# AN INVESTIGATION OF MICROBIAL DIVERSITY AND MICROBIOLOGICALLY INFLUENCED CORROSION IN AUTOMOTIVE FUEL ENVIRONMENTS

by

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ABSTRACT

Microbial contamination of fuels can cause issues such as biofouling, fuel degradation and microbiologically influenced corrosion (MIC). The focus of the research presented in this thesis was characterizing the microbial diversity of automotive fuels and automotive fuel environments in the United States via both molecular-based techniques as well as cultivation-based methods in order to gain insight into how this diversity is impacting fuels and fuel system infrastructure. A field survey of fuels including biodiesel, diesel, E10, E85, fuel-grade ethanol and gasoline was conducted; and 454 pyrosequencing of both 16S/18S rRNA genes as well as 16S/18S rRNA (transcribed into cDNA) was applied to identify both total and active microbial communities in these environments. Microbial communities in all fuel types were broadly similar, and prevalent phylotypes included *Halomonas* spp., *Pseudomonas* spp., *Shewanella* spp., *Corynebacterium* spp. and *Acetobacter* spp. Pyrosequencing libraries generated from cDNA and DNA indicated that the active and total communities of the sampled environments show significant overlap. The microbial communities of storage tanks containing fuel-grade ethanol and water were also characterized by molecular and cultivation-based techniques. Industry personnel have reported corrosion issues (suspected to be microbial corrosion) impacting storage tanks and other infrastructure exposed to fuel-grade ethanol and water, and acetic-acid-producing microbes were prevalent in samples collected from these environments. *Acetobacter* spp. and sulfate-reducing microbes were cultivated from samples collected from these storage tanks for laboratory corrosion testing. These corrosion tests (reported elsewhere) indicated that *Acetobacter* spp. increased pitting and cracking of carbon steels and that sulfate-reducing

microbes increased general corrosion rates as well as increased pitting and cracking of carbon steels. Additionally, a *Bacillus* sp. that produces spores that catalyze Mn(II) oxidation was isolated from an E10 fuel sample. The potential impact that these sorts of microbes may have on corrosion in fuel system infrastructure is discussed. Increased knowledge of the the microbial diversity associated with fuel system infrastructure will improve monitoring and prevention strategies and guide future research of issues such as microbial corrosion in fuel systems.

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LIST OF ACRONYMS

| | |
|---|---|
| API | American Petroleum Institute |
| BLAST | Bacic Local Alignment Search Tool |
| cDNA | complimentary deoxyribonucleic acid |
| DGGE | denaturing gradient gel electrophoresis |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| eSCC | ethanol stress corrosion cracking |
| HEPES | hydroxyethyl)piperazine-1-ethanesulfonic acid |
| LBB | leucoberbelin blue |
| MCO | multicopper oxidase |
| MIC | microbiologically influenced corrosion |
| MPN | most probable number |
| OTU | operational taxonomic unit |
| PCR | polymerase chain reaction |
| PMSF | phenylmethylsulfonyl fluoride |
| RNA | ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| SDS | sodium dodecyl sulfate |
| SSU | small subunit |

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# CHAPTER 1

## INTRODUCTION AND BACKGROUND

Microorganisms, or microbes, are literally everywhere, and these tiny organisms impact their environments immensely. Humans are often the beneficiaries of microbial activity as microbes are present in the human gut and aid in digestion, microbes in wastewater treatment facilities allow for the removal of undesirable components from wastewater, microbes inhabit root nodules and live syntrophically with plants promoting plant growth, and microbes aid in the production of foods such as cheese, yogurt and beer. While microbial activity is beneficial to humans in many ways, sometimes microbes and humans are at odds. A relatively small number of microbes are pathogenic to humans, and other microbes cause issues such as food spoilage or degradation of man-made structures such as stone monuments. Microbes can be particularly problematic in the context of industrial settings such as the production, transportation and storage of fuels due to product (fuel) degradation, biofouling and microbiologically influenced corrosion (MIC). The focus of this thesis is characterizing microbial communities present in automotive fuel environments and elucidating how these microbes may impact these environments. This chapter provides background information regarding molecular methods for investigating microbial diversity and how microbes impact environments associated with fuel production, storage and transportation.

## 1.1 Microbes and Molecular Methods

Traditionally, identifying microbes present in environments of interest required culturing a particular microbe in the laboratory and characterizing phenotypic traits. However, it has been estimated that less than one percent of microbes can be readily cultured in the laboratory (Amann et al. 1995). Thus, understanding the microbial diversity of many environments is not possible with cultivation-based methods alone. In the 1970's, Carl Woese and colleagues determined that the comparison of ribosomal ribonucleic acid (rRNA) sequences from different cultured microbes provided a way in which to determine the microbes' evolutionary relationships (Woese and Fox 1977). This discovery lead to the realization that life can be divided into three domains (the Bacteria, Archaea and Eucarya) based upon molecular phylogeny (Woese et al. 1990). The ability to determine how microbes are related by comparing rRNA sequences allowed for a survey of microbes present in environmental samples without the need to culture the microbes first (Olsen et al. 1986). As more and more rRNA sequences were produced and compared to each other a map of life based upon rRNA phylogeny (specifically, 16S/18S rRNA described below) began to take form (Figure 1.1, Pace 1997).

Molecular surveys of diversity are usually conducted by amplifying 16S or 18S small subunit (SSU) rRNA genes from nucleic acids extracted from an environmental sample using the polymerase chain reaction (PCR) (Mullis et al. 1986). Amplified gene sequences are then sequenced with one of a variety of sequencing technologies (e.g. Sanger sequencing, Roche 454 pyrosequencing or Illumina sequencing). The resulting sequences are quality checked, aligned to ensure that appropriate portions of a gene are being analyzed and compared to databases that have been accumulating for some 25 years. Molecular surveys often rely on 16S or 18S SSU rRNA for several reasons. 16S and 18S SSU rRNA in conjunction with proteins form the small

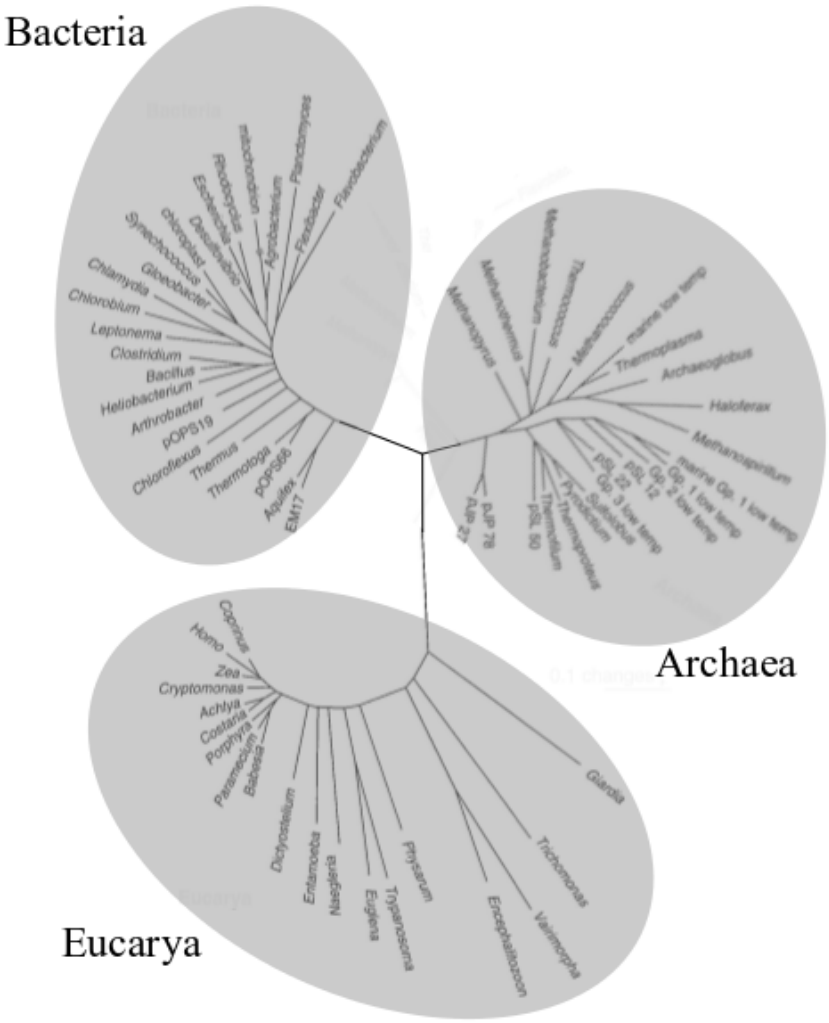


Figure 1.1: Illustration of the map of life - phylogenetic tree adapted from Pace (1997). Many molecular-based methods for surveying microbial diversity aim to determine where microbes in a particular environment fall on this map of life. Knowledge of closely related organisms can provide insight into how microbes are behaving in situ.

sub-units of ribosomes, which are structures that produce proteins in all organisms; thus, these SSU rRNA genes are found in all organisms and can be used to identify all life as we know it. The SSU rRNA is approximately 1500 nucleotides long, which provides sequences with an ample number of nucleotides for comparison. Also, portions of the SSU rRNA evolve at different rates allowing for comparison of both closely-related and distantly-related sequences (Woese 1987). While 16S/18S rRNA sequence comparisons are not sufficient to resolve all inter-organism relationships, much can be learned from these molecules. Molecular surveys of microbial diversity in environmental samples can provide information on the types of microbes present in an environment. Inferences can be made about the metabolic activities and functions of microbes closely related to cultured microbes whose genotypic and phenotypic traits are well known. Additionally, the relative abundance of SSU rRNA sequences recovered from an environment can serve as a proxy for the relative abundance of different types of microbes in an environment. However, estimations of relative abundance must be considered cautiously as not all microbes contain the same number of copies of SSU rRNA genes (Pace 2009; Klappenbach et al. 2001), and issues such as primer bias may impact the performance of amplicon-based gene surveys (Wang and Qian 2009; Hamady and Knight 2009). Primer bias may result in misrepresentations of the biodiversity in a surveyed environment due to insufficient coverage of the 16S/18S rRNA genes of the organisms present in that environment. Additionally, data analysis methods must be considered carefully in order to avoid introducing biases into molecular-based diversity surveys.

The advancement of DNA sequencing technologies and bioinformatic tools has occurred at a rapid pace allowing researchers to obtain greater and greater numbers of sequences (SSU rRNA gene sequences as well as many other types of genes and the sequencing of genomes and metagenomes) from environmental samples and to analyze these sequences with ever-developing

computational tools. In 1987 Woese (1987) envisioned one laboratory obtaining 100 new 16S rRNA sequences per year; whereas, today millions of sequences can be produced in less than a week. As sequencing technologies have improved, microbial ecologists have realized that many environments contain much greater microbial diversity than previously suspected (Hugenholtz et al. 1998; Ley et al. 2006). With increasing numbers of sequences, microbiologists can begin to understand the full microbial diversity of a single environment as well as more thoroughly compare microbial communities from different environments. Massive data sets have provided insight into the diversity of microbes in many systems (Caporaso et al. 2012; Gevers et al. 2012), and studies are moving past sequencing only 16S/18S rRNA genes to sequencing metagenomes of environmental samples (Canfield et al. 2010; Inskeep et al. 2010, Wright et al. - submitted) as well as genomes from single cells plucked from an environment (Stepanauskas 2012 and references therein). Though improved DNA sequencing technologies have greatly enhanced our ability to investigate microbial diversity without the need for cultivation, the need for rigorous cultivation-based microbial research is still paramount for fully understanding the roles microbes play in any environment.

## 1.2 Microbes and Fuel Environments

Microbes are common inhabitants of almost every environment on earth including the infrastructure involved with fuel production, storage and transportation. Though exposure to fuel may seem like a harsh environment in which to live, for many microbes these fuels can serve as a carbon source. Water tends to accumulate in tanks and pipelines, and electron donors and electron acceptors (e.g. iron species, sulfur species and oxygen present in the fuels as well as water that infiltrates fuel industry infrastructure and the materials, such as carbon steel, that are used to construct pipes and tanks) are often present, as well (Passman 2003; Lee et al. 2010).

Thus, microbes may find niches in fuel systems in which to thrive. While the presence of microbes in fuel systems is unavoidable and some microbes may not have any adverse effects when present in these environments, many issues may arise due to microbial contamination of fuels. Some of these issues are discussed in sections 1.2.1 and 1.2.2.

### 1.2.1 Microbiologically Influenced Corrosion (MIC)

Corrosion can be defined as the degradation or destruction of a metal or metal alloy due to electrochemical reactions with the environment. Extractive metallurgy requires significant energy input to transform minerals found in nature into metals or alloys, and corrosion processes release this energy as metals or alloys are returned to minerals. Basically, corrosion involves three components: an anode, a cathode and an electrolyte. Oxidation reactions and metal dissolution occur at the anodic site while cathodic reactions are reductive. Equation 1 displays the anodic dissolution of a metal.

$$Me \rightarrow Me^{n+} + ne^{-} \quad (1.1)$$

The interplay between anode, cathode and electrolyte can result in a variety of corrosion forms including uniform or general corrosion, pitting corrosion, galvanic corrosion, crevice corrosion, cracking and dealloying (Jones 1996). Corrosion is of enormous economic concern, and it has been estimated that corrosion-related issues cost approximately 3-4 percent of gross national product of industrialized nations (Revie 2011 and references therein). These costs include replacement of materials, monitoring costs, maintenance and repairs, insurance to guard against failures, redundant equipment and costs associated with remediation of spills caused by corrosion failures (Javaherdashti 2008). Of the billions of dollars lost to metallic corrosion, up to 20 percent of these costs may be attributed to microbiologically influenced corrosion (MIC)

(Flemming 1996). MIC (also referred to as biocorrosion or microbial corrosion) is the biologically-mediated deterioration of a material and can affect almost any metal or alloy as well as nonmetals. Microbes from all three domains of life enhance corrosion processes, and it is likely that corrosion in many environments is impacted by interactions of the microbial communities present. Microbial corrosion is often associated with localized corrosion such as pitting or cracking, but microbial activities can enhance other types of corrosion including general corrosion, as well. Microbial corrosion is a significant concern to many industries, and microbes have been implicated in many types of corrosion issues including failures of oil and water distribution systems, fuel storage tanks, and structures in seawater environments (e.g. ships and pilings) (Little and Lee 2007; Revie 2011 and references therein). Microbial metabolic activities and biofilm formation can impact corrosion processes in a number of ways and is dictated by many factors including the microbial diversity present, the chemical environment and the metal or alloy type. Some of the microbes, mechanisms and materials associated with MIC are discussed in sections 1.2.1.1 throught 1.2.1.4.

#### 1.2.1.1 Biofilms and Differential Concentration (Aeration) Cells

Microbes in many environments are attached to surfaces as biofilms (e.g. soap scum in a shower, plaque on teeth). Biofilms consist of microbial cells as well as microbially-produced extracellular polymeric substances, which form a matrix surrounding the cells. Biofilms provide microbes with protection from predation and toxic chemicals as well as the ability to remain stationary in nutrient-rich environments (Madigan et al. 2010). Natural biofilms often contain many types of microbes filling micro-niches resulting from heterogeneities within the biofilm. Biofilm formation on a metal surface can result in the creation of oxygen heterogeneities due to microbial metabolism (respiration) as well as differences in biofilm thickness and coverage.

Oxygen gradients along a metal surface may result in differential aeration cells and pitting corrosion (Lewandowski and Beyenal 2009). This mechanism of MIC, though demonstrated in the laboratory, is not often used to explain real-world microbial corrosion issues. A simplified illustration of this mechanism is displayed in Figure 1.2.

#### 1.2.1.2 Microbial Sulfate Reduction and Corrosion

Sulfate-reducing microbes include diverse members of the bacterial and archaeal domains that utilize sulfate ions as electron acceptors often resulting in the production of sulfides. Members of the deltaproteobacteria (e.g. *Desulfovibrio* spp.) are well-studied sulfate-reducers, though many other types exist including members of the firmicutes (see Chapter 3). Sulfate-reducing microbes have long been implicated as major contributors to MIC (Von Wolzogen Kuehr and van der Vlugt 1934; Lee et al. 1995; Little and Lee 2007), and research regarding sulfate-reducing bacteria has dominated much of the MIC literature. SRB are often associated with pitting corrosion of steel, but these microbes can also enhance environmental cracking when materials are under stress (Javaherdashti et al. 2006; Javaherdashti 2011). Wolzogen Kuehr and van der Vlugt (1934) first proposed the mechanism of cathodic depolarization to explain corrosion caused by microbial sulfate reduction. The authors theorized that sulfate-reducing bacteria oxidized cathodically generated hydrogen via hydrogenase enzymes while reducing sulfate to sulfide, which would consume products of the cathodic reaction and enhance corrosion.

In the years since this theory was first presented, it has become clear that this cathodic depolarization theory (or classical theory) does not adequately explain metallic corrosion caused by SRB (Dinh et al. 2004; Mori et al. 2010; Enning et al. 2012), and other mechanisms have been studied. Some of the mechanisms focus on how the metabolic byproducts of sulfate

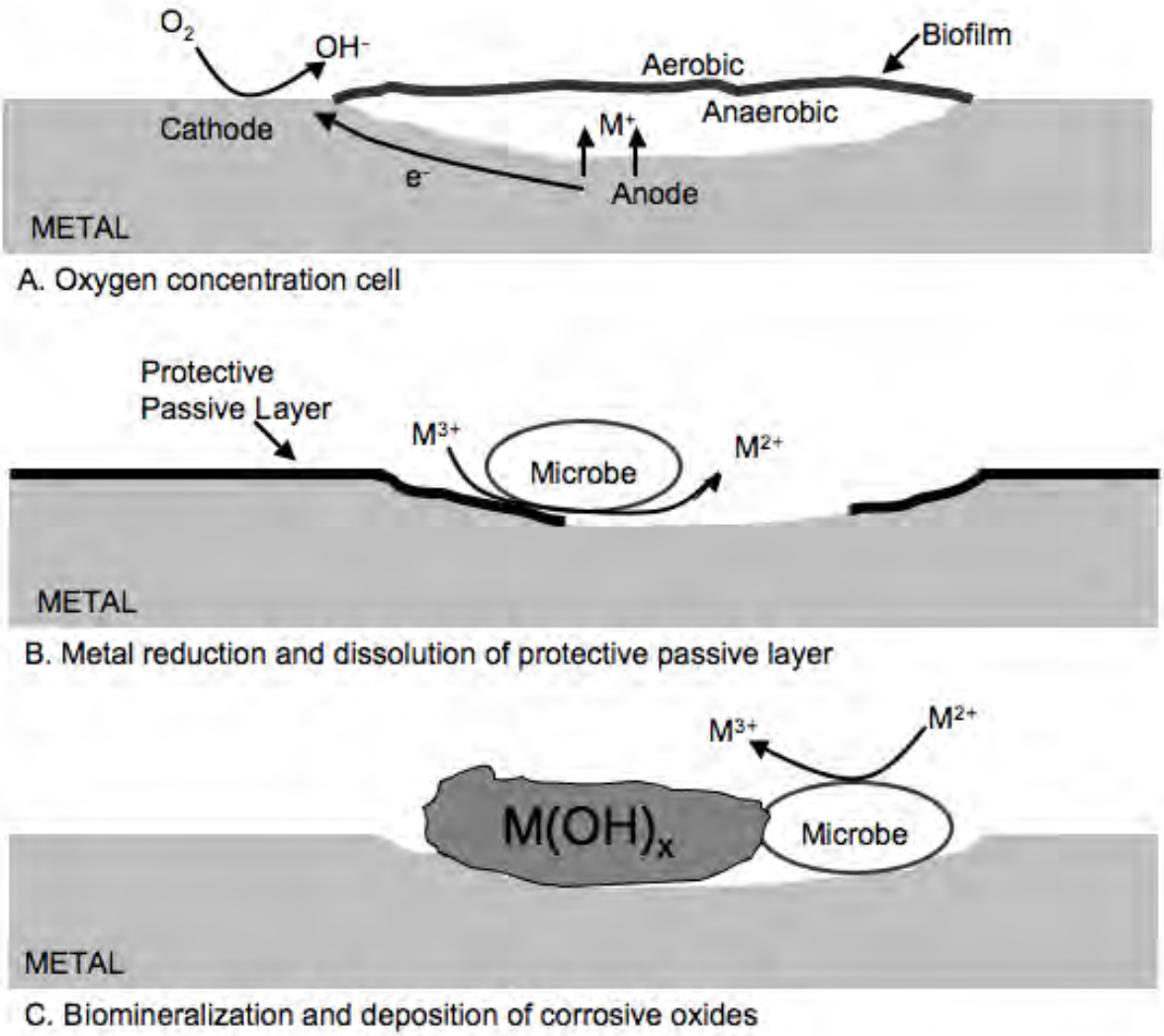


Figure 1.2: An illustration depicting three ways in which microbes can impact corrosion. A) Differential aeration cells (oxygen concentration heterogeneities. B) Dissolution of a protective layer via microbial metal reduction. C) Biomineralization and deposition of corrosion oxides onto a metal surface.

reduction interact with a steel surface. Hydrogen sulfide production can acidify localized environments on steel surfaces and act as a cathodic reactant, and iron sulfide production canresult in the formation of iron – iron sulfide galvanic couples (King et al. 1973; Lee et al. 1995; Javaherdashti 2008; Little and Lee 2007). Sulfate-reducing bacteria have also been shown to utilize metallic iron as an electron donor, which would result in the dissolution of metallic structures (Dinh et al. 2004; Enning et al. 2012). Interestingly, methanogenic archaea have also been shown to utilize elemental iron, suggesting these microbes can promote corrosion as well (Davidova et al. 2012; Dinh et al. 2004; Uchiyama et al. 2010).

#### 1.2.1.3 Microbial Production of Organic Acids

Many microbes produce organic acids (e.g. acetic acid, lactic acid) as metabolic byproducts, and these organic acids may increase corrosion of a variety of materials. Numerous studies have concluded that microbial acid production can increase corrosion rates of a variety of materials. Acetic acid production by *Acetobacter aceti* has been shown to accelerate corrosion of cathodically protected stainless steel (Little et al. 1988), and corrosion of carbon steel has also been associated with microbial acetic acid production (Pope 1988; Little et al.1992 and references therein). Fungal organic acid production has been attributed to corrosion of aluminum in aviation fuel environments (Salvarezza et al. 1983; McNamara et al. 2005) as well as degradation of concrete (Konhauser 2012).

