

**APPLICATION OF STABLE ISOTOPE PROBING AND HIGH-THROUGHPUT  
SEQUENCING TO IDENTIFY MICROORGANISMS IN MICROBIAL FUEL CELLS**

**By**

**Yang Song**

**A THESIS**

**Submitted to  
Michigan State University  
in partial fulfillment of the requirement  
for the degree of**

**Environmental Engineering – Master of Science**

**2014**

## ABSTRACT

### APPLICATION OF STABLE ISOTOPE PROBING AND HIGH-THROUGHPUT SEQUENCING TO IDENTIFY MICROORGANISMS IN MICROBIAL FUEL CELLS

By

Yang Song

Microbial fuel cells (MFCs) have the potential for use in both waste degradation as well as energy generation. An understanding of anode chamber microbial community could contribute to optimizing these applications. The combination of stable isotope probing (SIP) and high-throughput sequencing can provide information on the activity and abundance of microorganisms while existing in mixed cultures. For this study, eight sets of MFCs anode chamber microcosms were analyzed to profile the microbial community and identify the microorganisms involved in carbon uptake from the amended substrates. The MFCs were amended with labeled ( $^{13}\text{C}$ ) or unlabeled sodium acetate and glucose and the external resistance was manipulated to two levels (10 ohms and 1000 ohms). For the sodium acetate amended MFCs, the dominant phyla were the *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. For the glucose amended MFCs, similar dominant phyla were detected, except *Actinobacteria*. Through comparing enrichment factors between the labeled and unlabeled fractions, 14 phylotypes were found to be responsible for label uptake in the sodium acetate amended MFCs and 13 phylotypes were responsible in the glucose amended MFCs. Among these, *unclassified Parachlamydiaceae (Chlamydia)*, *Azospirillum (Proteobacteria)*, and *unclassified Rhodocyclaceae (Proteobacteria)* were the primary phylotypes uptaking the labeled carbon. To my knowledge, this is the first study to combine SIP with high-throughput sequencing to identify the active microorganisms in MFCs.

## ACKNOWLEDGEMENTS

I would like to acknowledge those people who helped make this thesis possible. Firstly, I would like to thank my parents for all their love and support during this research. Without their love, the research would be more stressful. Secondly, I owe a tremendous thank to my fellow graduate students, Indumathy Jayamani, Fernanda Paes as well as Yogendra Kanitkar who provide me with not only technical support but also spiritual encouragement to complete this research. I also appreciate the cooperation and help from Wisconsin University-Milwaukee environmental biotechnology & bioenergy laboratory. The MFCs were set up and operated by researchers under the direction of Dr. Zhen He (Associate Professor, University of Wisconsin-Milwaukee).

Finally, I would like to give my sincere thanks to my advisor Dr. Alison M. Cupples, who directed me through whole research and gave me valuable courage to finish this thesis. I would also like to thank other two committee members Dr. Susan J. Masten and Dr. Irene Xagorarakis.

## TABLE OF CONTENTS

LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
1. INTRODUCTION.....	1
1.1 Introduction to MFCs.....	1
1.2 High-throughput sequencing.....	5
1.2.1 Concept and principles.....	5
1.2.2 Categories.....	6
1.2.2.1 454 Pyrosequencing technology.....	6
1.2.2.2 Illumina's Solexa sequencing technology.....	7
1.2.2.3 SOLiD technology.....	8
1.2.3 Advantages.....	9
1.2.4 Potential drawbacks.....	9
1.3 Stable isotope probing.....	10
2. MATERIALS AND METHOD.....	13
2.1 Chemicals.....	13
2.2 Operation of MFCs.....	13
2.3 DNA extracts.....	15
2.4 Isopycnic centrifugation.....	15
2.5 High-throughput amplicon sequencing (Illumina MiSeq).....	16
3. RESULTS.....	18
3.1 Fraction generation and sequencing summary.....	18
3.2 Illumina sequencing results for total DNA.....	20
3.2.1 Phyla from total DNA extracts.....	20
3.2.2 Families from total DNA extracts.....	21
3.3 Identification of phylotypes responsible for label uptake.....	27
4. DISCUSSION.....	41
5. CONCLUSION.....	45
REFERENCES.....	46

## LIST OF TABLES

Table 1: Microbes used in MFCs.....	4
Table 2: MFCs running data summary.....	14
Table 3: Summary of MiSeq Illumina data generated from MFCs total DNA samples as well as fractions in labeled and unlabeled samples.....	19
Table 4: Buoyant density (BD) of fractions chosen for sequencing from MFCs sample (DUP is abbreviation of Duplicate).....	20
Table 5: Summary of genera enriched in both duplicates for sodium acetate (10 ohms/ 1000 ohms) and glucose (10 ohms/ 1000 ohms) fed MFCs samples.....	39

## LIST OF FIGURES

Figure 1: Working principle for MFCs.....	2
Figure 2: Summary of nucleic acid based isotope probing method.....	11
Figure 3: Comparison of relative abundance of sequences in total genomic DNA extracted from eight MFCs samples (A: Sodium Acetate; G: Glucose; 10: 10ohms; 1000: 1000ohms; L: Labeled; UL: Unlabeled; T: Total).....	23
Figure 4: Relative abundance of <i>Proteobacteria</i> (A), <i>Bacteroidetes</i> (B), <i>Firmicutes</i> (C), and <i>Actinobacteria</i> (D) from sodium acetate fed MFCs total DNA extracts classified at the family level (unless unclassified) (A: Sodium acetate; 10:10 ohms; 1000: 1000 ohms; L: Labeled; UL: Unlabeled; T: Total).....	24
Figure 5: Relative abundance of <i>Proteobacteria</i> (A), <i>Bacteroidetes</i> (B), and <i>Firmicutes</i> (C) from glucose fed MFCs total DNA extracts classified at the family level (unless unclassified) (G: Glucose; 10:10 ohms; 1000: 1000ohms; L: Labeled; UL: Unlabeled; T: Total).....	26
Figure 6: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled sodium acetate 10 ohms samples to the unlabeled sodium acetate 10 ohms samples for two duplicates (A and B).....	31
Figure 7: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled sodium acetate 1000 ohms samples to the unlabeled sodium acetate 1000 ohms samples for two duplicates (A and B).....	33
Figure 8: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled glucose 10 ohms samples to the unlabeled glucose 10 ohms samples for two duplicates (A and B).....	35
Figure 9: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled glucose 1000 ohms samples to the unlabeled glucose 1000 ohms samples for two duplicates (A and B).....	37

## **1. INTRODUCTION**

### **1.1 Introduction to MFCs**

Microbial fuel cells are devices that use microorganisms to transfer chemical energy to electrical energy. Typically, MFCs consist of anode and cathode chambers which are divided by one cation specific membrane. In the anode chamber, anaerobic conditions maximize reducing equivalent yield through promoting acidogenic fermentative metabolism. Organisms in anode chambers are capable of reproduction as well as transfer reducing equivalents to an exterior electron acceptor. Extracellular electron transfer can be achieved through fermentative pathways, acquisition by soluble electron shuttle compounds with reduction and through bacterial pili, i.e. nanowires (1). Electrons are transferred to cathode chambers with an external electric loop circuit. In the cathode chamber, electrons are consumed in oxidative conditions by terminal electron acceptors such as oxygen, nitrate and ferric ions (2). Figure 1 (3) illustrates a basic working principle for one MFCs with glucose as the electron donor.

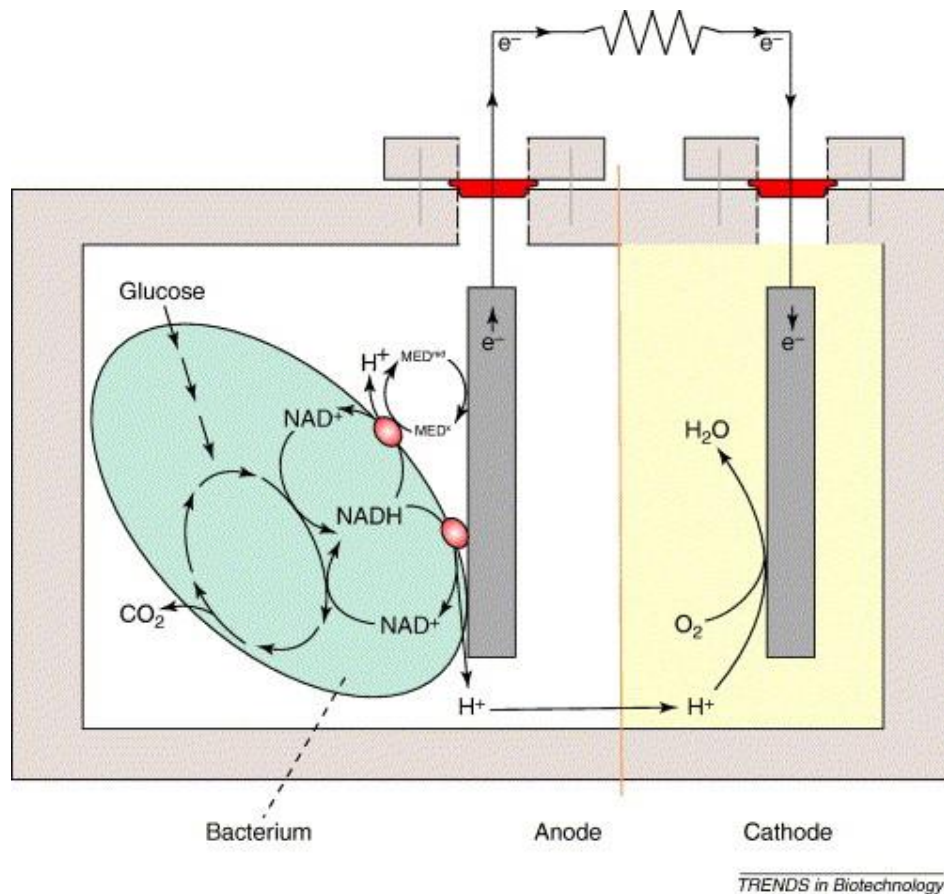


Figure 1: Working principle for MFCs (3)

Recently, there has been increasing attention towards MFCs due to their dual functionality for organic waste degradation as well as clean energy production. One ‘Scopus’ search survey on the keywords “microbial fuel cell” illustrates there has been an increase of ~60 fold in the papers published during 1998 to 2008 (4). During the past several years, multiple research areas have focused on MFCs as a new bioenergy resource. For example, researchers have focused on the comparison of anode bacterial communities based on different choices of electron donor (5); the use of new permeable membrane in microbial fuel cells (6); the influence of external resistance on electrogenesis (7); and the cathodic limitations in MFCs (8).



Under different substrate addition conditions, various MFCs power generation efficiencies have been reported. For instance, Logan reported that the power density produced in a graphite fiber brush anodes cube MFC with acetate substrate can be as much as 2400 mW/m<sup>2</sup> or 73mW/m<sup>3</sup> (9). Also, Rabaey indicated that a glucose fed-batch MFC using 100 mM ferric cyanide as cathode oxidant with power density up to 216 W/m<sup>3</sup> (10). Other substrates, such as lignocellulosic biomass, wastewater, landfill leachates, have also been explored (4). In most cases, acetate is preferred as the anode electron donor due to its high coulombic efficiency (CE). Glucose usually has high power density (PD) when used as an electron donor, while it generates much less CE than acetate due to its fermentable characteristic (11) . Researchers have also tested various inocula to MFCs for improving energy generation efficiency. *Clostridium cellulolyticum*, *G. sulfurreducens* (12), *Enterobacter cloacae* (13), *Schewanella* (14), domestic wastewater (15), or anaerobic sludge (16) have been added to MFCs to evaluate the corresponding current density generation.

Although researchers have already established the platform for MFCs improvement and industrial application, more knowledge is still needed to understand the microbial community structure as well as the significance of different microorganisms for improving energy production (17). Previous research has focused on the anode microbial community. For example, strain ISO2-3, affiliated with the *Aeromonas sp.* within the *Gammaproteobacteria* was reported to be important for current generation through oxidation of glucose or hydrogen (18). Moreover, Jung et al. reported that the characterization of MFCs under different substrate additions (acetate, lactate and glucose) with anaerobic sludge inoculum had shown

functional communities affiliated with *Geobacter sulfurreducens* based on 16S rDNA targeted denaturing gradient gel electrophoresis (DGGE) (5). In a recent study (19), with inoculum of primary clarifier effluent from a municipal wastewater plant, it was suggested that the families *Geobacteraceae* and *Desulfobulbaceae* correlate to electricity generation in the biofilm. Results of phylum level studies also showed *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were relatively abundant. A few papers also mentioned *Actinobacteria* could either act as a dominant phylum or at least be present in the tested microbial community (20, 21). A summary of microorganisms in MFCs is shown (Table 1) below (22).

Table 1: Microorganisms used in MFCs

Microbes	Substrate	Applications
<i>Actinobacillus succinogenes</i>	Glucose	Neutral red or thionin as electron mediator
<i>Aeromonas hydrophila</i>	Acetate	Mediator-less MFC
<i>Alcaligenes faecalis</i> , <i>Enterococcus gallinarum</i> , <i>Pseudomonas aeruginosa</i>	Glucose	Self-mediate consortia isolated from MFC with a maximal level of $4.31 \text{ W m}^{-2}$
<i>Clostridium beijerinckii</i>	Starch, glucose, lactate, molasses	Fermentative bacterium
<i>Clostridium butyricum</i>	Starch, glucose, lactate, molasses	Fermentative bacterium
<i>Desulfovibrio desulfuricans</i>	Sucrose	Sulphate/sulphide as mediator
<i>Erwinia dissolvens</i>	Glucose	Ferric chelate complex as mediators
<i>Escherichia coli</i>	Glucose sucrose	Mediators such as methylene blue needed
<i>Geobacter metallireducens</i>	Acetate	Mediator-less MFC

Table 1 (Cont'd)

Microbes	Substrate	Applications
<i>Geobacter sulfurreducens</i>	Acetate	Mediator-less MFC
<i>Gluconobacter oxydans</i>	Glucose	Mediator (HNQ, resazurin or thionine) needed
<i>Klebsiella pneumoniae</i>	Glucose	HNQ as mediator biomineralized manganese as electron acceptor
<i>Lactobacillus plantarum</i>	Glucose	Ferric chelate complex as mediators
<i>Proteus mirabilis</i>	Glucose	Thionin as mediator
<i>Pseudomonas aeruginosa</i>	Glucose	Pyocyanin and phenazine-1-carboxamide as mediator
<i>Rhodoferrax ferrireducens</i>	Glucose, xylose, sucrose, maltose	Mediator-less MFC
<i>Shewanella oneidensis</i>	Lactate	Anthraquinone-2,6-disulfonate (AQDS) as mediator
<i>Shewanella putrefaciens</i>	Lactate, pyruvate, acetate, glucose	Mediator-less MFC; but incorporating an electron mediator like Mn (IV) or NR into the anode enhanced the electricity production
<i>Streptococcus lactis</i>	Glucose	Ferric chelate complex as mediators

Many factors appear to affect the microbial community present, including substrate type, inoculum, cathode chamber types, and the experimental conditions. More information on the dominant microorganisms in the anode chamber has the potential to contribute to an understanding of the electron transfer process. Also, such information could potentially be used to optimize waste treatment with MFCs.

