace metals nutrients, phosphorus and salts. Based on the results of the ammonium measurement in experiment #4 nitrogen was not the defining nutrient to affect the balance between acetogenesis and methanogenesis. If it was not helping acetogenesis it was most likely harming acetogenic growth, so in these and all subsequent experiments, the added ammonium was doubled. Acetogenesis has key tungsten-dependent enzymes so it is unlikely that removing the tungstate-selenite from the medium aided acetogenesis. It was kept a normal (1X) levels. The volume of medium was decreased to 25 mL in a 50 mL serum bottle due to resource constraints which was initially considered to be a benign change. 100 mM of CaCO_3 was added without bicarbonate similar to experiment #4. Controls for low nutrient and high nutrient medium were included to account for changes in medium volume and increased nitrogen levels without other nutrient variations. Only a primary enrichment was performed and incubations ended after 60 days.

The high nutrient live control, which had increased nitrogen but was otherwise the same as previously used high nutrient medium, produced 345 μmol of acetic acid and 417 μmol of methane (Figure 50). This was half as much acetic acid and as third as much methane as in experiment #4 ($p < 0.05$). The low nutrient control, which had the increased nitrogen but a combination no trace metals and no phosphorus, produced 161 μmol of acetic acid and 24 μmol of methane. This is a fifth as much acetic acid and 42X less methane. Acetic acid levels declined

slowly after day 21 in all bottles, but the complete loss seen in previous high nutrient enrichments did not occur ($p < 0.05$). 76% of the acetic acid peak remained in the nutrient variant with the greatest decline, doubled trace metals, after 60 days. It is more likely that this is the result of doubling the nitrogen levels while changing the volume of the medium is responsible for the drop in actual production of acetic acid and methane. Increasing the nitrogen levels protects against the acetic acid loss.

Increasing the NaCl levels from created 394 μmol of acetic acid and 417 μmol of methane which was not significantly different from the high nutrient control ($p > 0.05$, Figure 50A). Decreasing MgCl_2 and CaCl_2 levels produced similar acetic acid (365 μmol) and methane (419 μmol) as the control ($p > 0.05$). Peak values for methane and acetic acid are seen in Figure 50B. Variations with no added phosphorus (406 μmol acetic acid and 420 μmol methane) and doubled phosphorus (371 μmol acetic acid and 392 μmol methane) were also tested (Figure 50E). Peak values for acetic acid and methane are shown in Figure 50F. Unfortunately, none of these variations were significantly different from the high nutrient control ($p > 0.05$).

Two more variations were performed, one with no trace metals added and one with double the usual trace metals added. When compared to the high nutrient control with normal “1X” amount of trace metals, no trace metals (250 μmol acetic acid, 430 μmol methane) and doubled trace metals (441 μmol acetic acid, 393 μmol methane) were not significantly different ($p > 0.05$, Figure 50C). However when compared directly to each other with analysis of variance, 2xTM produced more acetic acid than No TM with similar methane. Increasing trace metals appears to increase acetogenesis purely on a level of optimization since methanogenesis did not have a subsequent increase. A secondary enrichment should be done to test this effect is

synergistic with low nutrient medium effect. Peak values for acetic acid and methane are seen in Figure 50D.

The pH for most variations was similar to the pH of the controls and slowly rose from 6.7 to 7.5 ($p>0.05$, Figure 51). Little acetic acid loss occurred and related pH increases were observed so the pH rise is at least partially attributable to acetic acid loss and not CO₂ limitation. All bottles spiked above pH 8 on day 35 and then in the next week reduced back into the typical range. Since the sterile controls also increased, the spike is instrumental in origin.

The high nutrient control had less gas consumption than in experiment #4 (123 mL, $p<0.05$) and all nutrient variations were statistically the same ($p>0.05$, Figure 50). While there was no obvious association, gas consumption was more closely aligned with methane production than with acetic acid production. Acetic acid and methane production along with gas use were lower in all bottles in experiment #7 as compared to experiment #4 ($p<0.05$).

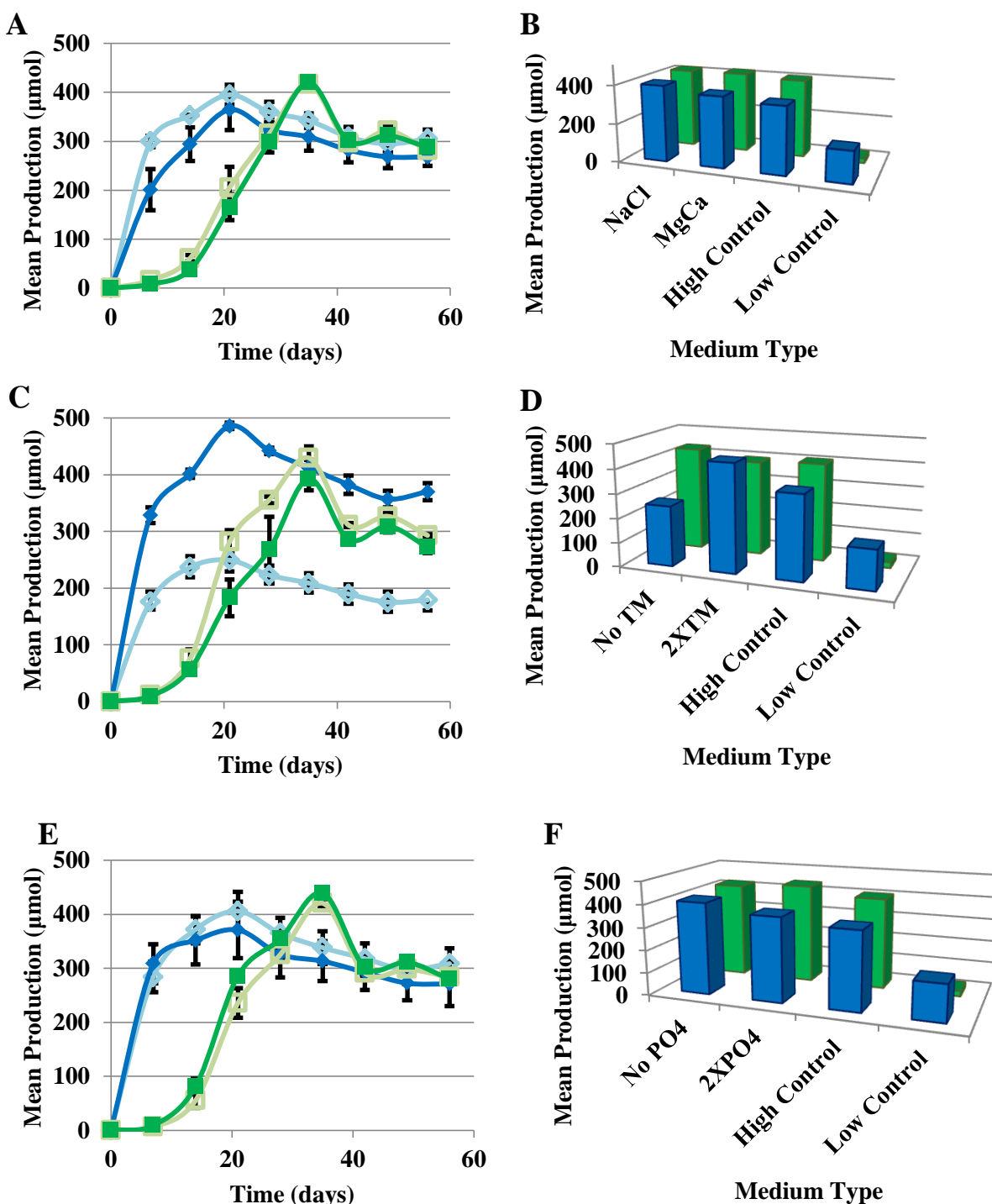


Figure 50. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #7. The levels of a single nutrient were varied one at a time. Ammonium was doubled in all variations. The top graphs show NaCl (hollow) and MgCa (solid) variations as (A) a function over time and (B) in peak values. The middle graphs show No TM (hollow) and 2xTM (solid) variations as (C) a function over time and (D) at peak values. The bottom graphs show No PO₄ (hollow) and 2xPO₄ (solid) as (E) a function over time and (F) at peak values. The error bars represent an average of 3 replicates. Controls (black) are a high nutrient and low nutrient version to account for changes due to increased nitrogen.

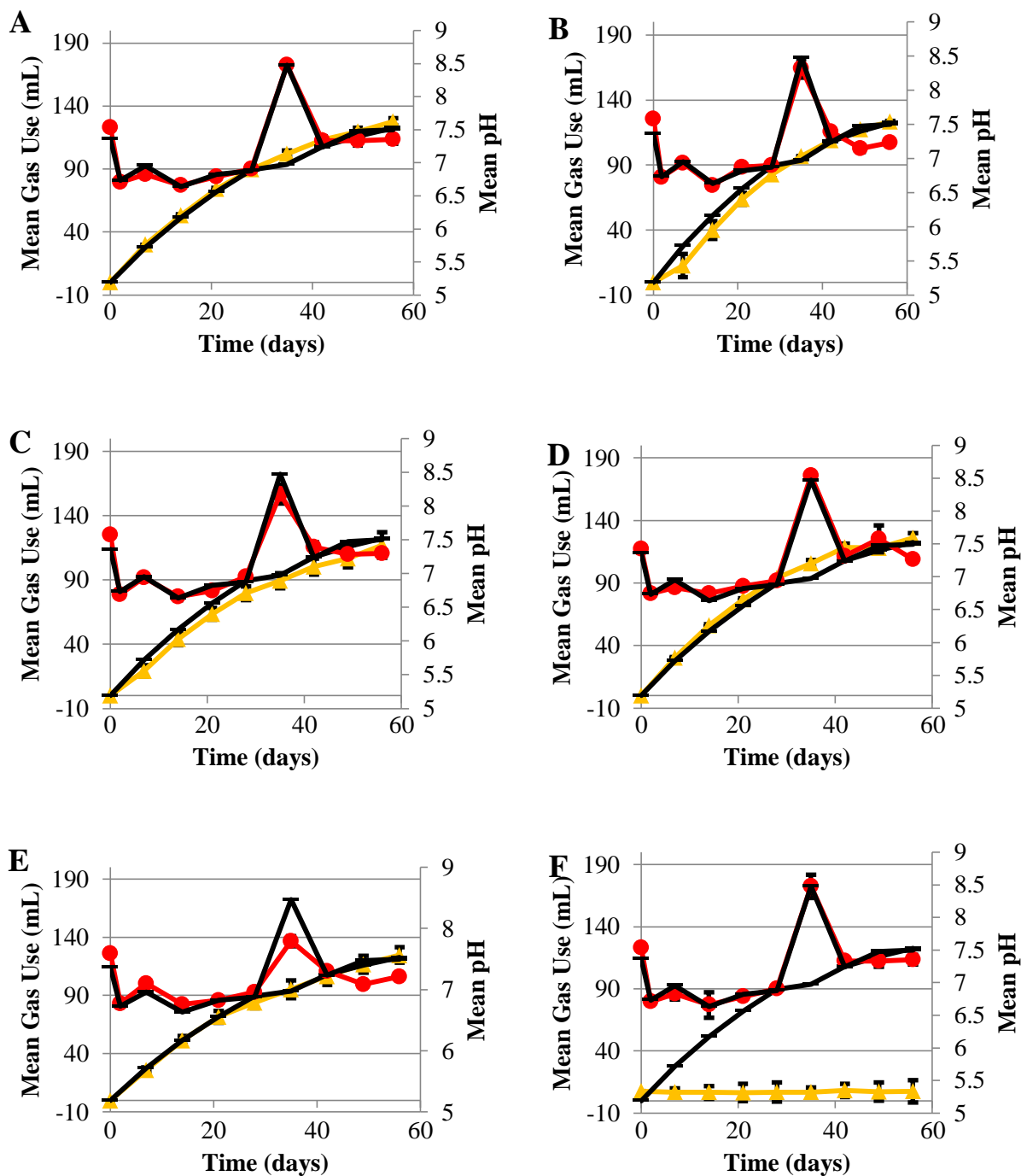


Figure 51. Mean pH (red) and mean gas consumption (yellow) as a function over time for experiment #7. The levels of a single nutrient were varied one at a time. Ammonium was doubled in all variations. The top graphs show (A) NaCl and (B) MgCa variations as a function over time. The middle graphs show (C) No TM and (D) 2xTM variations. The bottom graphs show (E) No PO_4 and (F) 2x PO_4 . The error bars represent an average of 3 replicates. Controls (black) are a high nutrient and low nutrient version to account for changes due to increased nitrogen.

4.7.2 16S rRNA microbial community analysis

All nutrient variations and controls were sequenced from samples taken on day 21 of the enrichment (Figure 52 and Table 34). Most of the nutrient variations were similar with *Acetobacterium* as the largest community component, ranging from 43.1 to 64.2%. The doubled trace metal variant “2xTM” also had 1.0% of *Acetohalobium*, a halophilic acetogen. Methanogenic communities were also present in all variants but in a wider range of proportions which is probably due to variation in DNA extraction and amplification. When the proportion of sequencing reads associated with *Methanobacterium*, *Methanocorpusculum*, *Methanobacteriales* and *Methanofollis* are combined, the methanogenic proportion ranged from 4.2 to 20.6%. NaCl and MgCa variants had no sulfur cycling community but they were major components in the phosphorus variants. *Arcobacter* was 29.9% of the No PO₄ reads and 10.1% of the 2xPO₄ reads. *Pseudomonas* was the remaining component with values ranging from 3.1 to 50.9%. The different community types were more mixed with less of a succession from one dominant type to another in experiment #7.

The low nutrient control produced less acetic acid and methane, but had a similar community composition as observed for the nutrient variants. The only differences were minor with the fermenter *Oscillibacter* (2.8%) and more syntrophs (3.5% *Proteiniphilum* and 1.5% *Sphingobacteriales*). On the other hand, even though the high nutrient control produced acetic acid and methane that were statistically the same as the nutrient variants, it had a drastically different microbial community dominated by 88.2% *Shigella*. This could be due to oxygen-exposure but it is more likely that contamination occurred during DNA processing.

Another sample was sent for 16S rRNA sequencing from the doubled trace metal variant from day 7, which is compared with the day 21 sample in Figure 53 and Table 35. Community succession is very clear. By day 7, smaller fractions of methanogens (2.1% *Methanoculleus*, 2.0% *Methanosaeta*) and syntrophs (8.0% *Sphingobacteriales*) were present but no acetogens are present though 328 μmol of acetic acid had already been produced. Most of the reads were comprised of the fermenter *Shigella* (22.2%) which may be a background precursor and a large assortment of microbes capable of forming carbon storage inclusion bodies (29.4% *Pseudomonas* with *Agrobacterium*, *Orchrobactrum*, *Corynebacterium*, *Shewanella*, *Achromobacter*, *Brevibacterium*, *Thauera* and *Stentrophomonas*; all with proportions ranging from 1-7%). This suggests that at this point the microbial community on the molecular level had not yet shifted to an actively growing acetogenic-methanogenic culture. The results seen in other sample at day 21 with mixed microbial communities may be limited to a certain time point during the incubation during transition from acetogen-dominant to methanogen-dominant.

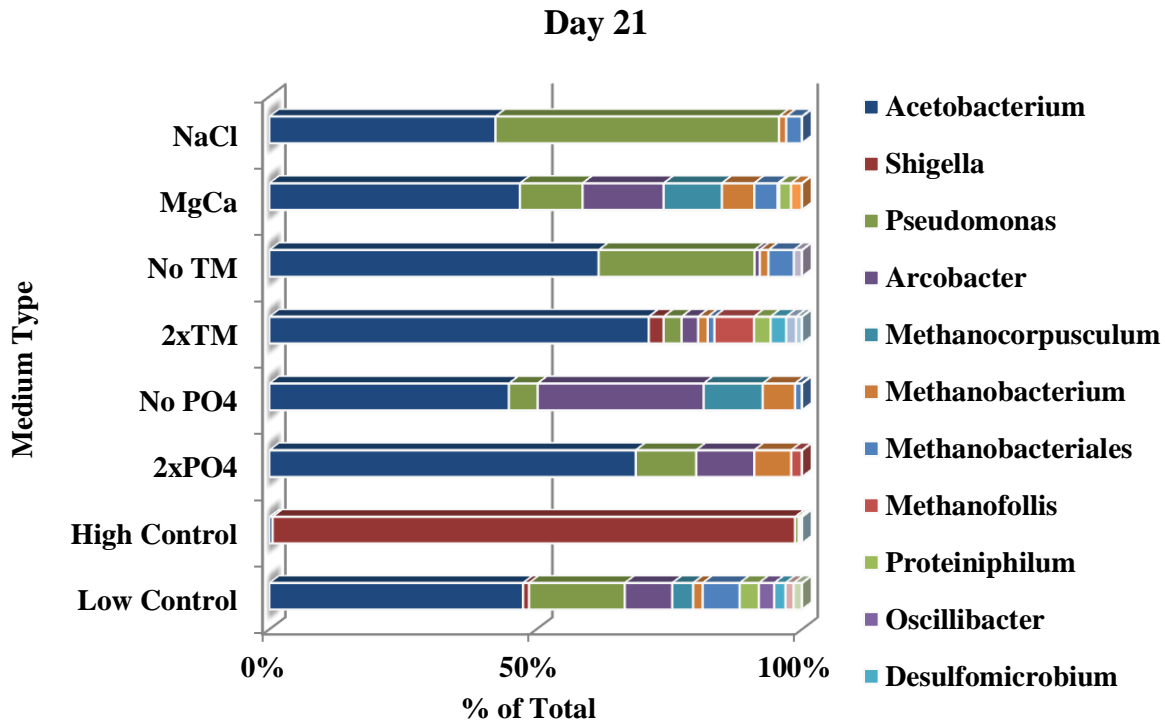


Figure 52. Experiment #7 16S rRNA pyrosequencing data at the genus level on day 21. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

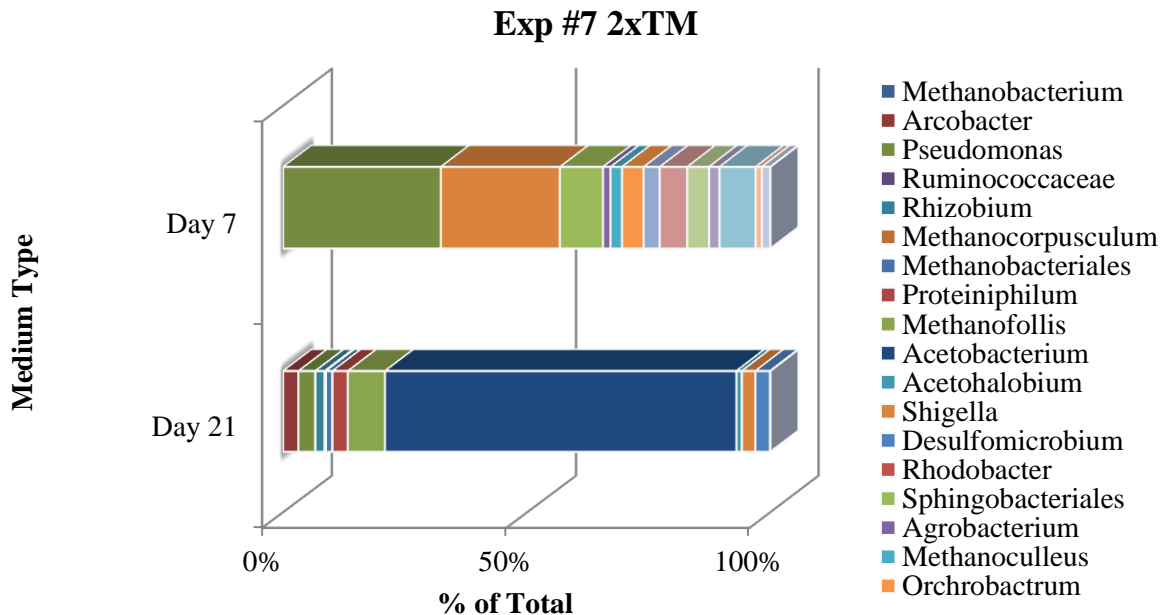


Figure 53. Experiment #7 16S rRNA pyrosequencing data at the genus level for the 2xTM variant on sampling days 7 and 21. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 34. Experiment #7 16S rRNA pyrosequencing data at the genus level on day 21. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	NaCl	MgCa	No TM	2xTM	No PO ₄	2xPO ₄	High Control	Low Control
<i>Methanobacterium</i>	1.31	5.79	1.55	1.64	5.87	6.42	0.10	1.75
<i>Arcobacter</i>	0.759	14.31	0.92	2.82	29.90	10.11	0.041	8.57
<i>Pseudomonas</i>	50.86	11.03	27.54	3.06	5.15	10.40	0.59	17.30
<i>Ruminococcaceae</i>	0	0.08	1.44	0.016	0	0	0	0
<i>Rhizobium</i>	0.037	0.36	0.27	1.68	0.09	0.66	0.061	0
<i>Methanocorpusculum</i>	0.089	10.34	0.13	0.27	10.74	0.15	0.061	3.80
<i>Methanobacteriales</i>	2.84	4.19	4.51	1.15	1.26	0.76	0.081	6.76
<i>Proteiniphilum</i>	0.23	2.11	0.16	2.83	0.37	0.78	0.041	3.521
<i>Methanofollis</i>	0.372	0.29	0.92	6.77	0.50	1.85	0.16	0
<i>Acetobacterium</i>	40.40	44.22	57.86	64.12	43.07	62.90	0.55	45.84
<i>Acetohalobium</i>	0	0	0	1.02	0	0	0	0
<i>Shigella</i>	0	0.018	0	2.51	0	0	88.20	1.09
<i>Desulfomicrobium</i>	0.052	0.65	0.088	2.68	0.21	0.36	0.02	2.06
<i>Rhodobacter</i>	0.022	1.93	0.89	0.051	0.008	0.054	0	0
<i>Bacteroides</i>	0	0	0	0	0	0	0	1.50
<i>Sphingobacteriales</i>	0	0	0	0	0	0	0	1.50
<i>Oscillibacter</i>	0	0	0	0	0	0	0	2.81