#### 1.2.1.4 Microbial Oxidation and Reduction of Metals

Iron and manganese species can serve as both electron donors and electron acceptors for many microbes (Lovley 1991; Madigan et al. 2010). Microbial metal reduction has been attributed to both increased and decreased corrosion rates. Fe(III)-reducing microbes have been

associated with increased metallic corrosion, and this phenomenon is explained by the removal of protective iron oxide layers from metal surfaces (Figure 1.2) (Obuekwe et al. 1981; Little et al. 1998). However, under static conditions microbial iron reduction has been observed to protect metals from corrosion by creating a reducing environment at the metal surface, which prevents oxygen from interacting with the metal and results in reduced corrosion (Potekhina et al. 1999; Dubiel et al. 2002; Lee and Newman 2003). Iron and manganese oxidizing bacteria have been shown to enhance corrosion of steel and stainless steel due to the deposition of corrosive oxides on metallic surfaces (Figure 1.2) (Dickinson et al. 1997; Olesen et al. 2000; Shi et al. 2002; Little and Lee 2007; Rajasekar et al. 2007; Ray et al. 2010).

### 1.2.2 Biofouling and Fuel Degradation

Biofouling is a term often used to describe clogging or fouling due to microbial activity (e.g. clogged fuel lines); fouling due to macroorganisms can also occur. As both aerobic and anaerobic microbes can metabolize hydrocarbons, fuels in tanks and pipelines are subject to biodegradation, which can result in fuel that must be discarded. Fuel contamination or degradation has been observed in a variety of fuels including aviation fuels, diesel, biodiesel, and gasoline (Neihof 1988 and references therein; Passman et al. 2001).

## 1.3 Methods for Studying Microbes in Fuel Environments

As microbial contamination of fuels can result in many adverse outcomes, it is important for industry personnel to understand the microbial diversity present within fuel industry infrastructure in order to make informed decisions regarding monitoring, mitigation and prevention. Traditionally, techniques employed for monitoring microbial fuel contamination have involved cultivation-based methods (ASTM Standard 6469). Methods such as most

probable number (MPN) and plate count techniques can provide information about microbial loads in fuels, and targeted cultivation efforts can attempt to answer questions about the types of metabolisms microbes present in an environment may employ. These sorts of cultivation-based methods have provided valuable insight into how particular microbes may impact fuel industry infrastructure. For instance, investigating how microbes influence corrosion processes often includes isolating a microbe from a corrosive environment (i.e. a fuel tank or pipeline) followed by a variety of corrosion testing methods. These corrosion testing methods may include immersion testing (Dinh et al. 2004; Little and Lee 2007; Davidova et al. 2012), mechanical testing (Javaherdashti et al. 2006; Jain 2011), or electrochemical testing (Mansfeld 2003; Frankel 2008; Mehanna et al. 2009; Revie 2011) of a material (e.g. steel) exposed to a microbial culture. These types of tests have provided great insight into how particular microbes impact corrosion processes in a number of environments. However, relating the information provided by these sorts of studies back to *in situ* conditions can be challenging. Additionally, as described above, cultivation-based methods may introduce biases as many microbes are not amenable to cultivation in a laboratory setting. Thus, it is important to investigate fuel system microbiology (or microbial diversity in any environment) with a variety of methods to elucidate how microbes are impacting fuels and fuel system infrastructure.

DNA sequencing technologies have shown rapid improvement in the past decades, and it is now technically feasible and cost-effective to survey environmental diversity via molecular methods. However, to date, relatively few studies have applied cultivation-independent, molecular surveys to fuel environments. Screening methods such as denaturing gradient gel electrophoresis (DGGE) and Sanger sequencing have been applied to survey diversity in gas industry pipelines, oil facilities and aviation fuel (Zhu et al. 2003; Duncan et al. 2009; Brown et al. 2010). Zhu et al. (2003) applied DGGE and subsequent Sanger sequencing analyses to

characterize microbial communities in gas industry pipelines and identified members of the gammaproteobacteria (e.g. *Pseudomonas* spp.), betaproteobacteria (e.g.*Ralstonia* spp.), deltaproteobacteria (*Desulfovibrio* spp.) and firmicutes (e.g. *Clostridia* spp.) as well as methanogenic archaea. Duncan and colleagues (2009) examined the microbial diversity of samples associated with an oil production facility via Sanger sequencing methodology. The authors identified microbes that could potentially impact corrosion processes including sulfate-reducing microbes, iron-reducing microbes and microbes capable of producing organic acids. Thermophillic microbes and methanogenic archaea were also identified. Brown and colleagues (2010) applied Sanger sequencing technology to identify microbes in aviation fuel samples. The authors identified *Pseudomonas* spp, *Methylobacterium* spp., *Bacillus* spp. and *Corynebacterium* spp. among others. Stevenson et al. (2011) applied Sanger sequencing methodologies as well as a 454 pyrosequencing approach to identify microbial communities present in samples collected from an oil production facility. The authors' analyses indicated that members of the firmicutes and synergistes were prevalent in these samples. The authors also identified sulfate-reducing and methanogenic microbes. White et al. (2011) investigated a variety of fuels via cultivation-based methods, DGGE and 454 pyrosequencing. Prevalent microbes identified by these methods included members of the firmicutes (*Clostridia* spp.), members of the betaproteobacteria (*Burkholderia* spp.) and members of the gammaproteobacteria (*Pseudomonas* spp., *Marinobacter* spp. and *Halomonas* spp.) However, only 4 samples were analyzed via the 454 pyrosequencing approach. Our knowledge of the microbial communities associated with environments associated with hydrocarbon production, transportation and storage will increase as microbial diversity surveys based upon molecular methodologies continue.

## 1.4 Research Motivation and Approach

Fuel grade ethanol production in the United States has increased dramatically over the past several decades (Figure 1.3) due, in part, to efforts to increase the use of alternative fuels such as ethanol and biodiesel via initiatives such as the Energy Independence and Security Act of 2007. As fuel-grade ethanol production has increased (and continues to increase), fuel transportation and storage infrastructure has been re-purposed for use with fuel-grade ethanol and ethanol fuel blends. Additionally, new infrastructure will be constructed to transport and store these fuels. This transition to fuel-grade ethanol and ethanol fuel blends has created an increased need to understand issues such as microbial corrosion and materials compatibility in the context of an evolving fuel/energy industry as increases in fuel-grade ethanol production have been accompanied by materials compatibility issues.

Ethanol stress corrosion cracking (eSCC) is a type of environmentally induced cracking phenomenon that affects steels when exposed to fuel-grade ethanol (Kane et al. 2005). This phenomenon has resulted in tank and pipeline failures and arose shortly after increased production of fuel grade ethanol. Investigations have indicated that factors such as water content, chloride levels, acidity and strain rate impact this cracking phenomenon (Lou et al. 2009; Lou et al. 2010; Lou and Singh 2010; Beavers et al. 2011; Kane 2007). This issue prompted the investigation of the potential for microbial corrosion to occur in environments exposed to fuel-grade ethanol as ethanol is known to serve as a carbon source for many microbes.

While corrosion and fouling issues arising from microbial activity in oil and natural gas production industry infrastructure have received much attention, less research has focused on these issues in the context of automotive fuel storage and transportation infrastructure even

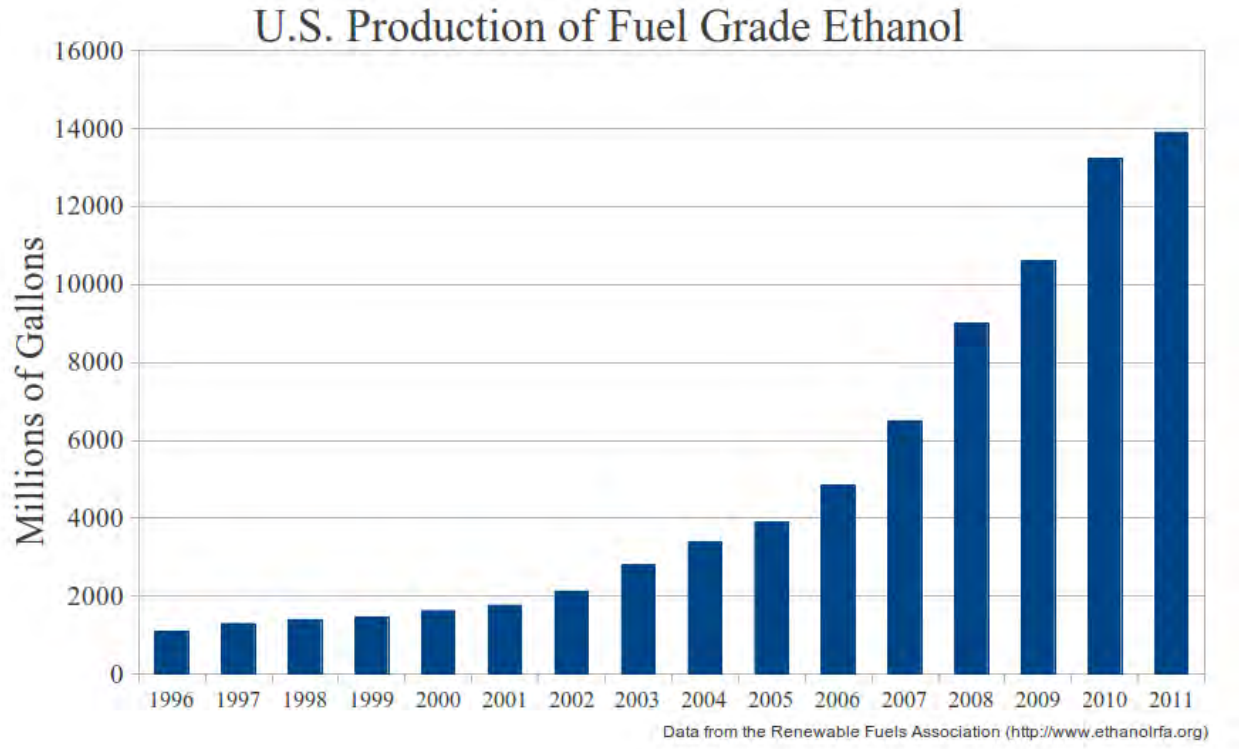


Figure 1.3: Fuel grade ethanol production in the United States since 1996. Data from the Renewable Fuels Association.

though the potential for microbial contamination in these environments has been documented for over half a century (Zobell 1946). The research presented in this thesis was inspired by the need to more thoroughly understand the microbial diversity and microbially-related issues such as MIC and biofouling impacting infrastructure involved with the transportation and storage of automotive fuels including fuel-grade ethanol and ethanol fuel blends. The research also contributes to understanding how laboratory corrosion tests relate to real-world environments as well as to assessing how cultivation-based methods of monitoring microbial contamination of fuels compare to molecular-based surveys.

## 1.5 Research / Thesis Outline

To investigate the microbial diversity of fuel environments and the impact of this diversity on corrosion, a three-pronged approach was employed. This approach included assessing microbial diversity via molecular techniques (Sanger sequencing and 454 pyrosequencing of 16/18S rRNA), cultivation-based methods designed to grow key microbes present in fuel industry infrastructure in the laboratory and corrosion testing to determine how key microbes impact corrosion of steels commonly used for construction of fuel system infrastructure. The corrosion testing was conducted in collaboration with corrosion engineers and scientists at the Colorado School of Mines (Luke Jain) as well as the National Institute of Standards and Technology (Jeff Sowards).

Chapter 2 describes a survey of microbial diversity associated with automotive fuels in the United States. 454 pyrosequencing of 16/18S rRNA genes as well as cDNA reverse transcribed from 16/18S rRNA extracted from fuel samples was employed to identify the types of microbes present in biodiesel, diesel, E10 (10 percent fuel-grade ethanol and 90 percent gasoline), E85 (85 percent fuel-grade ethanol and 15 percent gasoline), fuel-grade ethanol and gasoline. Knowledge of the biodiversity associated with these fuel environments can inform decisions about strategies of combating microbial contamination of fuels.

Chapter 3 describes an investigation of fuel-grade ethanol environments (also containing water) exhibiting severe corrosion damage suspected to be the result of microbial activity. A molecular survey of the microbial diversity associated with these samples as well as cultivation-based experiments indicate that *Acetobacter* spp. are prevalent in the fuel-grade ethanol environments sampled as part of this study. The corrosive nature of *Acetobacter* spp. as well as a sulfate-reducing bacterial consortium (including *Desulfosporosinus* sp.) cultivated from field

samples has been investigated and reported elsewhere (Jain 2011, Sowards et al. - in preparation).

Chapter 4 describes the isolation of a *Bacillus* sp. that produces spores capable of promoting the oxidation of manganese from samples of E10 fuel. These types of manganese-oxidizing microbes have been identified in other environments such as in marine sediments and the terrestrial subsurface, and the potential implications of the presence of these types of microbes in fuel environments is discussed.

Chapter 5 provides a summary and conclusion of the work presented in this thesis and discusses potential future work.

Appendix A includes a table describing the top Basic Local Alignment Search Tool (BLAST) hits for operational taxonomic units (OTUs) described in Chapter 2.

Appendix B includes information about a conference paper submitted to NACE 2013. Luke Jain is the first author of this paper, which describes electrochemical corrosion testing (open circuit potential (OCP), polarization resistance (PR), and electrochemical impedance spectroscopy (EIS)) of the effects of acetic-acid-producing and sulfate-reducing bacteria described in Chapter 3 on corrosion of API X52 and X70 steels. The *Acetobacter* sp. were shown to initiate pitting corrosion but did not increase general corrosion rates as monitored by electrochemical techniques. The sulfate-reducing consortium increased general corrosion rates and initiated pitting corrosion. I am a co-author of the manuscript and contributed to the paper through maintaining and monitoring bacterial cultures, aiding with corrosion test setup and conducting molecular microbiology work.

Appendix C includes information about a paper presented at the Department of Defense Corrosion Conference 2011. The first author of this paper is Jeff Sowards. This paper describes the crack growth behavior of API X52 and API X70 steels in the presence of simulated fuel-

grade ethanol as well as cultures of the *Acetobacter* sp. isolated from samples ECT.1 (Chapter 3). Fatigue crack growth rates were increased due to the presence of acetic-acid-producing bacteria. This research has continued to evaluate the impact of the sulfate-reducing consortium described in Chapter 3 on the crack growth behavior of pipeline and tank steels. A manuscript describing this work is currently in preparation. I am a co-author of the manuscript and contributed to the paper through maintaining and monitoring bacterial cultures, aiding with corrosion test setup and conducting molecular microbiology work.

Appendices D and E provide citation information for manuscripts regarding microbial diversity and sulfur cycling at a unique supraglacial sulfur spring in the Canadian High Arctic. Molecular-based techniques such as 16S rRNA gene pyrosequencing and metagenomic sequencing as well as cultivation-based methods have been employed to understand how microbes living in elemental sulfur deposits on the ice surface are utilizing sulfur species. Damhnait Gleeson and Katherine Wright are the lead authors on the manuscripts describing this unique sulfur-rich environment. I am a co-author on these manuscripts and contributed to the work by aiding with molecular lab work and bioinformatic analyses.

CHAPTER 2

# AN INVESTIGATION OF MICROBIAL DIVERSITY ASSOCIATED WITH AUTOMOTIVE FUELS IN THE UNITED STATES

## 2.1 Abstract

Microbial contamination of fuels can cause issues such as biofouling, fuel degradation and microbiologically influenced corrosion (MIC). Traditionally, cultivation-based methods have been used to assess the types of microbes present in these sorts of environments. Here we present a cultivation-independent microbial diversity survey of automotive fuels. We conducted a field survey of fuels including biodiesel, diesel, E10, E85, fuel-grade ethanol and gasoline; and used a 454 pyrosequencing approach to sequence both 16S/18S rRNA genes as well as 16S/18S rRNA (transcribed into cDNA) to identify both total and active microbial communities in these environments. Microbial communities in all fuel types were broadly similar, and prevalent phylotypes included *Halomonas* spp., *Pseudomonas* spp., *Shewanella* spp., *Corynebacterium* spp. and *Acetobacter* spp. Pyrosequencing libraries generated from cDNA and DNA indicated that the active and total communities of the sampled environments show significant overlap, though some of the most abundant phylotypes (*Halomonas* spp. and *Shewanella* spp.) present in the DNA libraries had lower relative abundances in the cDNA libraries, which may suggest that these microbes are less active members of the microbial communities in these environments. Increased knowledge of the the microbial diversity associated with fuel system infrastructure will

improve monitoring and prevention strategies and guide future research of issues such as microbial corrosion in fuel systems.

## 2.2 Introduction

Automotive fuels consumed in the United States include gasoline, diesel (ultra-low sulfur diesel), biodiesel, fuel-grade ethanol and ethanol fuel blends such as E10 (10 percent fuel-grade ethanol and 90 percent gasoline) and E85 (85 percent fuel-grade ethanol and 15 percent gasoline). Microbial contamination of automotive fuel systems can lead to issues such as biofouling, microbiologically influenced corrosion (MIC) and fuel degradation, and understanding the microbial diversity associated with these fuels is an important step towards improving monitoring, control and prevention of microbial contamination issues. Knowledge of the microbial diversity associated with automotive fuels and other hydrocarbons is largely based upon cultivation-based studies dating back many years (Zobell 1946; Gaylarde et al. 1999 and references therein). More recently molecular-based methods have been used to understand the the types of microbes present in some fuel systems, and many of these studies have involved fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE) or production of clone libraries via Sanger sequencing (Zhu et al. 2003; Jan-Roblero et al. 2004; Suflita et al. 2012). Recently, researchers have begun to apply so-called next-generation sequencing methodologies such as 454 pyrosequencing to investigate the microbial diversity of hydrocarbon environments such as oil production facilities and contaminated fuels (Stevenson et al. 2011; White et al. 2011). Here we present a microbial diversity survey of automotive fuels in the United States using Sanger sequencing and the 454 pyrosequencing technology (Roche). Our approach included sequencing both 16S/18S ribosomal ribonucleic acid (rRNA) (reverse transcribed into complimentary deoxyribonucleic acid (cDNA)) as a measure of the active

microbial communities in fuel system environments as well as 16S/18S rRNA genes to identify the total microbial communities. This approach of analyzing both DNA and cDNA sequences has been applied to other environments to assess active and total microbial communities in lakes (Jones and Lennon 2010), marine environments (Kamke et al. 2010) and stream biofilms (Besemer et al. 2012). To the authors' knowledge this approach has not been applied to environments associated with automotive fuels or other hydrocarbon environments such as oil production facilities.

## 2.3 Methods

The microbial diversity survey of automotive fuels presented in this chapter included collecting samples from a variety of fuel environments in the United States, extracting nucleic acids (DNA and RNA) from these samples and sequencing 16S/18S rRNA genes as well as 16S/18S rRNA (reverse transcribed into cDNA). The methods used for this investigation are described in sections 2.3.1 through 2.3.3.

### 2.3.1 Sample Collection

Fuel samples were collected from tanks containing a variety of fuels over a period of approximately three years. Fuel samples included E10 fuel (a blend of 90 percent gasoline and 10 percent fuel-grade ethanol), E85 (a blend of 15 percent gasoline and 85 percent fuel-grade ethanol), diesel and biodiesel samples from above-ground and below-ground tanks at production facilities, fueling terminals and retail fueling stations. Fuel samples were collected from sites across the Unites States including sites in the Rocky Mountain region, the midwest and the southeast. Multiple fuel types and multiple fuel samples of the same type were sampled at some sites, and at several sites fuel samples were collected multiple times. Fuel samples were

collected in sterilized 1-liter polycarbonate bottles. Bottles were field rinsed three times before collecting samples. Fuel samples were filtered through sterile, 0.2-micron polyethersulfone membranes (Pall Corporation) using a vacuum filtration system. All equipment was sterilized before use. Portions of filters were stored at -80 ºC for nucleic acid extraction. Samples from tanks containing fuel-grade ethanol included one tank bottoms sample, which included bulk solids and liquids scooped from the bottom of storage tank into a sterile 50-ml tube while maintenance procedures were being performed on the tank, as well as one sample of a filter designed to remove particulates from the fuel-grade ethanol before the ethanol is loaded onto tanker trucks. Filter units were collected and transported to the lab where a portion of the filter was removed from nucleic acid extraction. Nucleic acids were extracted directly from tank bottoms and filter samples collected from fuel-grade ethanol tanks.

### 2.3.2 Nucleic Acid Extraction, Sanger Sequencing and 454 Pyrosequencing

Nucleic acids were extracted from filter samples using a phenol:chloroform extraction protocol similar to the procedure described by Dojka et al. (1998). Sample material was suspended in 500 ul Buffer A (200 mM Tris [pH 8.0], 50 mM ethylenediaminetetraacetic acid (EDTA), 200 mM sodium chloride), 200 ul 20 percent sodium dodecyl sulfate and 500 ul phenol-chloroform-isoamyl alcohol (24:24:1). Borosilicate glass beads were added to the tubes to aid in cell lysis. The samples were reciprocated on a Mini-Beadbeater (Biospec) at maximum speed for one minute, extracted and then extracted again with phenol-chloroform-isoamyl alcohol. Nucleic acids were precipitated with one volume isopropanol and 0.3 M sodium acetate. Precipitated nucleic acids were washed with 70 percent ethanol and suspended in DEPC-treated water. Portions of the nucleic acid extracts were aliquoted for DNA and RNA analyses (only a subset of the samples collected in this study were used for RNA analyses). Aliquots for RNA

analysis were treated with RNase-free DNase I (Invitrogen) per the manufacturer's protocol to remove DNA. Removal of DNA was tested by polymerase chain reaction (PCR) of the DNase-I-treated nucleic acid aliquots. Failure of PCR amplification indicated elimination of DNA from the sample. RNA was reverse transcribed into complimentary DNA (cDNA) using the SuperScript VILO cDNA Synthesis Kit (Invitrogen) per the manufacturer's protocol.

As an initial screen of the microbial diversity present in fuel samples, PCR amplification and Sanger sequencing of 16S rRNA genes extracted from three E10 samples was conducted. 16S rRNA genes were amplified with 'universal' primers 515F (5′ – GTG CCA GCM GCC GCG GTA A – 3′) and 1391R (5′ - GAC GGG CGG TGW GTR CA – 3′ ) (Lane 1991). PCR, cloning, transformation and sequencing were carried out as described by Sahl et al. (2010). Sanger sequencing was conducted on a MegaBACE 1000 dye-terminating sequencer. Sanger reads were called with PHRED (Ewing et al. 1998; Ewing and Green 1998) and assembled with PHRAP (www.phrap.org) via Xplorseq (Frank 2008). Phylogenetic content of the samples was determined by aligning the sequences with the SINA aligner (Pruesse et al. 2012) and inserting (parsimony insertion with the pos_var_ssuref:bacteria filter) the sequences into the guide tree distributed with the Silva SSURef111_NR database (Pruesse et al. 2007) with the ARB software package (Ludwig et al. 2004).