## 1.2 High-throughput Sequencing

### 1.2.1 Concept and principles

High throughput sequencing is an efficient approach for obtaining large amounts of genetic information. The technique can follow DNA extraction and amplification of 16S rRNA genes (23). More specifically, high throughput sequencing involves two processes: i) the generation of DNA libraries through PCR clonal amplification; ii) DNA sequencing by synthesis which is determined by sequential addition of nucleotides to the complementary strand without a physical separation process.

### 1.2.2 Categories

Among the next generation sequencing platforms available, the Roche/454 FLX, the Illumina/Solexa Genome Analyzer, and the Applied Biosystems (ABI) SOLiD Analyzer are the predominant platforms that are broadly used (24). Other available platforms and developing ones are likely to become more popular in next few years due to their contribution to faster sequencing and lower prices (25). Newly emerging third-generation sequencing techniques could run without an initial DNA amplification process (26). An overview of the three platforms listed above is provided below.

#### 1.2.2.1 454 Pyrosequencing technology

Pyrosequencing is a method of DNA sequencing based on synthesis. It depends on the detection of photons produced during nucleotide incorporation (27). The Roche/454 FLX genome sequencer, based on pyrosequencing technology (28), was available for purchase in 2004. Major procedures in this application include sample library preparation, emulsion-based clonal amplification, bead recovery and enrichment, Pico TiterPlat™

preparation, sequencing and detection. Sample preparation in 454 pyrosequencing technology is more simplified than Sanger sequencing (traditional sequencing). To test the DNA sequence, the four nucleotides are sequentially provided through the plates with a polymerase enzyme and primer. Synthesis of the complementary strand can start and emit photons as a result of pyrophosphate release, which is captured by a CCD camera. By comparing the light intensity corresponding to different nucleotides addition sequence, strands sequences can be identified (24). According to one comparison with the advanced Sanger-based capillary electrophoresis platform, the 454 system can produce around 100 times higher throughput on sequence data (27). Currently, the limitation for the 454 system generators is that this technology can only contain relatively short read lengths and low ratings for some genomic regions' base reading accuracies.

#### 1.2.2.2 Illumina's Solexa sequencing technology

Since it became available on the commercial market in 2006, the Solexa sequencing platform has been broadly accepted as the most adaptable and easiest to use genomic analyzer. The gene library preparation process for Solexa sequencing is similar to 454 technology. However, the following substrate template capture and amplification procedure is different. It depends on solid-phase bridge PCR technique for amplifying targeted DNA that would randomly connect to adaptors and form clusters on the surface of a flow cell (27). After replicating cell clusters of approximately 1000 copies of one-stranded DNA fragments, the reaction mixture for sequencing and DNA synthesis is added onto the surface. The mixture pool for the following reactions contains primers, four reversible terminator nucleotides labeled with

fluorescent dye, and DNA polymerase. After integration with the DNA strands, the CCD camera can capture the images of fluorescent dye on the terminator nucleotides and the position where they incorporate with the DNA strands. Then the terminator group as well as the fluorescent dye are removed followed by the next round of synthesis reactions. There are reports showing that, at a minimum, 40 million pairs of strands can be synchronously determined in parallel resulting in high sequence throughput (24). Illumina's sequencing by synthesis (SBS) technology works both for single read and paired-end libraries. The SBS technology combines short inserts and longer reads, which increase the capability to fully characterize genomes. Its wide sample preparation process enables various sequencing applications, containing whole-genome sequencing, *de novo* sequencing, candidate region targeted resequencing, DNA sequencing, RNA sequencing, methylation analysis, and protein-nucleic acid interaction analysis ([http://www.illumina.com/technology/sequencing\\_technology.ilmn](http://www.illumina.com/technology/sequencing_technology.ilmn)). In 2008, the updated Genome Analyzer Illumina HiSeq 2000 was able to produce single reads of 2×100 base pairs, and around 200 giga base pair (Gbp) of short sequences each run. The Illumina MiSeq platform produces 250 bp paired reads.

#### 1.2.2.3 SOLiD technology

The Applied Biosystem SOLiD sequencing system based on ligation was launched in 2007. This technology applies an emulsion PCR approach with beads to amplify the DNA fragments for parallel sequencing. For the sequencing process, after a primer is attached to the adapter, a mixture of oligonucleotide octamers is hybridized to the DNA fragments

followed by adding the ligation mixture. The octamers are fluorescent labelled di-base probes that compete for ligation through interrogating the first and second bases in each ligation reaction. The first two bases are recognized by characterizing their corresponding fluorescent labels. Then, the fluorescent labels are removed enzymatically with the departure of the last three bases on the octamer. The hybridization and ligation cycles are then repeated, in which bases 6 and 7 as well as bases 11 and 12 can be determined. Moreover, the sequencing process can be continued through adding another primer, shorter by one base, to test the remaining bases, for example, bases 0 and 1, bases 5 and 6 etc. The primer offsetting scheme allows a universal primer to hybridize to DNA templates along the entire fragment within five cycles. Also, each base can be sequenced twice during the entire cycle. Because each base is determined by different fluorescent labels, the misreading rate is largely reduced and the accuracy rate can be as much as 99.94%. The SOLiD platform can provide accurate data, however the longer time needed for DNA library preparation can be a shortcoming (24, 25).

### 1.2.3 Advantages

High throughput sequencing can be fast and accurate. It can help to speed up the genotype characterization and also broaden the pools of determination targets. Researchers have used these approaches to investigate the expression and patterns of functional genes in microbial communities (29, 30). Moreover, transcriptomics (30-32) as well as plasmids (33) can also be targeted for high throughput sequencing.

### 1.2.4 Potential drawbacks

High throughput sequencing technologies are relatively expensive. The Roche 454 sequencing technology generates a smaller amount of data, which is usually between 0.25 and 1 Gbp sequence information per plate (34). Meanwhile, difficulties exist for 454 sequencing technology for dealing with homopolymeric DNA sequences. Both the Illumina Solexa sequencing system as well as the ABI SOLiD technology generate a larger amount of data. However, the Illumina Solexa sequencing system is limited by read lengths and the ABI SOLiD technology usually requires a longer time for sequencing. For this research, the Illumina platform was used because of local availability and support for the analysis of the data produced (Mothur, see methods section).

### 1.3 Stable Isotope Probing

Stable isotope probing is a molecular technique that allows identification of metabolically active microorganisms from diverse microbial communities through tracking the flow of isotopically labeled atoms incorporated into biomass (35, 36). According to a review on SIP, this technique broadens the scope for linking function with identification due to its independence from cultivation (37).

SIP involves the exposure of the microbial community to a labeled substrate. Microorganisms assimilate the stable isotope into biomass including their nucleic acids (37). Researchers have used three major biomarkers for detection during SIP: polar lipid derived fatty acids (PLFAs), DNA, and rRNA. Other biomarkers such as mRNA and proteins have recently been introduced for their strong sensitivity to isotopic enrichment. While both DNA and RNA are



taxonomically informative, DNA is more often employed (38).

DNA based SIP (DNA-SIP) dates back to 2000, when Radajewski et al. (39) detected two groups of bacteria,  *$\alpha$ -Proteobacteria* and *Acidobacterium*, which were responsible for the degradation of methanol.  $^{13}\text{C}$  is the most common isotope chosen for SIP based on the fact that carbon is the most abundant element in DNA. Even though buoyant density of DNA is affected by its guanine-cytosine (G+C) content, the heavy stable isotope components enhance the buoyant density of labeled DNA (40). Labeled DNA and unlabeled DNA are separated through isopycnic centrifugation based on different buoyant densities. The centrifugation is typically conducted in a CsCl solution and the heavier DNA can be found at the bottom of centrifugation tubes. It is also possible to visualize the DNA bands in tubes through adding ethidium bromide (EtBr) before centrifugation, which can show the location of bands under UV light. A figure summary of nucleic acid based stable isotope probing method (41) is shown here (Figure 2).

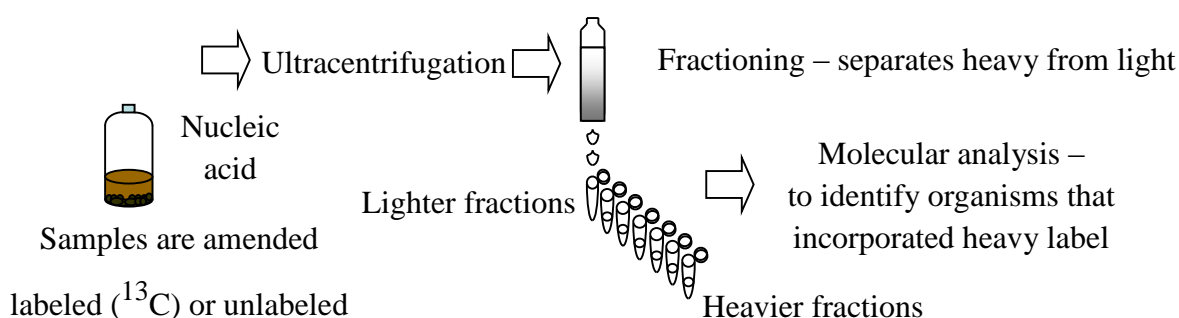


Figure 2: Summary of nucleic acid based isotope probing method

Despite its advantages, SIP also has some limitations, for instance, the limited availability

and high cost of labeled substrates. When designing SIP-based experiments, researchers also need to consider the proper substrate concentrations as well as the incubation times to minimize potential cross-feeding and over-enrichment (38).

In this study, SIP and high-throughput sequencing (Illumina) were used to profile the microbial community and identify the microorganisms involved in label uptake ( $^{13}\text{C}$ ) from eight sets of MFCs anode DNA samples. The MFCs were amended with labeled ( $^{13}\text{C}$ ) or unlabeled sodium acetate and glucose and the external resistance was manipulated to two levels (10 ohms and 1000 ohms). Initially TRFLP was coupled to SIP for this research, however, this method did not provide the resolution needed to identify the microorganisms that uptaking the label. Therefore, the molecular analysis approach was switched to high throughput sequencing.

This research is a collaboration with another research group. The MFCs were set up and operated by researchers under the direction of Dr. Zhen He (Associate Professor, University of Wisconsin-Milwaukee). These researchers removed the anode and sent them to MSU. All other activities (DNA extraction, SIP, Illumina sequencing, data analysis) were completed at MSU.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

Reagents were purchased from one or more of the following vendors: Fisher Bioreagent (Thermo Fisher Scientific, NJ, USA), Integrated DNA Technologies (Coralville, IA, USA) and Sigma-Aldrich (St.Louis, MO, USA).

### 2.2 Operation of MFCs

The MFCs operation and sample collection occurred at the University of Wisconsin-Milwaukee. The inoculum source of the two-chamber MFCs system was anaerobic sludge from a local municipal wastewater treatment plant. The anode material was carbon cloth with surface area of  $12\text{ cm}^2$  and the cathode material was carbon brush. Both electrodes were soaked in a 100 mL solution in the anode and cathode chambers. The solution in anode chamber contained 0.3 g/L  $\text{NH}_4\text{Cl}$ , 1 g/L  $\text{NaCl}$ , 0.03 g/L  $\text{MgSO}_4$ , 0.04 g/L  $\text{CaCl}_2$ , 0.2 g/L  $\text{NaHCO}_3$ , 5.3 g/L  $\text{KH}_2\text{PO}_4$ , 10.7 g/L  $\text{K}_2\text{HPO}_4$  and 1 mL/L trace solution. Trace solution contained 10000 mg/L  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$ ; 2000 mg/L  $\text{CoCl}_2\cdot 6\text{H}_2\text{O}$ ; 1000 mg/L EDTA; 500 mg/L  $\text{MnCl}_2\cdot 4\text{H}_2\text{O}$ ; 142 mg/L  $\text{NiCl}_2\cdot 6\text{H}_2\text{O}$ ; 123 mg/L  $\text{Na}_2\text{SeO}_3$ ; 90 mg/L  $\text{AlCl}_3\cdot 6\text{H}_2\text{O}$ ; 69 mg/L  $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ ; 50 mg/L  $\text{ZnCl}_2$ ; 50 mg/L  $\text{H}_3\text{BO}_3$ ; 38 mg/L  $\text{CuCl}_2\cdot 2\text{H}_2\text{O}$ ; 1 mL/L  $\text{HCl}$  (37.7% solution). The solution in the cathode chamber contained potassium ferricyanide at a concentration of 500 mM/L. All water was deionized water. The MFCs system in this study was the H-type system as described elsewhere (42). The anode and cathode chambers were separated by a cation exchange membrane (Ultex CMI 7000, Membranes International, Inc., Glen Rock, NJ, USA ).

The substrates for MFCs startup were unlabeled sodium acetate or glucose with an initial concentration of 1 g/L. After ~30 days operation, labeled ( $^{13}\text{C}$ ) and unlabeled substrates were amended in MFCs (1 g/L). Eight sets MFCs were amended with labeled ( $^{13}\text{C}$ ) or unlabeled sodium acetate and glucose and the external resistance was manipulated to two levels (10 ohms and 1000 ohms). All MFCs were operated at room temperature. After ~14 days, anode electrodes were collected from the MFCs and stored at -20 °C. A summary of the MFCs running characteristics is shown below (Table 2).

Table 2: MFCs running data summary

Samples	External resistance/ohms	Current/ mA	Coulombic efficiency/%	Power density/ W/m <sup>3</sup>
$^{12}\text{C}$ sodium acetate 10 ohms	10	4.17	37.8	1.74
$^{13}\text{C}$ sodium acetate 10 ohms	10	5.06	52.8	2.56
$^{12}\text{C}$ sodium acetate 1000 ohms	1000	0.62	11.8	3.84
$^{13}\text{C}$ sodium acetate 1000 ohms	1000	0.59	8.0	3.48
$^{12}\text{C}$ glucose 10 ohms	10	1.80	24	0.32
$^{13}\text{C}$ glucose 10 ohms	10	1.63	29.8	0.27
$^{12}\text{C}$ glucose 1000 ohms	1000	0.60	13.5	3.60
$^{13}\text{C}$ glucose 1000 ohms	1000	0.54	14.5	2.92

### 2.3 DNA extraction

Anode chamber samples were collected after two weeks of MFCs operation and were then sent to MSU. These samples were stored at -20 °C until DNA extraction occurred. Total genomic DNA was extracted using the Power Soil DNA extraction kit, following the manufacturer's instruction (MO BIO Laboratories, Inc. Carlsbad, CA). Eight samples were investigated, including materials obtained from a MFC amended with i) unlabeled sodium acetate operated at 10 ohms or 1000 ohms, ii) labeled sodium acetate operated at 10 ohms or 1000 ohms, iii) unlabeled glucose operated at 10 ohms or 1000 ohms, iv) labeled glucose operated at 10 ohms or 1000 ohms. Extracted DNA were quantified with the Nanodrop-1000 (Thermo Fisher Scientific Inc.).