Table 35. Experiment #7 16S rRNA pyrosequencing data at the genus level for the 2xTM variant on sampling days 7 and 21. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	Day 7	Day 21
<i>Methanobacterium</i>	0	1.64
<i>Arcobacter</i>	0	2.82
<i>Pseudomonas</i>	29.42	3.06
<i>Ruminococcaceae</i>	0	0.016
<i>Rhizobium</i>	0	1.68
<i>Methanocorpusculum</i>	0	0.27
<i>Methanobacteriales</i>	0	1.15
<i>Proteiniphilum</i>	0	2.83
<i>Methanofollis</i>	0	6.77
<i>Acetobacterium</i>	0	64.12
<i>Acetohalobium</i>	0	1.02
<i>Shigella</i>	22.21	2.51
<i>Desulfomicrobium</i>	0	2.69
<i>Rhodobacter</i>	0	0.051
<i>Bacteroides</i>	0	0
<i>Sphingobacteriales</i>	8.02	0
<i>Oscillibacter</i>	0	0
<i>Agrobacterium</i>	1.41	0
<i>Methanoculleus</i>	2.13	0
<i>Orchrobactrum</i>	4.05	0
<i>Corynebacterium</i>	2.99	0
<i>Shewanella</i>	5.14	0
<i>Achromobacter</i>	4.11	0
<i>Methanosaeta</i>	1.98	0
<i>Brevibacterium</i>	6.81	0
<i>Thauera</i>	1.18	0
<i>Stenotrophomonas</i>	1.58	0

4.7.3 CaCO₃ mass recovery

Mass recovery was performed for the CaCO₃. Sterile loss was simulated by scaling down a sterile control from experiment #4. Due to the decreased volume of medium, the amount of CaCO₃ added was scaled down from 1000 mg to 250 mg to maintain a concentration of 100 mM (Table 36). All bottles had some mass loss but the greatest loss was with the live controls including the low nutrient live control, which produced the least amount of acetic acid. All bottles had a net loss in the amount of mass recovered but this was not significantly different than the sterile control ($p>0.05$, Table 37).

In some of the bottles, an unknown dark gray-black precipitate formed that did not appear to follow a specific pattern related to nutrient type. This can be pictured in Figure 54, which compares images of two identical replicates from the same nutrient variation. One which had the dark precipitate and the other did not and showed only the typical suspended white CaCO₃ powder.

Table 36. CaCO₃ mass recovered from experiment #7. 250 mg ± 10 mg of ACS grade powdered CaCO₃ was originally added to bottles to create a concentration of 100 mM in suspension. Standard error and mean were calculated based on 3 replicates.

Sample ID	Mean CaCO ₃ Recovered (mg)	Mean Std Error (mg)	Mean % Recovered	Mean % Recovered Std Error	Ratio Active/Sterile
NaCl	224.17	11.42	89.67	4.57	0.93
MgCa	220.53	7.22	88.21	2.89	0.92
No TM	228.47	1.94	91.39	0.78	0.95
2XTM	227.33	2.76	90.93	1.10	0.94
No PO ₄	228.20	5.81	91.28	2.33	0.95
2XPO ₄	233.87	9.65	93.55	3.86	0.97
High Control	203.77	13.86	81.51	5.54	0.85
Low Control	207.97	2.40	83.19	0.96	0.92
Sterile Low	226.94	4.00	90.78	1.60	N/A
Sterile High	240.67	1.17	96.27	0.47	N/A

Table 37. Comparison of CaCO₃ mass change to peak acetic acid produced for experiment #7 after removal of abiotic loss as represented by loss in the sterile control. Precipitation occurred if the net change was positive and dissolution occurred if the net change was negative. The sterile control is calculated as the mean of sample loss found in the sterile control of experiment #6 scaled to 250 mg.

Sample ID	Mean Acetic Acid (mmol)	Mean CaCO ₃ Change (mg)	Mean CaCO ₃ change (mmol)	Mean CaCO ₃ change – sterile loss (mmol)	Precipitation or Dissolution
NaCl	0.39	-25.83	-0.26	-0.167	Dissolution
MgCa	0.36	-29.47	-0.29	-0.197	Dissolution
No TM	0.25	-21.53	-0.22	-0.127	Dissolution
2XTM	0.44	-22.67	-0.23	-0.137	Dissolution
No PO ₄	0.41	-21.8	-0.22	-0.127	Dissolution
2XPO ₄	0.37	-16.13	-0.16	-0.067	Dissolution
High Control	0.34	-46.23	-0.46	-0.367	Dissolution
Low Control	0.16	-42.03	-0.42	-0.327	Dissolution
Sterile	0.059	-9.33	-0.093	N/A	N/A

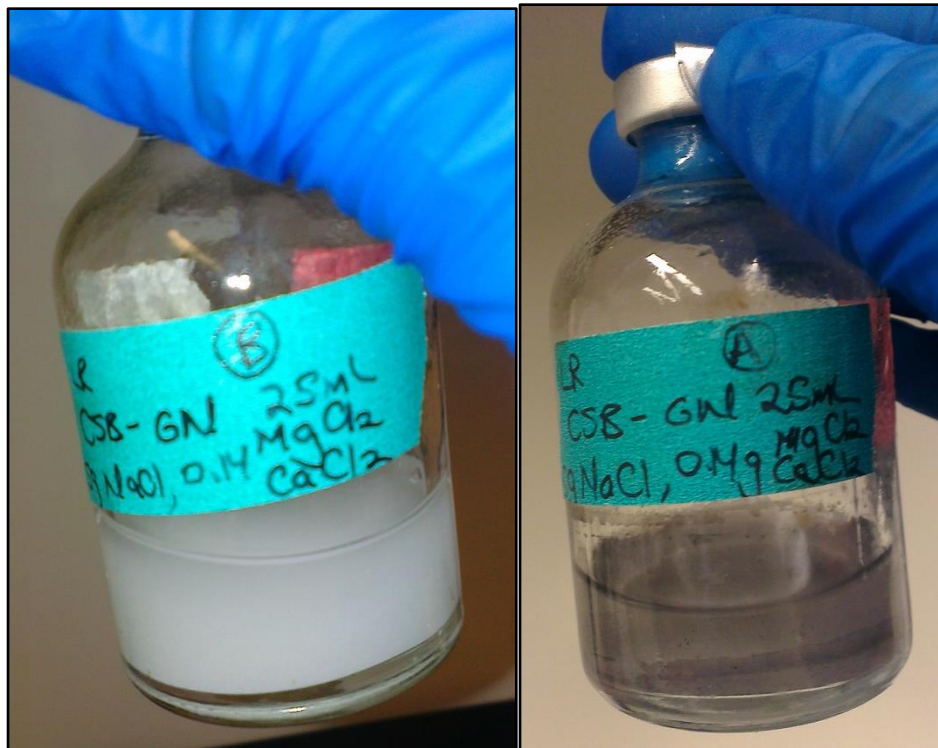


Figure 54. Two identical replicate bottles from experiment #7 both from the MgCa nutrient variation. Left: A replicate with no precipitate only the added white CaCO_3 suspended in the medium. Right: A replicate with an unidentified gray-black precipitate.

4.8 Experiment #8: 20 mM CaCO₃

4.8.1 Primary and secondary enrichments

The concentration of CaCO₃ within the bottles was adjusted from 100 mM to 20 mM to decrease the proportion of CaCO₃ present to acetic acid produced. A primary and secondary low nutrient enrichment was performed in an attempt to replicate the higher acetic acid in low nutrients as seen in experiment #3 through #6. No trace metal and doubled trace metal nutrient variations were also conducted. The primary and secondary enrichments did not produce enough acetic acid or methane to be considered significantly different from the sterile control ($p>0.05$). They had the same acetic acid (146 μmol) and methane (30 μmol , Figure 55A). This is similar to the low nutrient live control in experiment #7 ($p>0.05$) and still much less than experiment #4 through #6 ($p<0.05$). The restriction due to the decrease in medium volume makes observe grow in low nutrient medium untenable.

Acetic acid for incubations doubled trace metals (423 μmol) appeared to have more acetic acid than no trace metals (301 μmol) but the difference is not statistically significant. These variants produced the same acetic acid and methane as the same variants in experiment #7 so reducing the concentration of CaCO₃ at this level did not decrease microbial activity ($p>0.05$, Figure 55B). Methane was also the same, both at 157 μmol ($p>0.05$). Peak values for acetic acid and methane are shown in Figure 55C.

The pH of the active bottles was similar to the sterile control except for the trace metal variations ($p>0.05$, Figure 56). The doubled trace metals variant decreased from 7.2 to 6.2 by day 19 and then increased back up to 7.4 by the end of the experiment ($p<0.05$, Figure 56C). The no trace metals variations which followed a similar trend, but it only dropped to 6.6 ($p<0.05$, Figure

56D). These drops were not associated with acetic acid production as they occurred on day 19 more than a week before peak acetic production was reached on day 27. Gas use for the low nutrient enrichments was the same as for the low nutrient control in experiment #7 (less than 30 mL, $p>0.05$). Gas consumption for the trace metal variations associated with acetic acid production was less than in similar variants in experiment #7 though acetic acid production was the same so the drop is not associated with reduced methanogenesis.

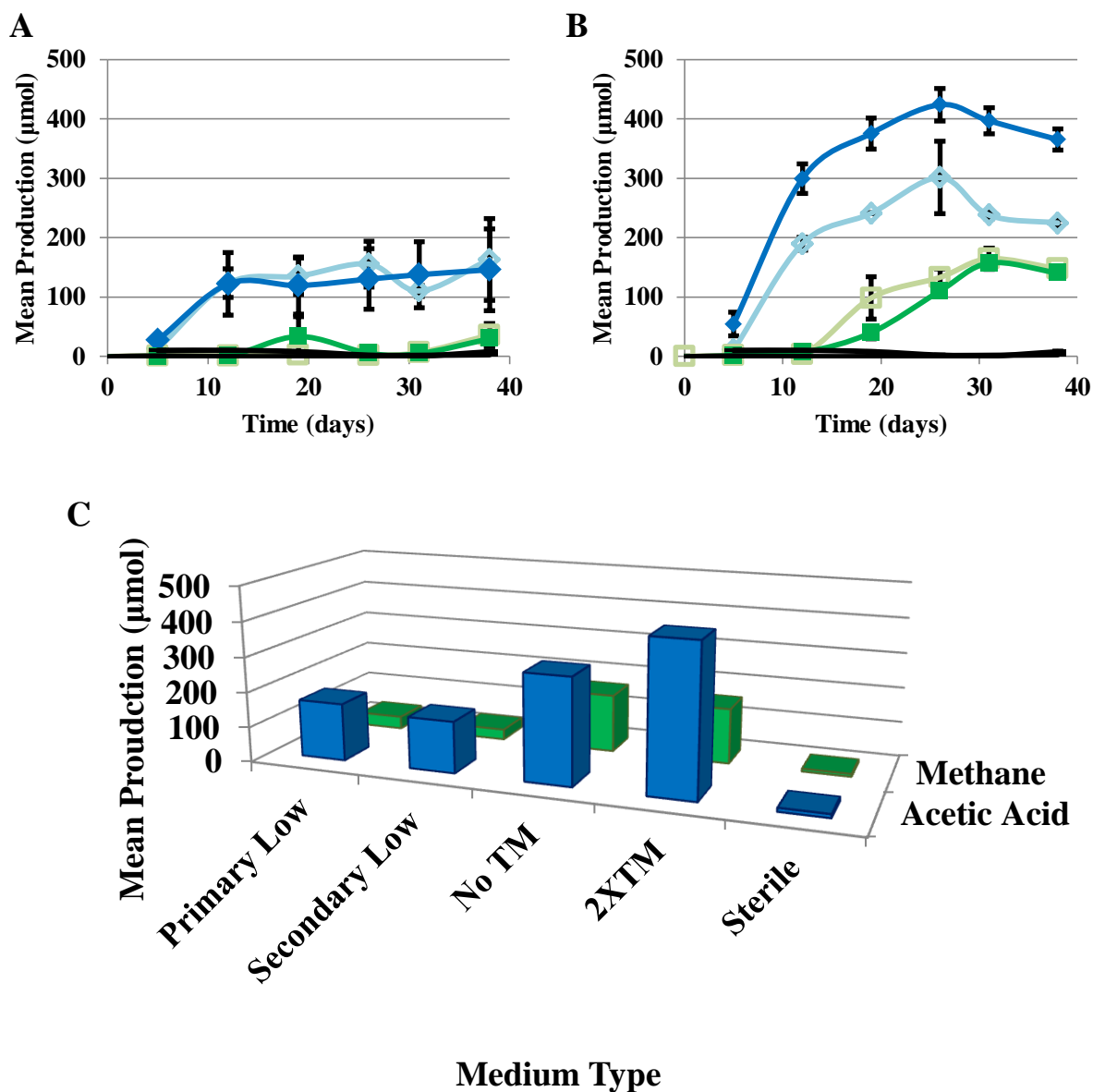


Figure 55. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #8. Concentration of CaCO_3 was decreased from 100 mM to 20 mM. The top graphs show (A) primary (hollow) and secondary (solid) enrichments with low nutrient medium and (B) No TM (hollow) and 2xTM (solid) variations. The bottom graphs show (C) peak values reached for mean acetic acid and methane in all three medium types. The error bars represent an average of 3 replicates. Controls (black) were sterilized by autoclaving.

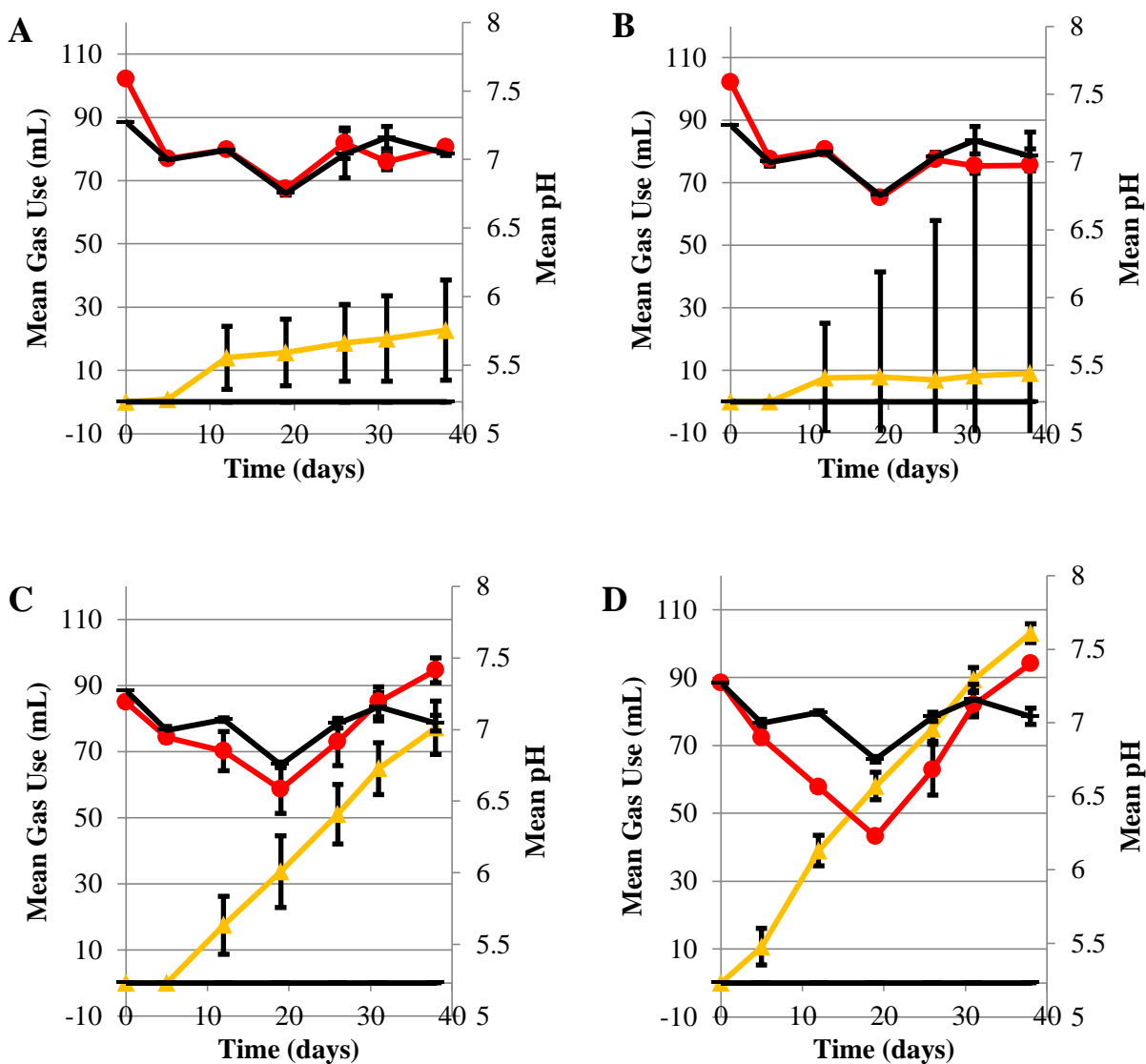


Figure 56. Mean pH (red) and mean gas consumption (yellow) as a function over time for experiment #8. Concentration of CaCO_3 was decreased from 100 mM to 20 mM. The top graphs show (A) primary (hollow) and (B) and secondary enrichments with low nutrient medium. The bottom graph shows and (C) No TM and (D) 2xTM variations. The error bars represent an average of 3 replicates.

4.8.2 16S rRNA microbial community analysis

CaCO₃ concentration was decreased from 100 to 20 mM. A PCR product was obtained and sequenced for the two enrichments in low nutrient medium with double trace metals (Figure 57 and Table 38). There were no acetogens or methanogens found on day 12 of the culture with doubled trace metals. The community was evenly split between microbes identified as potentially being able to store carbon as polyhydroxybutyrate (23.1% *Pseudomonas*, 9.6% *Alishewanella* and 7.15% *Acinetobacter*) and microbes capable of aerobic respiration (14.3% *Porphyrobacter*, 11.5% *Shigella*, 12.7% *Fontibacter*, 1.5% *Rhodobacter* and 1.8% *Cellvibrio*) (Hiraishi *et al.* 2002). Most of these organisms haven't been detected in previous experiments probably because they are mostly aerobic (some are facultative). This suggests oxygen ingress either by leakage or by photosynthesis, which would inhibit acetogenic and methanogenic growth.