DNA/cDNA was prepared for sequencing as described by Osburn and colleagues (Osburn et al. 2011) with the following exceptions: quantitative polymerase chain reaction (qPCR) reactions were performed in duplicate and pooled prior to normalization for sequencing. Each qPCR reaction contained 4 uL of template DNA. Primers 515f-modified (5' – GTG YCA GCM GCC GCG GTA A – 3') and 927r-modified (5' – CCG YCA ATT CMT TTR AGT TT – 3') (see Osburn et al. 2011), which demonstrate thorough *in silico* coverage of all three domains of life, incorporated adapter sequences for pyrosequencing on the GSFLX Titanium platform of the

Roche 454 Pyrosequencing technology. PCR amplicon concentrations were estimated via gel electrophoresis using the image analysis software ImageJ (http://rsbweb.nih.gov/ij/). These concentration estimations were used for normalization of PCR amplicons for the pyrosequencing run. Pooled amplicons were gel purified with the EZNA Gel Extraction Kit (Omega BioTek). Sequencing was completed on the Roche 454 FLX Titanium platform at the EnGenCOre facility at the University of South Carolina. All samples except E10.a, E10.b, E10.c, E.85a and E.85.b were sequenced on a single pyrosequencing run. These five samples were included on a separate 454 pyrosequencing run.

### 2.3.3 Sequence Analyses

Sequence analysis was carried out using the QIIME software package (Caporaso et al. 2010) and the mothur software package (Schloss et al. 2009). Initial quality filtering of the sequences was conducted in accordance with findings identified by Huse et al. (2007). Sequences with errors in barcodes or primers, homopolymer runs longer than 6 nucleotides, ambiguous base calls, or average quality scores less than 25 were removed from the data. Sequences shorter than 400 nucleotides or longer than 500 nucleotides were also discarded. Sequences were denoised with DeNoiser version 1.4.0 (Reeder and Knight 2010). Chimeric sequences were identified by UCHIME (Edgar et al. 2011) and removed. Reverse primers were removed with a custom python script written by Charles Pepe-Ranney. Remaining sequences were aligned with SSU-ALIGN (Nawrocki 2009). Sequences failing to align to the appropriate alignment space were removed, and remaining sequences were trimmed to overlapping alignment space.

Sequences were clustered into operational taxonomic units (OTUs) with the average neighbor clustering method in mothur (Schloss et al. 2009). Taxonomic classifications of

sequences were assigned using the naïve Bayesian algorithm described by Wang and colleagues (2007) as implemented in mothur (Schloss et al. 2009). The Silva SSURef111_NR database (Pruesse et al. 2007) was used as the training set for classifications. These reference sequences were quality filtered as described by Pepe-Ranney, et al. (2012) and trimmed to the primer-targeted region of the 16S rRNA gene (515F-927R). Pyrosequences were also subjected to the Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990) against the NCBI non-redundant nucleotide database (Benson et al. 2005) and the Silva SSURef111_NR database (Pruesse et al. 2007). Microbial community diversity was estimated with Catchall (Bunge et al. 2012). Alpha diversity metrics (Chao1 (Chao et al. 2010) and Shannon diversity index), rarefaction curves and beta diversity metrics were calculated in QIIME (Caporaso et al. 2010). For alpha and beta diversity analyses, a standardized number of sequences was used for each sample as some alpha and beta diversity metrics are sensitive to sampling effort (sequence number). Also, standardized sample sizes (sequence numbers) were used in an effort to minimize biases potentially introduced during DNA extraction, PCR amplification, sample pooling, sequencing and data analyses (Schloss et al. 2011). Sequences identified as chloroplasts or plant species were not considered for alpha and beta diversity measures as these sequences were not considered to be representative of relevant microbial communities. However, these sequences are included in Figure 2.3.

## 2.4 Results and Discussion

A survey of the microbial diversity of automotive fuel samples was conducted via sequencing of 16S/18S rRNA genes as well as 16S/18S rRNA (cDNA) via Sanger sequencing as well as 454 pyrosequencing. Knowledge of the microbial communities present in these environments will inform industry personnel on monitoring, mitigation and prevention strategies.

Table 2.1 displays sample information and pyrosequencing data such as sequencing depth, alpha diversity metrics and information on abundant phylotypes in each sample. 16/18S rRNA gene pyrosequencing libraries for 37 fuel samples included approximately 60000 16/18S rRNA gene sequences while cDNA pyrosequencing libraries included approximately 14000 sequences for 10 samples. Section 2.4.1 and Table 2.2 describe Sanger sequencing data.

### 2.4.1 Initial screen of microbial diversity - Sanger sequencing of 16S rRNA genes

As an initial screen of microbial diversity, clone libraries of 16S rRNA gene sequences from three E10 samples (E10.a, E10.b, E10.c) were created via Sanger sequencing. Table 2.2 displays the distribution of sequences for each sample determined by inserting the sequences into the guide tree distributed with the Silva 111 database (Pruesse et al. 2007). The most abundant phylotype in the clone libraries of samples E10.a and E10.b were identified as *Halomonas* spp., which is consistent with 454 pyrosequencing data (see section 2.4.2). *Halomonas* spp. have been associated with fuel environments, emulsifier production and hydrocarbon degradation (White et al. 2011; Martínez-Checa et al. 2002; Calvo et al. 2002; Mehdi and Giti 2008; Mnif et al. 2009; Mnif et al. 2011). The most abundant phylotype identified in the clone library generated from sample E10.c is a member of the comamonadaceae identified as an *Acidovorax* sp. Members of the comamonadaceae are also prevalent in pyrosequencing libraries generated from sample E10.c. *Acidovorax* spp. have been shown to metabolize hydrocarbons (Singleton et al. 2009). Additionally, phylotypes identified as *Pseudomonas* spp. and *Corynebacterium* spp. were identified in all three samples analyzed by Sanger sequencing. These phylotypes are also prevalent in pyrosequencing libraries.

Table 2.1: Sample information, pryosequencing library information, alpha diversity metrics and dominant phylotypes.

| Sample ID | FuelType | Region | DNA or cDNA | Sample Date | # of Sequences | # OTU's | Chao1 | Shannon ($H_{sh}$) | exp($H_{sh}$) | Dominant Phylotype | Dominant OTU |
|---|---|---|---|---|---|---|---|---|---|---|---|
| BD.2a | Biodiesel | Rocky Mountain | DNA | 2012 | 572 | 21 | 64.5 | 1.5 | 4.3 | Lactobacillus | 7 |
| BD.2b | Biodiesel | Rocky Mountain | cDNA | 2012 | 626 | 20 | 64.6 | 0.4 | 1.5 | Lactobacillus | 7 |
| BD.3a | Biodiesel | Rocky Mountain | DNA | 2012 | 625 | 53 | 66.1 | 2.7 | 15.5 | Halomonas | 19 |
| BD.3b | Biodiesel | Rocky Mountain | cDNA | 2012 | 941 | 115 | 143.5 | 4.6 | 101.4 | Clostridiales | 20 |
| D.1 | ULSD | Rocky Mountain | DNA | 2012 | 2796 | 117 | 96.6 | 3.4 | 30.9 | Staphylococcaceae | 6 |
| D.2 | ULSD | Rocky Mountain | DNA | 2012 | 586 | 33 | 46.6 | 2.1 | 8.6 | Halomonas | 19 |
| D.3 | ULSD | Rocky Mountain | DNA | 2012 | 1732 | 127 | 118.8 | 5.1 | 165.7 | Massilia | 211 |
| D.4a | ULSD | Rocky Mountain | DNA | 2012 | 1433 | 49 | 53.1 | 1.7 | 5.5 | Halomonas | 19 |
| D.4b | ULSD | Rocky Mountain | cDNA | 2012 | 1583 | 91 | 81.4 | 3.6 | 38.2 | Enterobacter | 9 |
| D.5a | ULSD | Rocky Mountain | DNA | 2012 | 942 | 58 | 104.8 | 3.1 | 22.5 | Halomonas | 19 |
| D.5b | ULSD | Rocky Mountain | cDNA | 2012 | 1768 | 52 | 57.9 | 1.5 | 4.7 | Pseudomonas | 703 |
| E10.1 | E10 | Rocky Mountain | DNA | 2011 | 1975 | 75 | 86.0 | 3.1 | 23.2 | Pseudomonas | 1 |
| E10.10a | E10 | Rocky Mountain | DNA | 2012 | 1092 | 45 | 50.9 | 3.2 | 23.9 | Halomonas | 19 |
| E10.10b | E10 | Rocky Mountain | cDNA | 2012 | 1632 | 47 | 64.9 | 2.6 | 12.9 | Halomonas | 19 |
| E10.11a | E10 | Rocky Mountain | DNA | 2012 | 1044 | 20 | 20.2 | 1.1 | 3.0 | Halomonas | 19 |
| E10.11b | E10 | Rocky Mountain | cDNA | 2012 | 1456 | 49 | 46.5 | 2.0 | 7.5 | Pseudomonas | 703 |
| E10.12a | E10 | Rocky Mountain | DNA | 2012 | 1498 | 21 | 33.3 | 1.3 | 3.7 | Halomonas | 19 |
| E10.12b | E10 | Rocky Mountain | cDNA | 2012 | 2246 | 66 | 77.2 | 1.6 | 5.2 | Pseudomonas | 703 |
| E10.13a | E10 | Rocky Mountain | DNA | 2012 | 621 | 50 | 68.5 | 3.2 | 24.9 | Halomonas | 19 |
| E10.13b | E10 | Rocky Mountain | cDNA | 2012 | 1135 | 127 | 143.7 | 4.3 | 71.5 | Pseudomonas | 1 |
| E10.14a | E10 | Rocky Mountain | DNA | 2012 | 1440 | 75 | 82.0 | 2.9 | 17.8 | Pseudomonas | 703 |
| E10.14b | E10 | Rocky Mountain | cDNA | 2012 | 2004 | 121 | 16.1 | 3.0 | 20.4 | Pseudomonas | 703 |
| E10.16 | E10 | Rocky Mountain | DNA | 2012 | 2778 | 75 | 67.7 | 3.1 | 22.5 | Corynebacterium | 2 |
| E10.17 | E10 | Rocky Mountain | DNA | 2012 | 2901 | 87 | 67.4 | 3.3 | 28.1 | Halomonas | 19 |

Table 2.1: continued.

| Sample ID | FuelType | Region | DNA or cDNA | Sample Date | # of Sequences | # OTU's | Chao1 | Shannon ($H_{sh}$) | exp($H_{sh}$) | Dominant Phylotype | Dominant OTU |
|---|---|---|---|---|---|---|---|---|---|---|---|
| E10.2 | E10 | Rocky Mountain | DNA | 2011 | 2753 | 25 | 17.7 | 1.2 | 3.2 | Halomonas | 19 |
| E10.3 | E10 | Rocky Mountain | DNA | 2011 | 2515 | 112 | 101.3 | 3.5 | 34.3 | Halomonas | 19 |
| E10.4 | E10 | Rocky Mountain | DNA | 2012 | 1911 | 191 | 195.0 | 4.8 | 118.3 | Pseudomonas | 1 |
| E10.5 | E10 | Southeast | DNA | 2011 | 1759 | 59 | 50.0 | 3.4 | 30.7 | Acetobacter | 47 |
| E10.6 | E10 | Midwest | DNA | 2011 | 1172 | 87 | 104.2 | 3.5 | 34.6 | Azospirillum | 13 |
| E10.7 | E10 | Southeast | DNA | 2011 | 538 | 33 | 42.8 | 3.2 | 24.6 | Halomonas | 19 |
| E10.8 | E10 | Rocky Mountain | DNA | 2012 | 1143 | 70 | 88.0 | 3.1 | 23.3 | Halomonas | 19 |
| E10.a | E10 | Rocky Mountain | DNA | 2010 | 3235 | 29 | 26.3 | 1.9 | 6.4 | Halomonas | 19 |
| E10.b | E10 | Rocky Mountain | DNA | 2011 | 3096 | 93 | 71.1 | 2.9 | 17.9 | Halomonas | 19 |
| E10.c | E10 | Rocky Mountain | DNA | 2011 | 3859 | 92 | 77.5 | 3.9 | 49.6 | Comamonadaceae | 707 |
| E100.1a | Ethanol | Rocky Mountain | DNA | 2009 | 725 | 108 | 140.7 | 5.1 | 161.7 | Halomonas | 19 |
| E100.b | Ethanol | Rocky Mountain | DNA | 2008 | 2763 | 9 | 6.4 | 0.8 | 2.3 | Halomonas | 19 |
| E85.1 | E85 | Rocky Mountain | DNA | 2011 | 1483 | 67 | 96.6 | 3.2 | 24.2 | Pseudomonas | 703 |
| E85.2 | E85 | Southeast | DNA | 2011 | 1417 | 134 | 147.7 | 2.8 | 15.8 | Acetobacter | 47 |
| E85.3 | E85 | Rocky Mountain | DNA | 2012 | 657 | 47 | 71.8 | 3.1 | 22.3 | Halomonas | 19 |
| E85.4 | E85 | Rocky Mountain | DNA | 2012 | 919 | 43 | 49.4 | 2.2 | 9.5 | Halomonas | 19 |
| E85.5a | E85 | Rocky Mountain | DNA | 2012 | 1209 | 81 | 90.9 | 2.9 | 18.8 | Halomonas | 19 |
| E85.5b | E85 | Rocky Mountain | cDNA | 2012 | 1307 | 157 | 161.2 | 4.8 | 126.1 | Cystobacterineae | 11 |
| E85.6a | E85 | Midwest | DNA | 2011 | 1167 | 132 | 145.2 | 4.5 | 88.5 | Halomonas | 19 |
| E85.a | E85 | Rocky Mountain | DNA | 2011 | 2526 | 33 | 29.2 | 3.8 | 46.7 | Halothiobacillus | 696 |
| E85.b | E85 | Rocky Mountain | DNA | 2011 | 1210 | 23 | 22.9 | 2.2 | 9.1 | Halomonas | 19 |
| G.1 | Gasoline | Rocky Mountain | DNA | 2011 | 1020 | 73 | 96.0 | 3.7 | 40.0 | Pseudomonas | 1 |
| G.2 | Gasoline | Rocky Mountain | DNA | 2011 | 975 | 126 | 167.6 | 4.5 | 87.4 | Halomonas | 19 |

Table 2.2: Distribution of phylotypes for three different E10 fuel samples determined by Sanger sequencing. Taxonomies were assigned by parsimony insertion of sequences into the Silva 111 database. Numbers indicate fraction of totatl sequences for each sample.

| | **Sample ID** | | |
|---|---|---|---|
| | E10.a | E10.b | E10.c |
| **Taxon Assignment (Silva 111)** | n=89 | n=64 | n=53 |
| **Gammaproteobacteria** | | | |
| Halomonas | 0.73 | 0.47 | 0.04 |
| Pseudomonas | 0.11 | 0.13 | 0.09 |
| Citrobacter | | 0.08 | |
| Stenotrophomonas | | 0.05 | 0.04 |
| Shewanella | | | 0.02 |
| **Betaproteobacteria** | | | |
| Acidovorax | 0.03 | 0.06 | 0.36 |
| Diaphorobacter | 0.01 | | |
| Simplicispira | 0.02 | 0.03 | 0.04 |
| Delftia | | | 0.02 |
| Ottowia | 0.01 | | |
| Rhodocyclales | 0.04 | | |
| **Alphaproteobacteria** | | | |
| Spingomonadales | | 0.02 | |
| **Fusobacteria** | | | |
| SHA-35 | 0.01 | 0.03 | |
| **Bacteroidetes** | | | |
| Proteiniphilum | | | 0.08 |
| **Firmicutes** | | | |
| Family XI Incertae Sedis | 0.01 | | 0.02 |
| Proteiniclasticum | | | 0.02 |
| Carnobacteriaceae | | 0.02 | |
| Streptococcus | | | 0.02 |
| **Actinobacteria** | | | |
| Corynebacterium | 0.01 | 0.09 | 0.21 |
| Trueperella | | 0.02 | 0.02 |
| Pilimelia | | 0.02 | |
| Coriobactericeae | | | 0.02 |
| **Eukaryota** | | | |
| Pezizomycotina | | | 0.02 |

### 2.4.2 Pyrosequencing of 16S/18S rRNA genes (DNA)

All 37 fuel sample pyrosequencing libraries of 16S/18S rRNA genes were dominated by bacterial sequences. Members of the betaproteobacteria and firmicutes are the most prevalent phylotypes identified in the fuel sample pyrosequencing libraries. Figure 2.1 displays a phylum-level distribution of 16S/18S rRNA genes for each sample. Members of the archaea represented approximately 0.5 percent of all sequences recovered, and only 22 eucaryal sequences were identified. The low number of archaeal sequences is not unexpected as 16S rRNA gene surveys of many environments indicate that archaea compose a relatively small portion of the microbial community. The very small number of eucaryal sequences was unexpected as *in silico* analyses of the primers used in this study show thorough coverage of all three domains of life and eucaryotes have long been associated with fuel environments (Zobell 1946; Salvarezza et al. 1983; McNamara et al. 2005; Little and Lee 2007). Also, banding patterns produced via gel electrophoresis of amplified rRNA genes from fuel samples indicated that PCR amplicons of two sizes were generated, which suggests that 18S rRNA genes (from eucarya) were present in the samples as 18S rRNA gene amplicons are generally longer than 16S rRNA gene amplicons. The small number of eucaryal sequences despite evidence of 18S rRNA gene amplification is likely explained by biases introduced during the 454 pyrosequencing process. The pyrosequencing process is biased towards short reads, which would result in more 16S rRNA gene sequences (shorter) than 18S rRNA gene sequences (longer). For molecular surveys of microbial diversity using the 454 pyrosequencing technology it may be advisable to sequence different sized amplicons on different sequencing runs; however, this adds significant costs to a research project.

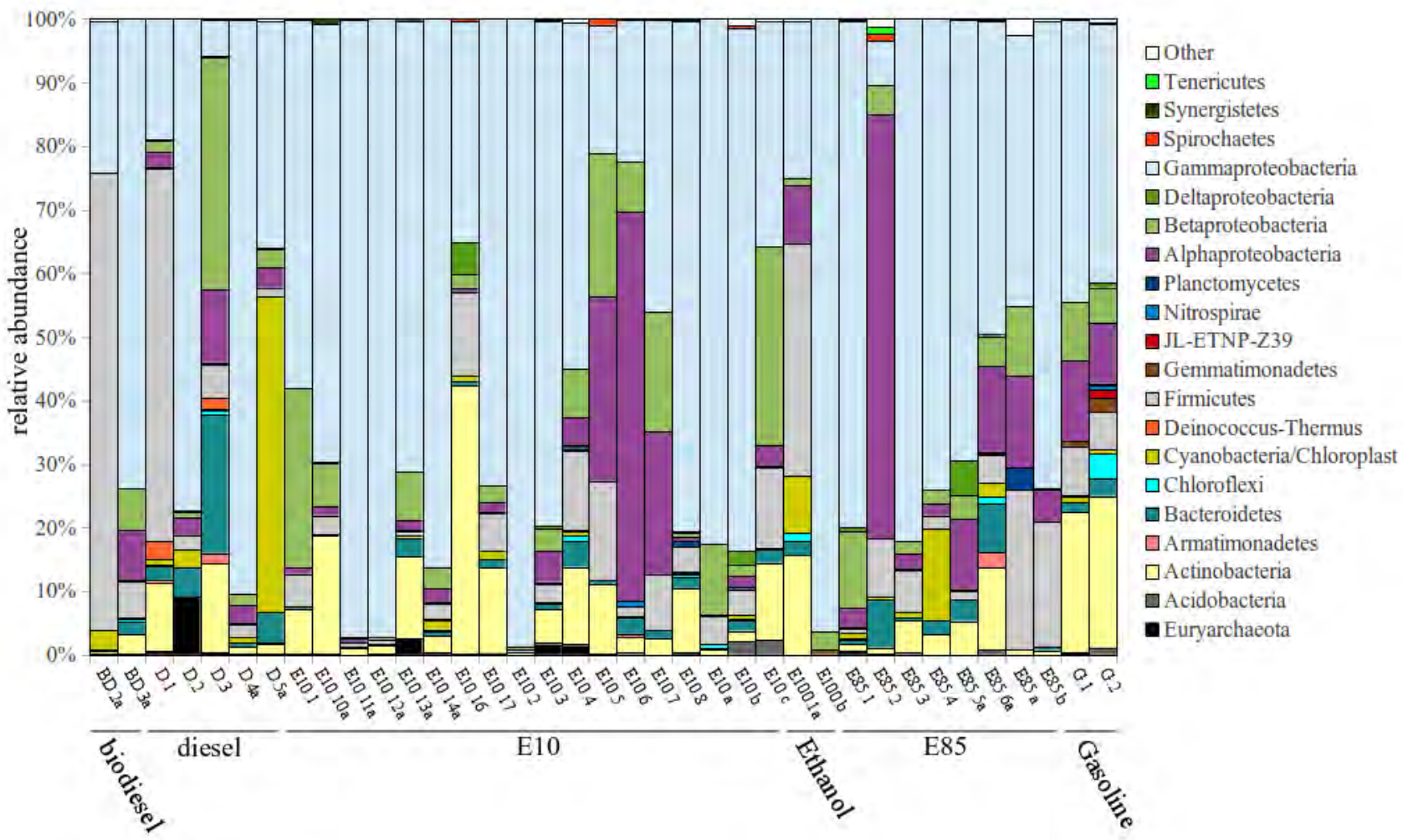


Figure 2.1: Distribution of rRNA gene pyrosequence reads for each sample library. Reads are distributed into phyla except for the proteobacteria, which are distributed into classes.

Of the 22 eucaryal sequences recovered in the pyrosequencing libraries, 19 sequences were classified as plants and are considered 'contamination' in the sense that these sequences likely originated from plant debris present in the fuels and do not represent potentially active microbes in these samples. The three remaining eucaryal sequences represent fungal species grouping within the saccharomycetales. Two of these sequences were found in the pyrosequencing libraries associated with a diesel sample (D.4a) and the remaining sequence was associated with a biodiesel sample (BD.2a). These sorts of microbes have been associated with fuel contamination and degradation of hydrocarbons (Atlas 1981; Little and Lee 2007) and may be under-estimated in this study.

The dominant phylotypes identified by 16S rRNA gene pyrosequencing include microbes that have been identified in fuel environments via culture-dependent methods as well as molecular techniques including sequencing of denaturing gradient gel electrophoresis (DGGE) bands and production of clone libraries via Sanger sequencing. Rarefaction curves (Figure 2.2) indicate that richness of fuel types is broadly similar, and many of the rarefaction curves seem to be approaching asymptotes which would suggest that the microbial diversity in these samples is well sampled. However, as mentioned above, the effectiveness with which our survey captured eucaryal diversity is unknown.