### 2.4 Isopycnic centrifugation

The extracted DNA was ultracentrifuged in cesium chloride gradients separately to obtain density-resolved gradients and fractions. For each MFC treatment, replicate DNA samples were subject to ultracentrifugation (16 DNA samples were ultracentrifuged). For each sample, approximately 10 µg of total genomic DNA (except for duplicate one of sodium acetate fed 1000 ohms which involved 20 µg) was mixed with a Tris-EDTA (pH 8.0) buffer and CsCl solution. This mixture was added to a 5.1 mL Quick-Seal polyallomer tubes (1.3 x 5.1 cm, Beckman Coulter) the buoyant density (BD) of this mixture was adjusted to around 1.72 g/mL using a model AR200 digital refractometer (Leica Microsystems Inc.) and then sealed using a tube topper (Cordless quick-seal tube topper, Beckman). The tubes were then centrifuged at 178,000 x g for 46 hours at 20 °C in a Wx Sorvall Ultra 80 ultracentrifuge

fitted with a Stepsaver 70 V6 Vertical Titanium Rotor (Thermo Fisher Scientific Inc.).

Each of the 16 ultracentrifuged samples were separated into 20 fractions (250  $\mu$ L) by displacing the samples with molecular grade water. A syringe pump attached to a needle (BD, 23G and 1 inch) was used to displace samples from the top of the tube. This resulted in fractions being collected from heavy to light BD values. The heavier BD fractions contained the labeled DNA. The BD of each fraction was calculated from the refractive index obtained using a refractometer. DNA from each of the fraction was recovered using a glycogen and ethanol precipitation. Precipitated DNA was then re-suspended in 30  $\mu$ L PCR grade water and stored at -20  $^{\circ}$ C for further analysis.

## 2.5 High-throughput amplicon sequencing (Illumina MiSeq)

For each isotope pair, and each duplicate, four heavy fractions from labeled sample and three heavy fractions from unlabeled sample were chosen for sequencing. In total, 56 fractions as well as 8 total DNA samples were submitted for Illumina sequencing. MFCs were amended with unlabeled substrate to provide a control against high GC content microorganisms that would naturally be found in all heavy fractions. By comparing the microorganism in the heavy fractions of the labeled amended samples to those in the heavy fractions of the unlabeled amended samples, this ensures only those involved in label uptake are identified. To quantitatively determine which phylotypes were more abundant in the heavy fractions of the samples (label amended) compared to the controls (unlabeled amended), an enrichment factor was calculated. The enrichment factor was obtained by dividing the % relative

abundance of the phylotype in the labeled fraction by the % relative abundance of phylotype in corresponding unlabeled fraction.

When the enrichment factor was larger than one, the phylotype was considered to be involved in carbon assimilation. Total DNA samples were also analyzed to profile the microbial community structure in microcosm.

PCR and Illumina sequencing were performed at RTSF (Research Technology Supply Facility) at Michigan State University using previously developed protocols (43). In short, this involved the amplification of V4 region from 16S rRNA gene, quantification of individual reactions (Picogreen assay), purifications with Ampure XP beads as well as gel purification, and finally use of the Illumina MiSeq platform using 2 × 250 bp paired end flow cell and reagent cartridge.

The data generated from Illumina sequencing was analyzed by Mothur (44) using the MiSeq standard operating procedure developed by the same laboratory (45). In brief, the analysis process involves the formation of contigs, removal of error sequences, chimera removal, sequences alignment for operational taxonomic units (OTUs), and taxonomical levels from group of OTUs. The data generated provided abundance data for the microbial community as well as data for the calculation of enrichment factors.

### 3. RESULTS

#### 3.1 Fraction generation and sequencing summary

Total DNA samples and the heavy fractions generated following ultracentrifugation were submitted for Illumina sequencing. For each pair (e.g.  $^{12}\text{C}$  10 ohms glucose and  $^{13}\text{C}$  10 ohms glucose) and duplicate, seven fractions were submitted for further analysis. In total, 56 fractions as well as 8 total DNA samples were submitted for Illumina sequencing and the data were analyzed through Mothur. One Mothur run included 8 total DNA samples and 8 other Mothur runs were for fractions from various treatments, which contained four labeled fractions and three unlabeled fractions. The sequencing results have been summarized (Table 3). A total of 10,218,342 sequences were obtained from the Illumina high-throughput sequencing for all samples. Approximately  $55.3 \pm 0.9\%$  of these sequences were excluded during sequencing analysis. Sequences were excluded because they were greater than 275 bp, they contained ambiguous bases, they contained homopolymer lengths of  $>8$  or they had a start position after 1968 or an end position before 11550. Further, additional edits were performed to remove chimeric sequences or to remove those belonging to mitochondria or chloroplast lineage. Following this, 4,119,441 sequences remained and 4.7% of these were unique sequences. These sequences were classified into OTUs and taxons through splitting into different bins and then clustering at the level of order with a 97% similarity cutoff level. Afterwards, three fractions pairs from the label amended and unlabeled amended anodes were chosen with similar BD values for the determination of enrichment factors (Table 4).



Table 3: Summary of MiSeq Illumina data generated from MFCs total DNA samples as well as fractions in labeled and unlabeled samples

	# of Sequences following make Contigs command	Final # of unique sequences	Final # of sequences	% Chimeric	OTUs per fraction or sample (average $\pm$ std dev)
Total DNA (8 MFCs samples from 10/1000 ohms labeled/unlabeled sodium acetate/glucose)	1,290,981	24,101	529,495	9.976	1439 $\pm$ 291
Sodium acetate 10 ohms duplicate 1 (7 fractions)	953,513	18,103	396,956	6.855	668 $\pm$ 250
Sodium acetate 10 ohms duplicate 2 (7 fractions)	1,092,795	20,727	427,028	13.873	1365 $\pm$ 167
Sodium acetate 1000 ohms duplicate 1 (7 fractions)	1,174,370	33,619	463,030	8.401	1261 $\pm$ 639
Sodium acetate 1000 ohms duplicate 2 (7 fractions)	1,017,669	18,092	404,495	12.209	753 $\pm$ 227
Glucose 10 ohms duplicate 1 (7 fractions)	1,092,399	19,492	432,749	9.100	744 $\pm$ 121
Glucose 10 ohms duplicate 2 (7 fractions)	977,908	18,080	400,811	9.427	654 $\pm$ 107
Glucose 1000 ohms duplicate 1 (7 fractions)	995,435	17,624	409,450	6.973	622 $\pm$ 81
Glucose 1000 ohms duplicate 2 (7 fractions)	1,623,272	27,100	655,427	9.545	956 $\pm$ 538

Table 4: Buoyant density (BD) of fractions chosen for sequencing from MFCs sample (DUP is abbreviation of Duplicate)

Samples	Fraction	BD (g/mL)		
		$^{13}\text{C}$	$^{12}\text{C}$	Difference
Sodium	F1	1.784	1.782	0.002
acetate 10	F2	1.778	1.774	0.004
ohms DUP1	F3	1.774	1.765	0.009
Sodium	F1	1.778	1.783	-0.004
acetate 1000	F2	1.772	1.775	-0.003
ohms DUP1	F3	1.765	1.768	-0.002
Sodium	F1	1.776	1.763	0.013
acetate 10	F2	1.766	1.757	0.010
ohms DUP2	F3	1.755	1.755	0.000
Sodium	F1	1.790	1.793	-0.002
acetate 1000	F2	1.782	1.783	-0.001
ohms DUP2	F3	1.772	1.773	-0.001
Glucose 10	F1	1.787	1.790	-0.003
ohms DUP1	F2	1.781	1.782	-0.001
	F3	1.773	1.773	0.000
Glucose	F1	1.783	1.780	0.003
1000 ohms	F2	1.778	1.772	0.007
DUP1	F3	1.772	1.764	0.008
Glucose 10	F1	1.784	1.784	0.000
ohms DUP2	F2	1.773	1.771	0.002
	F3	1.764	1.762	0.002
Glucose	F1	1.784	1.782	0.002
1000 ohms	F2	1.778	1.774	0.004
DUP2	F3	1.773	1.766	0.007

### 3.2 Illumina sequencing results for total DNA

#### 3.2.1 Phyla from total DNA extracts

Illumina Sequencing data from 8 total DNA ( $^{13}\text{C}$  sodium acetate 10 ohms/ 1000 ohms;  $^{12}\text{C}$

sodium acetate 10 ohms/ 1000 ohms;  $^{13}\text{C}$  glucose 10 ohms/ 1000 ohms;  $^{12}\text{C}$  glucose 10 ohms/ 1000ohms) showed a diverse phyla distribution (Figure 3). There were 17-21 phyla in each total DNA extract with a portion of unclassified sequences. The most abundant phylum was *Proteobacteria* with an average percentage of  $63.9 \pm 6.3\%$ . Other dominant phyla were *Bacteroidetes* and *Firmicutes*. *Actinobacteria* was over 1.5% in three out of four sodium acetate fed MFCs but only dominant in one glucose fed MFCs.

### 3.2.2 Families from total DNA extracts

The *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* were the most abundant phyla in sodium acetate fed MFCs microcosms. A family level classification within these four phyla has been generated (Figure 4). Within the *Proteobacteria* (Figure 4a), *Geobacteraceae* is the most abundant family, followed by *Rhodocyclaceae*, *Moraxellaceae*, and *Comamonadaceae*. Within *Bacteroidetes* (Figure 4b), the most abundant family for the sodium acetate fed 10 ohms unlabeled sample was *Flavobacteriaceae*. However, the dominance switched to *Porphyromonadaceae* for the other three samples of DNA extracts. Other abundant families included *Cryomorphaceae* for the 10 ohms samples and *unclassified Flavobacteriales* for the 1000 ohms samples. Within phylum of *Firmicutes*, families of *Clostridiales Incertae Sedis XI* and *Peptostreptococcaceae* together contributed to >50% abundance for the 10 ohms samples. While in the 1000 ohms samples, *Clostridiales Incertae Sedis XI*, *Gracilibacteraceae*, *Ruminococcaceae*, and *unclassified Clostridiales* were the most dominant families (Figure 4c). Family *Nocardiaceae* was the most dominant within the *Actinobacteria* in all four sodium acetate fed MFCs anode samples (Figure 4d).

For the glucose amended MFCs, three phyla exhibited the highest level of abundance, including *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Figure 5). Within the *Proteobacteria* phylum (Figure 5a), all of the glucose amended MFCs contained *Enterobacteriaceae* as the most abundant family except 1000 ohms labeled sample, which shifted to *Rhodocyclaceae*. Other families including *Geobacteraceae*, *Comamonadaceae*, and *Desulfovibrionaceae* were also present in these samples. In the *Bacteroidetes* phylum (Figure 5b), *Prophyromonadaceae*, *Bacteroidaceae*, *unclassified Bacteroidales*, *Flavobacteriaceae*, *unclassified Bacteroidetes*, *unclassified Flavobacteriales* were among the list of the most abundant families. *Bacteroidaceae* exhibited a higher abundance in the 10 ohms MFCs samples than those in the 1000 ohms samples. Also, *unclassified Flavobacteriales* was more dominant in the 1000 ohms MFCs samples than those in the 10 ohms MFCs samples. Within the phylum of *Firmicutes*, the dominant families were *Ruminococcaceae*, *Streptococcaceae*, *Clostridiales Incertae Sedis XI*, and *unclassified Clostridiales* (Figure 5c).

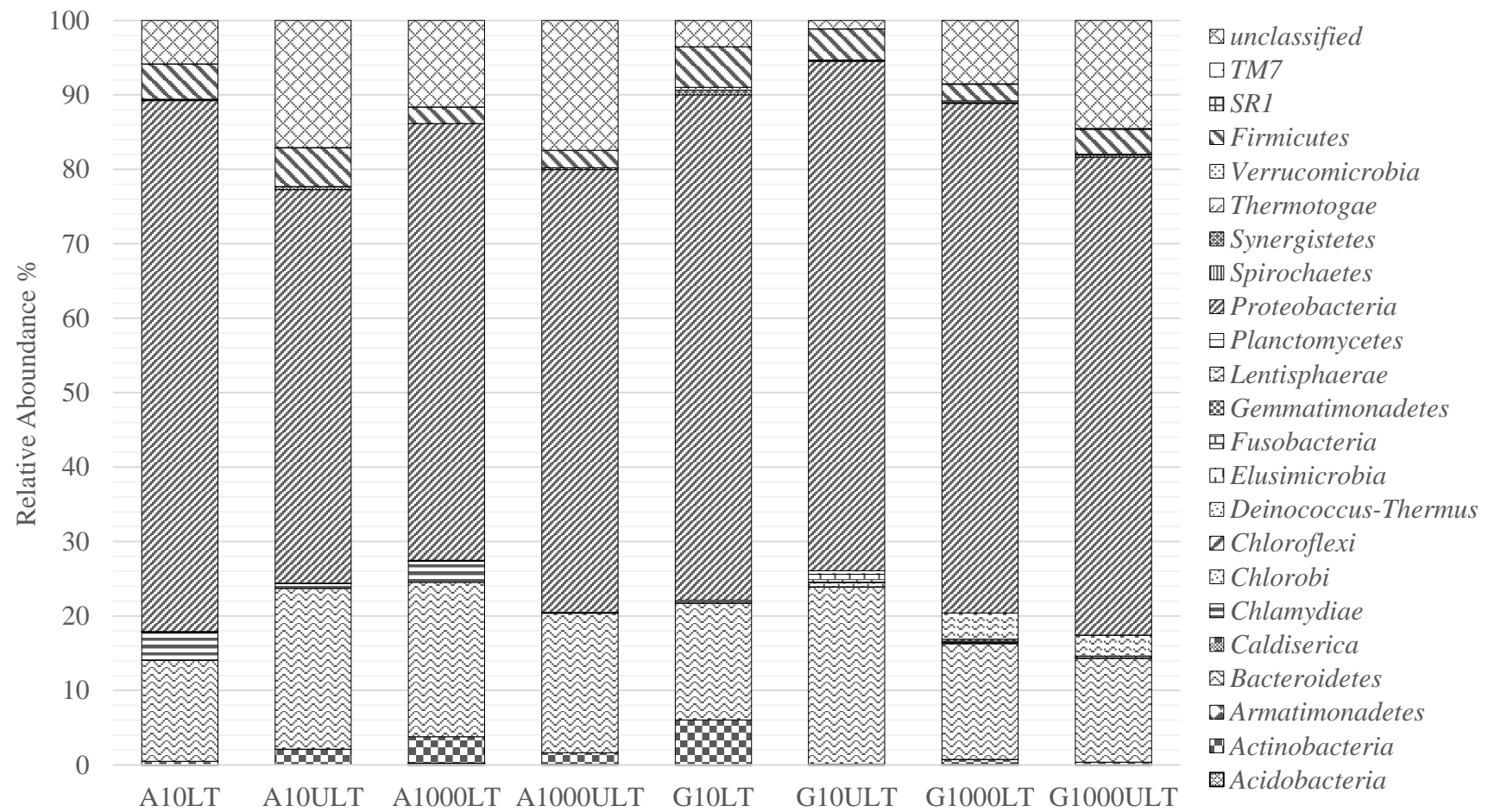


Figure 3: Comparison of relative abundance of sequences in total genomic DNA extracted from eight MFCs samples  
(A: Sodium Acetate; G: Glucose; 10: 10ohms; 1000: 1000ohms; L: Labeled; UL: Unlabeled; T: Total)