The primary enrichment of the low nutrient medium looked similar to the doubled trace metals variant with an approximately even split between carbon storing microbes (13.6% *Alishewanella*, 11.9% *Acinetobacter* and 9.1% *Comamonadaceae* and 11.3% *Pseudomonas*) and aerobes (2.4% *Tatumella*, 15.4% *Porphyrobacter*, 6.3% *Shigella* and 21.2% *Fontibacter*). Again the lack of methanogens and acetogens could be due to oxygen ingress and this is evidenced by the aerobic nature of the dominant groups.

The secondary enrichment of the low nutrient medium is much more anaerobic and similar to the microbial communities of previous experiments. The dominant organisms are *Methanohalophilus* (66.1%) and *Methanothermococcus* (3.8%). This microbial community is methanogenic and came from the same sampling date at which acetic acid production stopped.

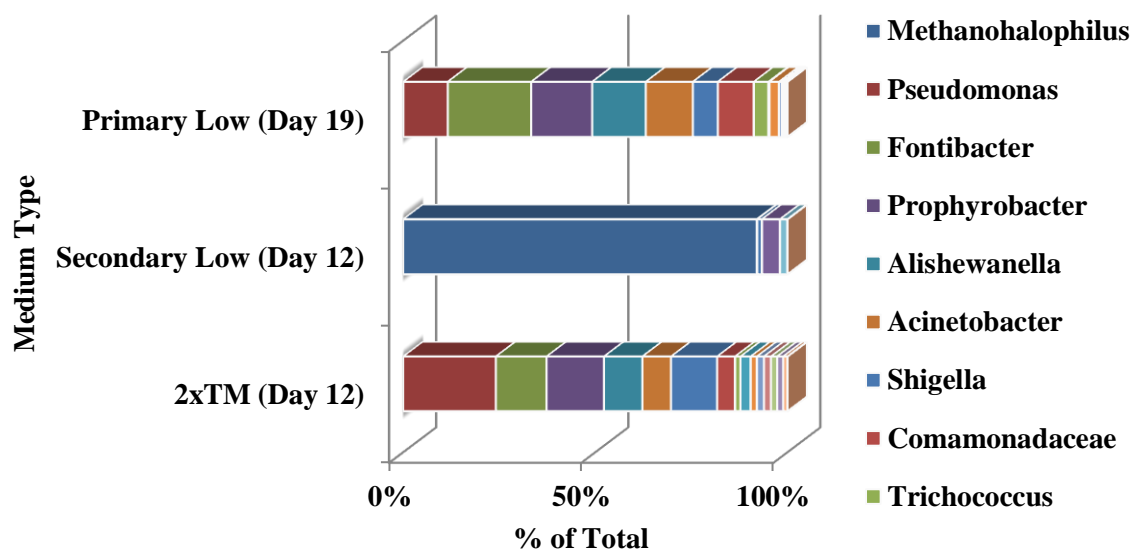


Figure 57. Experiment #8 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 38. Experiment #8 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	2xTM (Day 12)	Primary Low (Day 19)	Secondary Low (Day 12)
<i>Alishewanella</i>	9.55	13.58	0
<i>Methanothermococcus</i>	0	0	3.37
<i>Acetobacterium</i>	0	0	0.036
<i>Methanohalophilus</i>	0	0.015	66.09
<i>Trichococcus</i>	1.36	3.71	0
<i>Tatumella</i>	1.55	2.41	0
<i>Acinetobacter</i>	7.15	11.93	0.036
<i>Rhizobium</i>	2.54	0.26	0
<i>Comamonadaceae</i>	4.46	9.11	0
<i>Porphyrobacter</i>	14.32	15.43	0
<i>Shigella</i>	11.49	6.31	0.88
<i>Acidovorax</i>	1.06	0.18	0.018
<i>Fontibacter</i>	12.65	21.15	0
<i>Desulfohalobiaceae</i>	0	0	1.37
<i>Microcella</i>	1.75	0.72	0
<i>Pseudomonas</i>	23.11	11.28	0.036
<i>Paracoccus</i>	1.50	0.55	0
<i>Rhodobacter</i>	1.53	0.55	0
<i>Cellvibrio</i>	1.75	0.14	0

4.8.3 CaCO₃ mass recovery

The CaCO₃ mass was recovered from all serum bottles in experiment #8. No active bottles had significantly different mass recoveries from the sterile control and all had net positive recovery values ($p > 0.05$, Tables 39 and 40).

Table 39. CaCO₃ mass recovered from experiment #8. 50 mg ± 1 mg of ACS grade powdered CaCO₃ was originally added to bottles to create a concentration of 20 mM in suspension. Standard error and mean were calculated based on 3 replicates.

Sample ID	Mean CaCO ₃ Recovered (mg)	Mean Std Error (mg)	Mean % Recovered	Mean % Recovered Std Error	Ratio Active/Sterile
Primary Low	36.90	7.62	73.80	15.24	0.68
Secondary Low	63.53	8.66	127.07	17.32	1.17
No TM	62.10	10.57	124.20	21.13	1.14
2XTM	54.27	9.28	108.53	18.56	N/A
Sterile	48.93	14.26	97.87	28.52	0.90

Table 40. Comparison of CaCO₃ mass change to peak acetic acid produced for experiment #8 after removal of abiotic loss as represented by loss in the sterile control. Precipitation occurred if the net change was positive and dissolution occurred if the net change was negative.

Sample ID	Mean Acetic Acid (mmol)	Mean CaCO ₃ Change (mg)	Mean CaCO ₃ change (mmol)	Mean CaCO ₃ change – sterile loss (mmol)	Precipitation or Dissolution
Primary Low	0.163201	-13.1	-0.131	-0.024	Dissolution
Secondary Low	0.146002	+13.53	+0.1353	+0.146	Precipitation
No TM	0.301116	+12.1	+0.121	+0.1317	Precipitation
2XTM	0.010299	+4.27	+0.0427	+0.0534	Precipitation
Sterile	0.423782	-1.07	-0.0107	N/A	N/A

4.9 Experiment #9: Using solid MgCa carbonate core

4.9.1 Main enrichment

Solid carbonate rock core plugs taken from the Grosmont were incubated as the source of carbonate solids in 125 mL of medium in 250 mL serum bottles for 30 days in low and high nutrient media with a sterile and unamended control. Acetic acid production started on day 15 (Figure 58) in the unamended control, which suggests contamination. It is not used as a comparative control to the microbially active bottles. No secondary enrichment was performed.

526 μmol of acetic acid and 185 μmol of methane were produced in the low nutrient medium. High variation means that the acetic acid production was not different from the sterile control ($p>0.05$), but the methane production was greater ($p<0.05$, Figure 58A). For high nutrient medium, 1441 μmol of acetic acid and 848 μmol of methane were produced (Figure 58B). Visible turbidity formed within the bottle (Figure 60). High standard error in both the high and low nutrient medium renders them not statistically different ($p>0.05$) in acetic acid production though methane production was higher in the high nutrient medium ($p<0.05$). 1441 μmol of acetic acid was the most produced in a high nutrient medium that didn't contain BESA. Peak acetic acid and methane values are shown in Figure 58C.

pH for low nutrient medium was the same as the sterile control ($p>0.05$, Figure 58A). The pH dropped in high nutrient medium from 7.1 to 6.4 by day 8 and this was associated with acetic acid production. The pH then rose to 7.3 at the end of the experiment ($p<0.05$, Figure 58B). Gas use in high nutrient medium (296 mL, $p>0.05$, Figure 58B) was the same as gas consumption in the experiment #4 high nutrient medium. The low nutrient medium consumed less gas than in experiment #4 (66.5 mL, $p<0.05$, Figure 58A). Some of the carbonate rock

separated from the main core during incubation and was collected afterwards as “free mass”, pictured in Figure 60 (top left). Also visible within this free mass and in the cores themselves is more of the dark precipitate (Figure 60 bottom). This precipitate could have contributed to the high recovered masses seen in microbially active bottles.

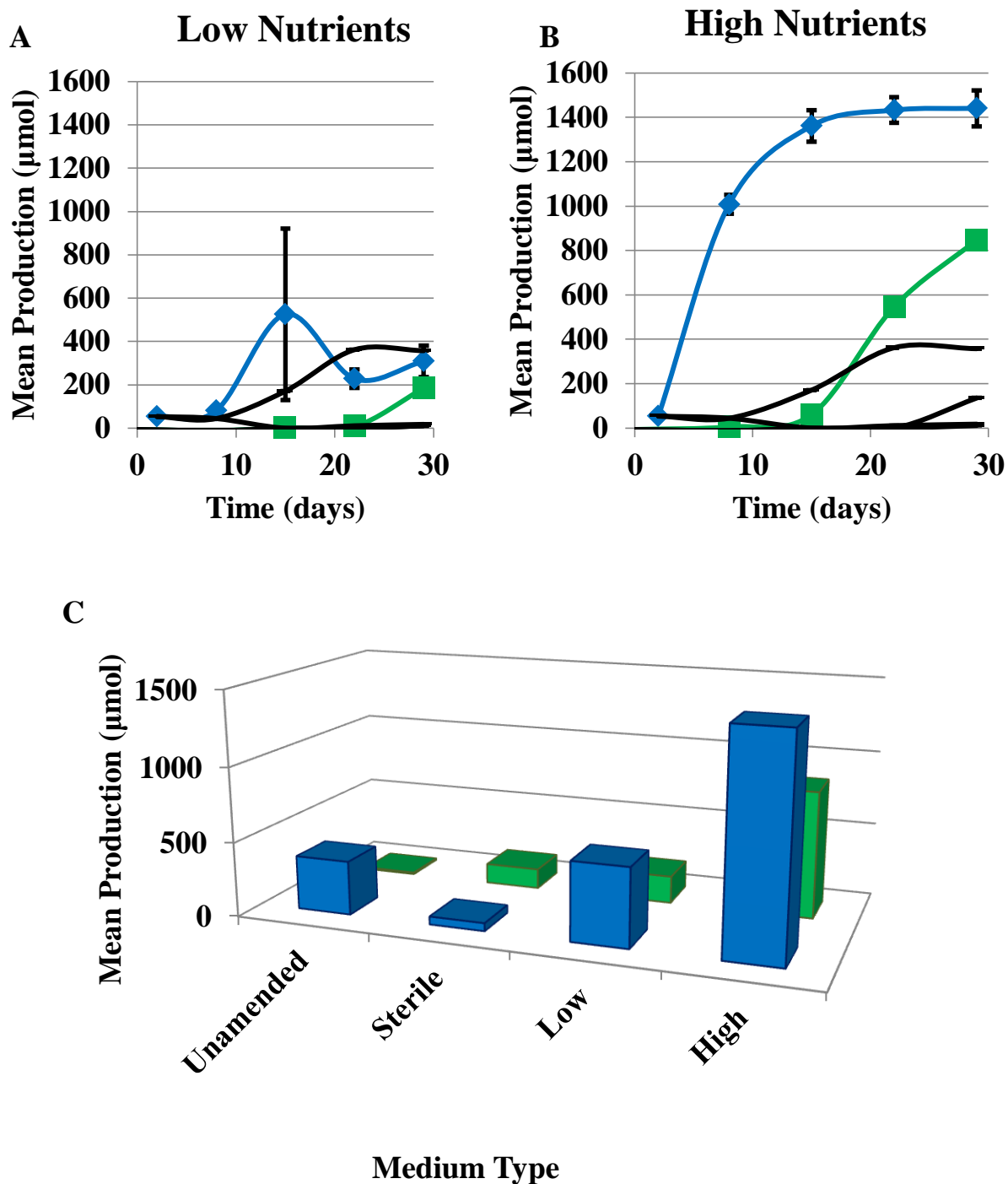


Figure 58. Mean acetic acid (blue) and mean methane (green) production as a function over time for experiment #9. Solid carbonate cores were used instead of crushed carbonate core. The top graphs show (A) low nutrient medium and (B) high nutrient medium. The bottom graph shows (C) peak values reached for mean acetic acid and methane in all medium types. The error bars represent an average of 3 replicates for low and high nutrient medium and one replicate for the unamended and sterile controls (black).

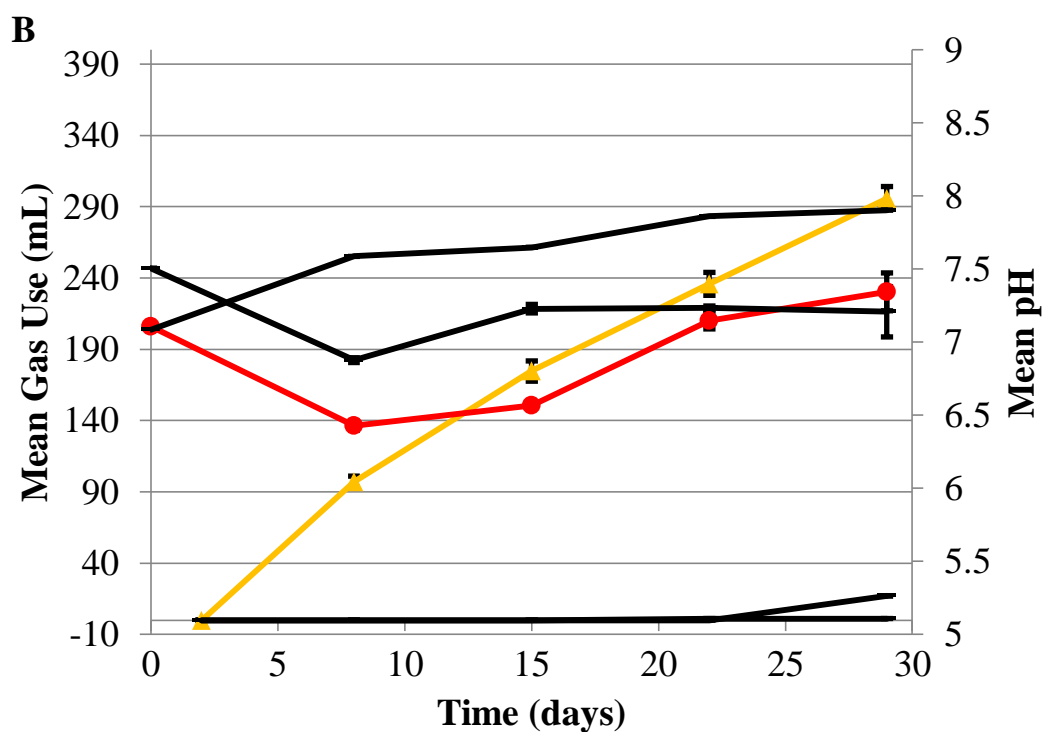
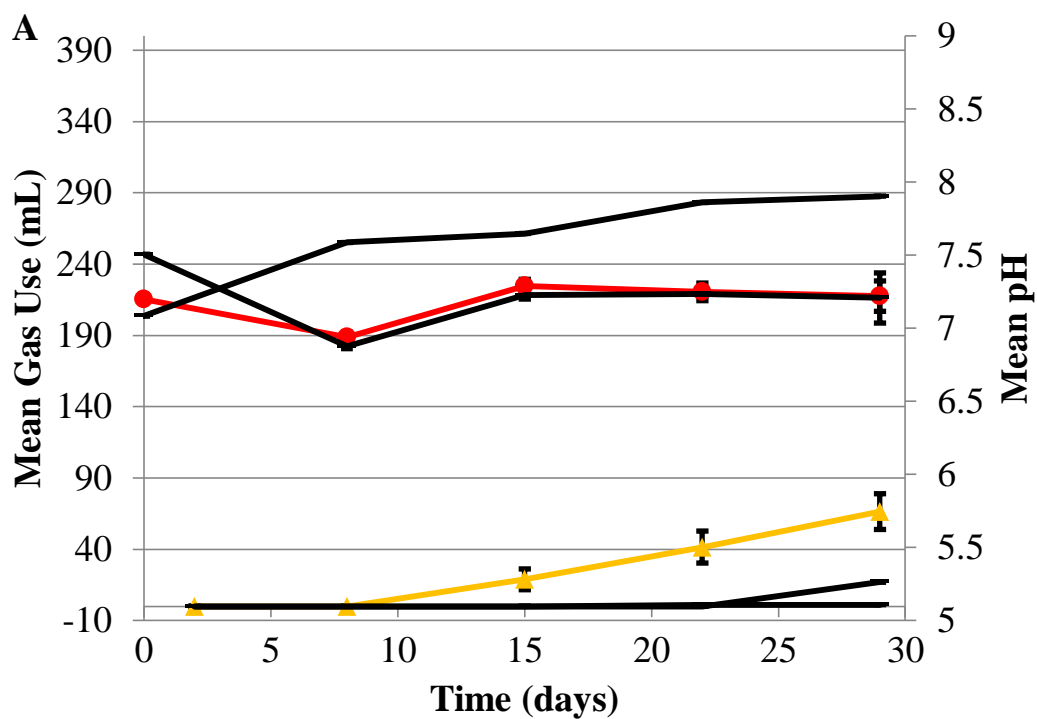


Figure 59. Mean gas consumption (yellow) and mean pH (red) for experiment #9. All variations had doubled nitrogen. Solid carbonate core instead of crushed carbonate core was used. The top graphs shows A) low nutrient enrichment and the bottom graph shows B) high nutrient enrichment. Error bars are calculated by as an average of 3 replicates. One control was inoculated and sterilized by autoclaving and the other control was not inoculated (unamended).



Figure 60. Experiment #9 at end of incubation. Top left: visible turbidity in the high nutrient medium. Top right: carbonate cores immediately after removal from the culture vessels. Bottom: Dark precipitate and free mass recovered from some bottles.

4.9.2 16S rRNA microbial community analysis

Metagenomic sequencing of the 16S rRNA gene for community analysis was only performed on the high nutrient medium (Figure 61 and Table 41). It is firmly within an acetogen-dominant stage of growth with 77.2% *Acetobacterium*. The only other significant microbes present are sulfide-oxidizing *Arcobacter* (16.3%) and *Shigella* (2.1%).

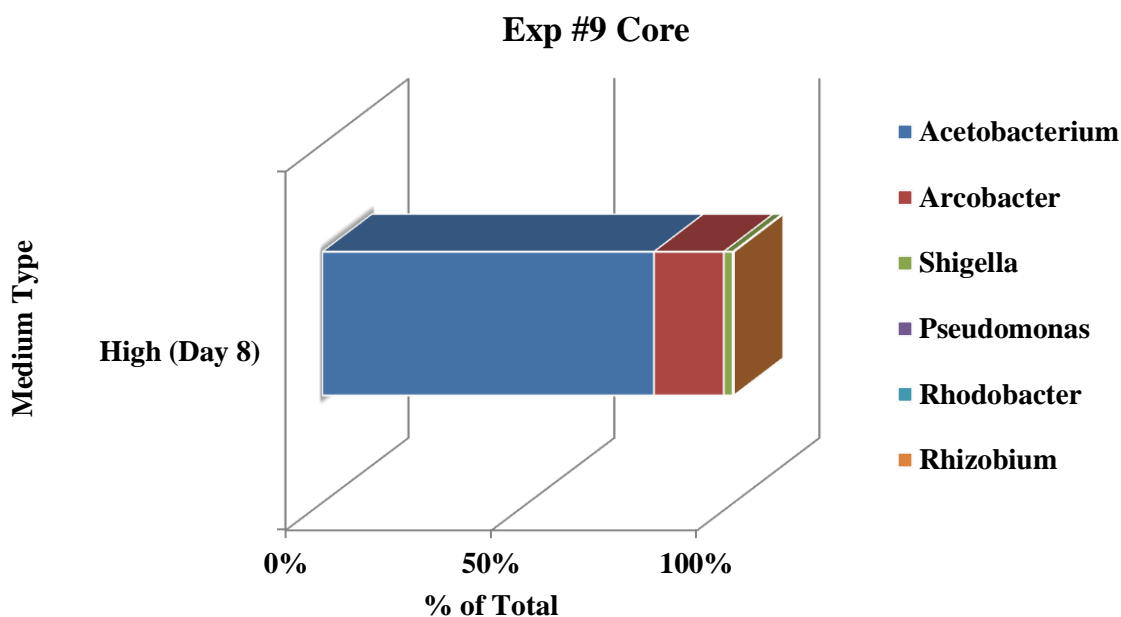


Figure 61. Experiment #9 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates.