Figure 2.3 displays the relative abundance of all taxa recovered in the 37 DNA-based pyrosequencing libraries, and Figure 2.4 displays a heat map of the 50 most abundant taxa identified in the fuel samples via 16S rRNA gene pyrosequencing. Appendix A provides the best BLAST hits for representative sequences for each OTU (BLASTed against the Silva SSURef111_NR database (Pruesse et al. 2007)). The most prevalent phylotype in 21 of the 37 16S rRNA gene pyrosequencing libraries shows greater than 99 percent sequence similarity to *Halomonas* spp. identified in an oil reservoir (accession number JQ690675), an asphalt seep

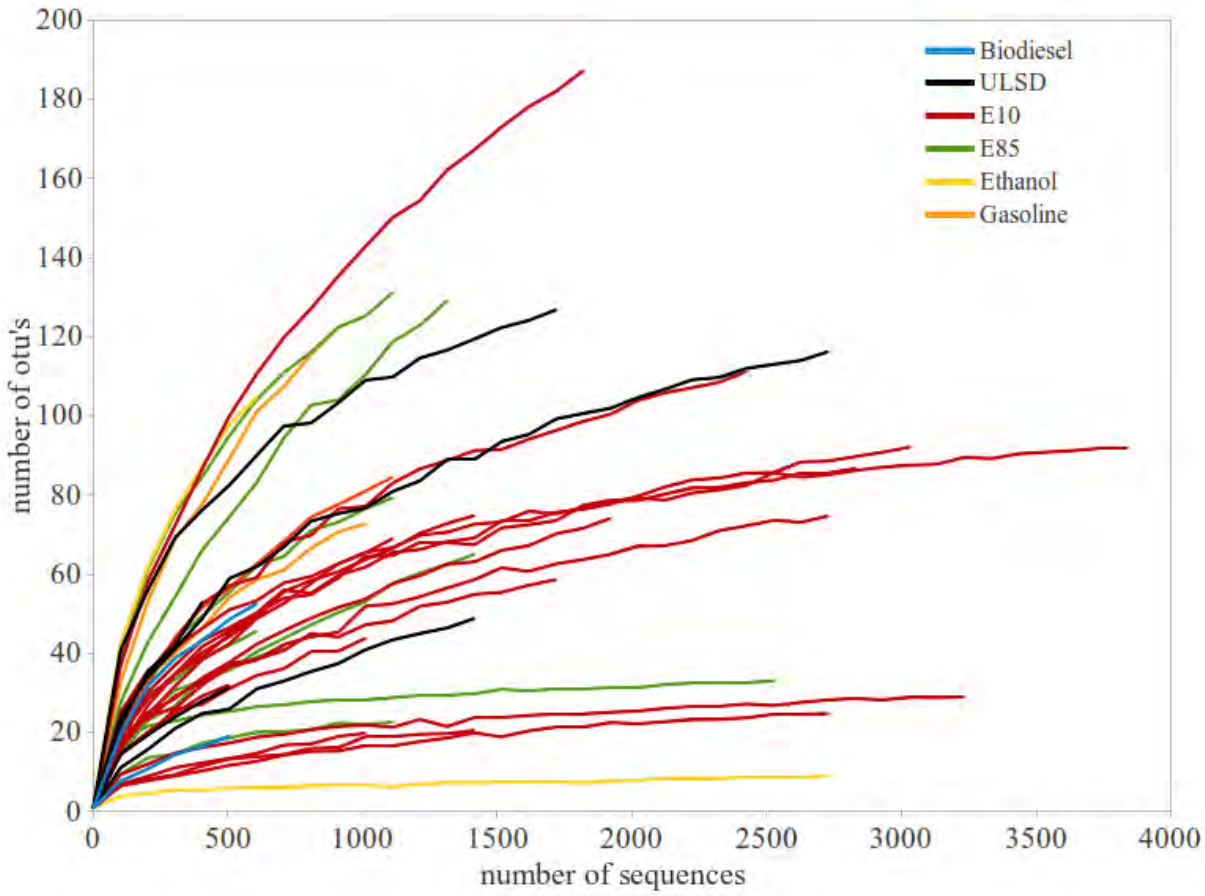


Figure2.2: Rarefaction analysis for DNA pyrosequencing libraries. Sequences were clustered with the average neighbor method in mothur (Schloss et al. 2009) at a distance of 0.03. Rarefaction curves were created with QIIME (Caporaso et al. 2010). Each color represents a fuel type.

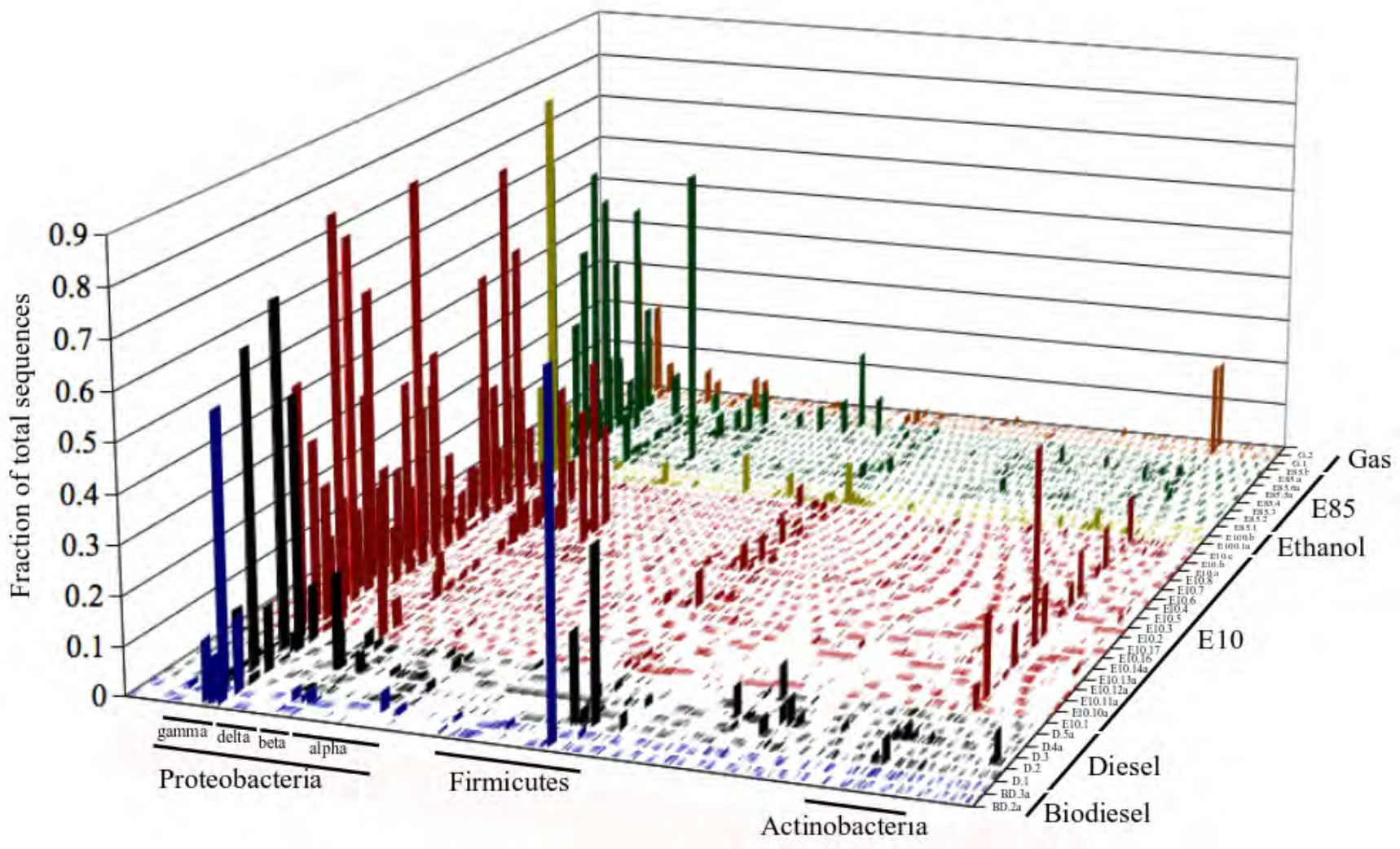


Figure 2.3: Distribution of taxa for each DNA pyrosequencing library.

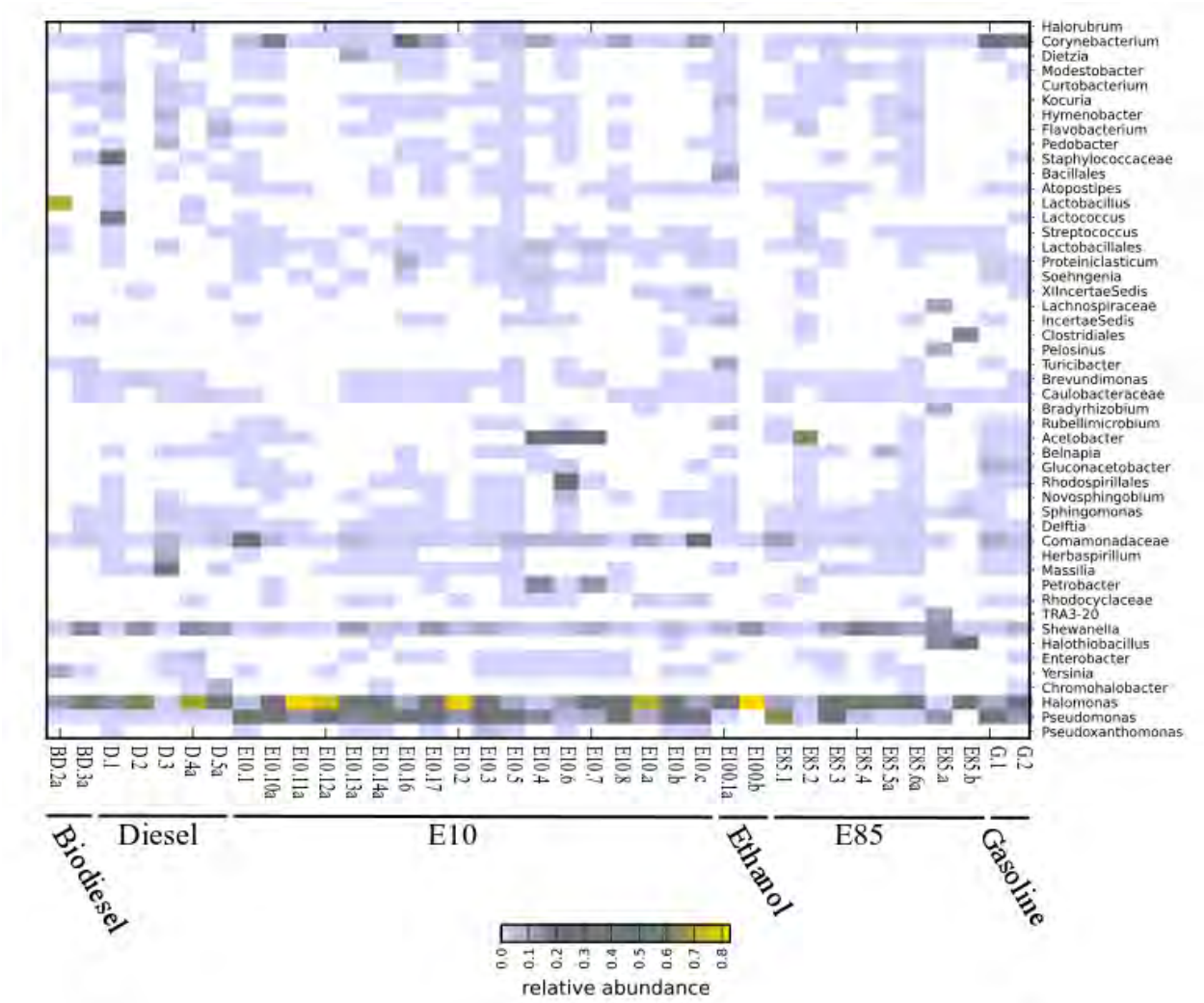


Figure 2.4: Heat map displaying the relative abundance of the 50 most abundant OTUs in the DNA pyrosequencing libraries. The relative abundance of each OTU is indicated by the color of the rectangles in the figure - yellow rectangles indicate more abundanct OTU's (see scale – bottom middle). Taxonomic classifications of OTU's are displayed to the right of the heat map.

(accession number DQ001684) and biodeteriorated chapel wall paintings (accession number AJ400546, Gurtner et al. 2000). This phylotype is present in all of the collected fuel samples and is the most abundant phylotype in the entire data set. *Halomonas* spp. are members of the gammaproteobacteria often associated with high-salt environments, though these microbes are known to thrive in a wide range of salt concentrations (Romano et al. 2005). Interestingly, a recent study published by White et al. (2011) identified *Halomonas* spp. in biodiesel, diesel and gas oil samples via 16S rRNA pyrosequencing as well as cultivation and denaturing gradient gel electrophoresis (DGGE) techniques. The *Halomonas* sp. comprised only small percentages of the pyrosequencing libraries for the biodiesel and diesel samples in that study but comprised over 60 percent of the library for the single gas oil sample collected from a tank on a ship. *Halomonas* spp. have been shown to produce emulsifying agents and degrade hydrocarbons (Martínez-Checa et al. 2002; Calvo et al. 2002; Mehdi and Giti 2008; Mnif et al. 2009; Mnif et al. 2011), which suggests that these microbes contribute to fuel degradation. To the authors' knowledge, the specific role of *Halomonas* spp. in metallic corrosion or biofouling has not been addressed; however, Lopez et al. (López et al. 2006) identified *Halomonas* spp. in biofilms associated with pitting corrosion of steel samples placed in seawater injection pipelines at an oil facility. Also, *Halomonas* spp. have been suggested to play a role in the deterioration of the RMS *Titanic* on the seafloor (Sánchez-Porro et al. 2010).

The second most abundant phylotype in the entire data set (approximately 16 percent of recovered sequences) is also a member of the gammaproteobacteria and is closely related (100 percent sequence identity) to *Pseudomonas* spp. identified in diverse environments including metal-working fluids (accession number HE575918, Lodders and Kämpfer 2012) and biological degreasing systems, which are designed to remove grease or oil from metal parts (accession number GQ417328, Boucher et al. 2011). This phylotype was the most prevalent phylotype in 5

of the 37 sample libraries. *Pseudomonas* spp. are commonly associated with hydrocarbon environments having been identified in kerosene, diesel, biodiesel, gas oil and other hydrocarbon fuels (Gaylarde et al. 1999; Zhu et al. 2003; Rodríguez-Rodríguez et al. 2009; Rajasekar et al. 2010; Brown et al. 2010; White et al. 2011) as well as fuel-contaminated soils (Whyte et al. 1997; Rahman et al. 2002). *Pseudomonas* spp. are well-known biofilm formers (Madigan et al. 2010) and have been associated with degradation of hydrocarbons (Yemashova et al. 2007; Mehdi and Giti 2008; Norman et al. 2002). Many investigations have addressed the corrosive impacts of *Pseudomonas* spp., and evidence suggests that these microbes can have a variety of impacts on corrosion of a number of different materials present in fuel systems including carbon steel, stainless steel and aluminum (Busalmen et al. 2002; Little and Ray 2002; Dagbert et al. 2006; Jayaraman et al. 1998; Morales et al. 1993; Yuan and Pehkonen 2007).

Phylotypes identified as *Shewanella* spp. (also members of the gammaproteobacteria) accounted for approximately six percent of the total recovered pyrosequences and were present in the 16S rRNA gene libraries of all fuel samples. *Shewanella* sp. are facultative anaerobes common in marine environments (Madigan et al. 2010). *Shewanella* spp. have been intensively studied in the context of extracellular electron transfer and iron cycling, and these microbes have been shown to both enhance and inhibit microbial corrosion of materials commonly used in fuel system infrastructure (Little et al. 1998; Dubiel et al. 2002).

*Corynebacterium* spp. (members of the actinobacteria) account for approximately four percent of the pyrosequencing libraries from all fuel samples and were found in all but three of the pyrosequencing libraries (the three samples in which *Corynebacterium* spp. were absent are samples D.2 and D.5a (diesel) and sample E100.b (fuel-grade ethanol)). *Corynebacterium* spp. have been identified in hydrocarbon contaminated soils and fuel systems and have been shown to degrade hydrocarbons (Gaylarde et al. 1999; Rahman et al. 2002; McGowan et al. 2004; White et

al. 2011). The corrosive impacts of *Corynebacterium* spp. have not been thoroughly investigated.

*Acetobacter* spp. account for approximately four percent of recovered pyrosequences for all samples and were the dominant phylotype in one E10 sample (E10.5) and one E85 sample (E85.2). *Acetobacter* spp. are present in the pyrosequencing libraries of 12 of the 37 fuel samples including E10, E85, diesel and gasoline samples. *Acetobacter* spp. convert ethanol to acetic acid (Kersters et al. 2006 and references therein), and these types of microbes have been suspected to contribute to microbial corrosion of tanks and equipment exposed to fuel-grade ethanol and water (Jain 2011, Sowards et al. - in preparation, Williamson et al. - in preparation, see Chapter 3, Appendices B and C). The presence of *Acetobacter* spp. suggests that microbial organic acid production could potentially contribute to corrosion issues in fuel systems. The presence of *Acetobacter* spp. in gasoline and diesel samples that have no additions of fuel-grade ethanol may be explained by cross-contamination of fuels due to shared infrastructure or by the microbial degradation of these fuels resulting in carbon sources readily used by these types of microbes. *Gluconacetobacter* spp., microbes closely related to *Acetobacter* spp. that also convert ethanol to acetic acid, were identified in 10 of the fuel sample pyrosequencing libraries.

In addition to the microbes discussed above, diverse phylotypes were identified in the fuel samples at lower relative abundances. Members of the firmicutes identified as *Lactobacillus* spp. are the most dominant phylotype in one biodiesel sample (BD.2a). Microbes classified as sulfate-reducing members of the Deltaproteobacteria (*Desulfovibrio* spp.), which are microbes that have long been associated with microbial corrosion, and anaerobic, spore-forming members of the firmicutes (clostridiaceae) are also present in the pyrosequencing libraries. Archaeal phylotypes including euryarchaeota identified as halophilic *Halorubrum* spp. as well as methanogenic *Methanobacterium* spp. are also identified in the sample libraries. Some

methanogenic archaea have been shown to increase corrosion due to direct oxidation of elemental iron (Dinh et al. 2004; Uchiyama et al. 2010). Though the microbes described here are less prevalent in pyrosequencing libraries, they may have significant impacts on fuels and fuel system infrastructure (e.g. enhancement of localized corrosion). Stevenson et al. ( 2011) analyzed microbial communities at an oil production facility via 16S rRNA gene pyrosequencing and determined that planktonic communities and communities associated with pipeline biofilms were similar in composition though not necessarily in structure; therefore, microbes identified in lower relative abundances in the bulk fuel samples collected in this study may be more significant biofilm community members.

### 2.4.3 Comparison of Microbial Communities in Different Fuel Types via 16S rRNA Gene (DNA) Pyrosequencing

We hypothesized that different fuel types may host different microbial communities due to factors such as carbon chain length, the presence or absence of ethanol in the fuel, the presence of fuel additives and water solubility. Figure 2.5 displays the distribution of taxa found in each type of fuel sampled. Inspection of this distribution of taxa suggests that microbial communities in different fuel types are similar. To compare microbial communities of different fuel types, beta diversity metrics including the Bray-Curtis, Marisita-Horn, unweighted UniFrac and weighted UniFrac metrics (Lozupone and Knight 2005) were computed and compared via principal component analysis. Figure 2.6 displays principal component analysis plots based upon the unweighted and weighted UniFrac metrics. These metrics measure the phylogenetic distance between all phylotypes in multiple samples based upon the branch length of phylogenetic trees containing all sequences from every sample being considered. These analyses

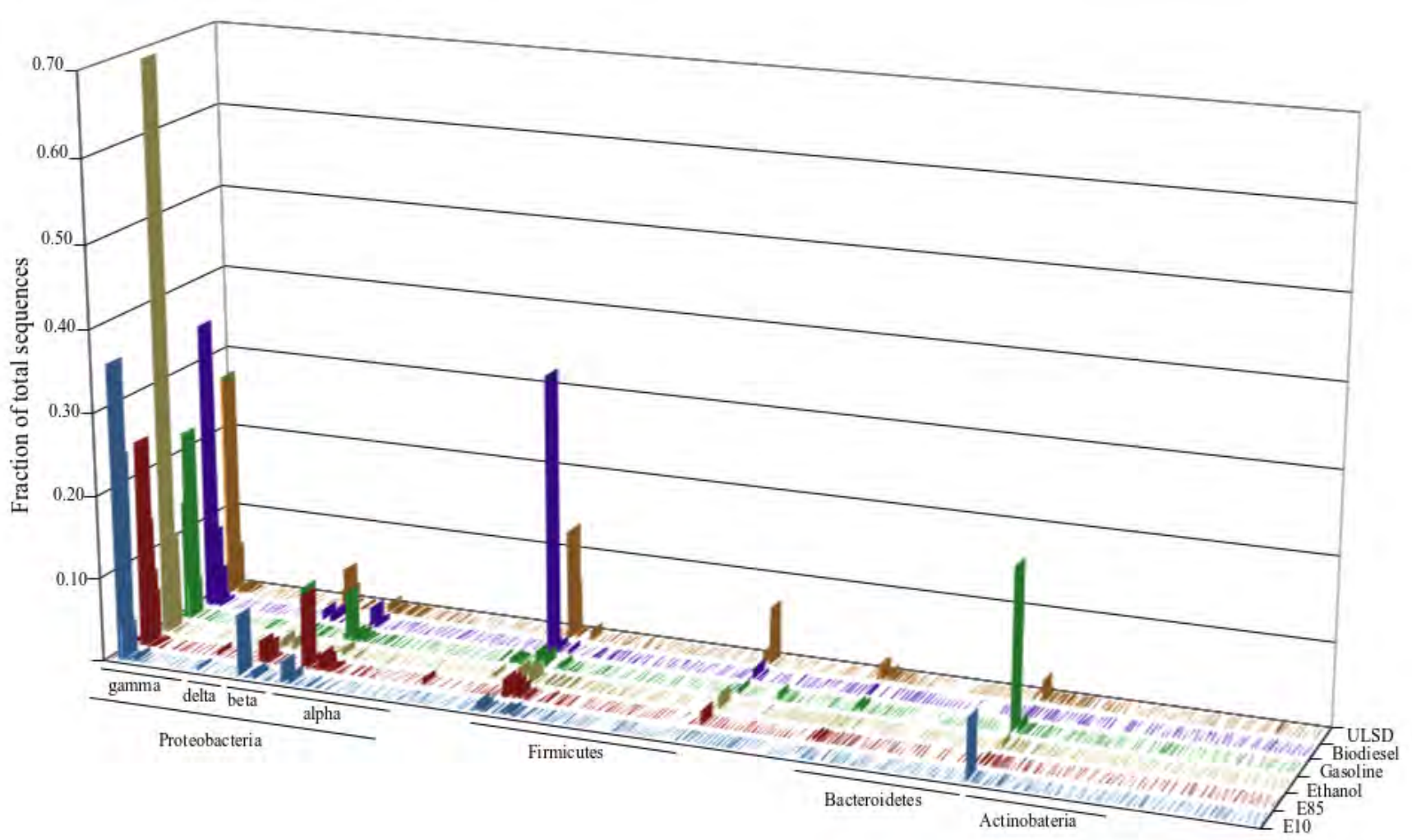


Figure 2.5: Distribution of taxa found in each fuel type sampled. Samples for each fuel type were pooled together for analysis.