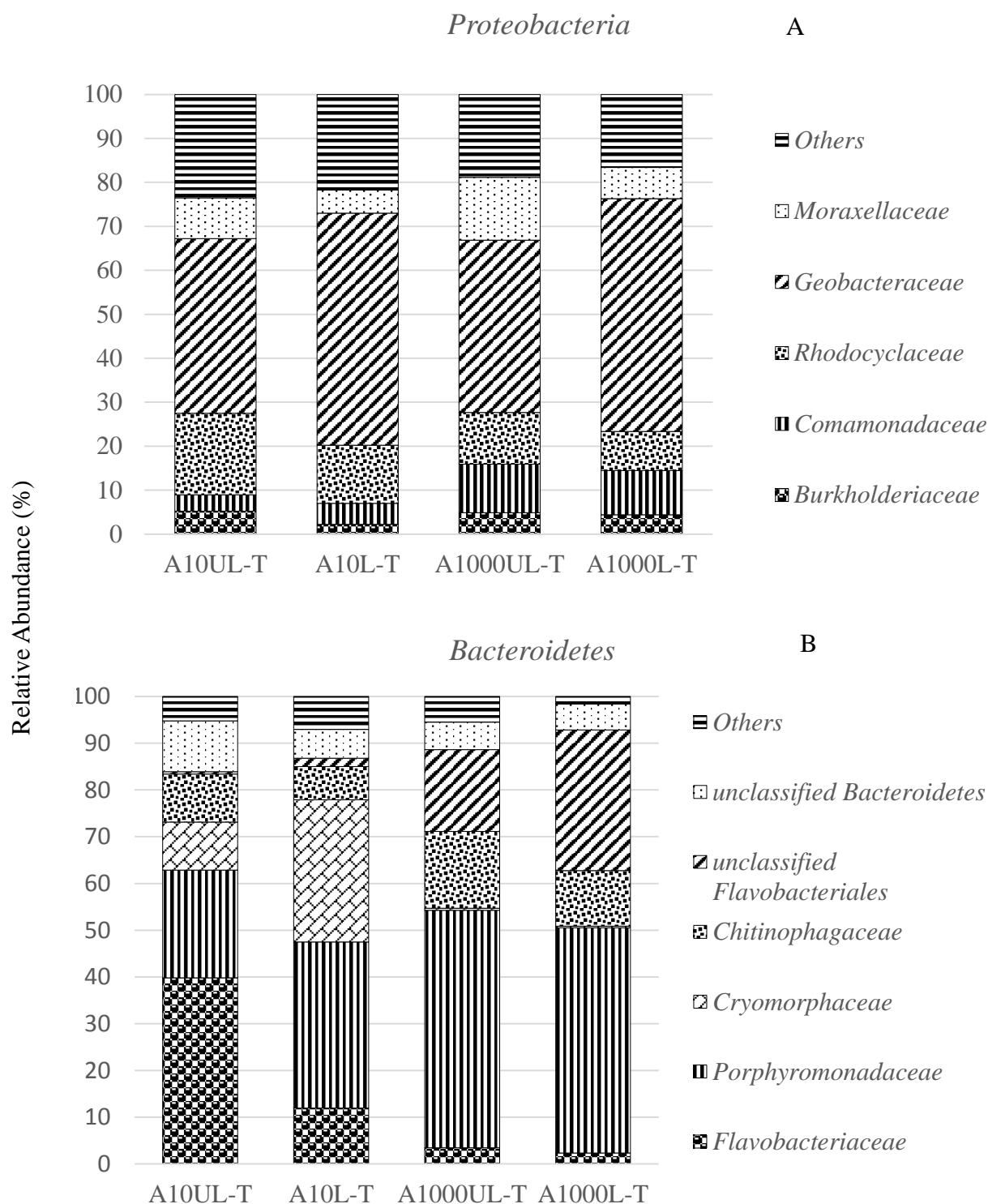
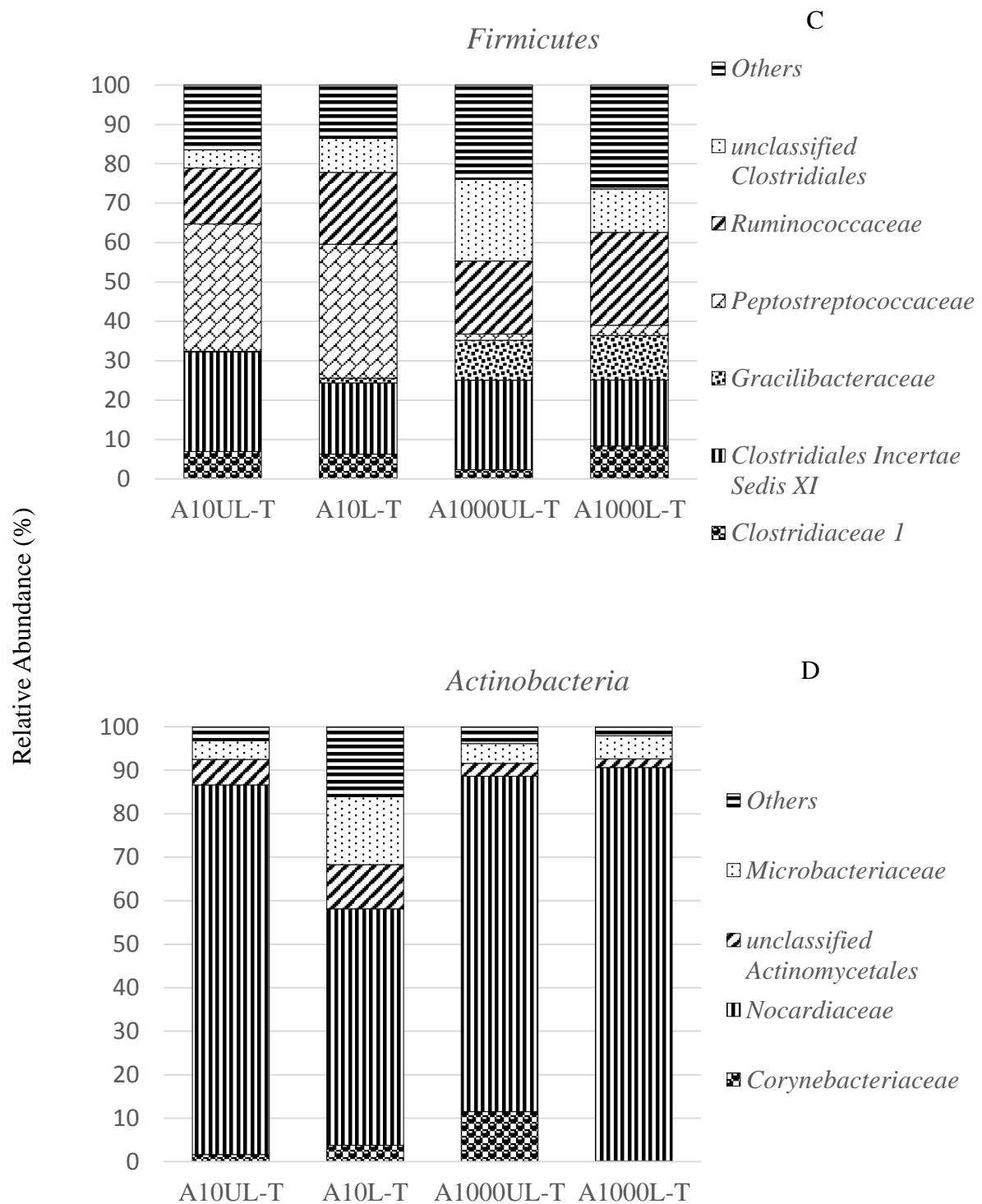


Figure 4: Relative abundance of *Proteobacteria* (A), *Bacteroidetes* (B), *Firmicutes* (C), and *Actinobacteria* (D) from sodium acetate fed MFCs total DNA extracts classified at the family level (unless unclassified) (A: Sodium acetate; 10:10 ohms; 1000: 1000 ohms; L: Labeled; UL: Unlabeled; T: Total)

Figure 4 (cont'd)



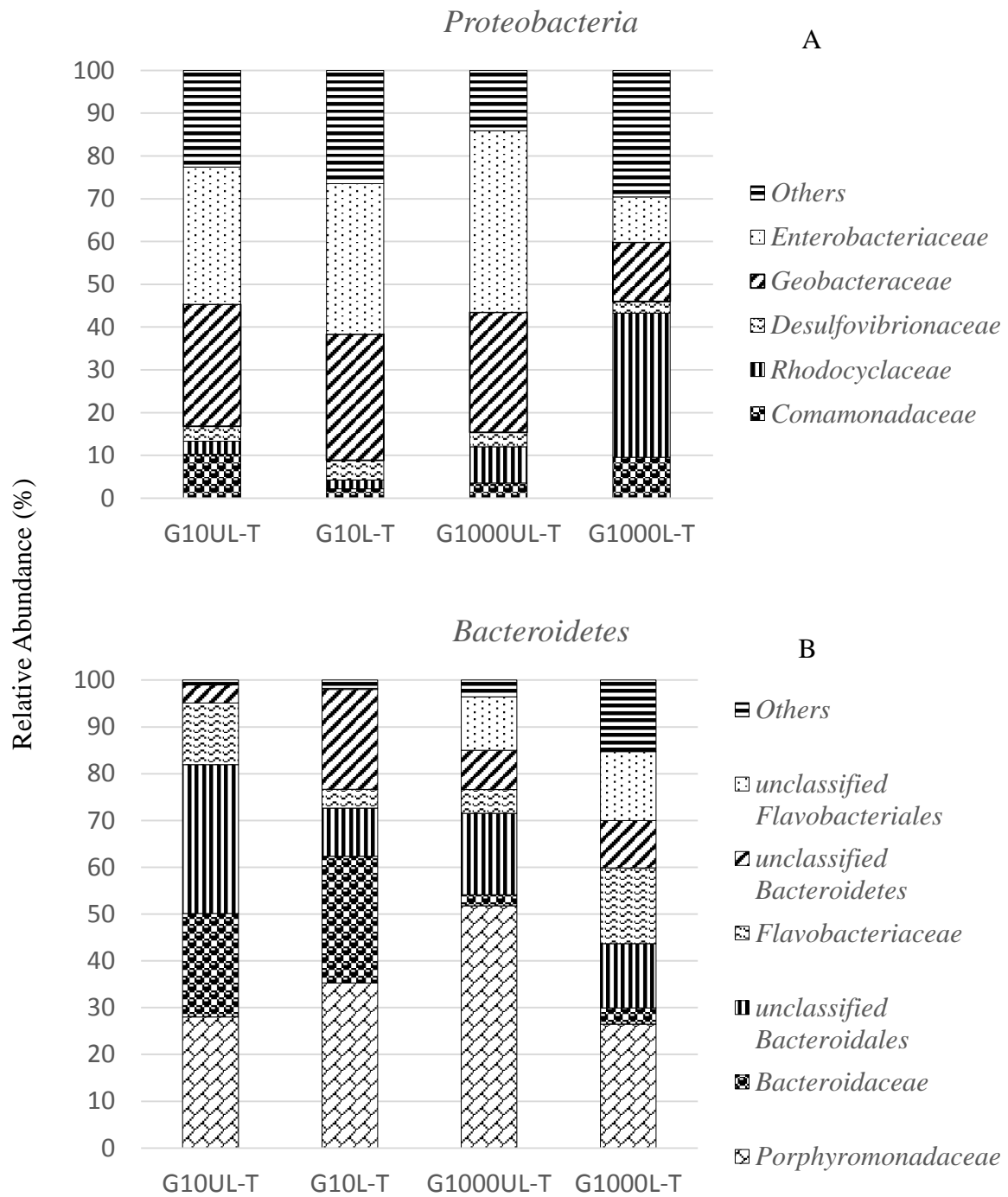
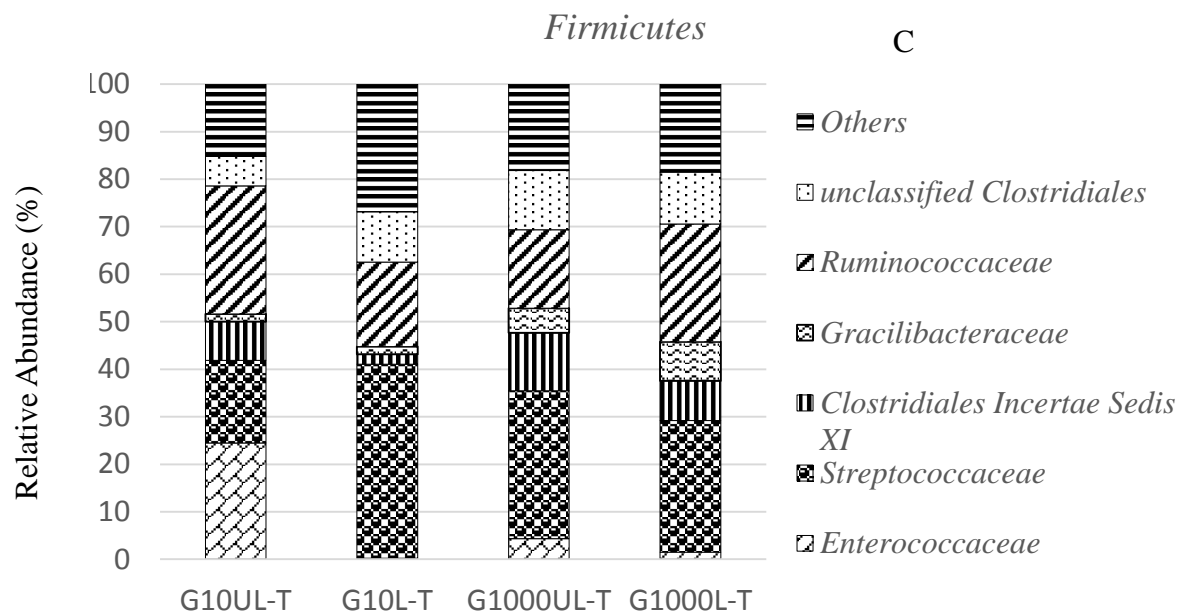


Figure 5: Relative abundance of *Proteobacteria* (A), *Bacteroidetes* (B), and *Firmicutes* (C) from glucose fed MFCs total DNA extracts classified at the family level (unless unclassified) (G: Glucose; 10:10 ohms; 1000: 1000ohms; L: Labeled; UL: Unlabeled; T: Total)



Figure 5 (cont'd)



### 3.3 Identification of phylotypes responsible for label uptake

To identify the microorganisms responsible for  $^{13}\text{C}$  uptake from the amended substrates (sodium acetate and glucose), enrichment factors for all phylotypes were calculated. For this, the relative abundance of each phylotype was compared between the heavy fractions of the samples (label amended) to heavy fractions of the controls (unlabeled amended). Specifically, the enrichment factor was obtained by dividing the % relative abundance of each phylotype in the fraction from the label amended substrate by the % relative abundance each phylotype in corresponding fraction from the unlabeled amended substrate. As discussed above, the comparison to heavy fractions from unlabeled amended samples is necessary to control for high GC content microorganisms (these would be found in all heavy fractions, regardless of label uptake).