Table 41. Experiment #9 16S rRNA pyrosequencing data at the genus level. Where no genus is identified, community components are identified at the next taxonomic level of identification. DNA was extracted from the cell pellet of 3 combined replicates. Major community components (>10%) are highlighted in bold.

	High (Day 8)
<i>Acetobacterium</i>	77.13
<i>Arcobacter</i>	16.30
<i>Shigella</i>	2.11
<i>Pseudomonas</i>	0.20
<i>Rhodobacter</i>	0.015
<i>Rhizobium</i>	0.015

4.9.3 CaCO₃ mass recovery

When the cores were recovered from the enrichments, they were weighed and compared to their pre-incubation weight. Some of the carbonate rock had separated from the main core and was moving freely in the medium. This was collected by vacuum filtration and included in the calculations (Table 42). These values are not comparable to other experiments which were based on a standardized added amount, but comparison can be made for the change in mass between active and sterile bottles. The sterile controls had greater mass loss (2.15% for sterile control, 2.30% for unamended control) than the active bottles (1.86% for low nutrients and 1.90% for high nutrients, Table 43) but this was not statistically significant ($p>0.05$). This may be an effect of precipitation of the black solid. This led to a net gain in rock mass once sterile controls were accounted for (Table 44).

Table 42. Free mass recovered that was had separated from the core in experiment #9. Standard error and mean were calculated based on 3 replicates.

Sample ID	Free Mass Recovered (mg)	Std Error (mg)	% of Core Mass	% Std Error
Unamended	18.7	0	0.0267	0
Sterile	23.4	0	0.0353	0
Low	24.53	7.93	0.0363	0.013
High	18.73	1.80	0.0286	0.0041

Table 43. Mass lost in experiment #9. Cores were pre-weighed on an analytical balance. Standard error and mean were calculated based on 3 replicates. Mass loss is based on the difference between initial weight and weight after incubation. Percent difference from the sterile control is calculated as the ratio of % mass loss of active bottle to % mass loss of sterile control.

Sample ID	Initial Core Mass (mg)	Mean Mass Loss (mg)	Mean Std Error (mg)	% Mass Loss	% Mean Mass Loss Std Error	% Difference from Sterile
Unamended	66198.8	1333.4	0	2.15	0	N/A
Sterile	70080.9	1328.9	0	2.30	0	N/A
Low	66299.2	1288.3	31.09	1.86	0.029	-13.5
High	69485.4	1252.13	27.42	1.90	0.082	-11.7

Table 44. Comparison of core mass loss to peak acetic acid produced for experiment #9 after removal of abiotic loss as represented by loss in the sterile control. Precipitation occurred if the net change was positive and dissolution occurred if the net change was negative. The net change also includes sample measured as free mass.

Sample ID	Mean Acetic Acid (mmol)	Mean CaCO ₃ Change (mg)	Mean CaCO ₃ change (mmol)	Mean CaCO ₃ change – sterile loss (mmol)	Precipitation or Dissolution
Unamended	0.36	-1333.4	-13.33	N/A	N/A
Sterile	0.054	-1328.9	-13.29	+0.04	Precipitation
Low	0.53	-1288.3	-12.88	+0.45	Precipitation
High	1.44	-1252.13	-12.52	+0.81	Precipitation

4.9.4 Experiment #9: porosity and permeability analysis on carbonate cores

The cores were analyzed for changes in porosity and permeability by analyzing them before (Figure 62 and Table 45) and after incubation (Figure 63 and Table 46). The cores from the controls had similar changes in porosity as the low nutrient and high nutrient medium ($p>0.05$). Cores from the controls and the low nutrient medium had an increase in gas permeability ranging from 0.16 to 0.24 mD (Table 47). Cores from high nutrient medium did not increase in gas permeability as much as the controls ($p<0.05$). Absolute permeability increased for all cores. Those treated in the high nutrient medium had a smaller increase but this was not statistically significant ($p>0.05$).



Figure 62. Carbonate cores before treatment in experiment #9.

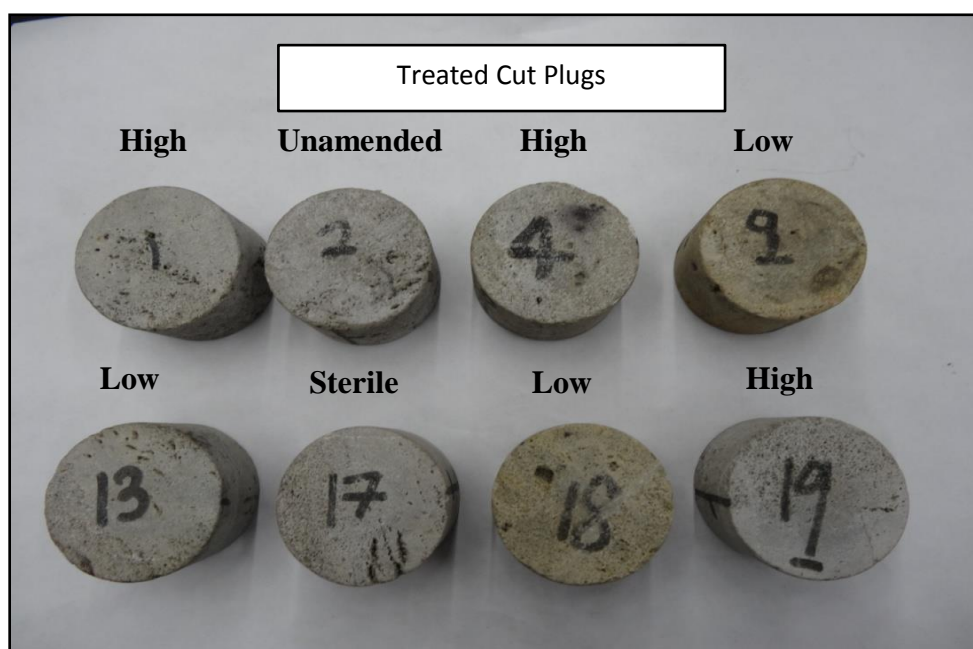


Figure 63. Carbonate cores after treatment in experiment #9.

Table 45. Analysis for porosity (%) and permeability (mD) as performed by CT scan and core-flow analysis of carbonate core before treatment in experiment #9. Low and high nutrient treatments are the mean of three replicates.

	Porosity (%)	Standard Error	Gas Permeability (mD)	Standard Error	Absolute Permeability (mD)	Standard Error
Sterile	5.258	0	9.498	0	6.66	0
Unamended	11.192	0	468.604	0	228.775	0
Low Nutrients	11.758	2.490	349.967	91.659	240.455	0
High Nutrients	9.135	2.344	340.580	188.691	108.645	60.233

Table 46. Analysis for porosity (%) and permeability (mD) as performed by CT scan and core-flow analysis of carbonate core after treatment in experiment #9. Low and high nutrient treatments are the mean of three replicates.

	Δ Porosity (%)	Standard Error	Δ Gas Permeability (mD)	Standard Error	Δ Absolute Permeability (mD)	Standard Error
Sterile	5.214	0	12.210	0	10.465	0
Unamended	10.953	0	560.857	0	378.128	0
Low Nutrients	11.807	2.562	455.574	118.431	345.700	0
High Nutrients	9.0384	2.315	341.837	182.089	137.895	84.285

Table 47. Analysis for standard change in porosity (%) and standardized change permeability (mD) as performed by CT scan and core-flow analysis of carbonate core after treatment in experiment #9. Low and high nutrient treatments are the mean of three replicates.

	Standardized Δ Porosity (%)	Standard Error	Standardized Δ Gas Permeability (mD)	Standard Error	Standardized Δ Absolute Permeability (mD)	Standard Error
Sterile	-0.00833	0	+ 0.222	0	+0.364	0
Unamended	-0.0218	0	+0.164	0	+0.395	0
Low Nutrients	+ 0.000856	0.00776	+0.237	0.0522	+0.304	0
High Nutrients	-0.0102	0.0123	+0.00948	0.0356	+0.168	0.0715

4.10. Summary

A comparison between different enrichments and experiments is summarized in Tables 48 and 49. Values were compared at peak values of absolute acetic acid and methane by percentage change which was also analyzed for statistical significance. For acetic acid production, significant differences are observed between the three types of medium, active versus sterile controls, primary versus secondary enrichment for high nutrient medium, headspace gas composition, medium volume, nitrogen levels and trace metals. Differences in methane are found for BESA medium versus high nutrient medium, active versus sterile, primary versus secondary enrichment for high nutrient medium, adding solids, adding bicarbonate, headspace gas composition, crushed versus powdered CaCO_3 , concentration of CaCO_3 , volume of medium, nitrogen levels, high nutrients versus low nutrients in a solid carbonate core. Acetic acid levels were significantly higher in the low nutrient versus high nutrient medium in only some of the tested conditions.

Factors found not to be significant for acetic acid production include primary versus secondary enrichment for low nutrients, added solids, added bicarbonate, crushed versus powdered CaCO_3 , concentration of CaCO_3 , phosphate levels, solid core versus powdered core for high nutrients, and high nutrients versus low nutrient for solid core. For the low nutrient medium it is important emphasize that there was no significant difference found between the primary and secondary enrichments when compared directly. Low nutrient medium had significantly more acetic acid than the high nutrient medium in secondary enrichment **only**. Factors found not to be significant for methane production included primary versus secondary enrichment for low nutrient medium, trace metals, phosphate levels and solid core versus powdered CaCO_3 in high nutrient medium.

Table 48. Comparison between experiments based on different comparable parameters for the change in mean acetic acid and mean methane. Other variables except the stated parameters were constant except where noted.

Parameter Comparison	Experiments	% Change in Mean Acetic Acid	Statistically Significant? (p<0.05)	% Change in Mean Methane	Statistically Significant? (p<0.05)
Low Nutrients vs High Nutrients	Exp. #3 High vs Exp. #3 Low (Primary)	-74.78	✓	+145.79	☒ (Significant for Exp. #6, 7, 8 and 9)
BESA vs High nutrients	Exp. #4 High vs Exp. #4 BESA (Primary)	+235.9	✓	-91.73	✓
Active vs Sterile	Exp. #3 High vs Exp. #3 High Sterile	+1735.12	✓	+2154.33	✓
Secondary vs Primary Enrichment (High Nutrients)	Exp. #3 High (Secondary) vs Exp. #3 High (Primary)	-71.53	✓	+12.20	✓
Secondary vs Primary Enrichment (Low)	Exp. #3 Low (Secondary) vs Exp. #3 Low (Primary)	+161.36	☒	-53.82	☒
Solid vs No Solid	Exp. #3 High vs Exp. #1 High (Primary)	+295.2	☒	+62.49	✓
Solid+ No HCO_3^- vs Solid+ HCO_3^-	Exp. #4 High vs Exp. #3 High (Primary)	-7.21	☒	+68.67	✓
100% H_2 vs 80% H_2 /20% CO_2	Exp. #5 H_2 High vs Exp. #4 High	-43.92	✓	-99.87	✓
100% CO_2 vs 80% H_2 /20% CO_2	Exp. #5 CO_2 High vs Exp. #4 High	-91.84	✓	-99.91	✓
Crushed carbonate core vs powdered CaCO_3	Exp. #6 High vs Exp. #4 High (Primary)	-9.94	☒	-16.78	✓
20 mM CaCO_3 vs 100 mM CaCO_3	Exp. #8 2XTM vs Exp. #7 2XTM	-4.12	☒	-60.00	✓

Table 49. Comparison between experiments with different comparable parameters for the change in mean acetic acid and mean methane continued. Other variables except the stated parameter were constant except where noted. *The effects of changing medium volume and doubling nitrogen cannot be differentiated.

**Medium volume and proportion to headspace gas volume was also changed and cannot be differentiated.

Parameter Comparison	Experiments	% Change in Mean Acetic Acid	Statistically Significant?	% Change in Mean Methane	Statistically Significant?
50 mL medium vs 100 mL medium*	Exp. #7 High Control vs Exp. #4 High *	-51.75	✓	-73.88	✓
2X Nitrogen vs 1X Nitrogen (High)*	Exp. #7 High Control vs Exp. #4 High*	-51.75	✓	-73.88	✓
2X Nitrogen vs 1X Nitrogen (Low)	Exp. #7 Low Control vs Exp. #4 Low*	-79.21	✓	-97.94	✓
2X Trace Metals vs No Trace Metals	Exp. #7 2XTM vs Exp. #7 No TM	+77.4	✓	-8.53	☒
2XPO₄ vs No PO₄	Exp. #7 2XPO ₄ vs Exp. #7 No PO ₄	-8.58	☒	+4.46	☒
Solid core vs powdered CaCO₃ (High)**	Exp. #9 High vs Exp. #4 High**	+101.64	☒	-91.07	☒
Solid core High vs Low	Exp. #9 High vs Exp. #9 Low	+173.79	☒	+359.30	✓

CHAPTER 5 DISCUSSION

The main goals of this research were to optimize the growth of acetogens relative to methanogens so that acetic acid production from H_2/CO_2 can be optimized. They were also evaluated for a potential biotechnological application to dissolve carbonate rock. It appears that several minor flaws in the experimental design that did not overtly affect the growth of these microbes, but had an affect on the comparability between experiments. This resulted in some trends becoming insignificant. Lack of statistical power is an issue that is difficult to address as lack of replicates is usually due to limitations in resources and time. Other confounding factors included the unexpectedly large effect of medium volume on levels of microbial growth. Also, even if a certain amount of acetic acid is considered to be high in an experimental context, it can only dissolve a small mass of carbonate. However, a review can still be made of the factors affecting or appearing to affect acetogenic growth and carbonate dissolution.

5.1 Acetogenesis Optimization

5.1.1 pH limitation

In the first experiment (Experiment #1), bicarbonate was not present in the medium to test the ability of acetogens to grow autotrophically from headspace CO_2 . Acetic acid and methane production occurred, but at a lower level than previous studies by the Voordouw lab (Nathoo *et al.* 2012, Folarin 2013). pH measurements were conducted on all samples and it was found that pH decreased to less than 6 for all media types and less than 5.2 for BESA medium. Removing the bicarbonate removed most of the pH buffering capacity and the medium became much more sensitive to pH changes. Acidity is a well-known factor for acetogenesis inhibition (Baronofsky *et al.* 1984). Until recently most acetogens were considered to have a pH growth

range of above pH 6 (Drake *et al.* 2006) though some acid-tolerant acetogens have since been found with a pH growth range of 4 to 6 (Göbner *et al.* 2008, Lee *et al.* 2007). Sodium 2-bromoethane sulfonate is also an acidic salt with a pKa of approximately 2.5 which caused an even larger drop in pH where it was added. Cultures with BESA added also had the highest amount of acetic acid was produced which is due to methanogenesis inhibition which removes the most direct competition for acetogens.

In the next comparable experiment, experiment #3, 100 mM of CaCO_3 and 30 mM of NaHCO_3 were added to buffer the pH. CaCO_3 is also a more controlled laboratory version of carbonate rock and this was a first step towards analysis of carbonate dissolution. This was not the goal of the experiment however, as the added bicarbonate suppressed dissolution by creating excess in the carbonic acid equilibrium. Because of this, CaCO_3 mass loss could not occur through increased acidity or $\text{CO}_2/\text{CO}_3^{2-}$ limitation. Excess pH buffering also limits carbonate dissolution. The ideal buffer would keep the pH between 6.0 and 6.5. Experiment #3 had strong pH buffering. Compared to experiment #1, acetic acid levels were the same for low nutrient medium in the primary enrichment, but higher in the secondary enrichment and much higher for BESA medium in both enrichments. In the earlier experiment, the levels of acetic acid produced a large enough pH drop so as to restrict further growth of the acetogens. Buffering the pH allowed the acetogens to grow for longer so they produced higher levels of acetic acid. Though the mean acetic acid appeared higher in the high nutrient medium, the difference was not statistically significant.

5.1.2 Differences between primary and secondary enrichments

Secondary enrichments (subculture) were performed for several experiments to account for the effects of nutrients in the inoculum waters and the unavoidable exposure to oxygen during transportation from the oil field to the lab. Allowing a period of growth and then transferring to fresh medium controls for these effects which are especially important for extremely oxygen-sensitive microbes such as methanogens. Some differences in growth were expected in the secondary enrichments but some unusual patterns were observed with the different medium types used.

The low nutrient medium had lower levels of added nutrients so lower levels of both acetic acid and methane production than high nutrient and BESA medium is a reasonable estimate of how these cultures would grow. This would be even more so in the secondary enrichment which would lack any extra nutrient present in the inoculum waters. Low nutrient would be associated with low acetic acid production, BESA medium with maximum acetic acid and high nutrient medium showing acetic acid somewhere in between. Low nutrients would produce low methane, high nutrients would have the highest methane and BESA medium would produce no methane due to the presence of the inhibitor. Experiment #1 generally had these expected trends.

This changed in experiment #3. Low nutrient medium did not follow the previous trend for acetic acid in the secondary enrichment. The mean acetic acid appeared to be higher than both the primary enrichment and the high nutrient medium. However, there were only two replicates and growth variation was high so these differences were not statistically significant though low nutrient medium was at least *the same* as the high nutrient medium. In the secondary

enrichment, the two bottles of low nutrient produced peaks of 646 and 343 μmol acetic acid respectively, one bottles was almost twice as much as the other. High nutrient medium produced 269 and 145 μmol of acetic acid. While at a glance the acetic acid in low nutrients is higher, but the variation renders the means not statistically different. The difference is large enough that simply increasing the statistical power of the experiment by doing a greater number of replicates may achieve a significant difference. This is seen in the experiments that followed. In experiment #4, the secondary enrichment of low nutrients had significantly higher acetic acid and similar methane to the high nutrient medium. Acetic acid and methane were the same in experiment #6 between low and high nutrient medium.

The effect of acetic acid being produced on at least a comparable level between low and high nutrients (and maybe more) could be a result of the chemistry of the produced waters used as the inoculum. As it is 10% of the overall medium, the chemistry of the waters could effect to the overall nutrient levels of the cultures as it would only be 10X diluted. If this does have an effect, than the primary and secondary enrichments are no longer two nutrient levels done twice in the same way, but four different nutrient levels (BESA is the same as high nutrients). The primary enrichment has higher levels of nutrients than the secondary enrichment. This translates to the primary enrichment of high nutrients having a “high” nutrient level and the primary enrichment with low nutrients would be a “low” level. In the secondary enrichment, high nutrients would only be a “medium” nutrient level and low nutrients in the secondary enrichment would be an “ultra-low” version (Figure 64).

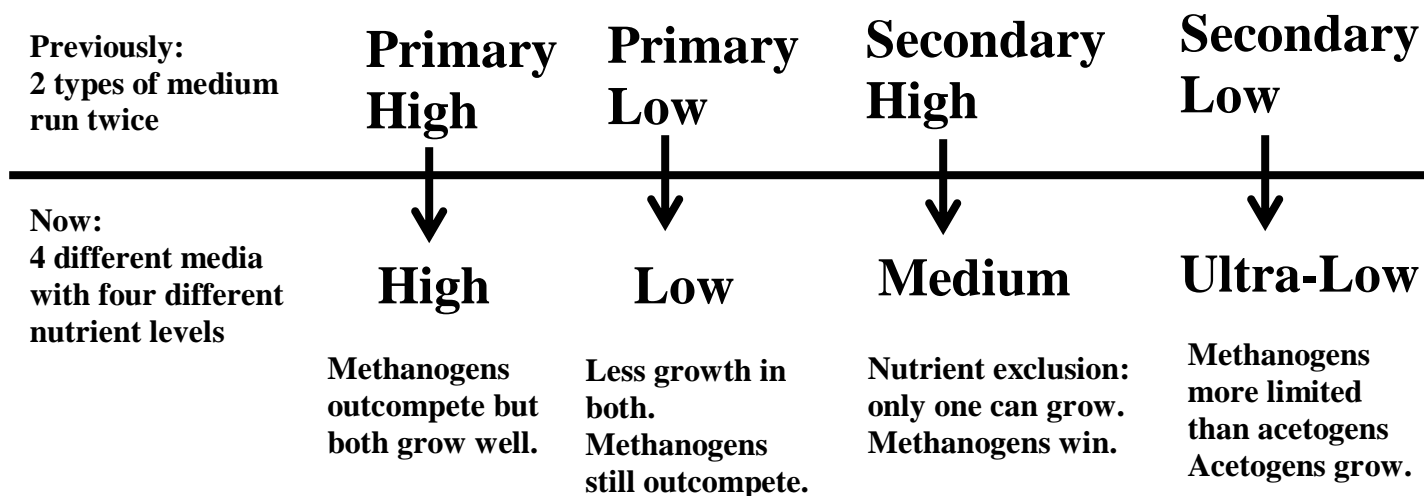


Figure 64. A representation of the changes in definition for nutrient levels when the influence of inoculum chemistry is accounted for.