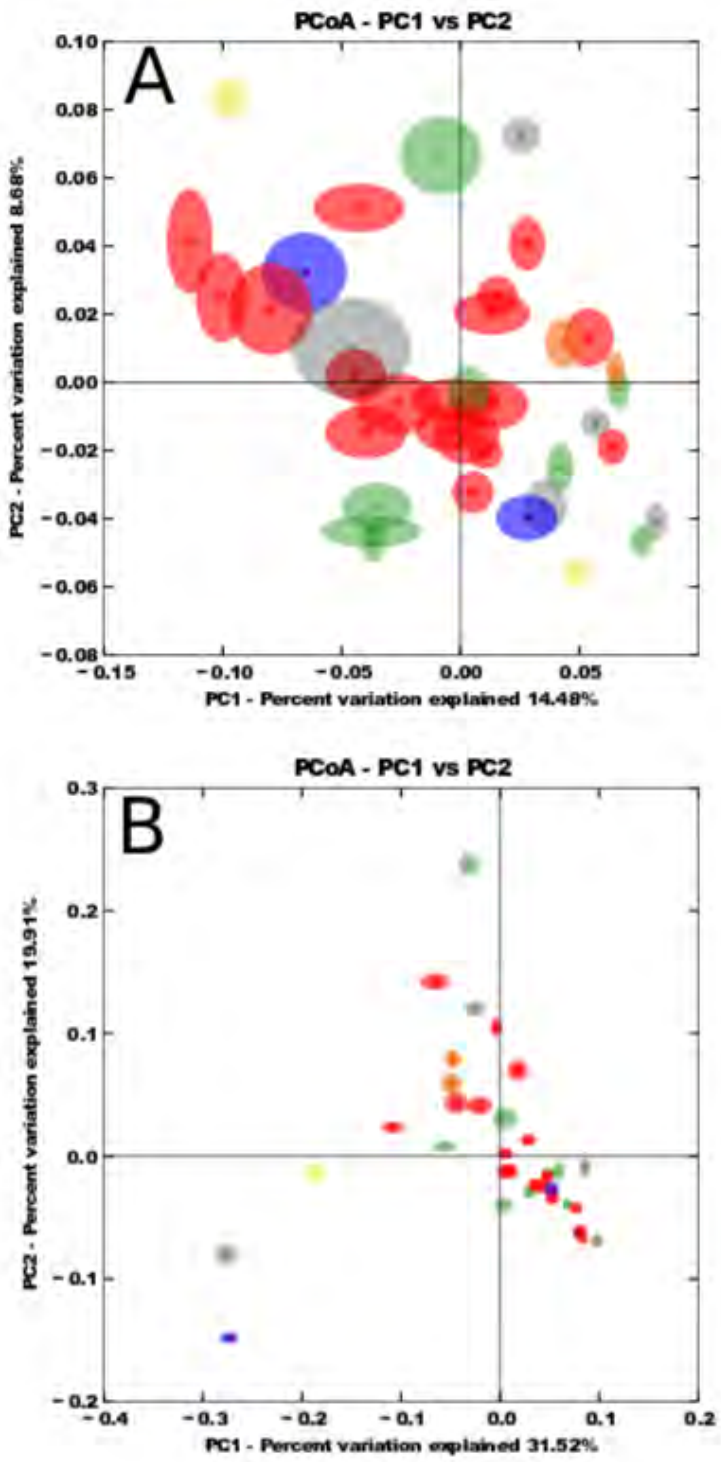


Figure 2.6: Principle coordinate analysisbased upon unweighted (A) and weighted (B) Unifrac distances. PC1 and PC2 are plotted on the X and Y axes. The percentage of variation explained by the plotted principle coordinates is displayed on the axes. Each color represents a different fuel type (red-E10, green-e85, yellow-ethanol, gray-diesel, blue-biodiesel, orange-gasoline).

demonstrate that fuel type does not separate microbial communities. Principal coordinates analysis based upon the Bray-Curtis and Marisita-Horn metrics show similar results.

Interestingly, many of the same types of microbes identified in fuel samples analyzed by White et al. (2011) are prevalent in the pyrosequencing libraries generated in this study. White et al. generated pyrosequencing libraries from 4 fuel samples including diesel, biodiesel and gas oil samples. In addition, the authors conducted cultivation-based assessments of diversity as well as denaturing gradient gel electrophoresis analyses of many fuels. The prevalence of similar phylotypes in the data generated in the study presented by White et al. and the study presented here suggests that microbial communities in fuels are consistent throughout fuel industry infrastructure. The fuels sampled by White et al. did not include fuel-grade ethanol or ethanol fuel blends; however, the authors identified a small number of sequences representing G*luconacetobacter* spp., microbes known to oxidize ethanol to acetic acid. No *Acetobacter* spp. were identified. Interestingly, *Acetobacter* spp. are present in 12 of the samples analyzed in this study and were the most prevalent members of two samples from ethanol fuel blend tanks (E10 and E85), which may suggest that the presence of fuel-grade ethanol provides an environment that will enrich for these types of microbes. Industry reports that acetic-acid-producing microbes may be enhancing corrosion of materials exposed to fuel-grade ethanol and water offer further evidence that these sorts of microbes are problematic in automotive fuel environments (see Chapter 3).

Schloss et al. (2011) discuss some of the potential biases and limitations that may be encountered when applying 454 pyrosequencing to 16S rRNA gene surveys. Some of these biases and limitations include PCR amplification bias, formation of chimeric sequences, batch effects, biases introduced by different bioinformatic analysis pipelines, and biases between different sequencing centers and between different sequencing runs (Schloss et al. 2011; Leek et

al. 2010). As many samples are often included on the same 454 pyrosequencing run via a barcoded approach (the data generated in this study used this approach – see methods), batch effects and cross-over of sequences between samples may be possible (unpublished data suggests cross-contamination can impact 454 pyrosequencing data). To attempt to combat these sorts of biases and limitations, a standardized number of sequences (a subset of the total number of sequences for each sample) was used for each sample for alpha and beta diversity analyses. 454 pyrosequencing data and Sanger sequencing data performed on the same samples show similar results, thus, it seems as though these biases and limitations may have been kept to a minimum. Additionally, similar phylotypes are present in 16S rRNA gene pyrosequencing libraries for E10 and E85 fuel samples produced from two separate 454 pyrosequencing runs (not replicate samples, but samples of the same fuel types).

### 2.4.4 Active vs. Bulk Microbial Communities (DNA vs. cDNA)

The concept of dormancy is thoroughly reviewed by Lennon and Jones (2011). Microbial communities in many environments combat adverse conditions by entering a state of reduced metabolic activity (dormancy) in order to survive until favorable conditions return. One common example of this strategy is the formation of highly resistant spores by many members of the firmicutes including *Bacillus* and *Clostridia* spp. Dormancy in the context of microbial communities in fuel environments is important to consider as microbes exposed to high levels of solvents (e.g. gasoline, ethanol or other fuels) may enter a state of dormancy in which metabolic activity may be greatly reduced. Thus, estimates of microbial diversity in these environments (cultivation-based techniques or molecular-based techniques relying on DNA sequencing) may not reflect the *in situ* active microbial communities, which has important implications for ensuring effective monitoring and mitigation strategies for combating microbial contamination

issues in fuels. A strategy that has been employed to identify both active and total communities in environmental samples is sequencing of both 16/18S rRNA genes as well as 16/18S rRNA (reverse transcribed to cDNA) (Lennon and Jones 2011 and references therein). This approach assumes that 16/18S rRNA genes serve as a proxy of the bulk microbial community present in an environment while the 16/18S rRNA serves as a proxy for active microbial members due to the correlation of cell activity and rRNA (DeLong et al. 1989). This approach is not without limitations. Factors such as 16S rRNA gene copy number, biases introduced during nucleic acid extraction and reverse transcription of rRNA into cDNA, and variations in rRNA concentrations in different types of microbes may impact results.

Studies employing the approach described above as well as other techniques designed to elucidate active and bulk microbial communities have demonstrated that active portions of bulk microbial communities may vary considerably in some environments (or with time in a single environment (Lennon and Jones 2011 and references therein)). Kamke and colleagues (2010) used Sanger sequencing of 16S rRNA (cDNA) and 16S rRNA genes to assess the microbial communities associated with marine sponges. The authors found that the active and bulk communities were similar. Jones and Lennon (2010) concluded that some abundant members of the bulk microbial communities in lake environments were inactive by comparing relative recovery of 16S rRNA gene pyrosequences and 16S rRNA (cDNA) pyrosequences. Logue and Lindstrom (Logue and Lindström 2010) investigated planktonic microbial communities in lakes with terminal restriction fragment length polymorphism methodology to conclude that 16S rRNA gene analysis and 16S rRNA (cDNA) analysis indicated bulk and active communities were similar.

To investigate the bulk and active communities of fuel samples, 16/18S rRNA from a subset of the samples collected in this study was pyrosequenced for comparison with 16/18S

rRNA gene pyrosequence data from the same samples. A total of ten samples were analyzed for both rRNA (cDNA) and rRNA genes (DNA) (see Table 2.1). The distribution of taxa recovered from cDNA and DNA libraries is displayed in Figure 2.7. Many of the same phylotypes are identified by each method suggesting that bulk and active communities overlap considerably. As mentioned above, the approach employed here is not without limitations; however, we consider the approach reasonable approximation of active and total microbial communities. The microbial diversity of pooled samples of each fuel type based upon both cDNA and DNA pyrosequencing data was estimated to determine if active communities were a subset of the bulk community. We hypothesized that if only a portion of the bulk community was active *in situ*, that diversity estimates would be lower for cDNA-based data than DNA-based data. Figure 2.8 displays the estimated total species and 95 percent confidence bounds produced by Catchall, open source software for analyzing population diversity developed by Bunge et al. (2012). These diversity estimates do not support the hypothesis that the active community is only a subset of the total microbial community. As displayed in Figure 2.8, estimations of the number of OTUs based upon cDNA-based data is similar to estimates base upon DNA data – and estimates are higher in some fuels. Alpha diversity metrics displayed in Table 2.1 also suggest that richness of active and bulk communities is similar. This suggests that much of the microbial community identified via DNA libraries is active *in situ*.

To determine which phylotypes have higher or lower relative abundances in DNA and cDNA pyrosequencing libraries, the change in relative recovery of abundant phylotypes for each fuel type was compared (Figures 2.9 and 2.10). *Shewanella* spp. and *Halomonas* spp. have higher relative abundances in the DNA pyrosequencing libraries than in the cDNA pyrosequencing libraries for all fuel types. If we consider phylotypes that show higher relative recovery in DNA libraries than in cDNA libraries to be inactive members of the microbial

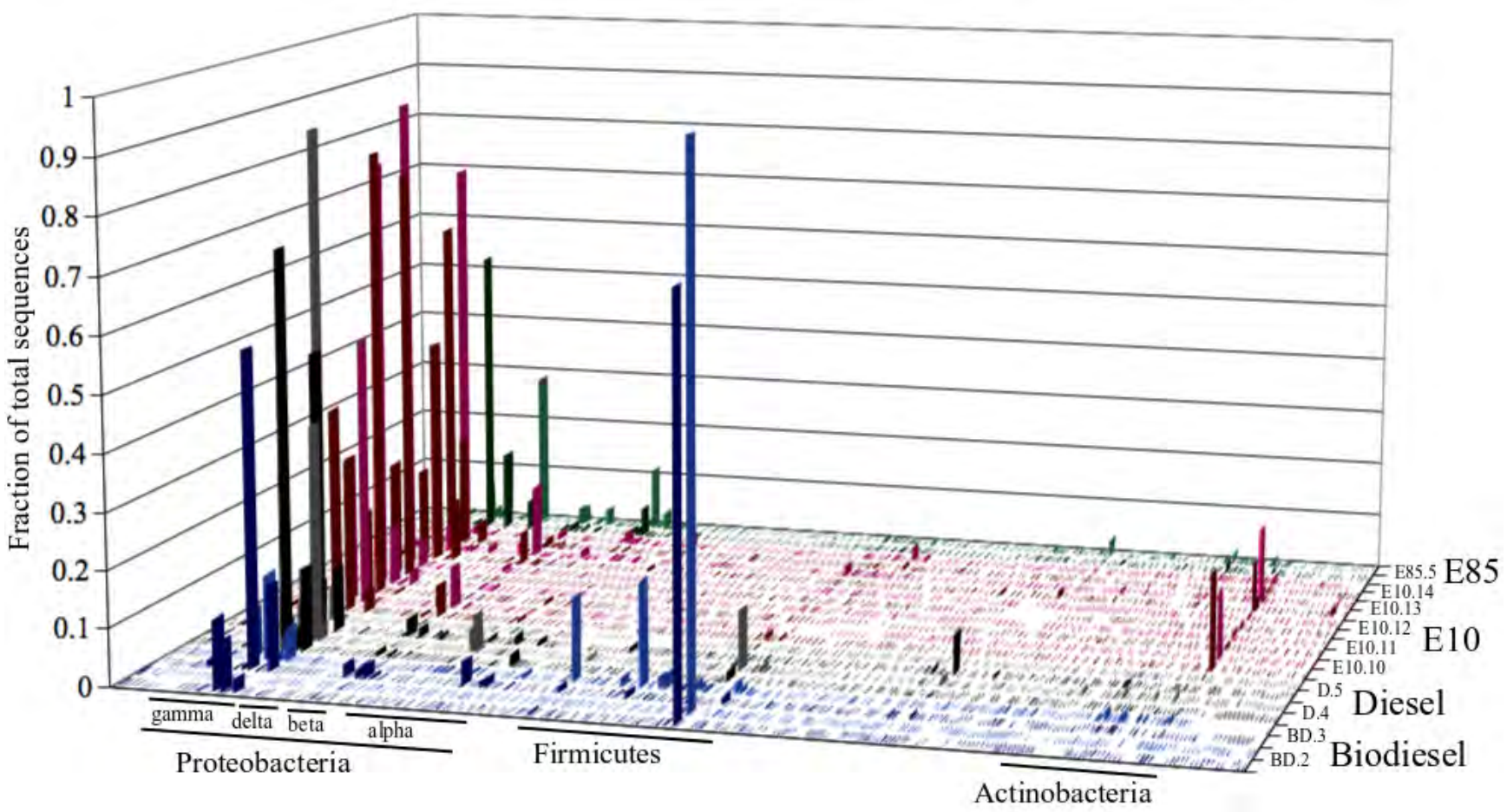


Figure 2.7: Distribution of taxa for DNA and cDNA pyrosequencing libraries. The DNA library for each sample is positioned closer to the reader and is colored in a darker color than the corresponding cDNA library. DNA and cDNA libraries for each sample are next to one another.

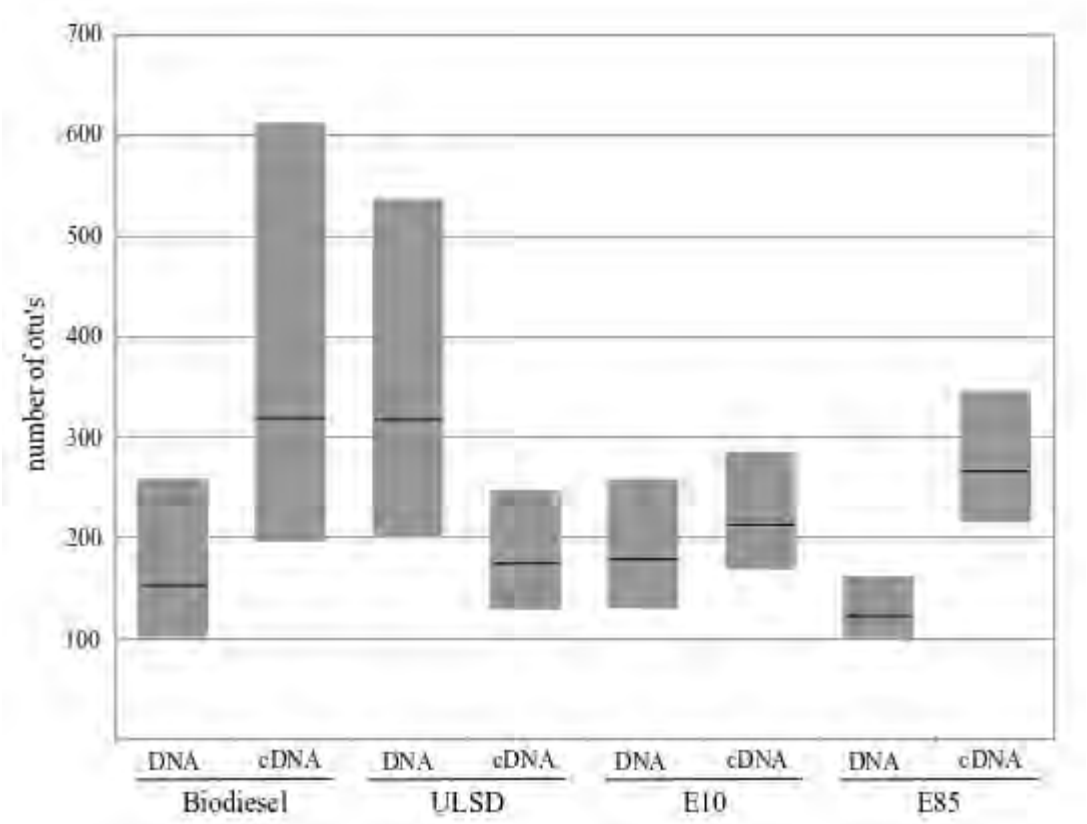


Figure 2.8: Estimation of richness for each fuel type based upon Catchall (Bunge et al. 2012) analysis of both DNA and cDNA pyrosequencing libraries.

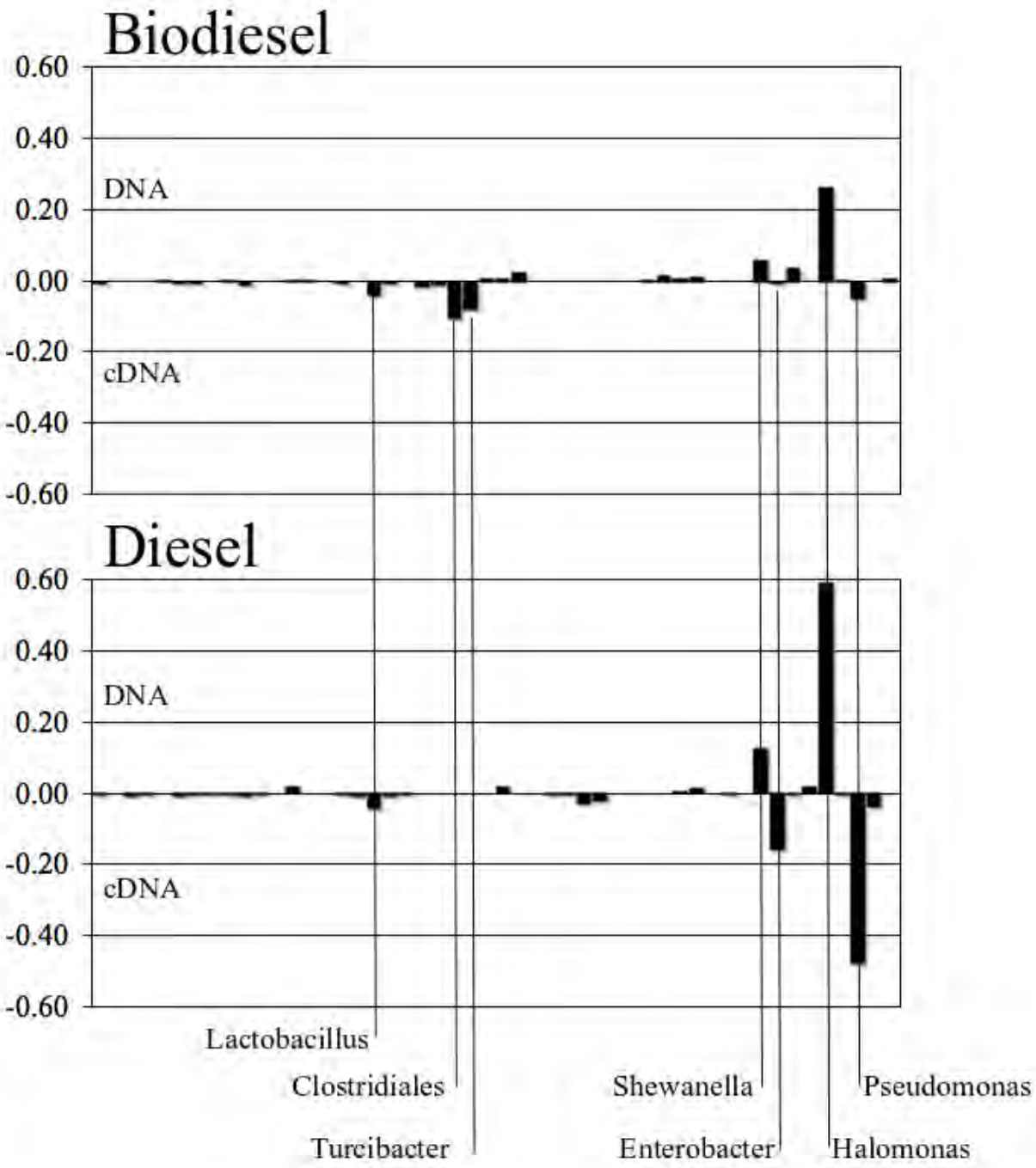


Figure 2.9: Comparison of relative abundances of phylotypes in DNA and cDNA libraries for biodiesel and diesel samples.