The analysis involved comparing three heavy fractions of similar buoyant density between the samples (label amended) and controls (unlabeled amended). The comparison was conducted for each duplicate for each treatment, resulting in eight comparisons. These comparisons involved  $^{13}\text{C}$  sodium acetate 10 ohms vs  $^{12}\text{C}$  sodium acetate 10 ohms,  $^{13}\text{C}$  sodium acetate 1000 ohms vs  $^{12}\text{C}$  sodium acetate 1000 ohms,  $^{13}\text{C}$  glucose 10 ohms vs  $^{12}\text{C}$  glucose 10 ohms,  $^{13}\text{C}$  glucose 1000 ohms vs  $^{12}\text{C}$  glucose 1000 ohms. Each comparison also involved a complete duplicate for the entire approach (starting from DNA extraction). The phylotypes enriched in the labeled fractions over the controls were responsible for  $^{13}\text{C}$  uptake from the added substrate. The phylotypes considered responsible for label uptake have been summarized for the sodium acetate 10 ohms treatment duplicates (Figure 6), sodium acetate 1000 ohms treatment duplicates (Figure 7), glucose 10 ohms treatment duplicates (Figure 8) and glucose 1000 ohms treatment duplicates (Figure 9).

For the sodium acetate amended 10 ohms MFCs anode comparisons for both replicates, 40 phylotypes exhibited an enrichment factor above 1 (Figure 6). Of these, 27 phylotypes belonged to *Proteobacteria*, 7 belonged to *Bacteroidetes*, 3 classified as *Firmicutes*, 2 classified as *Chlamydiae*, and 1 belonged to the phylum *TM7*. For these comparisons, the average enrichment factors varied between 1.1 and 99.2. In duplicate 1, the phylotypes with the highest enrichment factors included *unclassified Parachlamydiaceae (Chlamydiae)*, *Brevundimonas (Proteobacteria)*, *Azospirillum (Proteobacteria)*, *Azoarcus (Proteobacteria)*, and *Telmatospirillum (Proteobacteria)* (Figure 6a). In replicate 2, the most enriched phylotypes were *unclassified Parachlamydiaceae (Chlamydiae)*, *Thauera (Proteobacteria)*,

*Gracilibacter* (Firmicutes), *Castellaniella* (Proteobacteria), and *unclassified Rhodocyclales* (Proteobacteria) (Figure 6b).

For the sodium acetate amended 1000 ohms MFCs anode comparisons for both duplicates, 36 phylotypes exhibited an enrichment factor above 1 (Figure 7). Of these, 26 belonged to *Proteobacteria*, 4 to *Firmicutes*, 2 to *Actinobacteria*, 2 to *Chlamydiae*, 1 to *Lentisphaerae*, and 1 to *Planctomycetes*. The average enrichment factors from these comparisons varied between 1.2 and 115.7. In replicate 1, the five most enriched phylotypes were *unclassified Parachlamydiaceae* (Chlamydiae), *Azospirillum* (Proteobacteria), *Gordonia* (Actinobacteria), *Kaistia* (Proteobacteria), and *Shinella* (Proteobacteria) (Figure 7a). In replicate 2, the five most enriched phylotypes were *unclassified Parachlamydiaceae* (Chlamydiae), *Victivallis* (Lentisphaerae), *Rhizobium* (Proteobacteria), *Lactococcus* (Firmicutes), and *unclassified Gammaproteobacteria* (Proteobacteria) (Figure 7b).

For glucose amended 10 ohms MFCs anode comparisons for both replicates, 54 phylotypes exhibited an enrichment factor above 1 (Figure 8). Of these, 26 belonged to *Proteobacteria*, 12 classified as *Firmicutes*, 7 belonged to *Bacteroidetes* and 6 classified as *Actinobacteria*. The average enrichment factors from three fractions varied between 1.2 and 599.6. In replicate 1, the five most enriched phylotypes were *Corynebacterium* (Actinobacteria), *Sulfuricurvum* (Proteobacteria), *unclassified Verrucomicrobia* (Verrucomicrobia), *unclassified Betaproteobacteria* (Proteobacteria) and *Alistipes* (Bacteroidetes) (Figure 8a). In replicate 2, the five most enriched phylotypes were *unclassified Cellulomonadaceae*

(*Actinobacteria*), *unclassified Rhodocyclaceae* (*Proteobacteria*), *Aquabacterium* (*Proteobacteria*), *Ralstonia* (*Proteobacteria*), and *unclassified Hyphomicrobiaceae* (*Proteobacteria*) (Figure 8b).

For glucose fed 1000 ohms MFCs anode comparisons for both replicates, 62 phylotypes exhibited an enrichment factor above 1 (Figure 9). Of these, 38 belonged to *Proteobacteria*, 11 belonged to *Bacteroidetes* and 7 classified as *Firmicutes*. The average enrichment factors from these comparisons varied between 1.3 and 23.8. In replicate 1 the five most enriched phylotypes classified as *Bacteroides* (*Bacteroidetes*), *Aeromonas* (*Proteobacteria*), *unclassified Polyangiaceae* (*Proteobacteria*) *Chitinophaga* (*Bacteroides*), and *Lactococcus* (*Firmicutes*) (Figure 9a). In replicate 2, the five most enriched phylotypes classified as *Byssovorax* (*Proteobacteria*), *unclassified Parachlamydiaceae* (*Chlamydiae*), *Devosia* (*Proteobacteria*), *unclassified Polyangiaceae* (*Proteobacteria*), and *Sphingobacterium* (*Bacteroidetes*) (Figure 9b).

Notably, some phylotypes were highly enriched (enrichment factors of 100-1000) over the others. For example, *unclassified Parachlamydiaceae* were highly enriched in three of the four comparisons amended with acetate. A summary table has been provided that ranks the most enriched phylotypes as an average across both replicates (Table 5).

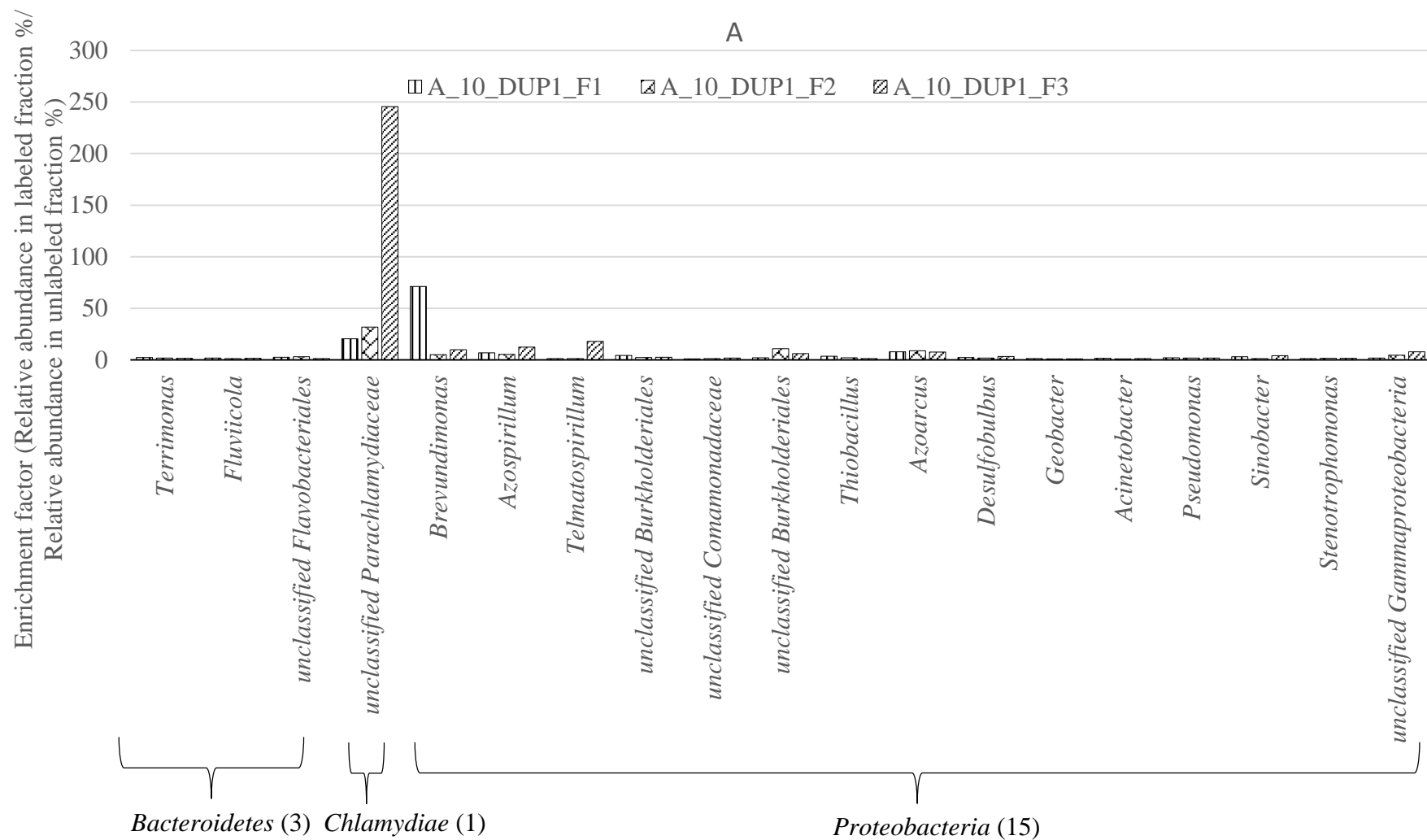
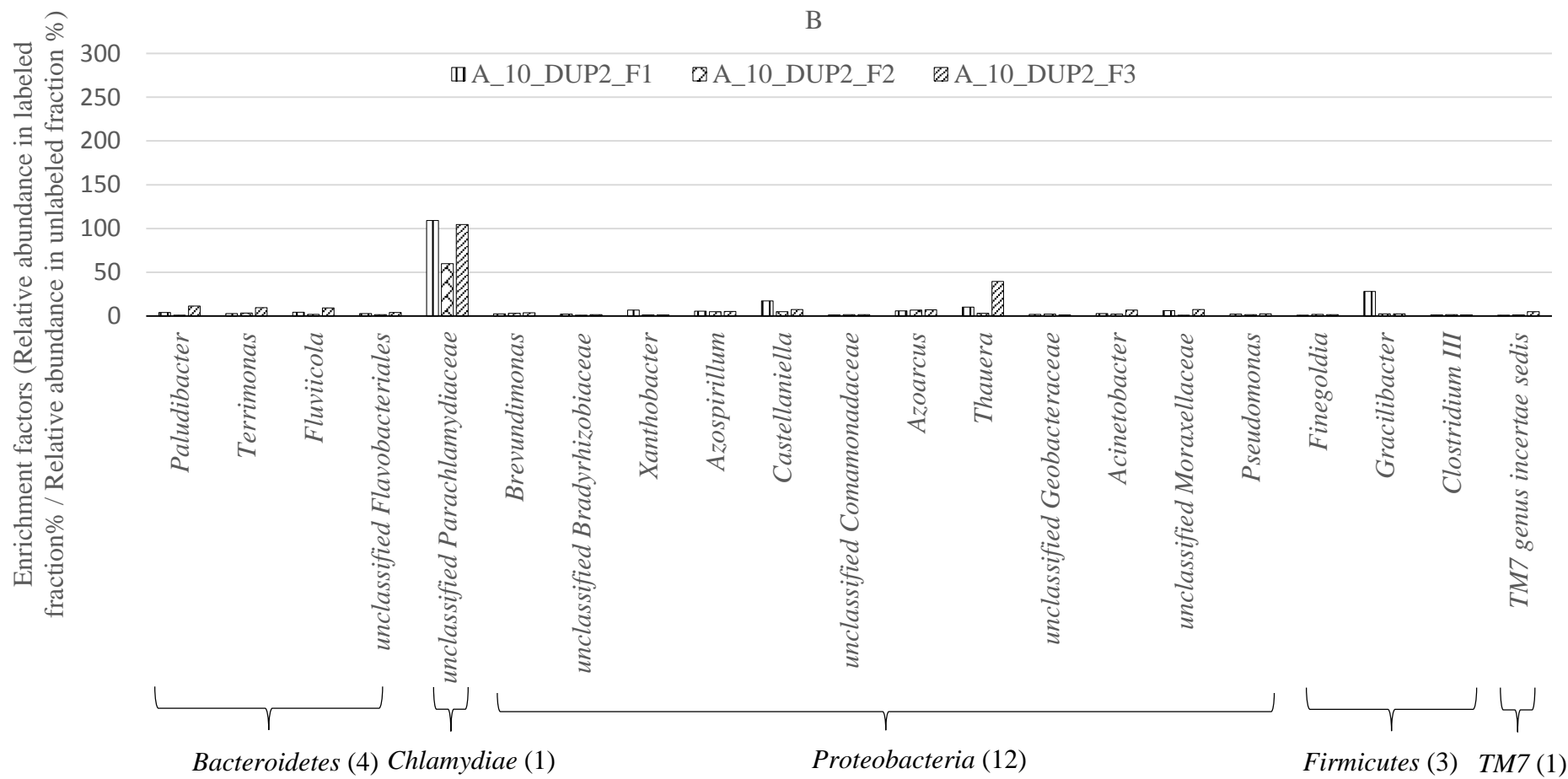


Figure 6: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled sodium acetate 10 ohms samples to the unlabeled sodium acetate 10 ohms samples for two duplicates (A and B)

Figure 6 (Cont'd)