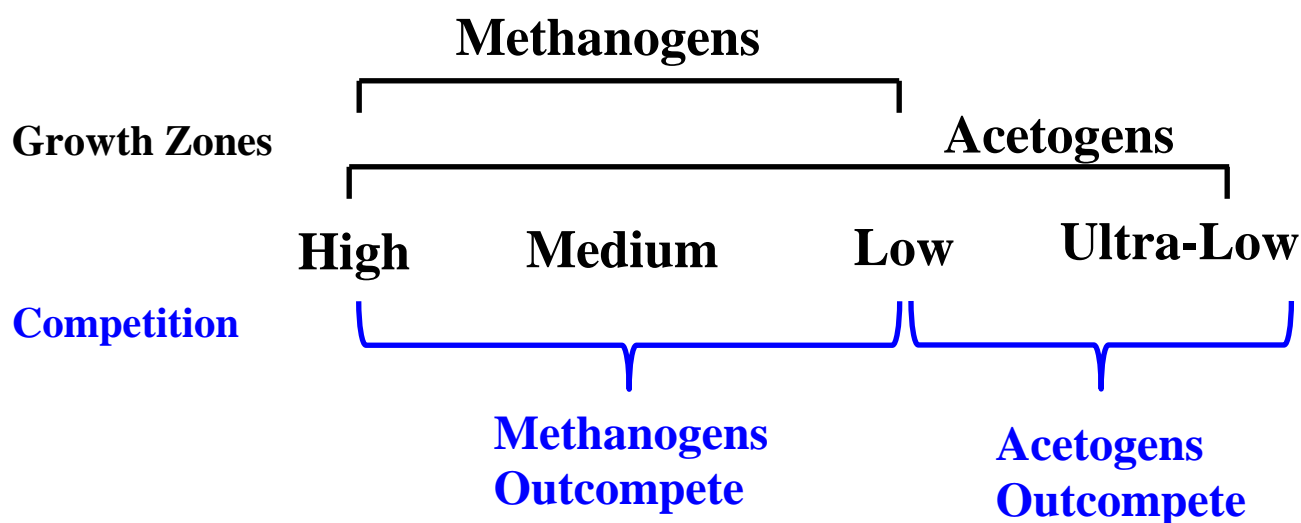


Figure 65. A representation of growth ranges and zones of competitiveness between acetogens and methanogens based on nutrient level.

With a new definition for nutrient levels it appears that the unusually high levels of acetic acid production are found in the low to ultra-low nutrient range. Acetogens have been posited to become competitive in extreme low nutrient environment for reasons such as psychro-tolerance or having the ability to metabolize a greater range of substrates than methanogens (Lever *et al.* 2010). As neither of these conditions are present in these experiments, other factors in low nutrient environments appear to play a part in successful acetogen competition. Figure 64 could also be rearranged to a model of a growth range (Figure 65) where acetogens are able to grow in environments that are too nutrient-limited for methanogens to grow in. This is also supported by the tendency for the methanogenic archaea to become less diverse as the culture goes from high nutrients to limited conditions. In the 16S rRNA analysis for experiment #3, when comparing the primary and secondary enrichments *Methanospirillum* appears to be the methanogen most able to grow under nutrient limitation before conditions become too severe for even that genus. Acetogens and other types of microbes are able to fill the gap. Experiment #1 is not comparable due to pH limitation and the numerous changes involved in adding the CaCO₃.

5.1.3 Acetic acid loss

In the high nutrient medium, acetic acid reaches a peak before day 30 in all experiments at which point it decreases back to zero by day 60. The exceptions are the acid-limited experiment #1 and experiment #2 which lack H₂. This could be the result of the metabolic activity of acetotrophic methanogenesis. Experiment #2 was conducted to understand the effects and timing of acetotrophic methanogenesis in the particular microbial community of the MHGC produced waters. To isolate only these effects which are not gas consuming, the headspace gas was switched from 80% H₂/20% CO₂ to 90% N₂/10% CO₂. Removing H₂ inhibits hydrogenotrophs including acetogens and hydrogenotrophic methanogens. Sodium acetate (5

mM) was added for acetate-utilizing methanogens and primary and secondary enrichments were performed. Controls with no acetate added were conducted along with low nutrient and BESA medium sets. Activity was only seen in high nutrient medium, acetate-amended bottles.

For this experiment (#2), acetic acid loss and methane production started at day 70 in the primary enrichment and day 40 in the secondary enrichment. Within this timeframe it appears that acetotrophic methanogenesis probably occurs too late in the incubation to account for the acetic acid loss. This loss always starts before day 30 and sometimes as early as day 7. Not enough methane was produced during the loss period to stoichiometrically account for the amount of acetic acid consumed. Approximately 740 μmol of acetic acid was lost in experiment #3 between days 24 and 55 and in that time period methane actually decreased 50 μmol (likely due to sampling and instrumental variation). As to whether this trend holds true for other experiments, the effect is obscured in experiments #4 and #6 due to the flushing of the headspace gas which increased hydrogenotrophic methanogenesis. Experiment #5 had plateaued levels of acetic acid with almost no methane production. Experiment #7 had much less acetic acid than other experiments (discussed below) and the rest were only run for 30 days.

Analysis of the 16S rRNA genes found in high nutrient medium of experiment #3 show that at the end of the experiment, the community composition had shifted from a mix of methanogenic archaea to over 85% of an unidentified species of the *Ralstonia* genus. This is even more pronounced in analysis of the secondary enrichment in which the proportion of *Ralstonia* grew from 40% to 84% by the end of the experiment. A review of the literature on *Ralstonia* finds that the commonly studied model organism for this genus, *Ralstonia eutropha* is known to be able to convert both inorganic carbon and acetate to poly-3-hydroxybutyrate (PHB). This is a form of carbon storage stress response that occurs under nitrogen-limiting conditions

(Volova *et al.* 2002, Lee *et al.* 1994, Uchino *et al.* 2007). A range of other microbes that are identified as being able to store carbon were found in other experiments, the major contributors are *Pseudomonas*, *Alishewanella*, and *Acinetobacter*.

As PHB is hydrophilic and is stored in large granules within cells, it can be directly examined under a light microscope at 1000X magnification when stained with the lipophilic Nile red fluorescent dye (Spiekermann *et al.* 1999). Examination of cells from experiment #4 (primary enrichment) was conducted under bright-field microscopy from the time points where acetic acid had peaked and started to decrease (Days 21 and 33). Clusters of cells are visible that contain large, hollow-looking inclusions consistent with lipid storage bodies. Nile red dye fluoresces red under blue-green fluorescent light. Upon examination with the epifluorescent component of the microscope, those same clusters had fluorescent clumps corresponding with the inclusions seen under bright-field setting.

PHB formed from acetate by carbon-storing microbes forming PHB in nutrient limited conditions is a plausible cause for the acetic acid loss. This is supported by the lack of acetic acid loss in low nutrient and BESA media in all experiments. BESA medium contains the same level of nutrients as high nutrient medium which suggests that the growth of methanogenic archaea somehow leads to stimulation of the carbon-storing microbial group. Low nutrient medium may work indirectly by limiting methanogenic growth. Most of the nutrient variants in experiment #7 are based directly off of high nutrient medium, but the variants as well as the high nutrient control showed only minor acetic acid loss after 60 days. The only genus present that contains some carbon storing members is *Pseudomonas*. However, *Pseudomonas* is a highly diverse genus and it is just as probable that it is not involved in carbon storage at all. There are two differences between experiment #7 and experiment #4 (which showed complete acetic acid loss).

One is the decrease in medium volume. The other is that experiment #7 had doubled the amount of added nitrogen (ammonium chloride). The extra nitrogen is probably what prevents the acetic acid loss as medium volume was changed between other experiments and did affect the loss.

5.1.4 16S rRNA microbial community analysis: Community succession

Each sample that was sequenced is a snap shot of particular moment during an enrichment. A model can be assembled based on the major functional groups of microbes present at different time points. This is most useful for experiments #3, #4 and #6 (Figures 66, 67 and 68). An overview of the sequencing results for all 16S rRNA genes shows a general trend of progression from an initial acetogen-dominant growth stage to a methanogenic stage but this varies based on medium composition. Acetogens may be more rapid growers than methanogens but the more likely explanation is they were more able to withstand the exposure to oxygen during transport from the field and needed less recovery time. The community then shifted to a stage with a high proportion of microbes found to store carbon (acetate and CO₂) in the form poly-3-hydroxybutyrate (PHB) and other polyhydroxyalkanoates.

The composition of a microbial population observed in a particular sample is strongly dependent on the time point that the sample was taken from. In experiment #3, the proportion of reads matched to *Acetobacterium* is small even though a significant amount of acetic acid was produced. This is because the sampling days used for analysis were fairly late in the overall course of the enrichment. This includes day 27 in the high nutrient medium which was selected because this sample was taken just after the acetic acid peaked. However, peak acetic acid means that the acetic acid producers have stopped growing. Changes in production activity probably start to occur before changes are seen in the proportion of detected sequencing reads, so an

acetogen-dominant community would be seen during the active production of acetic acid in the earlier days of the enrichment not after production has stopped. In experiments #4 and #6, the microbial groups overlap in slightly different ways but an overall succession is clear.

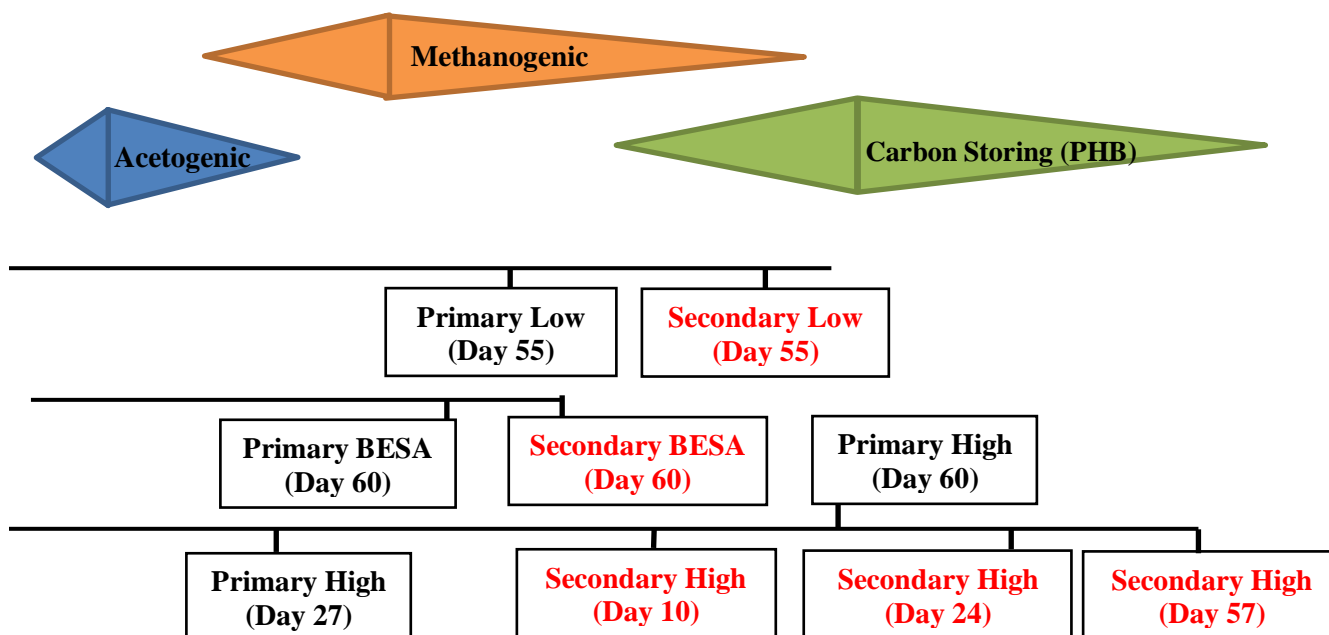


Figure 66. Model of microbial community succession for experiment #3 based on 16S rRNA molecular gene sequencing results. The primary enrichment was incubated for 60 days and the secondary enrichment was incubated for 62 days. The types of microbial functional groups and the order and degree of succession are represented as the coloured triangles. Samples are placed in time line as representation of an instantaneous measurement of community composition at that time point. Primary enrichment samples are in black text and secondary enrichment samples are in red text.

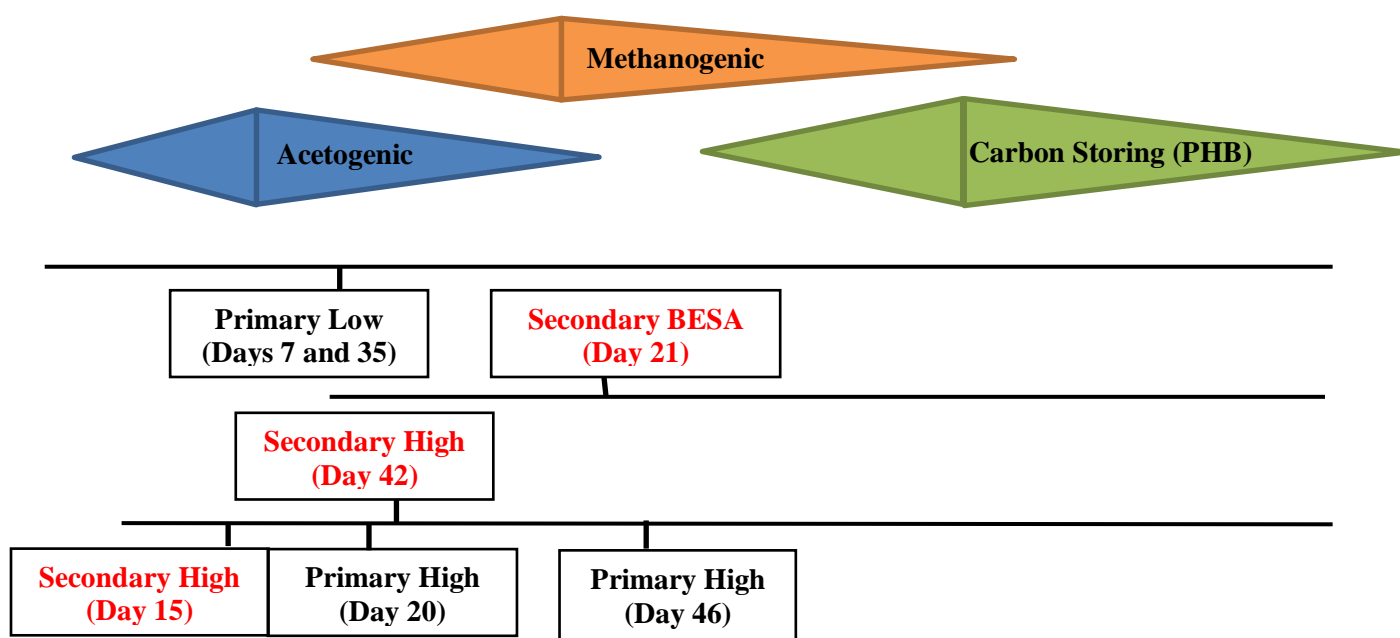


Figure 67. Model of microbial community succession for experiment #4 based on 16S rRNA molecular gene sequencing results. The types of microbial functional groups and the order and degree of succession are represented as the coloured triangles. Samples are placed in time line as representation of an instantaneous measurement of community composition at that time point. Primary enrichment samples are in black text and secondary enrichment samples are in red text.

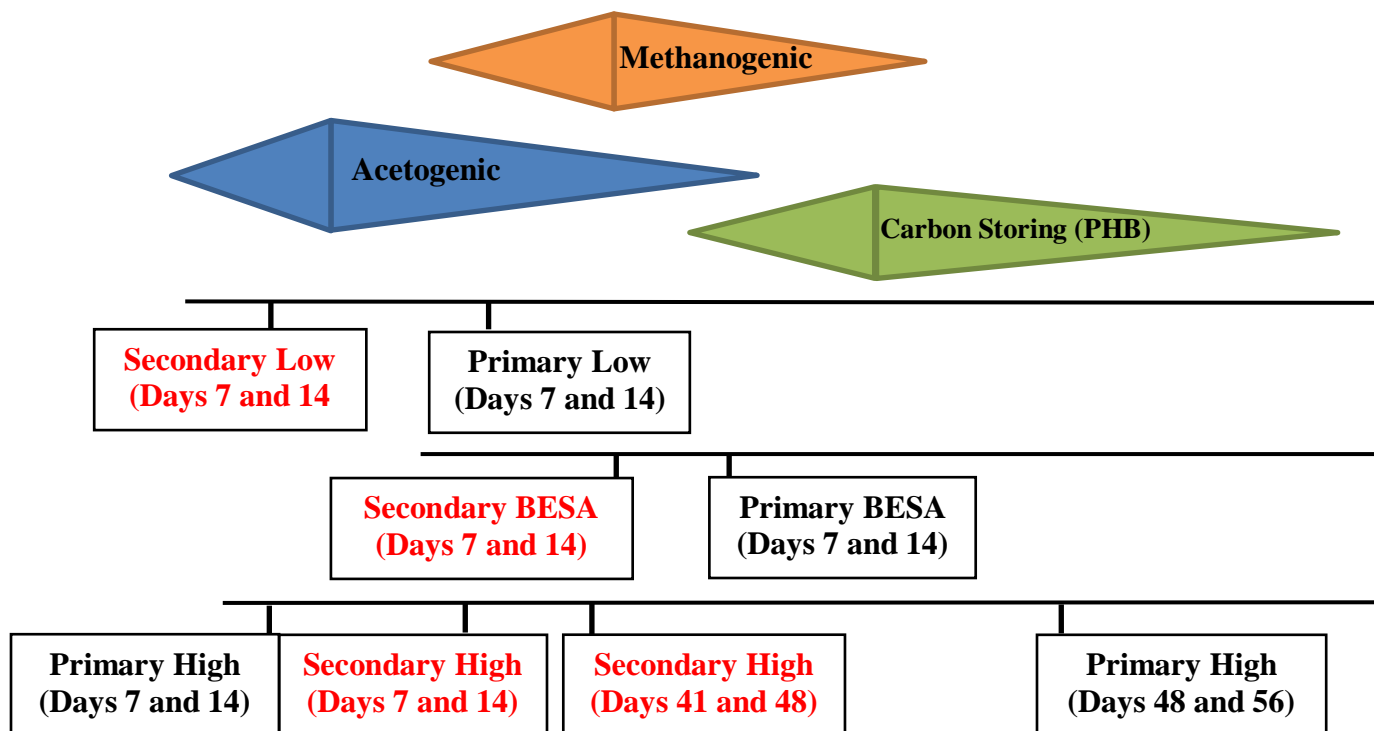


Figure 68. Model of microbial community succession for experiment #6 based on 16S rRNA molecular gene sequencing results. Both enrichments were incubated for 99 days. The types of microbial functional groups and the order and degree of succession are represented as the coloured triangles. Samples are placed in time line as representation of an instantaneous measurement of community composition at that time point. Primary enrichment samples are in black text and secondary enrichment samples are in red text.

5.1.5 16S rRNA microbial community analysis: Discrepancies with activity data and actual production of acetic acid and methane

Actual production is directly related to true microbial activity so it is the main point of comparison with all other data. There are some instances in which the methane and acetic acid production data do not match the DNA community analysis. In experiment #4 secondary enrichment, *Acetobacterium* persisted even after acetic acid was actively decreasing in the high nutrient enrichment. At a later time point “Secondary High Day 42”, the acetic acid had been reduced by half from its peak (from 560 to 219 μmol) but 64.8% of reads were still *Acetobacterium*. This is about the same as the earlier time point when acetic acid was still actively being produced. There are three possible explanations for this. There is supporting evidence for two of them and since they are not mutually exclusive both of them could be true.