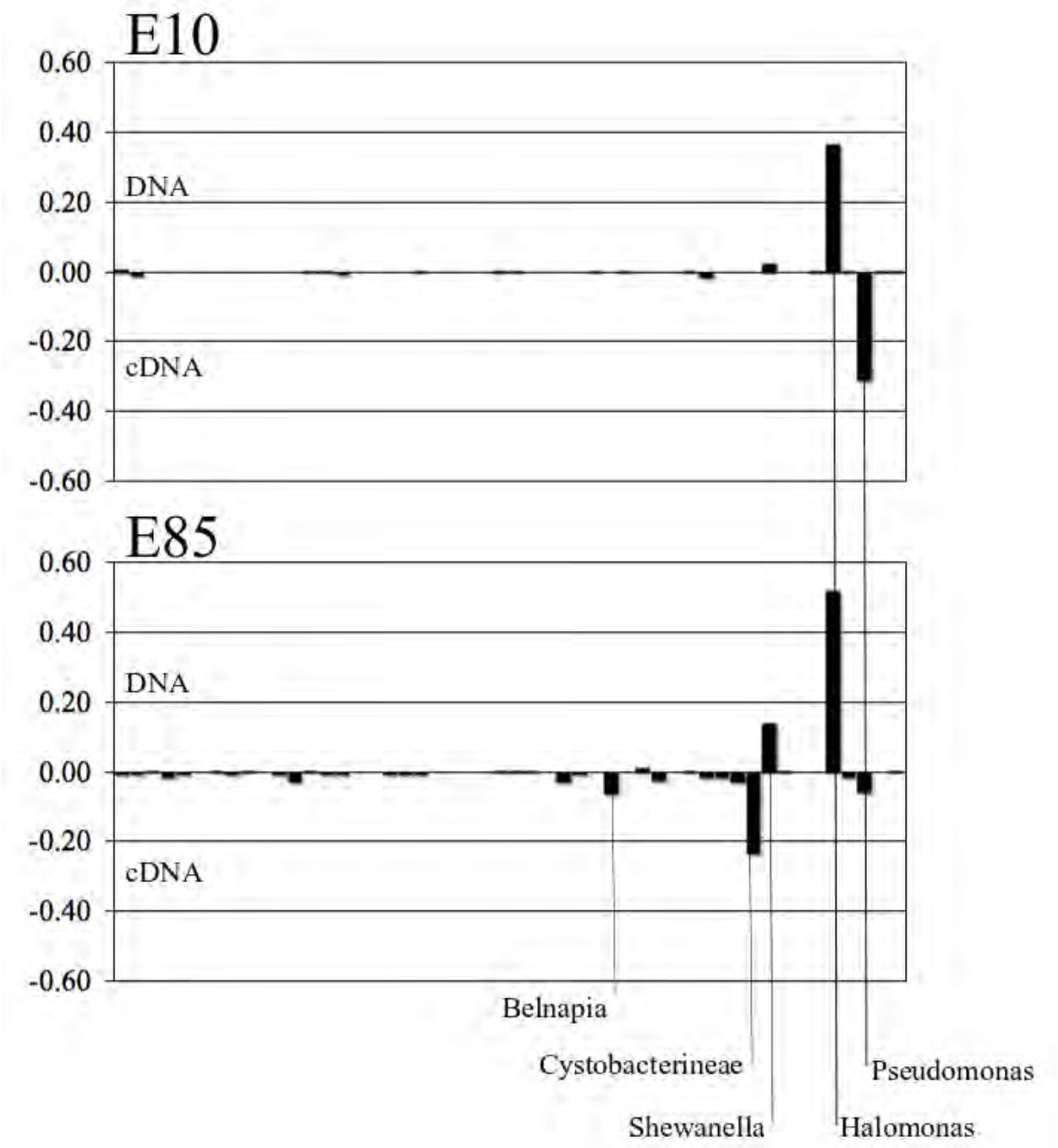


Figure 2.10: Comparison of relative abundances of phylotypes in DNA and cDNA libraries for E10 and E85 samples.

communities, the data suggests that these phylotypes, though prevalent in DNA pyrosequencing libraries, may be less active members of the community. However, as both cDNA and DNA-based pyrosequencing libraries identify similar prevalent phylotypes in all fuel types, DNA-based methods may provide an acceptable proxy for the active microbial communities in fuel environments. It is important to note that the preservation of microbial rRNA in the fuel environments is unknown; thus, these DNA-cDNA analyses may not accurately represent the active and dormant communities a at specific point in time. However, the recovery of rRNA from these samples suggests recent microbial activity.

### 2.4.5 Implications for Cultivation-based Monitoring

Cultivation-based methods are often employed to monitor microbial contamination of fuels (ASTM Standard D6469). Standard cultivation-based methods can be relatively straight-forward and inexpensive, but it has been demonstrated that these methods do not sufficiently capture microbial diversity in many environments (Amann et al. 1995). A recent study (White et al. 2011) aimed at determining if cultivation-based methods and molecular methods (DGGE as well as 16S rRNA gene pyrosequencing) capture similar diversity in fuel samples suggested that aerobic cultivation-based methods were able to recover the major aerobic phylotypes identified by molecular methods. However, the authors note that the cultivation methods applied were not applicable for the detection of anaerobic microbes and that certain aerobic members seemed to be over-represented by cultivation methods. As discussed in the previous sections, the dominant phylotypes identified in this study have been identified by both cultivation-based and molecular techniques in a number of fuel samples analyzed in other studies. This similarity of identified microbes may suggest that cultivation-based methods provide a relatively inexpensive option for routine monitoring of fuel contamination, especially when considering fuel degradation as the

most abundant microbes are likely to be consuming the most fuel. However, as over 100 different types of microbes (OTUs) are identified in some fuel samples analyzed in this study, cultivation-based methods are unlikely to capture a significant portion of the entire microbial communities present in these environments. Thus, molecular techniques may be warranted when attempting to more fully characterize the microbial contamination of fuels. This may be important when considering issues such a microbial corrosion, which may result from localized microbial communities present on metallic surfaces in fuel industry infrastructure. These communities likely include microbes employing both aerobic and anaerobic metabolisms, and the microbes may be present in very low relative abundances. Sampling procedures (i.e. where a sample is collected – bulk fluid, biofilm, tank bottoms) would also impact these sorts of monitoring procedures. The samples collected as part of this study include predominantly bulk fuel samples filtered through 0.2 micron filters. Additionally, one tank bottoms sample was collected from a storage tank containing fuel-grade ethanol, and one sample was collected from a filter associated with a fuel-grade ethanol tank. Interestingly, Stevenson et al. (2011) found that microbial communities in bulk fluid samples and biofilm samples collected from an oil production facility differed in structure but had similar compositions. This suggests that 16S rRNA-based molecular surveys of bulk fuel samples may provide some insight into the microbial community composition of biofilm communities provided adequate sequencing depth is achieved. While sampling only bulk fluids is less than ideal, often sampling opportunities of fuel systems is limited making acquiring biofilm samples in many areas of fuel industry infrastructure difficult. For instance, sampling biofilms associated with the internal surfaces of a fuel storage tank may require draining the tank, which may not be a routine procedure and may limit opportunities for sampling. Additionally, very few researchers have applied molecular methods

to exploring the microbial diversity of fuel systems; thus, further research is required to more fully understand fuel system microbial communities.

## 2.5 Summary

This survey of the microbial diversity associated with automotive fuels collected from across the United States indicates that microbes frequently associated with fuel system microbiology (e.g. *Pseudomonas* spp., *Corynebacterium* spp., *Lactobacillus* spp.) are identified via both 16S rRNA (cDNA) and 16S rRNA gene (DNA) pyrosequence analysis. Microbial communities identified by DNA-based pyrosequencing libraries and cDNA-based pyrosequencing libraries show considerable overlap, which suggests that the active microbial communities are similar to the total microbial communities in these fuels. Beta diversity metrics indicate overlap of microbial communities between fuel types. This overlap between communities from different fuel types is not unexpected as much of the fuel industry infrastructure is exposed to a variety of fuels. For example, a tanker truck or pipeline may be used to transport a variety of fuels as well as unrefined hydrocarbons. This in-depth microbial diversity survey of fuel environments provides a valuable view into the types of microbes present in fuel systems that can inform fuel contamination monitoring and prevention strategies as well as guide future research. For example, *Halomonas* spp. seem to be dominant members of microbial communities in fuel systems; however, to the authors' knowledge, little is known about how these microbes are actually impacting these environments. Also, as knowledge of the impacts of certain types of microbes or microbial communities on fuels and fuel system infrastructure accumulates, molecular methods may provide techniques to identify problematic microbial contamination in time to prevent negative impacts . Additionally, molecular methods

may be employed to monitor the efficacy of mitigation strategies such as biocide application to determine if problematic microbes or microbial communities are eliminated.

CHAPTER 3

# MICROBIAL COMMUNITIES ASSOCIATED WITH FUEL-GRADE ETHANOL ENVIRONMENTS: IMPLICATIONS FOR MICROBIOLOGICALLY INFLUENCED CORROSION

## 3.1 Abstract

Microbiologically influenced corrosion (MIC) is a costly problem that impacts hydrocarbon fuel production, transportation and storage infrastructure, water distribution systems, ships, railcars and many other types of metallic infrastructure. As the production and use of alternative fuels such as ethanol increase it is important to consider MIC in environments exposed to these fuels. Reports of suspected MIC in infrastructure exposed to fuel-grade ethanol and water prompted the investigation of the microbial diversity associated with such environments. Small subunit ribosomal RNA gene pyrosequencing indicates that acetic acid producing bacteria (*Acetobacter* spp. and *Gluconacetobacter* spp.) are prevalent members of fuel storage tanks that contain fuel-grade ethanol and water. In addition to these bacteria, other microbes that have been implicated in corrosion were identified including sulfate-reducing bacteria and methanogens. Acetic acid producing microbes and sulfate-reducing microbes were also cultivated from these environments, and the corrosive nature of these microbes was investigated and reported elsewhere (Jain 2011, Sowards et al. – in preparation). This study furthers our knowledge of the microbial diversity associated with fuel storage and transportation

systems and provides insight into the potential MIC issues that may impact alternative fuel systems.

## 3.2 Introduction

The detrimental effects of microbial contamination of fuel systems have been well described as microbes can cause biofouling, fuel degradation and microbiologically influenced corrosion (MIC) (Little and Lee 2007; Gaylarde et al. 1999; Rajasekar et al. 2012). MIC is the biologically-mediated deterioration of a material (e.g. carbon steel) and can be attributed to a number of different mechanisms including microbial production of corrosive substances such as organic acids and sulfides as well as microbially-mediated oxidation and/or reduction of metals (Little and Lee 2007; Lewandowski and Beyenal 2009). It has been estimated that MIC is responsible for up to 20 percent of corrosion damage in metal-containing infrastructure (Flemming 1996), and the development of effective strategies for the prevention and mitigation of MIC in many environments has proven difficult. While the corrosive impacts of microbes in oil and gas industry infrastructure have been well documented, MIC in 'alternative' fuel environments has been less extensively evaluated.

In the United States, fuel-grade ethanol production has increased due to efforts to use renewable fuels. Ethanol is added to gasoline as a low-level blend to offset fossil fuel consumption and to oxygenate the fuel for better combustion and better emissions (US DOE 2012). The increased use of fossil fuel alternatives such as fuel-grade ethanol requires an investigation of the impacts these fuels may have on storage and transportation infrastructure. For example, carbon steel is susceptible to stress corrosion cracking (SCC) when exposed to fuel-grade ethanol (Kane et al. 2005; Sridhar et al. 2006; Lou et al. 2009; Lou et al. 2010), and pitting corrosion of carbon steel in ethanolic environments has also been investigated (Lou and

Singh 2010). The potential for MIC in environments exposed to fuel-grade ethanol has not been widely considered; however, infrastructure exposed to fuel-grade ethanol (e.g. pipelines, tanks and equipment in pump sumps) have been subject to corrosion issues suspected to be microbial in nature (personal communication with industry personnel, US EPA 2011).

At high concentrations ethanol is thought to be toxic to microbes due to negative impacts on cell membranes and general dessication of the cell body (Taylor et al. 2012 and references therein); however, ethanol can also serve as a carbon source and/or electron donor for many microbes including sulfate reducing bacteria, acid producing bacteria and other microbes associated with MIC (Madigan et al. 2010). Additionally, microbial metabolism of ethanol results in the formation of metabolites such as acetic acid, propionate and hydrogen, which could subsequently be metabolized by other corrosion-inducing microbiota living as a communal biofilm on the surfaces of fuel transmission or storage infrastructure. As water is commonly found in pipelines, tanks and other areas in fuel storage and transportation systems (Passman 2003), the potential for MIC in fuel industry infrastructure exposed to ethanol must be considered.

The objective of this research was to investigate the microbial diversity that may impact corrosion in environments exposed to fuel-grade ethanol and water. As ethanol may serve as a carbon source or electron donor for many microbes and industry personnel have reported suspected MIC issues in some ethanolic environments, we hypothesized that environments exposed to ethanolic fuels and water host microbes capable of metabolizing ethanol and associated compounds resulting in MIC. To date, the microbial diversity of environments associated with ethanolic fuels and the potential for microbial corrosion in ethanolic environments has not been thoroughly investigated. Samples collected from fuel tanks containing ethanol and water or ethanol fuel blends were analyzed via both molecular techniques

(16S rRNA gene pyrosequencing) and cultivation methods to elucidate the types of microbes present in these systems and to provide insight into how these microbes may impact corrosion processes.

## 3.3 Materials and Methods

Pyrosequencing, Sanger sequencing and cultivation studies were used to characterize the microbial diversity of samples collected from tanks at fuel terminals. These methods are described in sections 3.3.1 through 3.3.3.

### 3.3.1 Sample Collection

Samples were collected from six ethanol containment tanks over a time period of approximately 15 months (November 2009-February 2011). These ethanol containment tanks collect fuel-grade ethanol as well as water (runoff and condensation of humidity) during normal operations at fueling terminals and ethanol production facilities. Samples from these tanks included bulk liquid and solids collected aseptically into sterile, 50 ml tubes. Samples were attained from the bottom of each tank. Sample pH was measured by paper. Additionally, one sample was collected from the exterior surface of an above ground storage tank containing E10 fuel (a blend of 90 percent gasoline and 10 percent fuel-grade ethanol) with obvious surface biofilms. A black substance had formed on the painted exterior of the tank near safety valves and pipes exiting the tank (points on the tank where vapors likely escape). The formation of this black substance on tanks containing fuel-grade ethanol or ethanol fuel blends is a common occurrence (personal communication with industry personnel). The black substance was collected aseptically by scraping the substance into a 2 ml cryovial. Samples for DNA extraction were held at -80 $^{o}$C until DNA was extracted. Samples for cultivation experiments were held at 4

°C until inoculation. Ethanol containment tank samples are labeled ECT.1 through ECT.6; the sample from the exterior surface of the E10 fuel tank is labeled EXT.1.

### 3.3.2 DNA Extraction, PCR and 454 Pyrosequencing

DNA was extracted from ethanol containment tank samples (ECT.1-6) using a phenol:chloroform extraction similar to the procedure described by (Dojka et al. 1998). Sample material was suspended in 500 ul Buffer A (200 mM Tris [pH 8.0], 50 mM ethylenediaminetetraacetic acid (EDTA), 200 mM sodium chloride), 200 ul 20 percent sodium dodecyl sulfate and 500 ul phenol-chloroform-isoamyl alcohol (24:24:1). The samples were reciprocated on a Mini-Beadbeater (Biospec) at maximum speed for one minute, extracted and then extracted again with phenol-chloroform-isoamyl alcohol. DNA was precipitated with one volume isopropanol and 0.3 M sodium acetate. Precipitated DNA was washed with 70 percent ethanol and suspended in nuclease-free water. DNA was extracted from sample EXT.1 using the MoBio Powersoil DNA extraction kit (MoBio, Carlsbad, CA); the 10-minute vortexing step was replaced by one minute of bead beating. DNA was prepared for sequencing as described by Osburn and colleagues (2011) with the following exceptions: Quantitative polymerase chain reaction (qPCR) reactions were performed in duplicate and pooled prior to normalization for sequencing. Each qPCR reaction contained 4 uL of template DNA. Primers 515f-modified (5' – GTG YCA GCM GC CGC GGT AA – 3') and 927r-modified (5' – CCG YCA ATT CMT TTR AGT TT – 3') (see Osburn et al. 2011) incorporated adapter sequences for pyrosequencing on the GSFLX Titanium platform of the Roche 454 Pyrosequencing technology. Amplicons for each sample were normalized for sequencing with the SequalPrep Normalization Plate Kit (Invitrogen), and pooled amplicons were gel purified with the EZNA Gel Extraction Kit (Omega

BioTek). Sequencing was completed on the Roche 454 FLX Titanium platform at the EnGenCOre facility at the University of South Carolina.

Sequence analysis was carried out using the QIIME software package (Caporaso et al. 2010) and the mothur software package (Schloss et al. 2009). Initial quality filtering of the sequences was conducted in accordance with findings identified by Huse and colleagues (Huse et al. 2007). Sequences with errors in barcodes or primers, homopolymer runs longer than 6 nucleotides, ambiguous base calls, or average quality scores less than 25 were removed from the data. Sequences shorter than 400 nucleotides or longer than 500 nucleotides were also discarded. Sequences were denoised with DeNoiser version 1.4.0 (Reeder and Knight 2010). Chimeric sequences were identified by UCHIME (Edgar et al. 2011) and removed. Reverse primers were removed with a custom python script written by Charles Pepe-Ranney. Remaining sequences were aligned with SSU-ALIGN (Nawrocki 2009). Sequences failing to align to the appropriate alignment space were removed, and remaining sequences were trimmed to overlapping alignment space.

Sequences were clustered into operational taxonomic units (OTUs) with the average neighbor clustering method in mothur (Schloss et al. 2009). Taxonomic classifications of sequences were assigned using the naïve Bayesian algorithm described by Wang and colleagues (Wang et al. 2007) as implemented in mothur (Schloss et al. 2009). The Silva SSURef108_NR database (Pruesse et al. 2007) was used as the training set for classifications. These reference sequences were quality filtered as described by Pepe-Ranney, et al. (2012) and trimmed to the primer-targeted region of the 16S rRNA gene (515F-927R). Pyrosequences were also subjected to the Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990) against the Silva SSURef108_NR database (Pruesse et al. 2007). Microbial community diversity was

estimated with Catchall (Bunge et al. 2012), and rarefaction curves were created in QIIME (Caporaso et al. 2010).

### 3.3.3 Cultivation and identification of acetic-acid-producing and sulfate-reducing consortia

Two of the samples collected from ethanol containment tanks (ECT.1 and ECT.2) were used to inoculate media intended to grow acetic acid producing bacteria and sulfate reducing bacteria. Acetic acid producing bacterial consortia were cultivated in an enrichment medium that contained glucose, 10 g/L; acetic acid, 3 mls/L; peptone, 15 g/L; yeast extract, 8 g/L; and ethanol, five percent (v/v) (Lisdiyanti et al. 2003). After several culture transfers, the consortium was transferred to a medium containing yeast extract, 0.5 g/L; peptone, 0.3 g/L; 1 g/L sodium chloride and ethanol, five percent (v/v) (this medium was used for corrosion testing – see Jain 2011; Sowards – in preparation, Appendices B and C). All inocula were incubated shaking (150 rpm) at room temperature.

To cultivate sulfate-reducing bacteria, samples were used to inoculate a modified Postgate B medium (Postgate 1979) that contained potassium dihydrogen phosphate, 0.5 g/L; ammonium chloride, 1 g/L; calcium sulfate, 1 g/L; magnesium sulfate 7-hydrate, 2 g/L; yeast extract, 1 g/L, ascorbic acid, 0.1 g/L; thioglycollic acid, 0.1 g/L; and iron sulfate 7-hydrate, 0.5 g/L. The pH of the media was adjusted to 7.5 with sodium hydroxide. Filtered ethanol (two percent v/v) was added as a carbon source after autoclaving. Cultures were incubated in serum vials with a nitrogen headspace at room temperature.

DNA was extracted from cultures with the phenol:chloroform extraction procedure described above. 16S rRNA gene amplification was carried out using primers 8F (5' – AGA GTT TGA TCC TGG CTC AF – 3') and 1492R (5' – GGT TAC CTT GTT ACG ACT T – 3')

(Lane 1991). PCR, cloning, transformation and sequencing were carried out as described by Sahl et al. (2010). Sanger reads were called with PHRED (Ewing et al. 1998; Ewing and Green 1998) and assembled with PHRAP (www.phrap.org) via Xplorseq (Frank 2008). Initial identification of cultivar sequences was accomplished by parsimony addition into the Silva SSURef108_NR database (Pruesse et al. 2007) and BLAST (Altschul et al. 1990) searches against the Silva SSURef108_NR database and the NCBI non-redundant nucleotide database (Benson et al. 2005). To more thoroughly establish phylogenetic relationships of cultivar and environmental sequences maximum-likelihood phylogenetic trees were created with RAxML (Stamatakis 2006). Closely-related, relevant sequences were retrieved from Genbank, binned (97 percent identity with UClust (Edgar et al. 2011)) in QIIME (Caporaso et al. 2010) and aligned and masked with SSU-ALIGN (Nawrocki 2009). Trees were created using the gamma distribution of rate heterogeneity and GTR substitution model, and the number of bootstrap replicates (rapid bootstrapping method (Stamatakis et al. 2008)) was determined by using the RAxML frequency-based criterion (Pattengale et al. 2010). Redundant sequences and pyrosequences with close identity to cultivar sequences were aligned and masked as described above and added to the phylogenetic tree with pplacer (Matsen, et al. 2010).

## 3.4 Results

The microbial diversity of tank samples was evaluated with 16S rRNA gene sequence analysis and cultivation of key microbes present in these samples. Experimental results are described below.

### 3.4.1 Sample Description

Samples collected from the bottoms of ethanol containment tanks (ECT samples) included bulk liquid and solids from tanks that contained fuel-grade ethanol and water (see Figure 3.1). pH values of these bulk samples ranged from 4 to 7 (see Table 3.1). Industry personnel noted the smell of vinegar (acetic acid) upon collecting some of the samples and noted marked visual corrosion damage on 4 of the 6 ethanol containment tanks. Sample EXT.1 included black solids scraped from painted, exterior tank surfaces near pressure-release valves (Figure 3.1).

### 3.4.2 Pyrosequencing Results

To identify microbes present in tank environments associated with fuel-grade ethanol, we generated small subunit ribosomal rRNA (16S rRNA) gene libraries via pyrosequencing technology. Pyrosequence libraries included a total of 15343 partial 16S rRNA gene sequence reads with an average of 2191 sequences per sample (range, 1077 to 3526), and 263 operational taxonomic units (OTUs) (average neighbor clustering algorithm at a distance of 0.03) were recovered from all seven samples combined. Libraries are labeled according to the sample from which they were produced (ECT – ethanol containment tanks, EXT – tank exterior biofilm). Sample descriptions, pyrosequencing library names and microbial diversity estimates are summarized in Table 3.1.