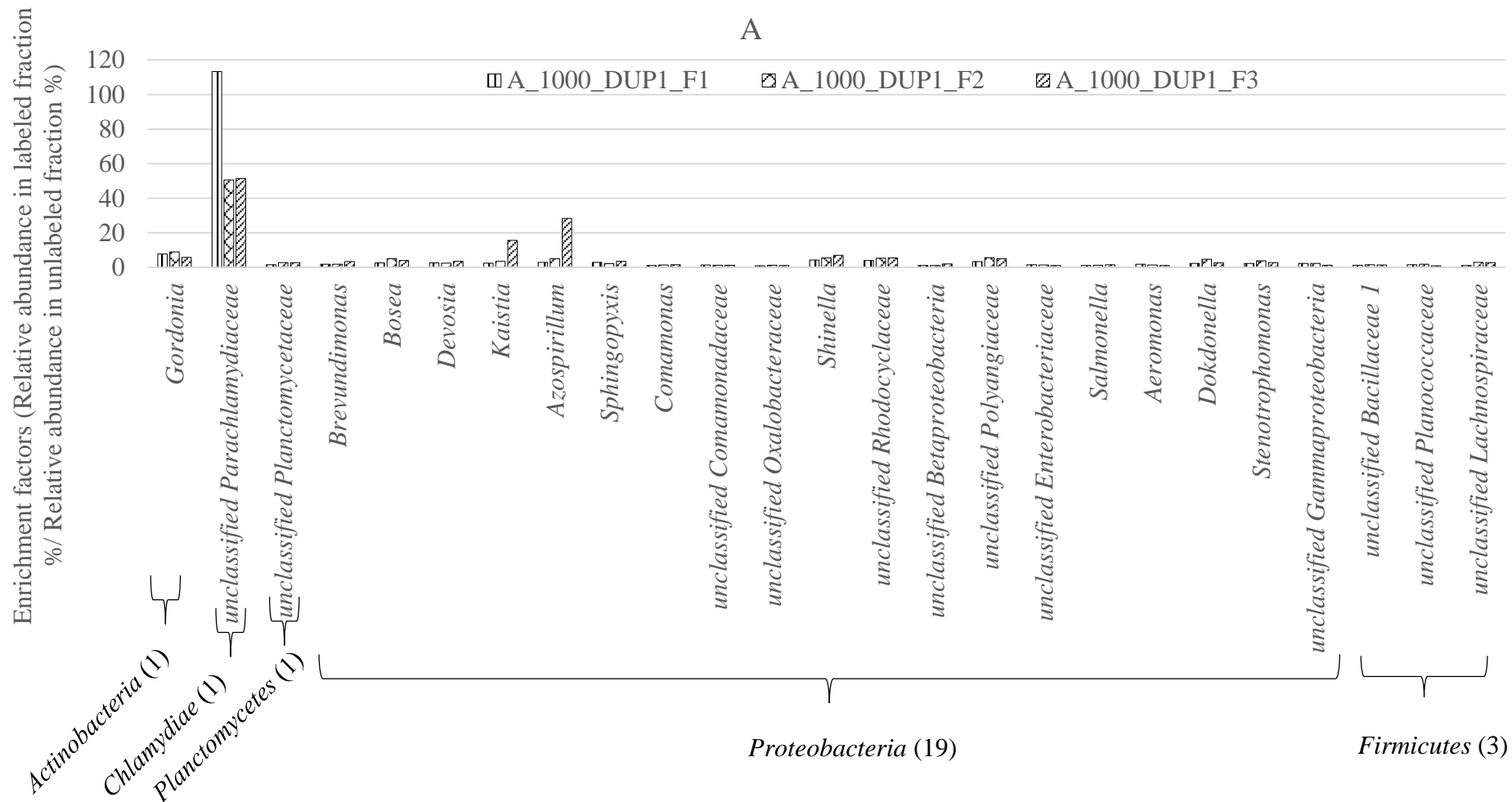
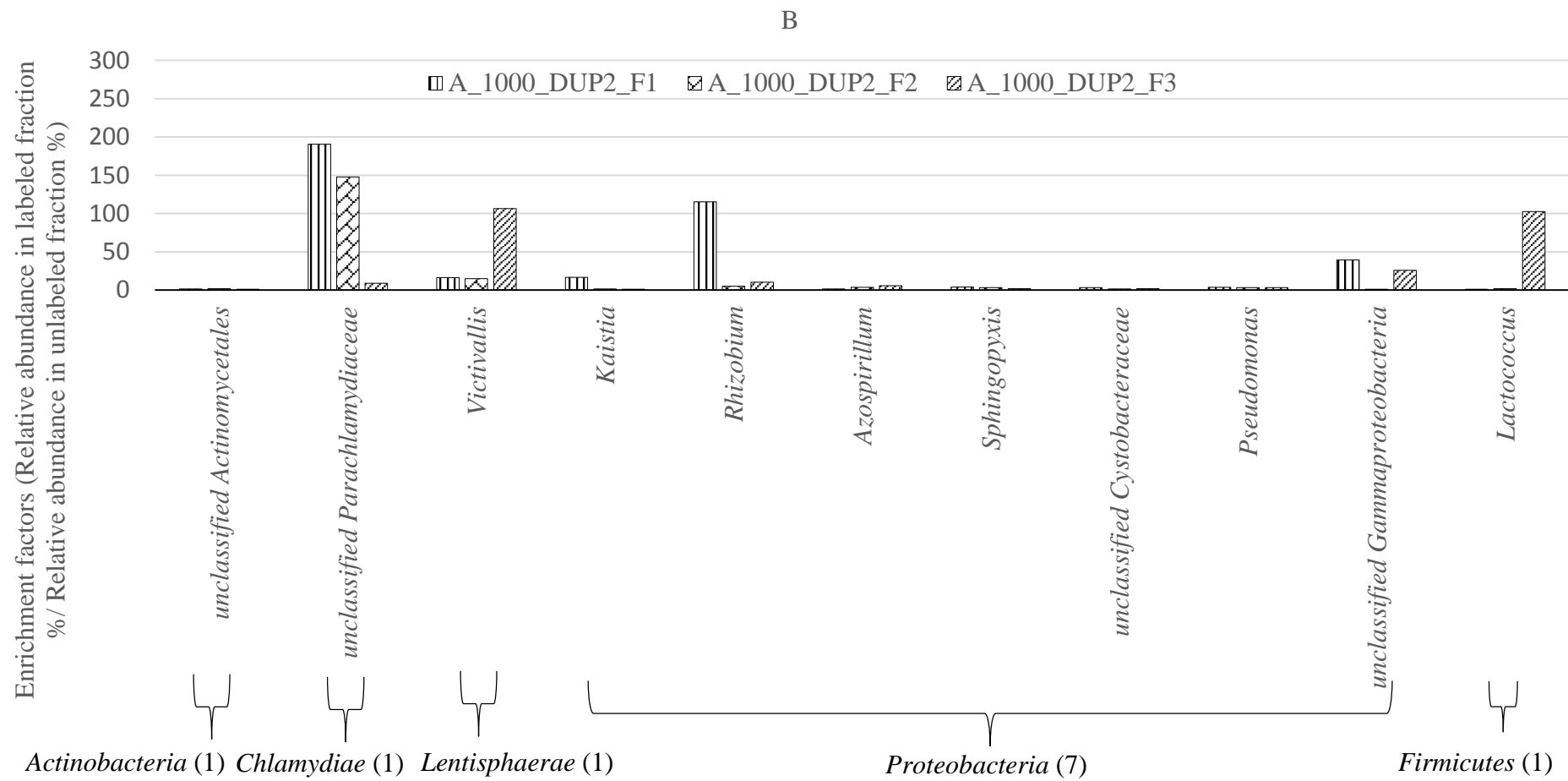


Figure 7: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled sodium acetate 1000 ohms samples to the unlabeled sodium acetate 1000 ohms samples for two duplicates (A and B)

Figure 7 (Cont'd)





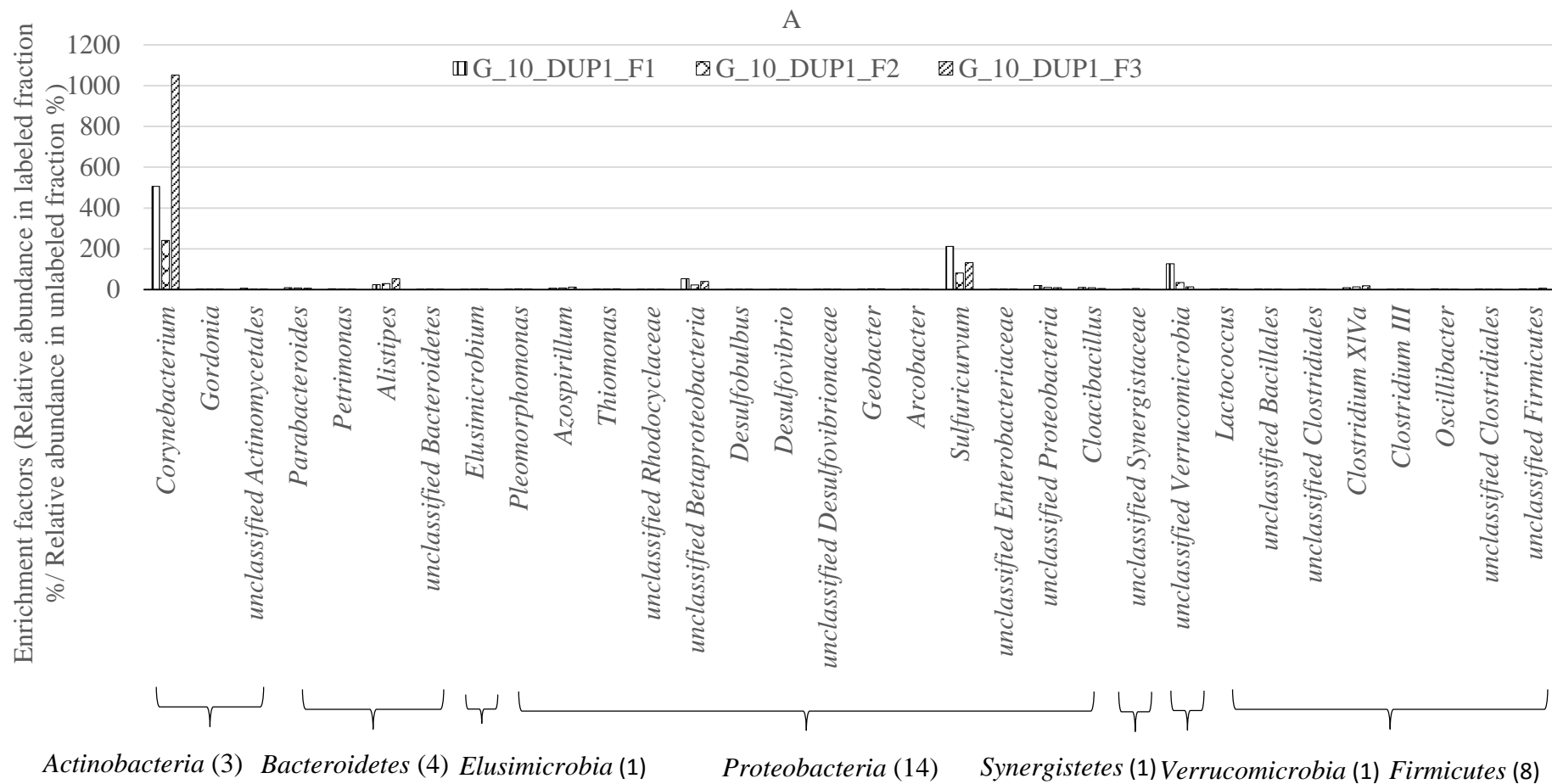
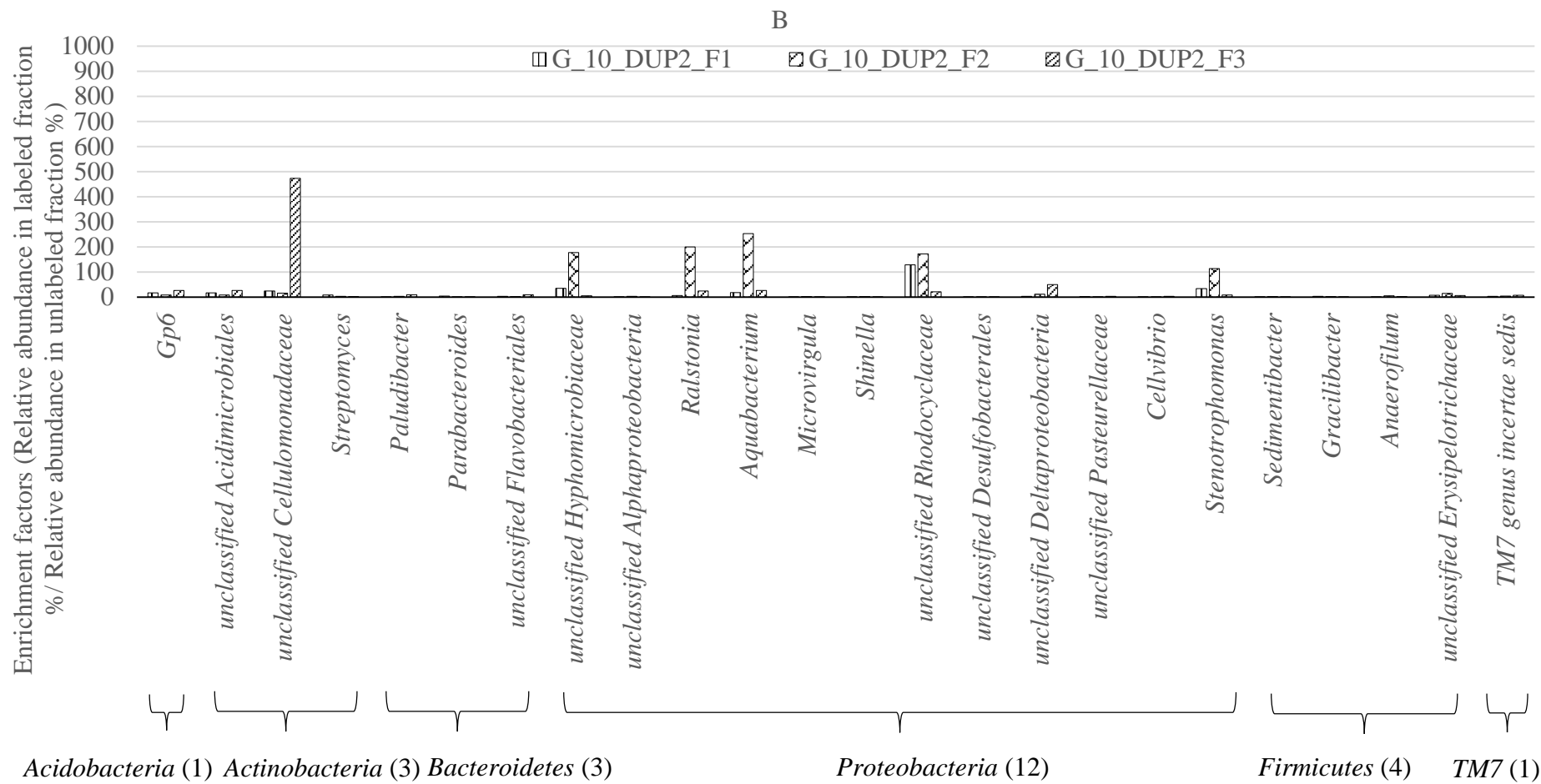


Figure 8: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled glucose 10 ohms samples to the unlabeled glucose 10 ohms samples for two duplicates (A and B)

Figure 8 (Cont'd)