The first explanation is that in most experiments, after active acetic acid growth stopped, acetogenic microbes either disappeared or their proportion of reads became smaller because of the increased growth of other microbes. Acetic acid consuming microbes only started to be detected after active production stopped. It may be that in experiment #4, *Acetobacterium* continued to produce acetic acid longer than in other experiments and continued even when the consumers started to grow. The consumers reduced acetic acid at a greater rate than the acetogens produced it with a net effect of acetic acid loss. The main problem with this is that carbon-consuming microbes are not detected in any significant amount. There is evidence of sulfur cycling microbes growing as part of an alternative metabolic cycle (discussed below) and they make up most of the remaining $\approx 30\%$ of reads. *Desulfovibrio desulfuricans* is present in a high proportion in this particular sample and it could be oxidizing the acetate, but this microbe is usually considered to convert lactate to acetate, producing acetate and not consuming it (Okabe

et al. 1992). However, when there are no other carbon molecules present that can act as both a carbon and electron source, the sulfide-oxidizer *Sulfurospirillum* has been found to use acetic acid and H₂ (Stolz *et al.* 1999, Grigoryan *et al.* 2008). *Sulfurospirillum* could be causing the acetate loss, but this is only partial evidence because sulfur cycling microbes tend exclude less energetically favourable metabolic lifestyles like acetogenesis and methanogenesis. In this case, they do not appear to be enough growth to suppress acetogenesis so it seems unlikely that it would cause major acetic acid loss.

The second possible explanation is that there is a lag between a change in microbial activity and detection by 16S rRNA molecular analysis. Changes in methane and acetic acid production and consumption may happen before such changes are detected in the community composition at the DNA level. Likewise, DNA from particular groups of microbes would linger after activity has stopped. This could explain the detection of acetogens after acetic acid production has stopped like in experiment #4. It could also help explain instances where acetic acid is actively produced but no acetogens are detected, even during exponential production at the beginning. In experiment #7, two samples were sequenced from different time points, day 7 and day 21. Acetic acid (328 μ mol) had been produced by day 7, but no *Acetobacterium* or other acetogens were detected. Day 21 was the week of peak acetic acid which means active production stopped, but 64.1% of reads were attributed to *Acetobacterium*. There is a lag in changes in the detected microbial community on the DNA level when compared to changes in microbial activity.

The last explanation is the logical conclusion if the other two are excluded. It is that this type of DNA analysis is semi-quantitative at best. DNA extraction and pyrosequencing have error that could be the source of these discrepancies. This includes different levels of success and

efficiency in the DNA extraction process for different types of microbes, known PCR errors and the 3% error rate of the pyrosequencing process.

5.1.6 16S rRNA community analysis: Biofilms and late sampling

In experiment #3, methane was significantly higher for all medium types, especially and surprisingly BESA medium. A comparison of DNA community analysis was made between the experiments #1 and #3 in the primary enrichment. It found that when the CaCO_3 and NaHCO_3 were added, the community in the BESA medium diversified from mostly *Methanobacterium*, *Methanobacteriales* and *Roseibacterium* to also include *Methanofollis*, *Methanospirillum*, *Methanosaeta* and *Methanosarcina*. Adding CaCO_3 not only buffered pH, but it also added a solid with a large amount of surface area since it was powdered. The new methanogenic archaea that were detected have all been found to be associated with biofilms (Fernandez *et al.* 2008, Rademacher *et al.* 2012, Raskin *et al.* 1996, Wu *et al.* 2013). The microbial community may have shifted towards biofilm-forming methanogens due to the greater available surface area. A biofilm could form a protective effect against BESA and this would explain the greater methanogenic production in this experiment.

Experiment #4 (secondary enrichment) had similar DNA results though with more *Methanofollis*. The primary enrichment in experiment #6 show that sample to be in transition from acetogen-dominant to a diverse methanogenic type of microbial community. The secondary enrichment became methanogenic more quickly. Both experiment #4 and #6 had better methane control than experiment #3 even though similar methanogenic communities were detected in the DNA. Bicarbonate was added in experiment #3 that was removed in subsequent cultures. The

methanogens need this bicarbonate to reach a critical biofilm mass to achieve sufficient protection from BESA and produce methane.

Finding so many methanogenic archaea in the DNA analysis becomes problematic in relation to finding acetogens and comparison with the activity data. The lack of detected acetogens, even though acetic acid was produced, may be explained by the late sampling dates used for DNA extraction, amplification and sequencing. Biomass was greatest at the end the experiments so samples from later days tended to be used for DNA extraction. For example, the sample for BESA medium was taken on day 60. However, acetic acid production peaked at day 30 for the primary enrichment BESA medium. Examining a sample from day 60 only shows the composition of the microbial community after acetogens have died out. This may also account for the lack of acetogens seen in experiment #3.

However this works *only* for experiment #3, later experiments used much earlier samples but did not many more acetogens in the sequencing reads. The secondary enrichment BESA medium of experiment #4 produced the most acetic acid of all experiments, but only 2% of reads associated with *Acetobacterium*. There was a higher proportion of methanogenic sequencing reads in BESA medium than in the low and high nutrient media even though less than 10 μmol had been produced by day 14. It could be that 2% of the reads translates to fewer but more active acetogens, but in experiment #6, BESA medium produced less acetic acid in the primary enrichment and had a higher proportion of *Acetobacterium* reads (27.8%).

And then the secondary enrichment produced more acetic acid than the primary enrichment, but the 16S RNA sequencing reads were even more methanogenic with only 6.4% *Acetobacterium*. This is not an issue of sampling dates used for sequencing as the samples from

the two enrichments in experiment #6 were from the same sampling date and the sample from experiment #4 was taken from day 21 during active acetic acid production. It appears that methanogen are growing and creating a larger proportion of DNA relative to *Acetobacterium*, but are not actively producing methane. Unless these microbes have found mode of growth not related to methanogenesis this is difficult to explain.

5.1.7 16S rRNA microbial community analysis: Alternative metabolic cycles

The acetogenic and methanogenic incubations were designed to prevent other more energetically favourable metabolic cycles from occurring. None of the more powerful alternative electron acceptors like oxygen, sulfate or nitrate were added. But due to some unrelated changes, these metabolisms have happened unintentionally.

Over time, needle punctures from sampling built up and increased the risk of oxygen leakage. This caused the resazurin dye to turn pink due to increased redox potential. Small amounts of sodium sulfide were added to act as an oxygen scavenger. Sulfide-oxidizing nitrate-reducing (so-NRB) microbes were known to exist in the inoculation waters, but with no obvious source of nitrate or other electron acceptors the growth of these organisms was unlikely. It appears that trace amounts of nitrate from the source water or oxygen from leaky stoppers may have been enough to stimulate their growth as the so-NRB *Sulfurospirillum* and *Arcobacter* were found to be enriched in many cultures where sodium sulfide was used. The level of growth does not appear to be excluding lower-energy microbes like *Acetobacterium* or methanogens. So while this growth is undesired, with one exception it does not appear to have had a major impact on acetogenesis and methanogenesis.

There may have been a larger source of oxygen ingress than leaky stoppers. Some forms of *Cyanobacteria* form oxygen as part of its metabolism and it was detected in several cultures in experiments #4 and #6. However this particular *Cyanobacteria* is unidentified past its phylum and some groups within *Cyanobacteria* are known to be non-photosynthetic. It is unknown whether stopper leakage or *Cyanobacteria* activity is the greater source of oxygen. This growth occurred in all nutrient types and enrichments and may have led to microaerophilic conditions. A substantial number of aerophiles and microaerophiles were detected including aerobes like *Ketogulonigenium*, *Cellvibrio* and *Citrobacter* along with facultative microbes like *Rhodobacter* and *Shigella*. Oxygen production may also have fed sulfide-oxidizing and sulfate-reducing microbes by providing a more powerful electron acceptor than trace nitrate (Figure 69). This is plausible as *Arcobacter* is a facultative anaerobe and it can grow under microaerophilic and aerophilic conditions (Fedorovich *et al.* 2009). If oxygen was produced, the amount was small enough that it didn't inhibit the growth of obligately anaerobic acetogens, methanogens and syntrophs.

Oxygen ingress appears to have caused at best microaerophilic conditions that should be further defined by future measurement of oxygen levels but around 1 to 5%. Overall, this was a minor issue except for experiment #8. This experiment was originally intended to test the effects of acetogenesis on lower concentrations of CaCO_3 . But an examination of the DNA results for the doubled trace metals variant and the low nutrient primary enrichment shows that most of the genera are obligate aerobic or facultative. This is creating completely different microbial metabolic cycles, but similar amounts of acetic acid were still produced in comparison with experiment #7. Half of the reads in the 2xTM variant of experiment #8 are associated with aerobes that typically degrade sugars, but are in some cases also able to degrade organic acids. It

is difficult to discern where these fermenters got their carbon from, but most of the rest of the reads are associated with PHB-forming microbes. Some of these microbes are autotrophic including *Pseudomonas* (Battley 1996) and *Acinetobacter* (Young and Kim 1993). It could be that microbes built up PHB stores which were then broken down into other compounds that fermenting microbes could degrade.

Fermenters grew in several enrichments, but except for experiment #7, they don't cause much of a problem in context with acetogenesis and methanogenesis. This was the experiment in which nutrient variations were done on the composition of high nutrient medium. The variants had reasonable levels of acetogenesis and detected *Acetobacterium*. The high nutrient control produced statistically the same acetic acid and methane as the nutrient variants, but it had a drastically different microbial community dominated by 88.2% *Shigella*. *Shigella* is a facultative anaerobe that is usually known as a digestive system pathogen (Zaika *et al.* 1993). Acetic acid production had stopped at by day 21, but methanogenesis continued for another 2 weeks so it is unlikely that oxygen ingress caused this problem. It could be the *Shigella* is interacting with carbon-storing microbes similarly to experiment #8, but there are few detected. The most likely interpretation is that the sample was contaminated with *Shigella* DNA sometime during sampling or processing.

There a few other minor contributors. *Proteiniphilum* and *Sphingobacteriales* were found in many of the cultures. They are identified as syntrophs capable of degrading fatty acids and polyaromatic compounds (respectively) in association with methanogens. They may be consuming trace amounts of oil sourced from the inoculum waters but they are mostly neutral in context with acetogenic and methanogenic growth. At worst they could be supporting more methanogenic growth.

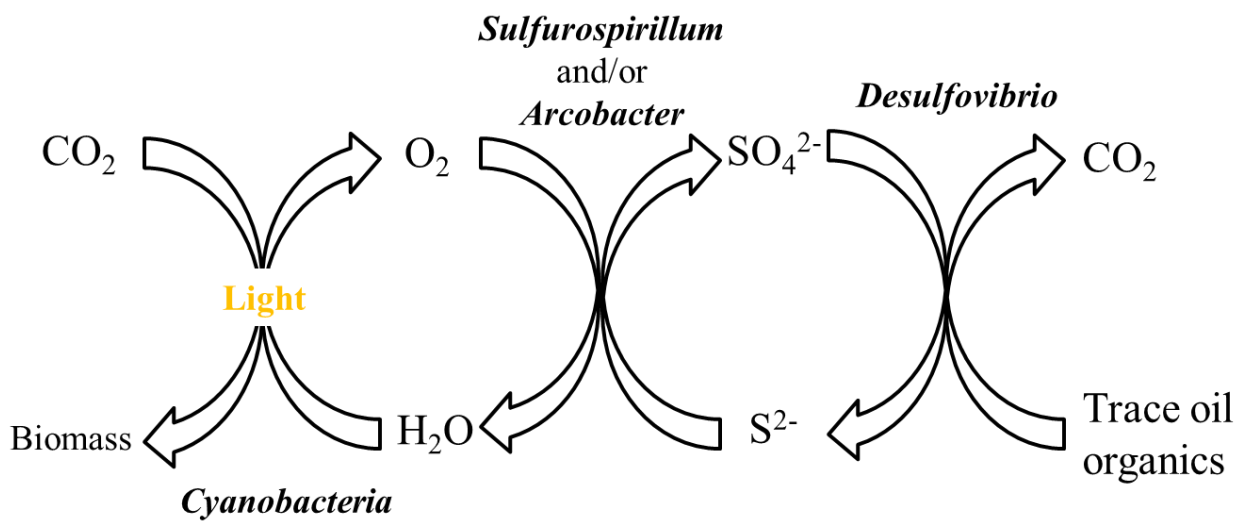


Figure 69. A model for an alternative metabolic cycle that may have formed due to sulfide addition and oxygen ingress based on 16S rRNA sequencing data. Nitrate may also have been used instead of oxygen. This cycle does not appear to have affect acetogenesis or methanogenesis.

5.1.8 Variations in headspace gas

In experiment #5, the 80% H₂/20% CO₂ headspace gas was switched to either 100% H₂ or 100% CO₂ creating CO₂-limited and H₂-limited set of bottles respectively. Minimal activity was observed in the 100% CO₂ environment and there was limited, but substantial activity with 100% H₂. H₂ is used as an energy equivalent to convert CO₂ into biomass. An excess H₂ environment with severely limited carbon could still utilize trace CO₂ and CO₂ could also be converted from the carbonate ion off of the CaCO₃ through the carbonic acid equilibrium. Very little microbial activity can happen in an environment with no H₂ for energy.

5.1.9 Carbon limitation

The pH of the high nutrient medium rose in both the primary and secondary enrichments of experiments #3, 4, 6, 8 and 9. Experiment #9 was finished early because the pH had started to rise so only the beginnings of this effect are seen. There are two plausible reasons for this. The pH drop is associated with acetic acid loss observed within the same time frame. Consumption of acetic acid could make the pH rise, but all of the above experiments had CaCO₃ added to create a pH buffering system and prevent acid limitation of acetogens. If the buffering system was sufficient enough to prevent a drop when the acetic acid was produced why would removing that same acetic acid cause a strong pH rise? The other likely possibility is that when carbon-storing microbes such as *Ralstonia* are actively taking up acetic acid they can also use inorganic CO₂. This active CO₂-consuming process could combine with other autotrophic microbes to cause a CO₂ limitation. As CO₂ is consumed, its acidifying effect in the medium is lost and pH rises.

Headspace gas was continuously replaced in the same proportion as the original gas composition (80% H₂/20% CO₂) for the purpose of prevents limitation for both CO₂ and H₂. This

proportion of H_2 to CO_2 was designed for a dominant methanogenic culture in which the proportion of gas consumed is 4:1 $H_2:CO_2$. But if the acetogenesis is the dominant gas-consuming process, then based on its chemical reaction (Equation 2), the proportion of gas consumed is 2:1 $H_2:CO_2$ or 66% H_2 : 33% CO_2 . If they are both occurring is similar in a mixed culture than the ratio of gas consumption is 8:3 $H_2:CO_2$ or approximately 72.7% H_2 to 27.3% CO_2 . Acetogenic-dominant and mixed acetogenic/methanogenic cultures are the most common scenarios in these experiments, so more CO_2 is being consumed than is being replaced.

This gradually leads to carbon limitation. Experiment #5 included a set of bottles with 100% H_2 , or near complete carbon limitation. This set had some microbial activity which was likely due to trace carbon from the inoculation waters, but acetic acid production was limited. pH was the highest out of all experiments, rising from 7.5 to 8.9. In experiments #4 and #6, the headspace gas was completely flushed at day 30 in an attempt to increase acetogenesis even some methane is lost. Flushing the headspace did not increase levels of acetic acid which still plateaued before day 30. The methane levels not only fully recovered but the overall peaks were higher. This may have been due to other differences in experiments (surface area, bicarbonate levels). But if it did contribute, it was through the removal of end product inhibition for methanogens. Future experiments could be improved by tracking CO_2 in the headspace. This is also useful in that it's a direct representation of the amount of carbon available to acetogens.

5.1.10 Nutrient variations

In experiments #3, #4 and #6, there is an effect rendered by different variations of low and high nutrient medium. Low nutrient medium had no added nitrogen, phosphorus, trace metals and tungstate-selenite. Changing four of the nutrients masked whether the increased

acetogenesis was due to a specific nutrient or a combination thereof. Some of the removed nutrients may only be harming the overall growth of the culture and are indistinguishable from those that promote acetogenic growth. Experiment #7 was conducted as more detailed study of the effects of each of these nutrients on acetogenesis.

Ammonium was assayed in experiment #4 to see whether the nitrogen was becoming limited. As expected, the levels of ammonium were higher for high nutrient and BESA medium and low nutrients were essentially zero. More importantly, these values were fairly steady over time making it unlikely that this is the limiting nutrient. Previous work by Y. Folarin (2013) and by Nathoo *et al.* (2012) enriched acetogens in medium with twice as much ammonium as the CSB-K variants used in this research. The levels of ammonium for all of the variants in experiment #7 were increased from 3.5 g/L NH_4Cl to 7.0 g/L. Tungsten is a co-factor essential for the Wood-Ljungdahl biochemical pathway so removing tungstate-selenite is probably harmful to acetogens. Tungstate-selenite was kept at normal concentration in all variants.

Since powdered CaCO_3 was used in the bottles, the comparable experiment is experiment #4. The high nutrient control in experiment #7 produced less than half of the acetic acid and less than a third of the methane than experiment #4. Since previous work with the higher nitrogen level produced more acetic acid it is unlikely that it's toxic this situation. The difference is due to the decreased volume of medium used in experiment #7, because though the absolute amount of acetic acid was lower the concentration was higher. The higher absolute amount of acetic acid seen in the high nutrient medium of experiment #9 could be due to the greater medium volume and headspace gas. The medium volume was increased from 100 mL to 125 mL and the headspace gas volume was increased from 50 mL to 125 mL. This explains why the concentration is lower (11 mM) though the absolute production was the highest at 1428 μmol . It

is important to use an absolute (μmol) value for measuring acetic acid levels so it can be directly compared to methane levels. Comparing concentration does not account differences in volume associated between aqueous acetic acid and gaseous methane.

Variations in salts do not appear to make any difference in acetogenic or methanogenic growth. There were no differences seen when NaCl concentration was increased or MgCl_2 and CaCl_2 concentrations were decreased. Removing or doubling added phosphorus also does not change growth of acetogens. Variations in trace metals showed a trend. Removing trace metals decreased acetogenesis and doubling trace metals increased acetogenesis but these two variations were not different from the high nutrient control which contains 1X (the regular level) trace metals. Methane is unaffected. More replicates may be needed when comparing 1X trace metals to 0X trace metals or 1X trace metals to 2X trace metals to achieve a significant difference. When compared directly, 0X trace metals is significantly less than 2X trace metals. This effect is not as large as the one seen when using low nutrient medium and methanogenesis doesn't decrease like it did for the low nutrient secondary enrichments for experiments #3, #4 and #6.

5.2 Evaluation as a Method for Microbially Enhanced Oil Recovery

5.2.1. Carbon dissolution: Absolute mass loss

CaCO_3 was originally added as a pH buffer but this also a prototype model for carbonate dissolution. Experiment #3 had solids, but added excess bicarbonate pushed the solubility equilibrium against dissolution. Following experiments were adjusted to become more and more representative of carbonate rock dissolution when exposed to the microbial acetic acid.

Experiment #4 is the first experiment where mass recovery measurements are viable as the

bicarbonate was removed to encourage dissolution. The number of replicates was increased to three to improve statistical power.

This means that there would be a lower level of inorganic carbon present in the medium and that carbon limitation might happen sooner. Unexpectedly, the levels of acetic acid and methane in the media were either the same as in experiment #3 or higher. The exception was methane production in BESA bottles which was less than in experiment #3. Methanogens and acetogens may not be adapted to excess carbon; it's still not as harmful as limited carbon. In removing the bicarbonate that the buffering capacity of medium would be reduced and acid-limitation would become a problem again. The pH did drop more than experiment #3, but it was still more buffered than experiment #1. pH remained in the 6.5 to 6.9 range which is a more favourable range for CaCO_3 dissolution.