Rarefaction curves (Figure 3.2) indicate that samples ECT.4 and ECT.5 have the highest species (OTU) richness of the collected samples. Interestingly, these two samples had pH values of approximately seven while the other tank samples had pH values of 4 – 4.5. Samples ECT.1, ECT.6 and EXT.1 exhibit lower species richness than other samples, and it appears as though most of the diversity in these samples has been observed with the generated sequence number

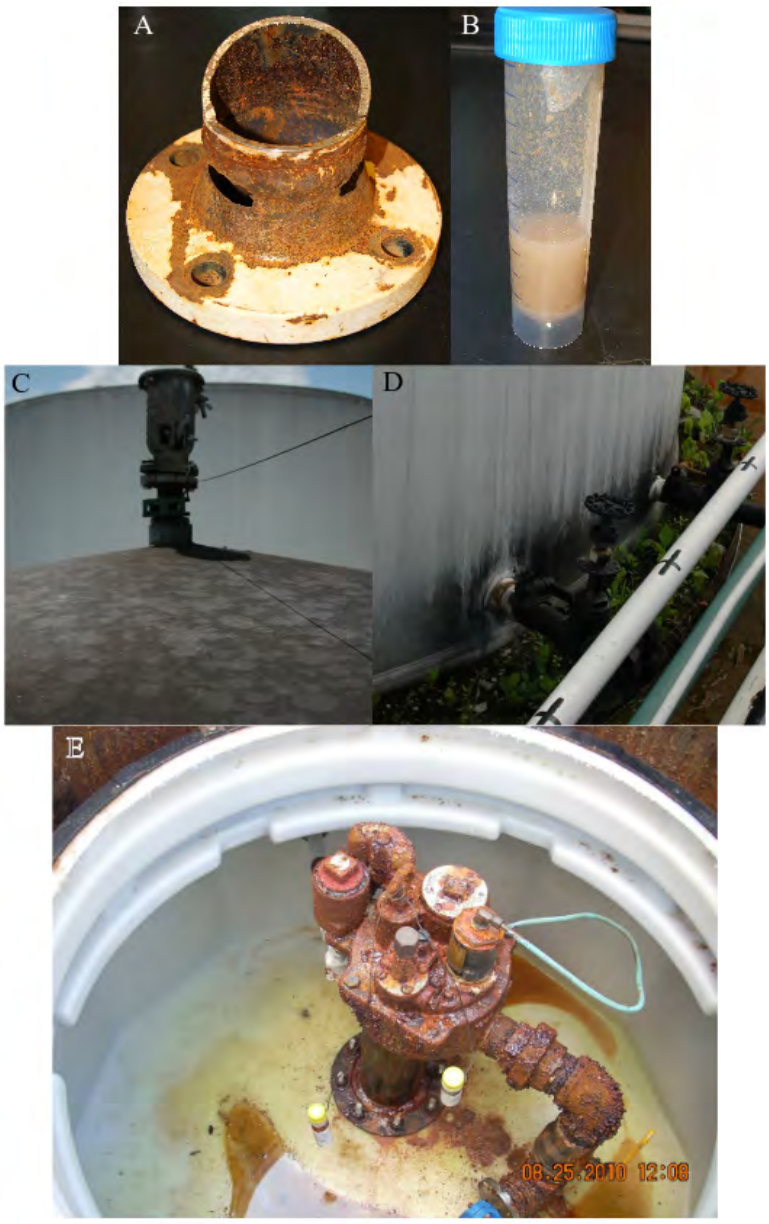


Figure 3.1: Images of infrastructure associated with fuel-grade ethanol. A) Corroded pipe flange removed from service after exposure to fuel-grade ethanol and water. B) Bottoms/bulk liquid sampled from a tank containing fuel-grade ethanol and water. C,D) Black biofilms formed on the outer surface of tanks containing E10. E) Corrosion of piping and equipment in a pump sump at a fueling station. Corrosion in some of these types of sumps is suspected to be impacted by ethanol and acetic acid. Picture E courtesy of Ed Fowler.

Table 3.1: Sample information, pyrosequencing information, alpha diversity metrics.

| Sample ID/ Library Name | Tank Material | Corrosion Damage | Dominant Phylotype | pH | # of sequences | Observed OTUs | Estimated Total Species | Lower CB[a] | Upper CB[a] |
|---|---|---|---|---|---|---|---|---|---|
| ECT.1 | Carbon Steel | Yes | Acetobacter | 4 | 3526 | 32 | 32.1 | 22.6 | 57.3 |
| ECT.2 | Carbon Steel | Yes | Acetobacter | 4.5 | 3235 | 104 | 132.3 | 96.2 | 208.7 |
| ECT.3 | Carbon Steel | No[1] | Acetobacter | 4.5 | 1624 | 57 | 94.2 | 70.8 | 139.5 |
| ECT.4 | Fiberglass | No | Sediminibacterium | 7 | 1197 | 139 | 585.9 | 297.4 | 1377.2 |
| ECT.5 | Carbon Steel | Yes | Denitratisoma | 7 | 1077 | 77 | 177.5 | 126.6 | 280.9 |
| ECT.6 | Carbon Steel | Yes | Acetobacter | 4.5 | 3309 | 30 | 25.5 | 20.7 | 38.5 |
| EXT.1 | Carbon Steel | No[2] | Modestobacter | NA | 1375 | 18 | 19.5 | 18.3 | 24.7 |

[1]New tank, [2]Painted surface, [a]Catchall 95% confidence bound (Bunge et al. 2012)

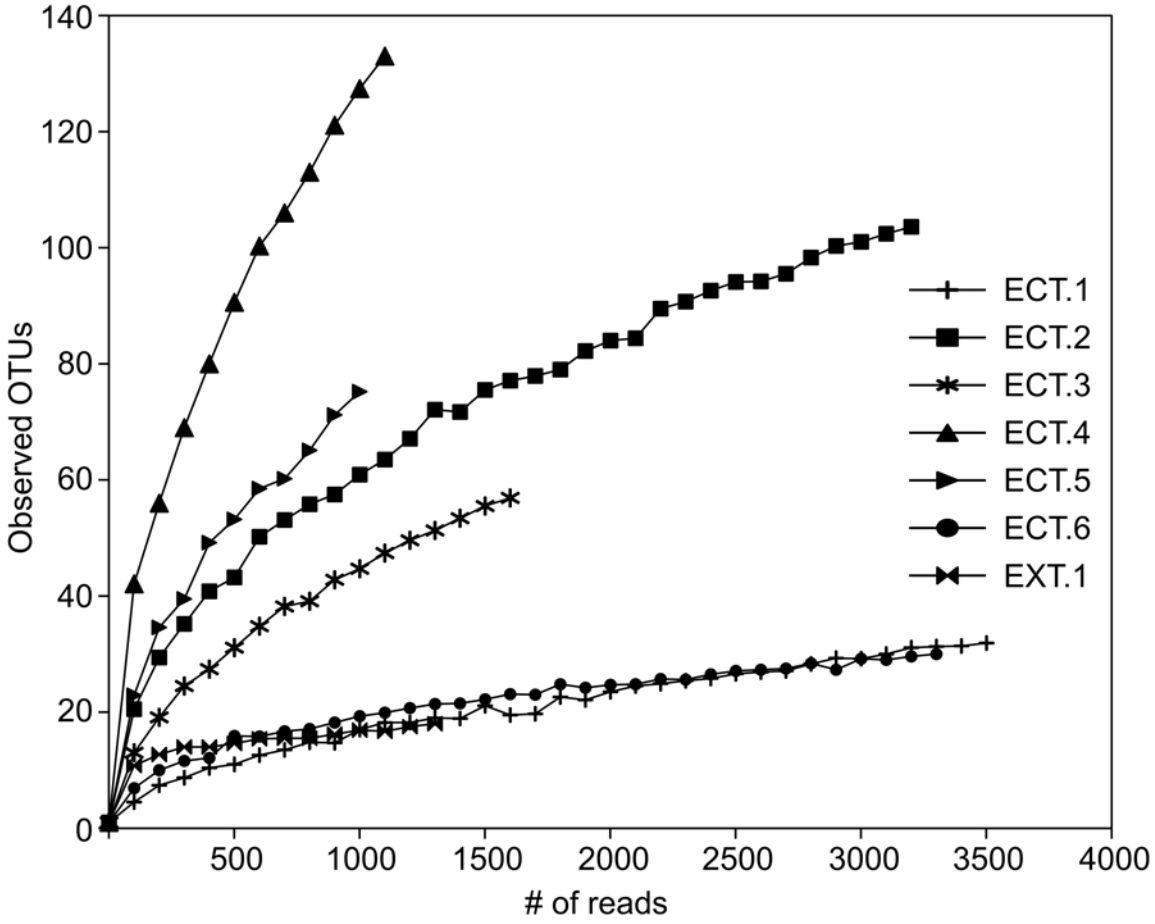


Figure 3.2: Rarefaction analysis for pyrosequencing libraries. Sequences were clustered with the average neighbor method in mothur (Schloss et al. 2009) at a distance of 0.03. Rarefaction curves were created with QIIME (Caporaso et al. 2010).

per sample. Parametric estimates of microbial diversity for each sample were estimated with Catchall (Bunge et al. 2012) and are displayed in Table 3.1. These estimates also indicate that samples ECT.4 and ECT.5 are the most diverse samples, while samples ECT.1, ECT.6 and EXT.1 have lower richness.

The majority of the pyrosequences in all 6 ethanol containment tank sample libraries represent members of the proteobacteria, and four of the six ethanol containment tank samples (ECT.1, 2, 3 and 6) are dominated by members of the alphaproteobacteria that include microbes closely related to *Acetobacter* spp. and *Gluconacetobacter* spp. (Figure 3.3 and 3.4). These types of acetic-acid-producing microbes are also present in the ECT.4 and ECT.5 samples. The most abundant phylotype (OTU 1) in samples ECT.1, ECT.2, ECT.3 and ECT.6 has 100 percent sequence similarity to *Acetobacter lovaniensis* (accession number AJ419837, Cleenwerck et al. 2002). Abundant acetic-acid-producing bacterial sequences also include OTU 4, which is greater than 99 percent similar to *Acetobacter aceti* (accession number X74066, Sievers et al. 1994), and OTU 2, which is greater than 99 percent similar to *Gluconacetobacter liquefacians* (accession # X75617, Sievers et al. 1994).

The pH of samples ECT.4 and ECT.5 was approximately 7, and neither of the most abundant phylotypes in these samples are acetic-acid-producing microbes. The most prevalent phylotype in the ECT.4 pyrosequencing library (OTU 8) is a member of the chitinophagaceae family of the bacteroidetes that is greater than 99 percent similar to an uncultured bacterium identified in a low-sulfate, freshwater lake (accession # GQ390233, (Biderre-Petit et al. 2011)). The second most abundant phylotype (OTU 10) in ECT.4 is a member of the acidobacterium with greater than 99 percent similarity to an uncultured acidobacterium identified in a microbial fuel cell (accession # AB286269, (Ishii et al. 2008)). The ECT.4 pyrosequencing library also includes sequences closely related to *Methanobacterales* spp. (OTU 2a), which are

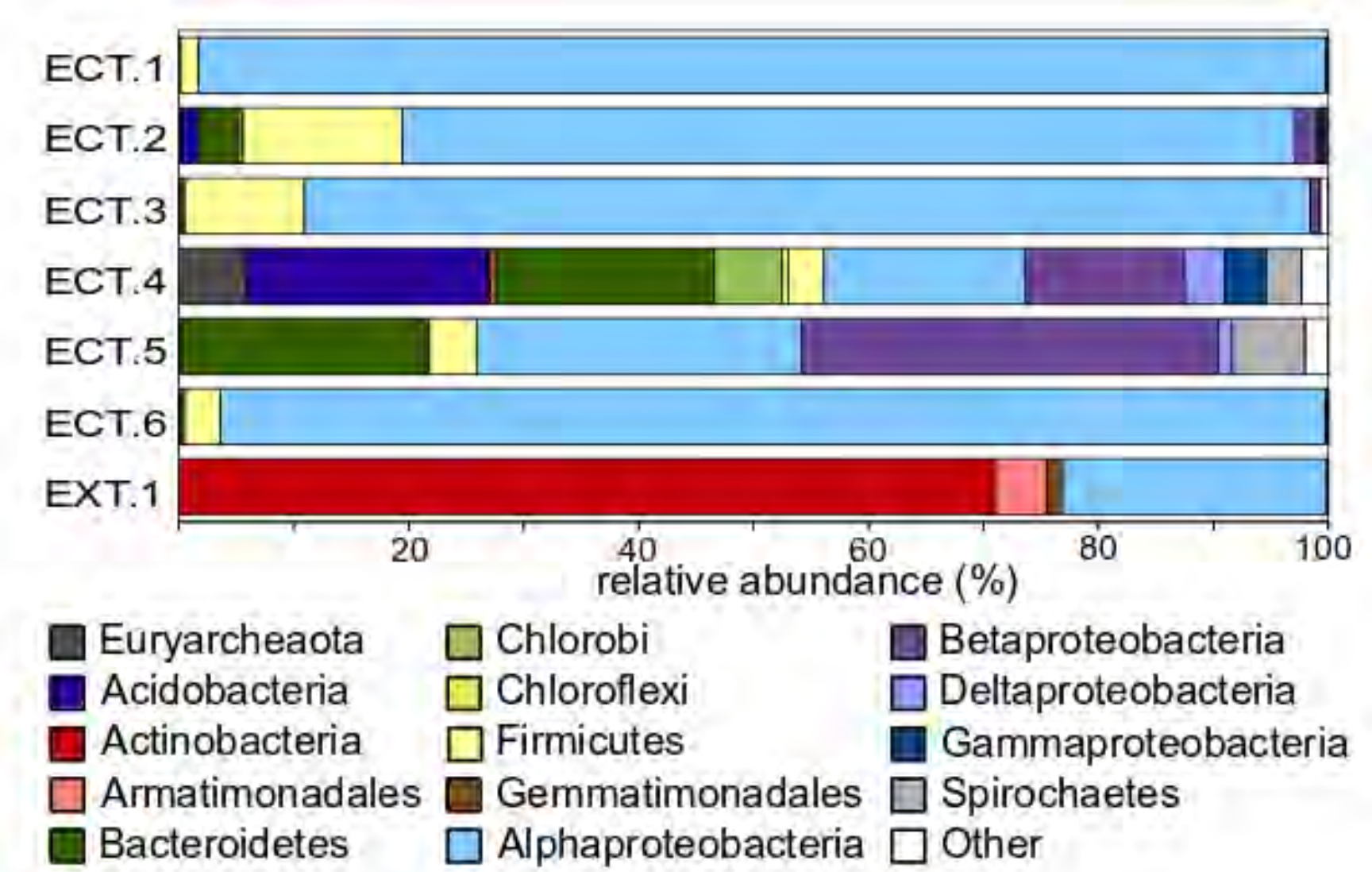


Figure 3.3: Distribution of 16S rRNA gene pyrosequence reads for each sample library. Reads are distributed into phyla except for the Proteobacteria, which are distributed into classes.

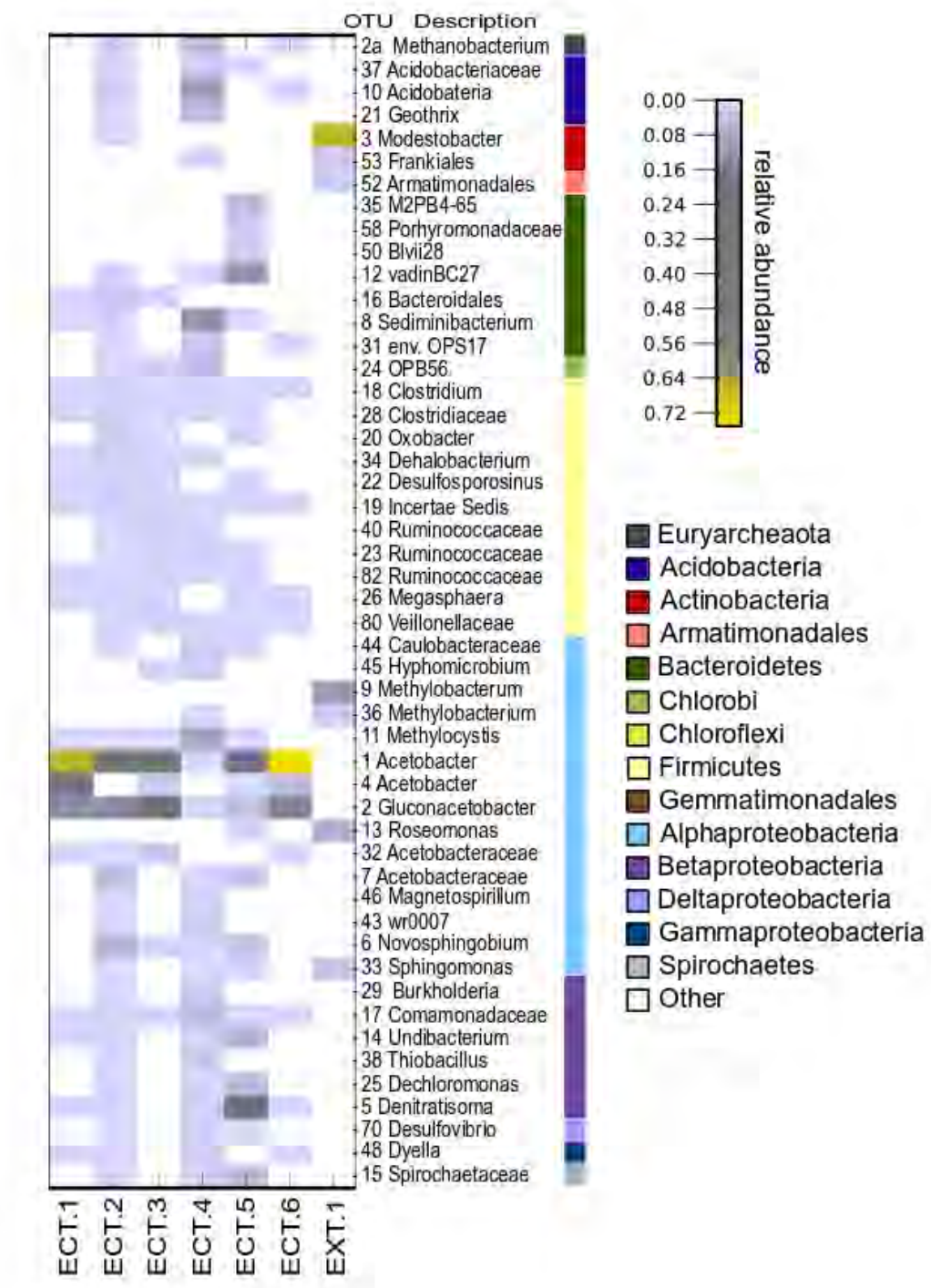


Figure 3.4: Heat map displaying the abundance of the 50 most abundant OTUs in the pyrosequencing libraries. The relative abundance of each OTU is indicated by the color of the rectangles in the figure - yellow rectangles indicate more abundanct OTU's (see scale - top right). Taxonomic classifications of OTU's are displayed to the right of the heat map.

methanogenic members of the euryarcheaota and have been found in environments including low-pH, mining water treatment reactors (greater than 99 percent similar to accession # EU717093). Sample ECT.4 also contains sequences (OTU 11) closely related to *Methylocystis* spp. that have been identified in soils at landfills (greater than 99 percent similar to accession # EU647259, (Lin et al. 2009)) and microbial fuel cell anodes (greater than 99 percent similar to accession # JF326062). *Methylocystis* spp. are methanotrophic members of the alphaproteobacteria (Bowman et al. 1993).

The most abundant phylotype in the ECT.5 pyrosequencing library (OTU 5) is a member of the betaproteobacteria classified as a member of the denitratisoma. This phylotype has greater than 99 percent similarity to a bacterium isolated from wild rice (accession # AY235688). OTU 1 (100 percent similarity to *Acetobacter lovaniensis*) is the second most abundant phylotype in sample ECT.5. The third most abundant phylotype (OTU 12) in sample ECT.5 is a member of the bacteroidetes (classified as a member of the Rikenellaceae family) that is closely related to microbes identified in iron-reducing consortia (accession # DQ676996, Lin et al. 2007), microbes that are found in urban storm water environments (accession # HE659028, Badin et al. 2012), and microbes that are present in environments contaminated with chlorinated hydrocarbons (accession # HM481376, Lee et al. 2012; accession # JF502581, Wei and Finneran 2011).

In addition to the microbes described above, the libraries from the ECT samples include numerous phylotypes capable of a number of different metabolisms. Microbes that utilize ethanol or metabolic products related to ethanol (e.g. acetate) are common. For instance, members of the clostridiales order (within the firmicutes phylum) including *Clostridia* spp., *Desulfosporosinus* spp. and members of the ruminococcaceae family, which have been identified in bioreactors designed to convert ethanol into n-caproic acid (Agler et al. 2012), are present in

the ECT libraries. Members of the clostridiales order include spore-forming microbes capable of surviving harsh conditions such as exposure to high concentrations of solvents (e.g. fuel-grade ethanol) (Vos et al. 2009).

The library from the biofilm sample obtained from a painted exterior surface of a tank that contained E10 fuel (EXT.1) is dominated by members of the actinobacteria and alphaproteobacteria (Figure 3.3). The most abundant phylotype (OTU 3, approximately 68 percent of the library) is a member of the actinobacteria classified as a *Modestobacter* spp. and shares greater than 99 percent sequence identity to an uncultured bacterium identified in subsurface sediments (accession # HM186639, Lin et al. 2012). The pyrosequencing library from sample EXT.1 also contains members of the alphaproteobacteria that include *Methylobacterium* spp. (OTU 9 – greater than 99 percent similar to a *Methylobacterium* spp. identified in a concrete surface biofilm - accession # JN020186, Ragon et al. 2011) and a member of the Roseomonas (OTU 13) that has greater than 99 percent sequence similarity to an uncultured bacterium identified in epilithic biofilms (accession # JQ627531, Ragon et al. 2012). Alphaproteobacterial members of sample EXT.1 also include sequences closely related to *Sphingomonas* spp. (OTU 33 – greater than 99 percent similarity to a groundwater biofilm clone, accession # FJ204468). The microbes identified in sample EXT.1 are closely related to chemoorganoheterotrophs, and phototrophic microbes are not prevalent in the pyrosequencing libraries.

### 3.4.3 Cultivation of acetic-acid producing bacteria

Acetic-acid-producing bacteria were successfully cultivated from both ECT samples (ECT.1 and ECT.2) used for inoculation. Decreasing pH values were indicative of acetic acid production (though other organic acids may also have been produced). pH values of cultures

decreased to approximately pH 3. Small subunit (SSU) rRNA gene sequencing indicated that cultivars from both samples are closely related to *Acetobacter* spp. (Figure 3.5). Both cultivar sequences are closely related to sequences in pyrosequencing libraries produced from environmental samples. The sequence from the ECT.1 sample acetic-acid-producing culture (sequence ECT.1c1) has greater than 99 percent identity to *Acetobacter aceti* (accession # D30768) and is closely related to OTU 4 (greater than 99 percent identity), which comprises approximately 18 percent of the pyrosequencing library generated from sample ECT.1. The sequence from the ECT.2 sample acetic-acid-producing culture (sequence ECT.2c1) has 100 percent BLAST identity to *Acetobacter lovaniensis* (accession # AF419837, Cleenwerck et al. 2002), and is related to OTU 1 (100 percent identity), which accounts for approximately 53 percent of the pyrosequencing library from sample ECT.2.