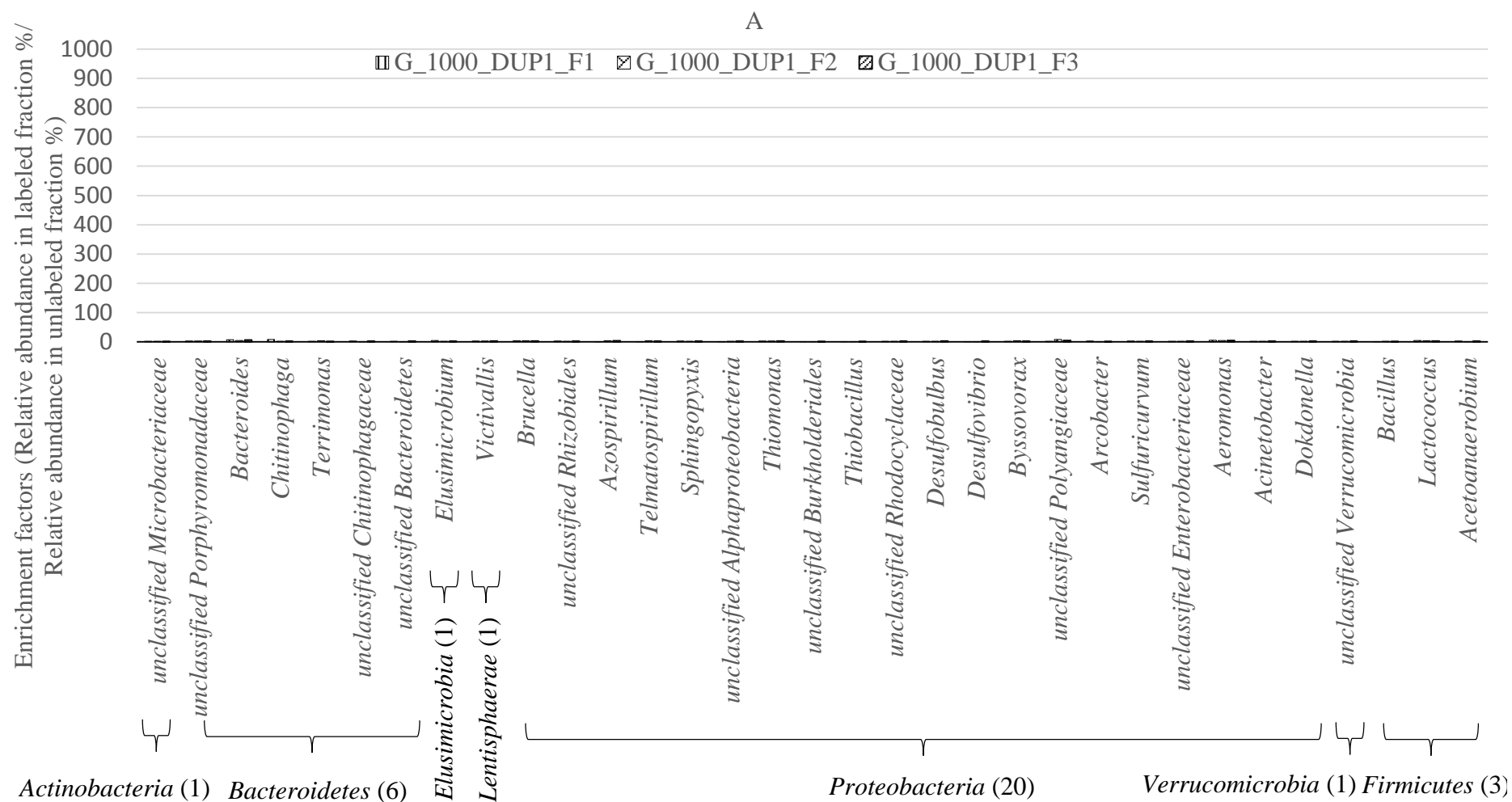


Figure 9: Enrichment factor of select OTUs (at genus level unless unclassified) in the heavy fractions of the labeled glucose 1000 ohms samples to the unlabeled glucose 1000 ohms samples for two duplicates (A and B)

Figure 9 (Cont'd)

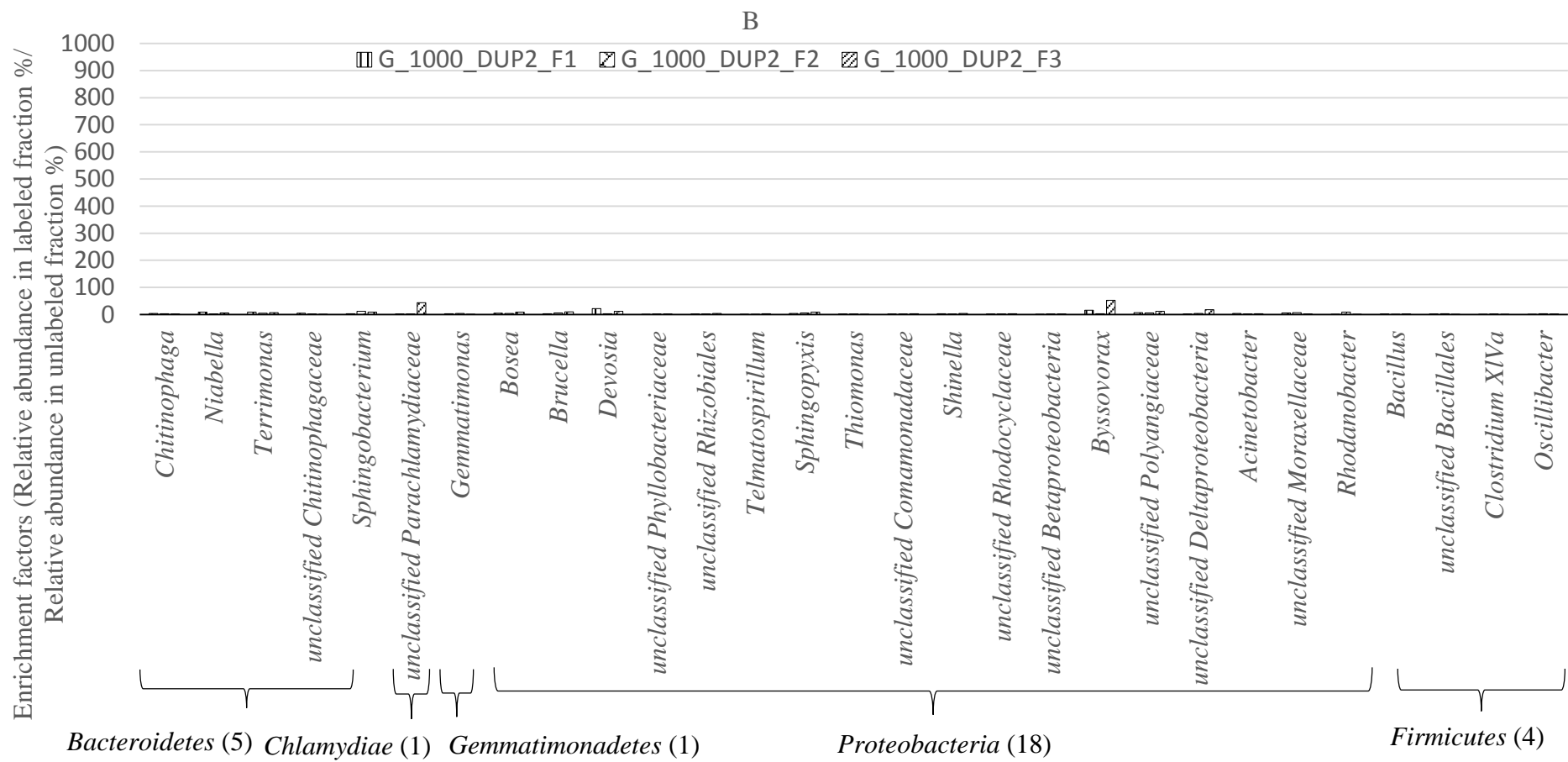


Table 5: Summary of genera enriched in both duplicates for sodium acetate (10 ohms/ 1000 ohms) and glucose (10 ohms/ 1000 ohms) fed MFCs samples

Sample	Phylum	Genus	Average enrichment factor
Sodium acetate 10 ohms	<i>Chlamydiae</i>	<i>unclassified Parachlamydiaceae</i>	95.2
	<i>Proteobacteria</i>	<i>Brevundimonas</i>	16.0
	<i>Proteobacteria</i>	<i>Azoarcus</i>	7.4
	<i>Proteobacteria</i>	<i>Azospirillum</i>	6.8
	<i>Bacteroidetes</i>	<i>Terrimonas</i>	3.7
	<i>Bacteroidetes</i>	<i>Fluviicola</i>	3.4
	<i>Proteobacteria</i>	<i>Acinetobacter</i>	2.7
	<i>Bacteroidetes</i>	<i>unclassified Flavobacteriales</i>	2.6
	<i>Proteobacteria</i>	<i>unclassified Comamonadaceae</i>	1.4
Sodium acetate 1000 ohms	<i>Chlamydiae</i>	<i>unclassified Parachlamydiaceae</i>	93.7
	<i>Proteobacteria</i>	<i>unclassified Gammaproteobacteria</i>	12.0
	<i>Proteobacteria</i>	<i>Azospirillum</i>	7.9
	<i>Proteobacteria</i>	<i>Kaistia</i>	6.8
	<i>Proteobacteria</i>	<i>Sphingopyxis</i>	2.9
Glucose 10 ohms	<i>Proteobacteria</i>	<i>unclassified Rhodocyclaceae</i>	54.4
	<i>Bacteroidetes</i>	<i>Parabacteroides</i>	5.1
Glucose 1000 ohms	<i>Proteobacteria</i>	<i>Byssovorax</i>	13.1
	<i>Proteobacteria</i>	<i>unclassified Polyangiaceae</i>	6.4
	<i>Proteobacteria</i>	<i>Brucella</i>	4.7
	<i>Bacteroidetes</i>	<i>Terrimonas</i>	4.6
	<i>Proteobacteria</i>	<i>Sphingopyxis</i>	4.0
	<i>Bacteroidetes</i>	<i>Chitinophaga</i>	3.3
	<i>Bacteroidetes</i>	<i>unclassified Chitinophagaceae</i>	2.3
	<i>Proteobacteria</i>	<i>Thiomonas</i>	2.1
	<i>Proteobacteria</i>	<i>Telmatospirillum</i>	1.9
	<i>Proteobacteria</i>	<i>unclassified Rhodocyclaceae</i>	1.8
	<i>Firmicutes</i>	<i>Bacillus</i>	1.3

The phylotypes in Table 5 were dominant in label uptake and were therefore important microorganisms for MFC function. Within the MFCs amended with sodium acetate, phylotypes *unclassified Parachlamydiaceae* (*Chlamydia*) and *Azospirillum* (*Proteobacteria*)

were enriched in both 10 ohms and 1000 ohms replicates. And within MFCs amended with glucose, *unclassified Rhodocyclaceae* were enriched in both 10 ohms and 1000 ohms replicates.

#### 4. DISCUSSION

To the author's knowledge, this is the first study that combines SIP with high-throughput sequencing to identify active community members in MFCs. SIP, a cultivation independent technique, enables researchers to identify microorganisms involved in label uptake.

High-throughput sequencing provides a greater amount of data compared to traditional sequencing methods. The combination of these two methods enables the investigation of potentially low abundance, but functionally important microorganisms. Although MiSeq sequencing provides shorter reads than 454 pyrosequencing, this study as well as other research demonstrated the availability to apply paired-end MiSeq sequencing to identify diverse microbial communities (24, 46).

The sequencing results for the overall microbial community of the MFCs anodes illustrates a diverse collection of microorganisms. The major phyla were *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* for sodium acetate amended MFCs. *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were also the dominant phyla within glucose amended MFCs. The dominant abundance of *Proteobacteria* is consistent with prior research results (Table 1). For example, *Aeromonas hydrophila* (*Gammaproteobacteria*), *Geobacter metallireducens* (*Deltaproteobacteria*), and *Geobacter sulfurreducens* (*Deltaproteobacteria*) were found in acetate amended MFCs. Further, *Alcaligenes faecalis* (*Betaproteobacteria*), *Pseudomonas aeruginosa* (*Gammaproteobacteria*), *Gluconobacter oxydans* (*Alphaproteobacteria*), *Klebsiella pneumoniae* (*Gammaproteobacteria*), *Proteus mirabilis* (*Gamaproteobacteria*),

and *Pseudomonas aeruginosa* (Gammaproteobacteria) were identified in glucose fed MFCs (22).

The occurrence of microorganisms in the phyla *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* in MFCs anode chambers was previously reported. One project reported *Bacteroidetes* could contribute to the power generation in granular semicoke anodic MFC with sodium acetate as the substrate (47). Also, *Bacteroidetes* and *Firmicutes* were detected within air-cathode MFCs fed with acetate (48). *Actinobacteria* and *Firmicutes* were reported on the anode biofilm in MFC fed with acetate and propionate (49). In addition, *Bacteroidetes* and *Firmicutes* were detected in a membrane-less MFC with glucose as the carbon source (50). Jung indicated that *Firmicutes* were only found within glucose fed MFCs (5). Another former study showed most *Firmicutes* phylum were related to glucose (22). In the current study, both glucose and sodium acetate fed MFCs microcosms contained *Firmicutes*. Phyla of *Bacteroidetes*, *Firmicutes*, and *Actinobacteria* in MFCs anode chamber were also identified with other substrates additions (20, 51).

Comparing relative abundance between the eight microcosms, the *Proteobacteria* in the glucose amended MFCs had a relatively higher abundance than those in sodium acetate amended MFCs. In contrast, microorganisms within the phylum *Actinobacteria* were more abundant in the sodium acetate amended MFCs. Glucose is a fermentative substrate and sodium acetate is a non-fermentative substrate. Fermentation acts as important role in the utilization of complex substances in MFCs. The differences of fermentative and



non-fermentative substrates would have an effect on the structure of anode microbial community (52). Some organisms in *Proteobacteria* would participate in the process of degrading complex substrate into simple substances. Comparing the results from the two types of external resistance, the phyla percentage change was complex. The only obvious trend was that *Firmicutes* exhibited a higher abundance at the lower external resistance (10 ohms).

A different trend was described in a study using azo dye as substrate in MFCs. They found no species belonging to *Firmicutes* phylum with the external resistance of 10 ohms. Whereas, some microorganisms were identified as phylum of *Firmicutes* with 510 ohms external resistance (53).

At the genus level, *unclassified Parachlamydiaceae (Chlamydia)*, *Azospirillum (Proteobacteria)* were enriched in all sodium acetate amended MFCs anodes, and *unclassified Rhodocyclaceae* were enriched in all glucose fed MFCs anodes. To my knowledge, the family *Parachlamydiaceae* has not been identified as an important phylotype in MFCs in previous studies. Previously, researchers identified the *Parachlamydiaceae* in drinking water, which may have been attributed to an infectious bovine abortion (54).

*Parachlamydiaceae*, often existing as endosymbionts of amoebae, have also been found in waste water treatment plant samples (55). Consistent with this, the MFCs in the current study were inoculated with activated sludge samples. The current study suggests microorganisms in this family are important for MFCs electricity generation when acetate is the added substrate.

Interestingly, *Azospirillum* has previously been linked to electrochemical activity. This phylotype was enriched in microbial electrolysis cell (MEC) bio-cathodes, which were transferred from sediment MFC bio-anodes (56). Microorganisms from the family *Rhodocyclaceae* were also noted in MFCs anode chambers from several previous studies (48, 57).