All bottles had $1000 \text{ mg} \pm 10 \text{ mg}$ of CaCO_3 (experiment #4 and #5) or crushed carbonate rock (experiment #6) measured out before the start of the experiment. Sterile controls were crucial for detection of significant microbially caused dissolution because there was a strong possibility of abiotic loss due to sampling loss and chemical dissolution, especially for the powdered CaCO_3 . In the primary enrichment, the recovered material in the microbially active part of the experiment ranged from 828 to 949 mg. At first glance this suggests loss, but unfortunately the sterile controls had recovered values ranging from 824 to 971 mg. After statistical comparison the losses observed in the active bottles were not significantly different from the sterile controls. When net values were calculated by removing the control loss, low nutrient medium was positive suggesting a net gain, high nutrient medium was near zero and the BESA medium (which had the highest acetic acid levels and most acidity) was negative.

In the secondary enrichment of experiment #4, once sterile mass loss was accounted for all net changes in CaCO_3 were positive. The only active set that was significantly different from its control was the low nutrient medium in the secondary enrichment. Significantly *more* mass was recovered from microbially active low nutrient medium than the sterile control.

5.2.2 Chemical loss

Experiment #6 was similar to experiment #4 and #5 in terms of the low and high nutrient medium, but with significant mass losses in the BESA medium. This is true even though less acetic acid was produced in the BESA medium for experiment #6 than experiment #4. The main change for experiment #6 was going from powdered laboratory CaCO_3 to crushed Grosmont carbonate core, so it is likely that far less abiotic loss happened during sampling. Fine powdered CaCO_3 readily enters the sampling needle even with great care, whereas the larger carbonate core chunks were too large to go into the needle.

A potential source of chemical CaCO_3 mass loss is the increased partial pressure of CO_2 in headspace and medium. This would cause a higher level of carbonic acid to form in the medium and lead to more dissolution. Experiment #5 informs towards this as it uses sets of bottles where no CO_2 is present (100% H_2) and where maximum CO_2 is present (100% CO_2). There was a significant loss for in mass for low nutrient and BESA with 100% CO_2 headspace as compared to 100% H_2 . Chemical loss may contribute to mass loss in sterile controls, but 100% CO_2 resulted in a 7% dissolution at the greatest and the proportion of CO_2 used in the other experiments was a fifth of that (20%). Sampling loss is probably a larger source of error than chemical loss, but chemical loss could also contribute.

5.2.3. Sampling loss

The effect of such large sample loss in sterile controls can be observed when comparing the changes in mass to the amount of acetic acid produced. From a culturing perspective, producing nearly 2400 μmol of acetic acid or nearly 28 mM in medium (secondary enrichment BESA medium, experiment #4) is a success. Since the dissolution reaction is 1:1 in stoichiometry 2400 μmol of CaCO_3 should dissolve. 2400 μmol of CaCO_3 converts to 2.4 mmol which is 240 mg of CaCO_3 lost. About 760 mg of CaCO_3 should be recovered. Without sampling loss, this would mean 24% dissolution.

In the sterile control for that particular experiment, only 919 mg of CaCO_3 out of 1000 were recovered. Subtracting that 81 mg of sterile loss from the theoretical 240 mg of CaCO_3 dissolved gives 159 mg lost or 841 mg of CaCO_3 recovered which is 15.9% dissolution. An average of 887.8 ± 13.17 mg of CaCO_3 was actually recovered for that set of bottles which not significantly different from the mean sterile value of 919 mg. If the confounding effects of sampling loss and high variation could be contained, it is still possible that dissolution could be observed.

Another issue is that 1000 mg of CaCO_3 converts to 10 mmol which is 10,000 μmol . Looking for a loss of 1590 μmol (159 mg) loss in an original mass of 10,000 μmol could be masked by the high variation seen in the three replicates. Raw mass recovery values for the samples presented above were 911, 887 and 865.4 mg. The sterile recovery values were 995.5, 856.4, and 906.4 mg. In experiment #6 statistically significant loss was observed. 867.2 mg (13.3% loss) was recovered in the active bottles and 929 mg in the sterile controls (7.1% loss).

This is a net recovery of 938.2 mg (6.1% net loss). The amount of mass lost was smaller than observed in experiment #4, but experiment #6 probably had lower sampling loss.

A comparative example can be taken from previous work by Nathoo *et al.* (2012). Crushed carbonate core (0.1 g) was added to 50 mL of medium in which 17 mM of acetic acid was produced. An average weight loss of 25% was observed. When converted to micromoles, 17 mM of acetic acid in 50 mL of medium is 850 μmol of acetic acid. This amount of CaCO_3 converts to 100 mg or 1000 μmol of carbonate rock. The means that 850 μmol is the maximum amount of carbonate that could have been dissolved out of a total of 1000 μmol of CaCO_3 . The amount of carbonate that could be dissolved versus the total amount present is a ratio of 0.85 to 1. A maximum dissolution of 85% could have been observed. The lower dissolution value that was actually achieved is reasonable as crushed core has lower surface area and is composed mostly of dolomite which is more difficult to dissolve. In experiment #4, this ratio is 0.24 to 1 (2400 to 10,000). It used powdered CaCO_3 that has a greater surface area and is more easily dissolved. If a lower initial amount of CaCO_3 had been used the loss of mass may have been statistically significant.

5.2.4 Re-precipitation

A third issue with dissolution is re-precipitation. In some of the later experiments that used smaller amounts of CaCO_3 , a dark unidentified precipitate was observed forming in the bottles. This precipitate may have also occurred in earlier bottles but was concealed by the higher amount of CaCO_3 . If it was present it could have added mass to the bottles. Precipitation only occurred in some bottles, irrespective of medium type. Some identical replicates had it and others didn't, so sterile controls would not account for this.

Precipitation is especially seen in experiment #8. $50 \text{ mg} \pm 5 \text{ mg}$ of CaCO_3 was used or $500 \text{ } \mu\text{mol}$ (20 mM). In the doubled trace metal variation, $424 \text{ } \mu\text{mol}$ of acetic was produced. In the active 2xTM bottles, an average of 54.3 mg of CaCO_3 was recovered, with 48 mg recovered in the sterile control. The mass loss was less than 2 mg in the sterile control, but precipitation appears to have caused an increase in mass in all bottles. For the three replicates, 64.5 , 51.9 and 46.4 mg of CaCO_3 were recovered in the active bottles and 35.5 , 47.4 and 63.9 mg were recovered in the sterile controls. Precipitation may have occurred, especially for the values above 60 mg that were greater than instrumental error ($\pm 5 \text{ mg}$). Even if those two greater than 60 mg values were removed, the additional sterile control loss prevents a significant loss of CaCO_3 mass from being found.

5.2.5 Carbonate core dissolution

In experiment #9 mass loss was calculated instead of mass recovery for the cores. Porosity and permeability were also determined. Sampling loss is unlikely with the large solid core and large chunks of free mass. Since the masses of the cores could not be standardized, the amount of mass lost from each core was by dividing the changes in mass by the final core mass. The high nutrient medium produced $1428 \text{ } \mu\text{mol}$ of acetic acid. The initial masses of the cores in the high nutrient enrichment were all over 60 grams or $\approx 600,000 \text{ } \mu\text{mol}$. A mean of $118 \pm 21.8 \text{ mg}$ was lost from these cores or $0.162 \pm 0.03\%$. 118 mg is $1180 \text{ } \mu\text{mol}$ of CaCO_3 or $639.9 \text{ } \mu\text{mol}$ of dolomite ($\text{MgCa}(\text{CO}_3)_2$). Even though the amount dissolved is tiny, without abiotic loss this could be considered reasonable dissolution for the amount of acetic acid that was produced.

However 76.2 mg or 0.109% mass was lost in the sterile control. The amount lost in the high nutrient medium is still not significantly more than the loss in the sterile control. Also not

accounted for is the free mass recovered separately from the core. Subtracting 24.5 ± 0.59 mg of free mass from the 118 mg only drives that value closer to that of the sterile control. Precipitation was also observed in one replicate of the high nutrient medium.

Porosity and permeability were measured before and after microbial treatment. Porosity was unaffected. Permeability increased in both medium types and with the controls. There was a substantial increase in permeability with the controls. It is possible that most of the increase is due to chemical dissolution of cores due to incubation in water with a high $p\text{CO}_2$. But the results of experiment #5 do not support this, so it is mostly likely a result of dry CaCO_3 coming into contact with water. The only statistically significant difference from the controls was that high nutrient medium had a significantly lower increase in permeability than the control. It appears that microbial activity counteracted most of the sample loss and acted more as a form of biopugging than acidic carbonate dissolution.

CHAPTER 6 CONCLUSIONS

6.1. Acetogenesis optimization

In terms of the objective of acetogenesis optimization, it appears the factors most important to increasing acetogenesis are sufficient pH buffering, the addition of solids, subculturing (making a secondary enrichment) and removing bicarbonate. Adding BESA definitely increases acetogenesis, but adding methanogenic inhibitors would not be feasible to a field-wide *in-situ* treatment especially considering BESA has an ionic chemical structure which means that it is not oil soluble and would not penetrate into the reservoir.

Nutrient restriction is important, but the specific nature of this effect and exactly what the ideal combination of nutrients is, is still unclear. The severe carbon limitation in experiment #5 affected methanogenesis more than acetogenesis, but acetogenesis is still strongly limited and limiting CO₂ would defeat the purpose of using this microbial reaction for a CO₂ capture biotechnology. No ammonium or nitrogen restriction was found, but doubling the ammonium had a protective effect against acetic acid loss. There was also no loss in medium where methanogenesis was inhibited (low nutrients, BESA) but enrichment of acetotrophic methanogens showed to late within the enrichment to account for the acetic acid loss.

Adding CaCO₃ increased the production of both acetic acid and methane. It may be that it was pH buffering that increased acetogenesis and that it was the higher available surface area of CaCO₃ that increased methanogenesis. This is reasonable as other experiments found that decreasing the available surface area decreased methanogenesis more than acetogenesis. It may be useful to find a way to separate these two effects to increase acetogenesis without a concurrent increase in methanogenesis. Otherwise, only subculturing in the low nutrient medium

(to create an ultra-low nutrient environment) gave acetogens a true advantage over methanogens. The other factors altered the activity of both groups of microbes.

16S rRNA microbial analysis shows the succession of microbial communities grouped by function. When interpreted alongside with activity data it shows that acetogenesis is dominant in the first two weeks of growth which is reasonable since most acetic acid is produced during this time. The microbial community then changes to become composed mostly of methanogens and then the community shifts to microbes that are capable of building up stores of carbon by consuming acetate to create polyhydroxyalkanoates like polyhydroxybutyrate (PHB) in intracellular inclusions bodies. An acetogenesis-related treatment should be confined to within 7 to 14 days. This would also minimize the problem of concurrent methane production which would allow the use of methods that promote both types of growth. The DNA-related results were useful for confirming certain trends, but they have some limitations and do not perfectly aligned with changes in microbial activity. There were other metabolic processes in the bottles related to oxygen ingress and the use of sulfide as an oxygen scavenger. This invalidated the results in a few cases, but these effects were minor overall and did not appear to suppress acetogenic or methanogenic activity.

6.2. Evaluation as a potential microbial enhanced oil recovery

The capacity for acetogenic activity to dissolve carbonate rock is inconclusive. Sampling loss is definitely obscuring any dissolution that may be taking place. Re-precipitation also needs to be confirmed. If it is happening it could be an important part of the reason that absolute mass loss is showing no difference from control even where substantial acetic acid is being produced. Another experimental issue has been the proportion of CaCO_3 added to acetic acid that is

produced in these cultures. The chances that dissolution will be indistinguishable from statistical noise and variation increased when 10,000 μmol of CaCO_3 is used when the highest acetic acid level that can be achieved is 2400 μmol . More problematic is that even if full dissolution is occurring and 2400 μmol of carbonate rock is dissolved, this is only 240 mg of rock in cores that are over 70 g in mass (70,000 mg). Less than 0.5% sample loss is the maximum that could be achieved even under ideal conditions with no sample loss and no re-precipitation.

6.3. Future work

If absolute dissolution experiments are to be repeated, the amount of CaCO_3 added should be closer to the anticipated amount of acetic acid. 100 mg or 1000 μmol of CaCO_3 (or carbonate rock) would be reasonable. Efforts should also be taken to minimize sampling loss. Probably the best method for this would be to minimize the number of sampling days, perhaps only sampling at the beginning of the experiment and after 20 days. Incubations longer 30 days are unnecessary for analyzing acetogenesis as it typically peaks within 2 weeks. Further study is also warranted to look for differences between biofilm and planktonic cultures for acetogens and methanogens.

Microbial culture appears to plug cores with biomass and reduce permeability more than acetic acid dissolves rock. However, these two effects are not necessarily antagonistic. If the acetic acid *is* causing dissolution, the microbial biomass could be useful in pushing displaced bitumen from the rock. But this is not detectable with the current analyses, so bitumen-containing carbonate cores are probably required. Re-precipitation could be confirmed through examination with scanning electron microscopy (SEM).

It appears that acetic acid levels need to be increased if dissolution is to overcome the biopugging effect of microbial mass. One way to further increase acetic acid levels would be to closely monitor CO₂ levels to ensure carbon limitation does not occur as this appears to inhibit both acetogenesis and methanogenesis. Re-flushing the headspace gas once limitation has occurred appears to be ineffective at increasing acetic acid and increases methanogenesis. Adding back CO₂ manually would be more effective or the experimental setup could be moved from batch culture to continuous culture.

Another possibility is studying the effect of medium volume and headspace gas. Medium volume has a much stronger effect on the level of acetic acid production than originally expected. A comparison of three experiments of similar medium (without BESA) is demonstrative. A proportion of 70 mL of headspace to 50 mL of medium (a ratio of 1.4) was used in Nathoo *et al.* (2012) which used the same medium. 850 µmol of acetic was produced with 490 µmol of methane. Experiment #4 (No bicarbonate) of this thesis used a ratio of 50 mL headspace to 100 mL of medium (a ratio of 0.5) and produced a maximum of 776 µmol acetic acid and 1155 µmol of methane in the low nutrient medium (slightly less acetic acid, more methane). Experiment #9 (Core) produced the most acetic acid (1428 µmol) and the least amount of methane (138 µmol) in any non-BESA medium with 125 mL of medium with 125 mL of headspace gas (a ratio of 1). It appears that the ideal ratio to achieve maximum acetic acid and minimum methane is somewhere around 1, but it is definitely between 0.5 and 1.4.

The overall volume of medium appears to be important as well. Experiment #7 used 25 mL of medium with 25 mL of headspace gas, the same ratio as experiment #9, but it produced a maximum of only 442 µmol of acetic acid. Further study is required to attain a more precise

measurement of the best volume of medium and ratio of medium to headspace to promote acetogenesis.

CHAPTER 7 REFERENCES

- Alberquerque, L., Tiago, I., Nobre, I.F., Veríssimo, A., and da Costa, M.S. 2013. *Cecembia calidifontis* sp. nov., isolated from a hot spring runoff, and emended description of the genus *Cecembia*. *Int. J. Syst. Evol. Microbiol.* 63:1431-1436.
- Andreesen J.R., Ljungdahl L.G. 1973. Formate dehydrogenase of *Clostridium thermoaceticum*: incorporation of selenium-75, and the effects of selenite, molybdate, and tungstate on the enzyme. *J. Bacteriol.* 116:867-873.
- Bachu, S., Underschultz, J.R., Hitchon, B., Cotterill, D. 1993. Regional-scale subsurface hydrogeology in northeast Alberta. *Alberta Research Council Bull.* 61.
- Baronofsky, J.J., Schreurs, W.J.A. and Kashket, E.R. 1984. Uncoupling by acetic acid limits growth of and acetogenesis by *Clostridium thermoaceticum*. *Appl Environ Microbiol.* 48:1134-1139.
- Battley, E.H. 1996. On the thermodynamics of autotrophic and heterotrophic growth of *Pseudomonas saccharophila*. *Can. J. Microbiol.* 42:38-45.
- Bernard, L. Mougél, C., Maron, P. Nowak, V., Lévêque, J., Henault, C., Haichar, F.Z., Berge, O., Marol, C., Balesdent, J., Gibiat, F., Lemanceau, P., and Ranjard, L. 2007. Dynamics and identification of soil microbial populations actively assimilating carbon from ¹³C-labelled wheat residue as estimated by DNA- and RNA-SIP techniques. *Env. Microbiol.* 9(3):752-764.
- Bogdahn, M., Andreesen, J.R., Kleiner, D. 1983. Pathways and regulation of N₂, ammonium and glutamate assimilation by *Clostridium formicoaceticum*. *Arch. Microbiol.* 134: 167-169.
- Chen, S., and Dong, X. 2005. *Proteiniphilum acetatigenes* gen. nov., sp. nov., from a UASB reactor treating brewery wastewater. *Int. J. Syst. Evol. Microbiol.* 55:2257-2261.
- Chou, L., Garrels, R.M., and Wollast, R. 1989. Comparative study of the kinetics and mechanisms of dissolution of carbonate minerals. *Chem. Geol.* 78:269-282.
- Davydova-Charakhch'yan, I.A., Mileeva, A.N., Mityushina, L.L. and Belyaev, S.S. 1993. Acetogenic bacteria from oil fields of Tataria and western Siberia. *Microbiol.* 61:306-315.
- Drake, H.L., Daniel, S.L., Küsel, K., Matthies, C., Kuhner, C., Braus- Stromeyer, S. 1997. Acetogenic bacteria: what are the *in situ* consequences of their diverse metabolic versatilities? *Biofactors.* 6: 13-24.
- Drake, H.L., Gobner, A.S., Daniel, S.L. 2008. Old Acetogens New Light. *Ann. N.Y. Acad. Sci.* 1125:100-128.

- Drake, H.L., Hu, S.I., Wood, H.G. 1981. Purification of five components from *Clostridium thermoaceticum* which catalyze synthesis of acetate from pyruvate and methyltetrahydrofolate. Properties of phosphotransacetylase. *J. Biol. Chem.* 256:11137–11144.
- Drake, H.L., Küsel, K. and Matthies, C. 2006. Acetogenic prokaryotes. The Prokaryotes (Balows, A., Trüper, H.G., Dworkin, M., Harder, W. and Schleifer, K.H., eds), pp. 354–420. Springer, New York.
- Edmunds, N., Barrett, K., Solanki, S., Cimolai, M., Wong, A. 2008. Prospects for commercial bitumen recovery from the Grosmont carbonate, Alberta, 2008 *Cdn Int. Petrol. Conf.* Paper 2008-154. pp. 12.
- El Ghazzawi, E. 1967. Neuisolierung von *Clostridium aceticum* Wieringa und stoffwechselphysiologische untersuchungen. *Arch. Microbiol.* 57: 1–19.
- Ezeuko, C.C., Kallos, M.S., Gates, I.D. 2013. Object characterization and simulation of thermal recovery from karstified, brecciated and fractured bitumen carbonate reservoirs. *Int. Petrol. Tech. Conf.* IPTC 16860.
- Federovich, V., Knighton, M.C., Pagaling, E., Ward, F.B., Free, A., and Goryanin, I. 2009. Novel electrochemically active bacterium phylogenetically related to *Arcobacter butzleri* isolate from a microbial fuel cell. *App. Env. Microbiol.* 75(23):7326-7334.
- Fernandez, N., Diaz, E.E., Amils, R., Sanz, J.L. 2008. Analysis of microbial community during biofilm development in an anaerobic wastewater treatment reactor. *Microbiol. Ecol.* 56:121-132.
- Ferry, J.G. 1999. Enzymology of one-carbon metabolism in methanogenic pathways. *FEMS Microbiol. Ecol.* 23:13–38.
- Fischer, F., Lieske, R., Winzer, K. 1932. Biologische Gasreaktionen. II. Über die Bildung von Essigsäure bei der biologischen umsetzung von kohlenoxyd und kohlensäure mit wasserstoff zu *Methan.* *Biochem. Z.* 245: 2–12.
- Folarin, Y.O. 2013. Roles of acetogens and methanogens in carbon capture and storage [dissertation]. University of Calgary. 10 p.
- Folarin, Y., An, D., Caffrey, S., Soh, J., Sensen, C.W., Voordouw, J., Jack, T., and Voordouw, G. (2012). Contribution of make-up water to the microbial community in an oilfield from which oil is produced by produced water re-injection. *Int. Biodeter. & Biodeg.* XXX. 1-7.
- Fontaine, F.E., Peterson, W.H., McCoy, E., Johnson, M.J. 1942. A new type of glucose fermentation by *Clostridium thermoaceticum* n. sp. *J. Bacteriol.* 43: 701–715.
- Fujita, M., Ike, M., Tachibana, S., Kitada, G., Kim, S.M., and Inoue, Z. 2000. Characterization of a bioflocculant produced by *Citrobacter* sp-. TKF04 from acetic and propionic acids. *J. Biosci. Bioeng.* 89(1):40-46.