Notably, the enrichment factors from the glucose amended high external resistance (1000 ohms) MFCs were lower than other three treatments. One possible reason could be that glucose contributes to other microbial processes, such as fermentation, and methanogenesis under higher external resistance values and using terminal electron acceptors that do not lead to electricity generation (58). This explanation would be also evidenced by the relative low coulombic efficiency of higher resistance (glucose 1000 ohms) compared with lower resistance (glucose 10 ohms) in Table 2. This change might transfer carbon to other forms (e.g. CO<sub>2</sub>) rather than retain the carbon within the cells in the MFC.

## 5. CONCLUSION

Stable isotope probing (SIP) and high-throughput sequencing were used to i) profile overall microbial community present and ii) identify the microorganisms responsible for carbon uptake at the MFC anode over four experimental treatments (with replication). This involved the analysis of eight samples of DNA extracted from the MFCs anodes. The MFCs samples differed from each other by substrate type (labelled and unlabeled acetate or glucose) and external resistance (10 ohms and 1000 ohms). The results illustrated the anodes consisted of a diverse microbial community. For the sodium acetate amended MFCs, the dominant phyla were *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*. For the glucose amended MFCs, in addition to the above three phyla, *Actinobacteria* was also detected as important phylum. Through comparing enrichment factors between the labeled and unlabeled fractions, 14 phylotypes were enriched in both replicates of the acetate amended MFC anodes (including both resistance levels). Also, 13 phylotypes were enriched in both replicates of the glucose amended MFC anodes (including both resistance levels). Among these, *unclassified Parachlamydiaceae* (*Chlamydia*), *Azospirillum* (*Proteobacteria*), and *unclassified Rhodocyclaceae* (*Proteobacteria*) were the dominant label uptaking phylotypes at the MFCs anode. This study demonstrated that combination of SIP and high-throughput sequencing is a useful tool to characterize microbial community and identify functional organisms in MFCs.

## **REFERENCES**

## REFERENCES

1. **Ahmad F, Atiyeh MN, Pereira B, Stephanopoulos GN.** 2013. A review of cellulosic microbial fuel cells: Performance and challenges. *Biomass & Bioenergy* **56**:179-188.
2. **Jang JK, Ryou YS, Kim JG, Kang YK, Lee EY.** 2012. Use of Nitrate and Ferric Ion as Electron Acceptors in Cathodes to Improve Current Generation in Single-cathode and Dual-cathode Microbial Fuel Cells. *Korean Journal of Microbiology and Biotechnology* **40**:414-418.
3. **Rabaey K, Verstraete W.** 2005. Microbial fuel cells: novel biotechnology for energy generation. *Trends in Biotechnology* **23**:291-298.
4. **Pant D, Van Bogaert G, Diels L, Vanbroekhoven K.** 2010. A review of the substrates used in microbial fuel cells (MFCs) for sustainable energy production. *Bioresource Technology* **101**:1533-1543.
5. **Jung S, Regan JM.** 2007. Comparison of anode bacterial communities and performance in microbial fuel cells with different electron donors. *Applied Microbiology and Biotechnology* **77**:393-402.
6. **Pant D, Van Bogaert G, De Smet M, Diels L, Vanbroekhoven K.** 2010. Use of novel permeable membrane and air cathodes in acetate microbial fuel cells. *Electrochimica Acta* **55**:7710-7716.
7. **Jung S, Regan JM.** 2011. Influence of External Resistance on Electrogenesis, Methanogenesis, and Anode Prokaryotic Communities in Microbial Fuel Cells. *Applied and Environmental Microbiology* **77**:564-571.
8. **Rismani-Yazdi H, Carver SM, Christy AD, Tuovinen IH.** 2008. Cathodic limitations in microbial fuel cells: An overview. *Journal of Power Sources* **180**:683-694.
9. **Logan B, Cheng S, Watson V, Estadt G.** 2007. Graphite fiber brush anodes for increased power production in air-cathode microbial fuel cells. *Environmental Science & Technology* **41**:3341-3346.

10. **Rabaey K, Lissens G, Siciliano SD, Verstraete W.** 2003. A microbial fuel cell capable of converting glucose to electricity at high rate and efficiency. *Biotechnology Letters* **25**:1531-1535.
11. **Chae K-J, Choi M-J, Lee J-W, Kim K-Y, Kim IS.** 2009. Effect of different substrates on the performance, bacterial diversity, and bacterial viability in microbial fuel cells. *Bioresource Technology* **100**:3518-3525.
12. **Ren Z, Steinberg LM, Regan JM.** 2008. Electricity production and microbial biofilm characterization in cellulose-fed microbial fuel cells. *Water Science and Technology* **58**:617-622.
13. **Rezaei F, Xing DF, Wagner R, Regan JM, Richard TL, Logan BE.** 2009. Simultaneous Cellulose Degradation and Electricity Production by *Enterobacter cloacae* in a Microbial Fuel Cell. *Applied and Environmental Microbiology* **75**:3673-3678.
14. **Debabov VG.** 2008. Electricity from microorganisms. *Microbiology* **77**:123-131.
15. **Ahn Y, Logan BE.** 2010. Effectiveness of domestic wastewater treatment using microbial fuel cells at ambient and mesophilic temperatures. *Bioresource Technology* **101**:469-475.
16. **Li XM, Cheng KY, Selvam A, Wong JWC.** 2013. Bioelectricity production from acidic food waste leachate using microbial fuel cells: Effect of microbial inocula. *Process Biochemistry* **48**:283-288.
17. **Xing DF, Cheng SA, Regan JM, Logan BE.** 2009. Change in microbial communities in acetate- and glucose-fed microbial fuel cells in the presence of light. *Biosensors & Bioelectronics* **25**:105-111.
18. **Chung KM, Okahe S.** 2009. Characterization of Electrochemical Activity of a Strain IS02-3 Phylogenetically Related to *Aeromonas* sp Isolated From a Glucose-Fed Microbial Fuel Cell. *Biotechnology and Bioengineering* **104**:901-910.
19. **Ishii Si, Suzuki S, Norden-Krichmar TM, Wu A, Yamanaka Y, Nealson KH, Bretschger O.** Identifying the microbial communities and operational conditions for optimized wastewater treatment in microbial fuel cells. *Water Research*.

20. **Zhang GD, Zhao QL, Jiao Y, Wang K, Lee DJ, Ren NQ.** 2012. Biocathode microbial fuel cell for efficient electricity recovery from dairy manure. *Biosensors & Bioelectronics* **31**:537-543.
21. **Phung NT, Lee J, Kang KH, Chang IS, Gadd GM, Kim BH.** 2004. Analysis of microbial diversity in oligotrophic microbial fuel cells using 16S rDNA sequences. *FEMS Microbiology Letters* **233**:77-82.
22. **Du Z, Li H, Gu T.** 2007. A state of the art review on microbial fuel cells: A promising technology for wastewater treatment and bioenergy. *Biotechnology Advances* **25**:464-482.
23. **Fremont M, Coomans D, Massart S, De Meirleir K.** 2013. High-throughput 16S rRNA gene sequencing reveals alterations of intestinal microbiota in myalgic encephalomyelitis/chronic fatigue syndrome patients. *Anaerobe* **22**:50-56.
24. **Ansorge WJ.** 2009. Next-generation DNA sequencing techniques. *New Biotechnology* **25**:195-203.
25. **Zhang J, Chiodini R, Badr A, Zhang G.** 2011. The impact of next-generation sequencing on genomics. *Journal of Genetics and Genomics* **38**:95-109.
26. **Di Bella JM, Bao Y, Gloor GB, Burton JP, Reid G.** 2013. High throughput sequencing methods and analysis for microbiome research. *Journal of Microbiological Methods* **95**:401-414.
27. **Strausberg RL, Levy S, Rogers Y-H.** 2008. Emerging DNA sequencing technologies for human genomic medicine. *Drug Discovery Today* **13**:569-577.
28. **Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, Berka J, Braverman MS, Chen YJ, Chen ZT, Dewell SB, Du L, Fierro JM, Gomes XV, Godwin BC, He W, Helgesen S, Ho CH, Irzyk GP, Jando SC, Alenquer MLI, Jarvie TP, Jirage KB, Kim JB, Knight JR, Lanza JR, Leamon JH, Lefkowitz SM, Lei M, Li J, Lohman KL, Lu H, Makhijani VB, McDade KE, McKenna MP, Myers EW, Nickerson E, Nobile JR, Plant R, Puc BP, Ronan MT, Roth GT, Sarkis GJ, Simons JF, Simpson JW, Srinivasan M, Tartaro KR, Tomasz A, Vogt KA, Volkmer GA, Wang SH, Wang Y, Weiner MP, Yu PG, Begley RF, Rothberg JM.** 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature* **437**:376-380.

29. **Li B, Zhang XX, Guo F, Wu WM, Zhang T.** 2013. Characterization of tetracycline resistant bacterial community in saline activated sludge using batch stress incubation with high-throughput sequencing analysis. *Water Research* **47**:4207-4216.
30. **Yoder-Himes DR, Chain PSG, Zhu Y, Wurtzel O, Rubin EM, Tiedje JM, Sorek R.** 2009. Mapping the *Burkholderia cenocepacia* niche response via high-throughput sequencing. *Proceedings of the National Academy of Sciences of the United States of America* **106**:3976-3981.
31. **Marguerat S, Wilhelm BT, Bahler J.** 2008. Next-generation sequencing: applications beyond genomes. *Biochemical Society Transactions* **36**:1091-1096.
32. **Passalacqua KD, Varadarajan A, Ondov BD, Okou DT, Zwick ME, Bergman NH.** 2009. Structure and Complexity of a Bacterial Transcriptome. *Journal of Bacteriology* **191**:3203-3211.
33. **Li LL, Norman A, Hansen LH, Sorensen SJ.** 2012. Metamobilomics--expanding our knowledge on the pool of plasmid encoded traits in natural environments using high-throughput sequencing. *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases* **18 Suppl 4**:5-7.
34. **van Vliet AHM.** 2010. Next generation sequencing of microbial transcriptomes: challenges and opportunities. *Fems Microbiology Letters* **302**:1-7.
35. **Friedrich MW.** 2006. Stable-isotope probing of DNA: insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. *Current Opinion in Biotechnology* **17**:59-66.
36. **Madsen EL.** 2006. The use of stable isotope probing techniques in bioreactor and field studies on bioremediation. *Current Opinion in Biotechnology* **17**:92-97.
37. **Uhlik O, Jecna K, Leigh MB, Mackova M, Macek T.** 2009. DNA-based stable isotope probing: a link between community structure and function. *Science of the Total Environment* **407**:3611-3619.
38. **Uhlik O, Leewis MC, Strejcek M, Musilova L, Mackova M, Leigh MB, Macek T.** 2013. Stable isotope probing in the metagenomics era: A bridge towards improved bioremediation. *Biotechnology Advances* **31**:154-165.



39. **Radajewski S, Ineson P, Parekh NR, Murrell JC.** 2000. Stable-isotope probing as a tool in microbial ecology. *Nature* **403**:646-649.
40. **Radajewski S, McDonald IR, Murrell JC.** 2003. Stable-isotope probing of nucleic acids: a window to the function of uncultured microorganisms. *Current Opinion in Biotechnology* **14**:296-302.
41. **Cupples AM.** 2011. The use of nucleic acid based stable isotope probing to identify the microorganisms responsible for anaerobic benzene and toluene biodegradation. *Journal of Microbiological Methods* **85**:83-91.
42. **Logan BE, Hamelers B, Rozendal RA, Schrorder U, Keller J, Freguia S, Aelterman P, Verstraete W, Rabaey K.** 2006. Microbial fuel cells: Methodology and technology. *Environmental Science & Technology* **40**:5181-5192.
43. **Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M.** 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* **6**:1621-1624.
44. **Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF.** 2009. Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology* **75**:7537-7541.
45. **Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD.** 2013. Development of a Dual-Index Sequencing Strategy and Curation Pipeline for Analyzing Amplicon Sequence Data on the MiSeq Illumina Sequencing Platform. *Applied and Environmental Microbiology* **79**:5112-5120.
46. **Liang B, Cheng H, Van Nostrand JD, Ma J, Yu H, Kong D, Liu W, Ren N, Wu L, Wang A, Lee D-J, Zhou J.** 2014. Microbial community structure and function of Nitrobenzene reduction biocathode in response to carbon source switchover. *Water research* **54**:137-148.
47. **Sun Y, Wei J, Liang P, Huang X.** 2011. Electricity generation and microbial community changes in microbial fuel cells packed with different anodic materials. *Bioresource Technology* **102**:10886-10891.

48. **Borole AP, Hamilton CY, Vishnivetskaya T, Leak D, Andras C.** 2009. Improving power production in acetate-fed microbial fuel cells via enrichment of exoelectrogenic organisms in flow-through systems. *Biochemical Engineering Journal* **48**:71-80.
49. **Zhang GD, Zhao QL, Jiao Y, Wang K, Lee DJ, Ren NQ.** 2012. Efficient electricity generation from sewage sludge using biocathode microbial fuel cell. *Water Research* **46**:43-52.
50. **Aldrovandi A, Marsili E, Stante L, Paganin P, Tabacchioni S, Giordano A.** 2009. Sustainable power production in a membrane-less and mediator-less synthetic wastewater microbial fuel cell. *Bioresource Technology* **100**:3252-3260.
51. **Zhang Y, Min B, Huang L, Angelidaki I.** 2011. Electricity generation and microbial community response to substrate changes in microbial fuel cell. *Bioresource Technology* **102**:1166-1173.
52. **Quan XC, Quan YP, Tao K, Jiang XM.** 2013. Comparative investigation on microbial community and electricity generation in aerobic and anaerobic enriched MFCs. *Bioresource Technology* **128**:259-265.
53. **Fernando E, Keshavarz T, Kyazze G.** 2014. External resistance as a potential tool for influencing azo dye reductive decolourisation kinetics in microbial fuel cells. *International Biodeterioration & Biodegradation* **89**:7-14.
54. **Wheelhouse N, Sait M, Gidlow J, Deuchande R, Borel N, Bailly J, Caldow G, Longbottom D.** 2011. Molecular detection of Chlamydia-like organisms in cattle drinking water. *Veterinary Microbiology* **152**:196-199.
55. **Horn M, Wagner M.** 2001. Evidence for additional genus-level diversity of Chlamydiales in the environment. *Fems Microbiology Letters* **204**:71-74.
56. **Pisciotta JM, Zaybak Z, Call DF, Nam JY, Logan BE.** 2012. Enrichment of Microbial Electrolysis Cell Biocathodes from Sediment Microbial Fuel Cell Bioanodes. *Applied and Environmental Microbiology* **78**:5212-5219.
57. **Quan X-c, Quan Y-p, Tao K.** 2012. Effect of anode aeration on the performance and microbial community of an air-cathode microbial fuel cell. *Chemical Engineering Journal* **210**:150-156.

58. **Logan BE, Regan JM.** 2006. Electricity-producing bacterial communities in microbial fuel cells. *Trends in Microbiology* **14**:512-518.