- Göbner, A.S., Devereux, R., Ohnemüller, N., Acker, G., Stackebrandt, E., Drake, H.L. 1999. *Thermicanus aegyptius* gen. nov., sp. nov., isolated from oxic soil, a fermentative microaerophile that grows commensally with the thermophilic acetogen *Moorella thermoacetica*. *Appl. Environ. Microbiol.* 65: 5124–5133.
- Göbner, A.S., Picardal, F., Tanner, R.S. and Drake, H.L. 2008. Carbon metabolism of the moderately acid-tolerant acetogen *Clostridium drakei* isolated from peat. *FEMS Microbiol. Letters.* 287(2):236-242.
- Gottschalk, G., Braun, M. 1981. Revival of the name *Clostridium aceticum*. *Int. J. Syst. Bacteriol.* 31: 476.
- 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 Microbiol. Ecol.* 54:427-443.
- Grasby, S. E., Chen, Z. 2005. Subglacial recharge into the western Canada sedimentary basin—Impact of Pleistocene glaciation on basin hydrodynamics, *Geol. Soc. Am. Bull.*, 117(3/4), 500–514.
- Gray, M.R., Yeung, A., Foght, J.M., Julia, M., Harvey, Y.W. 2008. Potential microbial enhanced oil recovery processes: A critical analysis. *Soc. of Petrol/ Eng.* SPE 114676.
- Grigoryan, A.A., Cornish, S.L., Buziak, B., Lin, S., Cavallaro, A., Arensdorf, J.J., and Voordouw, G. 2008. Competitive oxidation of volatile fatty acids by sulfate- and nitrate-reducing bacteria from an oil field in Argentina. *App. Env. Microbiol.* 74(14):4324-4335.
- Herman, M., Vandecasteele, J.P., Ballerini, D. 1992. Anaerobic microflora of oil reservoirs – Microbiological characterization of samples from some production wells. In: *Bacterial Gas* (Vially, R., Ed.), pp. 223–234. Editions Technip, Paris.
- Hiraishi, A., Yonemitsu, Y., Matsushita, M., Shin, Y.K., Kuraishi, H., and Kawahara, K. 2002. Characterization of *Porphyrobacter sanguineus* sp. nov., an aerobic bacteriochlorophyll-containing bacterium capable of degrading biphenyl and dibenzofuran. *Arch. Microbiol.* 178:45-52.
- Hubert, C., and Voordouw, G. 2007. Oil field souring control by nitrate reducing *Sulfurospirillum* spp. that outcompete sulfate-reducing bacteria for organic electron donors. *App. Env. Microbiol.* 73(8):2644-2652.
- Hui, L., Mingqiang, A., Siqin, H., Yanling, G., Nana, L., Rongjiu, S., Xiangling, Z., Shengbing, L., Jinyi, Z., Xiaobo, L., and Ying, Z. 2012. Microbial diversity and functionally distinct groups in produced water from the Daqing Oilfield, China. *Pet. Sci.* 9:469-484.

- Huse, S.M., Huber, J.A., Morrison, H.G., Sogin, M.L., and Welch, D.M. 2007. Accuracy and quality of massively parallel DNA pyrosequencing. *Genome Biology*. **8**:R143 (doi:10.1186/gb-2007-8-7-r143).
- Jarrahan, K., Seiedi, O., Sheykhan, M., Vafaie Sefti, M., Ayatollahi, S. 2012. Wettability alteration of carbonate rocks by surfactants: a mechanistic study. *Colloids and Surfaces A: Physicochem. Eng. Aspects*. 410:1-10.
- Jiang Q. J., Yuan J., Russel-Houston J., Thornton B., Squires A. 2010. Evaluation of recovery technologies for the Grosmont carbonate reservoirs. *J. Cdn Petrol. Tech.* 49:56–64.
- Khan, A.W. 1977. Anaerobic degradation of cellulose by mixed culture. *Can. J. Microbiol.* 23:1700-1705.
- Kotsyurenko, 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 Microbiol. Ecol.* 38:153-159.
- Kowalewski, E., Rueslåtten, I., Steen, K.H., Bødtker, G., and Torsæter, O. 2006. Microbial improved oil recovery – bacterial induced wettability and interfacial tension effects on oil production. *J. Petrol. Sci. Eng.* 52:275-286.
- Kryachko, Y., Dong, X., Sensen, C.W., and Voordouw, G. 2012. Composition of microbial communities associated with oil and water in a mesothermic oil field. *Anton. van Lee.* 101:493-506.
- Langmuir, D. 1997. Aqueous Environment Geochemistry. The University of California (CA): Prentice Hall.
- Lakshman, K., Rastogi, N.K., and Shamala, T.R. 2004. Simultaneous and comparative assessment of parent and mutant strain of *Rhizobium meliloti* for nutrient limitation and enhanced polyhydroxyalkanoate (PHA) production using optimization studies. *Proc. Biochem.* 39:1977-1983.
- Larrabee, A.R., Rosenthal, S., Cathou, R.E., Buchanan, J.M. 1963. Enzymatic synthesis of the methyl group of methionine. Isolation, characterization, and role of 5-methyl tetrahydrofolate. *J. Biol. Chem.* 238:1025.
- Lapado, J., Whitman, W.B. 1990. Method for isolation of auxotrophs in the methanogenic archaeobacteria: role of the acetyl-CoA pathway of autotrophic CO₂ fixation in *Methanococcus maripauludis*. *Proc. Natl. Acad. Sci. USA.* 87:5598–5602.
- Lee, S.Y., Lee, K.M., Chang, H.N., and Steinbüchel, A. 1994. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly-(3-hydroxybutyric acid) and morphological changes. *Biotechnol. Bioeng.* 44:1337-1347.

- Lee, Y.J., Romanek, C.S., and Wiegel, J. 2007. *Clostridium aciditolerans* sp. nov., an acid-tolerant spore-forming anaerobic bacterium from constructed wetland sediment. *Int. J. Syst. Evol. Microbiol.* 57:311-315.
- Lever, M.A., Heuer, V.B., Morono, Y., Masui, N., Schmidt, F., Alperin, M., Inagaki, F., Hinrichs, K., and Teske, A. 2010. Acetogenesis in deep subseafloor sediments of the Juan de Fuca ridge flank: a synthesis of geochemical, thermodynamic, and gene-based evidence. *J. Geomicrobiol.* 27:183-211.
- Ljungdahl, L., Irion, E., Wood, H.G. 1966. Role of corrinoids in the total synthesis of acetate from CO₂ by *Clostridium thermoaceticum*. *Fed. Proc.* 25:1642–1648.
- Ljungdahl, L.G., Drake, H.L. 1994. The acetyl-CoA pathway and the chemiosmotic generation of ATP during acetogenesis. In *Acetogenesis*. New York (NY): Chapman & Hall. 63–87 p.
- Lodwig, E.M., Leonard, M. Marroqui, S., Wheeler, T.R., Findlay, K., Downie, J.A., and Poole, P.S. 2005. Role of polyhydroxybutyrate and glycogen as carbon storage compounds in pea and bean bacteroids. *Am. Phytopath. Soc.* 18(1):67-74.
- Ma, J. (2011). Methanogenesis Mechanical Pathway Map [Internet]. University of Minnesota. [cited 2013 March 12]. Available from http://umbbd.ethz.ch/meth/meth_image_map.html [2011 January 26].
- Machel, H.G., Borrero, M., Dembicki, E., Huebscher, H., Luo, P., Zhao, Y. 2012. Advances in carbonate exploration and reservoir analysis, The Grosmont: the world's largest unconventional oil reservoir hosted in carbonate rocks. *Geo. Soc, London, Special Publications.* 370: 49-81.
- Machel, H.G., Borrero, M., Dembicki, E., Heubscher, H., Ping, L., Zhao, Y. 2012. The Grosmont: a complex heavy oil reservoir in a Devonian carbonate-evaporite platform. *GeoConvention 2012: Vision.*
- Majone, M., Beccari, M., Gregorio, S.D., Dionisi, D., and Vallini, G. 2006. Enrichment of activated sludge in a sequencing batch reactor for polyhydroxyalkanoate production. *Water Sci. Tech.* 54(1):119-128.
- Mao, J., Luo, Y., Teng, Y., and Li, Z. 2012. Bioremediation of polycyclic aromatic hydrocarbon-contaminated soil by a bacterial consortium and associated microbial community changes. *Biodet. Biodeg.* 70:141-147.
- Mayumi, D., Hanako, M., Hideyoshi, Y., Susumu, S., Haruo, M., Yoshihiro, M., Masayuki, I., Mio, T., Yoichi, K.. 2010. Evidence for syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis in the high-temperature petroleum reservoir of Yabase oil field (Japan). *Environ. Microbiol.* 13(8):1995-2006.
- McDougall, M., Alvarez, J.M., Isaacs, E. 2008. Alberta Carbonates: the Third Trillion. *One Petro.* 19-0925, July 2008.

Mori, K., Tsurumaru, H., and Harayama, S. 2010. Iron corrosion activity of anaerobic hydrogen-consuming microorganisms isolated from oil facilities. *J. Biosci. Bioeng.* 110(4):426-430.

Mossop, G. D., Shetsen, I. 1994. Introduction to geologic atlas of the Western Canada sedimentary basin. *Canadian Society of Petroleum Geologists and Alberta Research Council*.
Müller, V. 2003. Energy conservation in acetogenic bacteria. *Appl. Environ. Microbiol.* 69: 6345–6353.

Nathoo, S., Folarin, Y., and Voordouw, G. 2012. Potential of microbial formation of acetic acid from hydrogen and carbon dioxide for permeability modification in carbonate reservoirs. *World Heavy Oil Congress*. Aberdeen, UK, Paper WHOC-12.

Numata, K., Morisaki, K., Tomizawa, S., Ohtani, M., Demura, T., Miyazaki, M., Nogi, Y., Deguchi, S., and Yoshiharu, D. 2013. Synthesis of poly- and oligo(hydroxyalkanoate)s by deep-sea bacteria, *Colwellia* spp., *Moritella* spp., and *Shewanella* spp. *Polymer J.* 45:1094-1100.

Okabe, S., Nielsen, P.H., and Characklis, W.G. 1992. Factors affecting microbial sulfate reduction by *Desulfovibrio desulfuricans* in continuous culture: limiting nutrients and sulfide concentration. *Biotech. Bioeng.* 40:725-734.

Park, E.Y., Clark, J.E., DerVartanian, D.V., Ljungdahl, L.G. 5,10-methylenetetrahydrofolate reductases: iron-sulfur-zinc flavoproteins of two acetogenic clostridia. 1991. Chemistry and Biochemistry of Flavoenzymes. Boca Raton (FL): CRC Press. 389-400 p.

Pierce, E., Xie, G., Barabote, R. D., Saunders, E., Han, C. S., Detter, J. C., Richardson, P., Brettin, T. S., Das, A., Ljungdahl, L. G., Ragsdale, S. W. 2008. The complete genome sequence of *Moorella thermoacetica* (f. *Clostridium thermoaceticum*). *Environ. Microbiol.* 10:2550–2573.

Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J., and Glockner, F.O. 2007. SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. *Nucleic Acids Research.* 35(21):7188-7196. (doi:10.1093/nar/gkm864).

Rademacher, A., Zakrzewski, M., Schlüter, A., Schönberg, M., Szczepanowski, R., Goesmann, A., Pühler, A., and Klocke, M. 2012. Characterization of microbial biofilms in a thermophilic biogas system by high-throughput metagenome sequencing. *FEMS Microbiol. Ecol.* 79:785-799.

Ragsdale, S.W. 1997. The Eastern and Western branches of the Wood/Ljungdahl pathway: how the East and West were won. *BioFactors.* 9:1–9.

Ragsdale, S.W. 2008. Catalysis of methyl group transfers involving tetrahydrofolate and B12. Vitamins and Hormones, Folic Acid and Folates. Amsterdam (The Netherlands): Elsevier, Inc.

Ragsdale, S.W. 2006. Metals and their scaffolds to promote difficult enzymatic reactions. *Chem Rev.* 106:3317–3337.

Ragsdale, S. W., Pierce, E. 2008. Acetogenesis and the Wood-Ljungdahl pathway of CO₂ fixation. *Biochim. Biophys. Acta*. 1784:1873-1898.

Raskin, L., Rittmann, B.E., and Stahl, D.A. 1996. Competition and coexistence of sulfate-reducing and methanogenic populations in anaerobic biofilms. *App. Env. Microbiol.* 62(10):3847-3857.

Riedel, T., Fiebig, A., Göker, M., and Klenk, H. 2013. Complete genome sequence of the bacteriochlorophyll a-containing *Roseibacterium elongatum* type strain (DSM 19469^T), a representative of the *Rosebacter* group isolated from Australian coast sand. *Stand. Genomic. Sci.* 9:3.

Sarafzadeh, P., Hezave, A.Z., Ravanbakhsh, M., Niazi, A., Ayatollahi, S. 2013. *Enterobacter cloacae* as a biosurfactant producing bacterium: Differentiating its effects on interfacial tension and wettability alteration. Mechanisms for oil recovery during MEOR process. *Colloids and Surfaces B: Biointerfaces*. 105:223-229.

Schidlowski, M., Hayes, J.M., Kaplan, I.R. 1983. Earth's Earliest Biosphere: Its Origin and Evolution. Princeton (NJ): Princeton Univ. Press. 1983. 149-186 p.

Schloss, P.D., Westcott, S.L., Ryabin, T., 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 mother: open-source, platform-independent, community-supported software for describing and comparing microbial communities. *App. and Env. Microbiol.* 75(23):7537-7541. (doi: 10.1128/AEM.01541-09).

Seifritz, C., Daniel, S.L., Göbner, A., Drake, H.L. 1993. Nitrate as a preferred electron sink for the acetogen *Clostridium thermoaceticum*. *J. Bacteriol.* 175: 8008– 8013.

Soh, J., Dong, X., Caffrey, S.M., Voordouw, G., Sense, C.S. 2013. Phoenix 2: a locally installable large-scale 16S rRNA gene sequence analysis pipeline with Web interface. *J. Biotechnol.* 167:393-403.

Kulik, M.M. 1995. The potential for using cyanobacteria (blue-green algae) and algae in the biological control of plant pathogenic bacteria and fungi. *E.J. Plant. Path.* 101:585-599.

Spiekermann, P., Rehm, B.H.A., Kalscheuer, R., Baumeister, D. and Steinbüchel, A. 1999. A sensitive, viable-colony staining method using Nile red for direct screening of bacteria that accumulate polyhydroxyalkanoic acids and other lipid storage compounds. *Arch. Microbiol.* 171:73-80.

Stanton, M.S. 2004. Origin of the Lower Cretaceous Heavy Oil (“Tar Sands”) of Alberta. *Search and Discovery*. Article No. 10067.

- Stolz, J.F., Ellis, D.J., Blum, J.S. Ahmann, D., Lovley, D.R. and Oremland, R.S. 1999. *Sulfurospirillum barnesii* sp. nov. and *Sulfurospirillum arsenophilum* sp. nov., new members of the *Sulfurospirillum* clade of the ϵ proteobacteria. *Int. J. Syst. Bacteriol.* 49:1177-1180.
- Thevenieau, F., Fardeau, M.L., Ollivier, B., Joulain, C., and Baena, S. 2007. *Desulfomicrobium thermophilum* sp. nov., a novel thermophilic sulphate-reducing bacterium isolated from a terrestrial hot spring in Colombia. *Extremophiles.* 11:295-303.
- Uchima, K., Saito, T., Gebauer, B., and Jendrossek, D. 2007. Isolated poly(3-hydroxybutyrate) (PHB) granules are complex bacterial organelles catalyzing formation of PHB from acetyl coenzyme A (CoA) and degradation of PHB to acetyl-CoA. *J. Bacteriol.* 189(22):8250-8256.
- Urbance, J.W. Bratina, B.J. Stoddard, S.F., and Schmidt, T.M. 2001. Taxonomic characterization of *Ketogulonigenium vulgare* gen. nov., sp. nov. and *Ketogulonigenium robustum* sp. nov., which oxidize L-sorbose to 2-keto-L-gulonic acid. *Int. J. Syst. Evol. Microbiol.* 51:1059-1070.
- Volova, T.G., Kalacheva, G.S., and Altukhova, O.V. 2002. Autotrophic synthesis of polyhydroxyalkanoates by the bacteria *Ralstonia eutropha* in the presence of carbon monoxide. *App. Microbiol. Biotechnol.* 56:675-678.
- Wieringa, K.T. 1936. Over het verdwijnen van waterstof en koolzuur onder anaerobe voorwaarden. *Antonie Leeuwenhoek.* 3: 263–273.
- Wirsén, C.O., Sievert, S.M., Cavanaugh, C.M., Molyneux, S.J., Ahmad, A., Taylor, L.T., DeLong, E.F., and Taylor, C.D. 2002. Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. *App. Env. Microbiol.* 68(1):316-325.
- Wo, E., Song, L., Hurst, T., Sitek, N. 2011. Geological review and bitumen resource appraisal of the Grosmont formation within the Athabasca Oil Sands Area. Search and Discovery Article no. 80130 [Internet]. Available at: <http://www.searchanddiscovery.com/documents/2004/stanton/images/stanton.pdf>.
- Wood, H.G., Ljungdahl, L.G. Autotrophic character of acetogenic bacteria. 1991. *In Variations in Autotrophic Life*. San Diego (CA): Academic Press. 201-250 p.
- Wu, J., Chuang, H., Hsu, M., and Chen, W. 2013. Use of a hierarchical oligonucleotide primer extension approach for multiplexed relative abundance analysis of methanogens in anaerobic digestion systems. *App. Env. Microbiol.* 79(24):7598-7609.
- Xie, C., and Yokota, A. 2005. Reclassification of *Alcaligenes latus* strains IAM 12599T and IAM 12664 and *Pseudomonas saccharophila* as *Azohydromonas lata* gen. nov., comb. nov., *Azohydromonas australica* sp. nov. and *Pelomonas saccharophila* gen. nov., comb. nov., respectively. *Int. J. Syst. Evol. Microbiol.* 55:2419-2425.

- Yokoi, H., Mori, S., Hirose, J., Hiyashi, S., and Takasaki, Y. 1998. H₂ production from starch by a mixed culture of *Clostridium butyricum* and *Rhodobacter* sp. M-19. *Biotech. Letters*. 20(9):895-899.
- Young, R.T., and Kim, Y.M. 1993. Constitutive expression of carbon monoxide dehydrogenase in *Acinetobacter* sp. strain JC1 DSM 3803. *Kor. J. Microbiol.* 31(3):214-217.
- Youssef N., Elshahed M.S., McInerney, M.J. 2009. Chapter 6: Microbial processes in oil fields: culprits, problems, and opportunities. *Adv Appl Microbiol.* 66:141–251.
- Zhao, D., Liu, C., Liu, L., Zhang, Y., Liu, Q., and Wu, W. 2011. Selection of functional consortium for crude oil-contaminated soil remediation. *Int. Biodeter. & Biodeg.* 65:1244-1248.
- Zhao, Y., and Machel, H.G. 2011. Biodegradation characteristics of bitumen from the Upper Devonian Grosmont Reservoir, Alberta, Canada. *Bulletin Cdn Petrol. Geol.* 59:112–130.
- Zhao, Y., and Machel, H.G. 2012. Viscosity and other rheological properties of bitumen from the Upper Devonian Grosmont reservoir, Alberta, Canada. *AAPG Bulletin.* 96(1):133-153.
- Zaika, L.L., Moulden, E.E., Weimer, L., Phillips, J.G., and Buchanan, R.L. 1993. Model for the combined effects temperature, initial pH, sodium chloride and sodium nitrite concentration on anaerobic growth of *Shigella flexneri*. *Int. J. Food Microbiol.* 23:345-358.