### 3.4.4 Cultivation of sulfate-reducing consortium

Blackening of the culture medium was indicative of growth of sulfate-reducing microbes, and sulfate reduction was observed only in vials inoculated with sample ECT.2. Sanger sequence analyses indicated that the sulfate-reducing consortium included microbes closely related to *Desulfosporosinus* spp. as well as *Clostridia* spp., both of which are present in the ECT.2 pyrosequencing library (Figure 3.6). Four phylotypes were identified in the sulfate-reducing consortium (sequences ECT.2c2, ECT.2c3, ECT.2c4 and ECT.2c5). Sequence ECT.2c2 is greater than 99 percent identical to *Desulfosporosinus auripigmenti* (accession # AJ493051 Stackebrandt et al. 2003) and is closely related (97.75 percent identity) to OTU 22, which comprises approximately two percent of the ECT.2 pyrosequencing library. Sequences ECT.2c3, ECT.2c4, and ECT.2c5 group within the *Clostridium* genus. Sequences ECT.2c3 and ECT.2c4 are less than 97 percent identical to any OTU present in the ECT.2 pyrosequencing library.

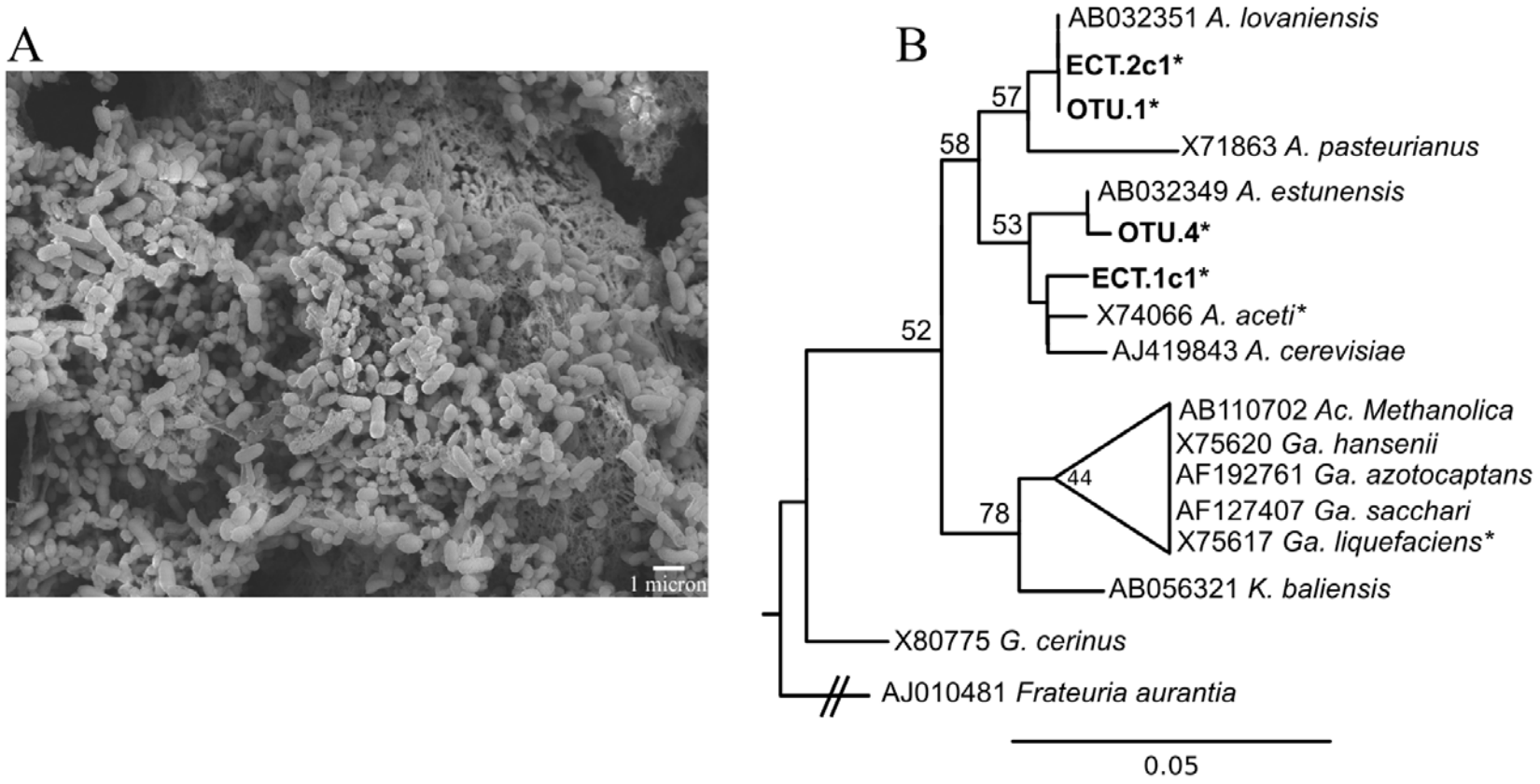


Figure 3.5: A) Scanning electron micrograph of the acetic-acid producing culture from sample ECT.1 on a steel coupon. B) Maximum likelihood phylogenetic tree of members of the Acetobacteriaceae. Sequences produced in this study are in bold. Starred sequences were inserted with pplacer (Matsen et al. 2010). Sequences representing members of the acetic-acid producing cultures are labeled ECT.1c1 and ECT.2c1. OTUs from pyrosequencing libraries that are closely related to cultivar sequences are also included. Clades with bootstrap values below 50% were collapsed. *Frateuria aurantia* (accession number AJ010481) was used as the outgroup.

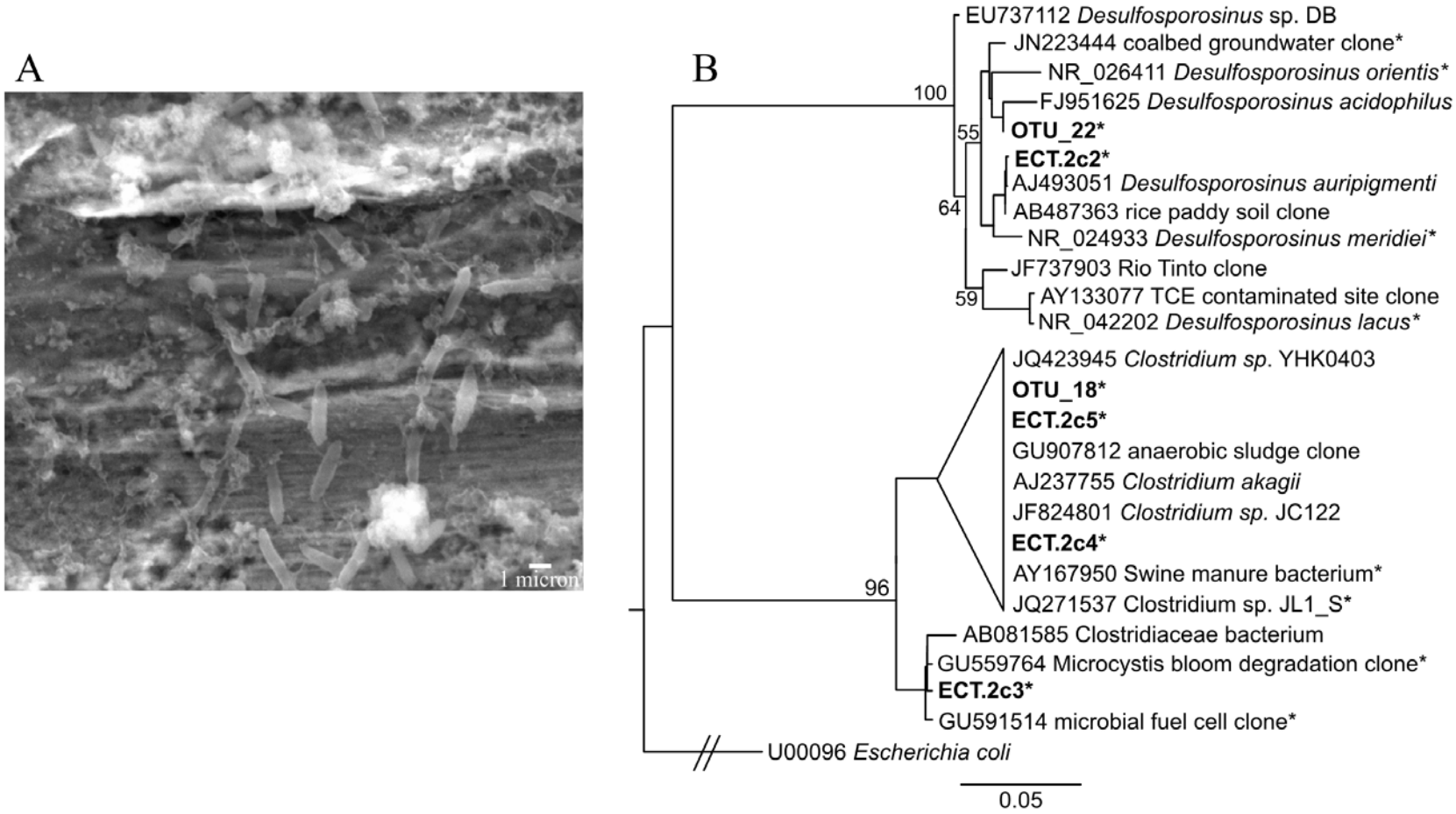


Figure 3.6: A) Scanning electron micrograph of the sulfate-reducing consortium on a steel coupon. B) Maximum likelihood phylogenetic tree of members of the Firmicutes. Sequences produced in this study are in bold. Starred sequences were inserted with pplacer (Matsen et al. 2010). Sequences representing members of the sulfate-reducing consortium are labeled ECT.2c2-5. OTUs from pyrosequencing libraries that are closely related to cultivar sequences are also included. Clades with bootstrap values below 50% were collapsed. Escherichia coli (accession number U00096) was used as the outgroup.

Sequence ECT.2c3 is closely related (greater than 99 percent identity) to an uncultured bacterium clone identified from an anaerobic fermentation of a Microcystis bloom (accession # GU559764, Xing et al. 2011) and sequence ECT.2c4 is greater than 99 percent identical to a bacterium isolated from a swine manure storage pit (accession # AY167950, Whitehead and Cotta 2004). Sequence ECT.2c5 is greater than 99 percent identical to a *Clostridium* spp. isolated from under a corroding gas pipeline (accession # JQ423945) and is closely related (greater than 99 percent identity) to OTU 18, which comprises approximately one percent of the ECT.2 pyrosequencing library.

## 3.5 Discussion

Reports of suspected MIC issues in environments in which carbon steel and other metal alloys are exposed to fuel-grade ethanol and water prompted the examination of the microbial diversity associated with these types of environments. As ethanol may serve as a carbon source and/or electron donor for microbial metabolisms, we hypothesized that these environments host microbial life capable of impacting corrosion processes in a number of ways, including the production of organic acids. 16S rRNA gene pyrosequencing analyses indicate that microbes suspected to be capable of utilizing ethanol (and metabolites of ethanol) are present in all sampled environments. Additionally, cultivation experiments demonstrate that both acetic-acid-producing bacteria and sulfate-reducing bacteria inhabit these environments. Both culture-independent and culture-dependent methods identified microbes that have been associated with microbial corrosion (e.g. acid-producing and sulfate-reducing microbes).

Acetic-acid-producing microbes (*Acetobacter* spp. and *Gluconacetobacter* spp.) were the dominant members of most (4 of 6) of the ECT pyrosequencing libraries and were present in all of the ECT samples. Also, *Acetobacter* spp. were cultivated from both ECT samples (ECT.1 and

ECT.2) used for inoculation. *Acetobacter* spp. and *Gluconacetobacter* spp. are strictly aerobic microbes capable of oxidizing ethanol to acetic acid and may also convert acetic acid to carbon dioxide and water (Kersters et al. 2006 and references therein). The tank samples in which acetic-acid producing microbes were the most prevalent phylotypes in the pyrosequencing libraries had pH values of approximately 4, indicating that the activities of these microbes had a significant impact on the internal tank environment. It is important to note that the chemical environment of tank contents may be different than the conditions at the fuel-water-tank interface where biofilm formation may have occurred. It is this interface that is key for the MIC process to initiate and persist as all of the necessities of life are present; a carbon source, water, an electron donor (the hydrocarbon/ethanol blend and/or metals in the tank) and an electron acceptor, $O_2$. The presence of acetic-acid producing bacteria in these environments is consistent with reports of the presence of these types of microbes in pump sumps exposed to fuel-grade ethanol (US EPA 2011). *Acetobacter* spp. are present in a variety of environments and have been reported to contaminate ethanolic environments such as fuel-grade ethanol fermentations (Heist 2009) and wines (Du Toit and Pretorius 2002; Bartowsky and Henschke 2008); thus, the presence of these microbes in fuel industry infrastructure exposed to ethanol and water seems likely.

Organic acids, including acetic acid, are metabolic byproducts of many microbes, and microbial organic acid production has been shown to enhance corrosion or deterioration of a number of materials (Little and Lee 2007). Fungal and bacterial organic acid production has been associated with fuel degradation and corrosion of carbon steel, stainless steel, aluminum and other materials that are used to construct fuel storage and transportation infrastructure (Salvarezza et al. 1983; Little et al. 1992; Little et al. 1995; McNamara et al. 2005). Acetic acid production by *Acetobacter aceti* has been shown to accelerate corrosion of cathodically protected

stainless steel (Little et al. 1988), and corrosion of carbon steel has also been associated with microbial acetic acid production (Little et al. 1992 and references therein). The corrosive nature of the *Acetobacter* spp. cultivated in this study are described elsewhere (Jain 2011, Jain et al. - submitted to NACE 2013, Sowards et al. - in preparation, see Appendices B and C). Jain (2011) found that carbon steel exposed to the acetic-acid-producing bacterial culture experienced pitting corrosion, and Sowards and colleagues (in preparation) demonstrated that the *Acetobacter* spp. culture enhanced fatigue crack growth rates of carbon steels commonly used for pipelines and tanks. Interestingly, acetic acid has been shown to impact the corrosion and cracking behavior of carbon steel in fuel-grade ethanol environments containing low amounts of water (Lou et al. 2009; Lou and Singh 2010). The research of Lou and colleagues did not address MIC; however, one could speculate that microbial acetic acid production could impact these phenomena.

While fuels contain dissolved oxygen and oxic conditions are likely present in parts of a fuel tank environment (Passman 2003), anoxic micro-niches are likely to occur due to microbial oxygen consumption. The presence of anoxic micro-environments and anaerobic microbes suggests that interactions of many types of microbial metabolisms may impact corrosion in these environments. Anaerobic microbes identified in the ECT samples include members of the proteobacteria and firmicutes. *Clostridia* spp. were identified in ECT sample pyrosequencing libraries as well as the ECT.2 sulfate-reducing consortium. Some *Clostridia* spp. are known to produce acetic acid from hydrogen and carbon dioxide (Braun et al. 1981), compounds likely to be present in the tanks due to the activity of *Acetobacter* spp. and *Gluconacetobacter* spp. Additionally, these aerobic, acetic-acid producing microbes are likely to consume oxygen and create conditions suitable for the growth of anaerobic *Clostridia* spp. as well as sulfate-reducing bacteria such as *Desulfosporosinus* spp., which were also identified in pyrosequencing libraries as well as the sulfate-reducing consortium cultivated from sample ECT.2. The impact of the

sulfate-reducing consortium cultivated in this study are examined elsewhere (Jain 2011, Jain et al. – submitted to NACE 2013, Sowards et al. - in preparation, see Appendices B and C). Jain et al. found that the sulfate-reducing consortium increased general corrosion rates of X52 and X70 linepipe steel, and Sowards et al. demonstrated increased fatigue crack growth rates of A36, X52 and X70 steels in the presence of the sulfate-reducing consortium. *Clostridia* spp. have been associated with corrosion of carbon steels in oil and gas industry environments (Zhu et al. 2003; Jan-Roblero et al. 2008; Monroy et al. 2011), and microbial sulfate-reduction has long been associated with MIC of many materials (Von Wolzogen Kuehr and van der Vlugt 1934; Little and Lee 2007; Javaherdashti 2008; Enning et al. 2012; Venzlaff et al. 2013), which likely means that even if the corrosive impact of microbial acetic acid production was neutralized, microbial corrosion issues would persist by other means. It is interesting to note that while sulfate-reducing microbes are often associated with oil industry environments (Cord-Ruwisch et al. 1987; Magot et al. 2000; Stevenson et al. 2011), these types of microbes are also present in these fuel-grade ethanol environments.

Methanogenic archaea were identified in 4 of the 6 ECT sample pyrosequencing libraries. The most abundant methanogen identified (OTU 2a) is closely related to members of the methanobacterium, and the most closely related cultured representative of this OTU is *Methanobacterium congolense* (Cuzin et al. 2001), which produces methane from carbon dioxide and hydrogen. Acidophilic methanogens have been described (Bräuer et al. 2006), and Kotsyurbenko and colleagues (2007) described an acid-tolerant, hydrogenotrophic methanogen of the methanobacterium genus isolated from acidic peat bogs. These types of methanogens may thrive in ethanolic environments also containing acid producing microbes. Methanogenic archaea have been linked to elemental iron oxidation and corrosion (Dinh et al. 2004; Uchiyama et al. 2010), and Zhang and colleagues (2003) cultivated both acetotrophic and hydrogenotrophic

methanogens from a marine biofilm sample and concluded that the hydrogenotrophic methanogens were directly responsible for mild steel corrosion while the acetotrophic methanogens contributed only indirectly to corrosion by growing synthrophically with sulfate reducing bacteria. Nelson and colleagues (2010) linked the stimulation of methanogenesis to the conversion of ethanol to acetic acid in soil column experiments designed to investigate the impact of ethanol-based fuel on microbial communities. Environments present in ethanolic fuel conveyance systems may provide niches in which methanogens thrive; though, the impact of methanogens on corrosion and deterioration of fuel industry infrastructure is not currently well understood. With methanogens present there is not only the worry of their contribution to MIC but also of their consumption of substrate in a tank or pipeline and the subsequent production of volatile methane.

The pyrosequencing library created from the biofilm found on the external surface of the E10 fuel tank (sample EXT.1) is comprised of chemoorganoheterotrophic phylotypes that have been associated with epilithic biofilms. The most prevalent OTU is closely related to *Modestobacter sp.*, an actinobacterium, that has been identified on degrading stone surfaces (Eppard et al. 1996; Urzì et al. 2001). A *Modestobacter* sp. has been shown to produce pigments in darkly colored desert soil cryptobiotic crusts (Reddy et al. 2007), which may explain the black color of the biofilms formed on the tank surface. Ragon and colleagues (2011) identified *Methylobacterium* spp., *Roseomonas* spp. and *Sphingomonas* spp., which were also present in the EXT pyrosequencing library, in biofilms sampled from concrete surfaces. Gundlapally and colleagues (2006) identified *Modestobacter* spp., *Methylobacterium* spp., *Roseomonas* spp. and *Sphingomonas* spp. in soil crusts. While some *Methylobacterium* spp. and *Sphingomonas* spp. have been shown to metabolize ethanol (Šmejkalová, Erb, and Fuchs 2010; Zhao et al. 2008), it is unclear if the biofilms forming on these tanks are utilizing fuel escaping the tanks, and

phylotypes known to convert ethanol to acetic acid are not found in the EXT sample. While some *Methylobacterium* spp. and *Sphingomonas* spp. have been associated with copper corrosion (Pavissich et al. 2010), the corrosive nature of the biofilms sampled in this study have not been investigated.

### 3.6 Summary

Reports of suspected MIC impacting infrastructure exposed to fuel-grade ethanol and water prompted the investigation of microbial communities present in such environments. Microbial communities associated with tanks that contain fuel-grade ethanol and water included microbes capable of metabolizing ethanol and producing corrosive organic acids as well as microbes associated with other biocorrosion mechanisms (e.g. sulfate reduction). Though low water availability and high solvent content (fuel) may inhibit microbial activity under ideal operating conditions in many parts of fuel storage and transportation systems, microbial conversion of ethanol to acetic acid could potentially enhance corrosion of steels and other materials in areas of fuel industry infrastructure (e.g. tanks, pipes, pump sumps) in which fuel-grade ethanol and water (even in trace amounts) are present. *Acetobacter* spp. and *Gluconacetobacter* spp. are prevalent in pyrosequencing libraries created from samples of tanks containing fuel-grade ethanol and water, though the presence of anaerobic microbes such as sulfate-reducing bacteria suggest that microbial communities living syntrophically may impact corrosion in these environments.

CHAPTER 4

# SPORE-INDUCED MANGANESE OXIDATION BY A BACILLUS SP. ISOLATED FROM E10 GASOLINE TANKS

## 4.1 Abstract

Spore-forming microbes belonging to the bacterial phylum firmicutes are present in many environments, including industrial environments such as fuel industry infrastructure. The spores of these microbes are highly resistant and are likely to persist in these environments despite efforts to remove microbial contamination from these systems (e.g. the use of biocides). In this study, we describe the isolation of *Bacillus* spp. that produce spores that catalyze Mn(II) oxidation from an E10 fuel sample. 16S rRNA gene analysis indicated that the *Bacillus* spp. are closely related to other *Bacillus* spp. capable of promoting manganese oxidation. Genes (mnxG genes) coding for putative manganese-oxidizing enzymes were amplified and sequenced. The potential impact that these sorts of microbes may have on corrosion in fuel system infrastructure is discussed.

## 4.2 Introduction

Members of the firmicutes (e.g. *Bacillus* spp. and *Clostridia* spp.) are common inhabitants of many environments including engineered systems such as water distribution systems and oil and gas industry infrastructure. Due to the ability of these microbes to form endospores, resistant structures formed during periods of stress, these types of microbes can

persist in many 'harsh' environments (Nicholson et al. 2000 and references therein). Molecular surveys as well as cultivation-based approaches have identified *Bacillus* spp. in water distribution systems (Tokajian et al. 2005; Szabo et al. 2009; Cerrato et al. 2010; Revetta et al. 2010; White et al. 2011; Liu et al. 2012) and in fuel storage and transportation infrastructure (DeGray and Killian 1960; Edmonds and Cooney 1967; López et al. 2006; Rodríguez-Rodríguez et al. 2009; Rajasekar et al. 2010). Additionally, the authors' many attempts to cultivate numerous types of microbes from storage tanks containing a variety of fuels have resulted in thriving cultures of *Bacillus* spp. Though these microbes may not be the most prevalent microbes in many industrial environments, the fact that they may persist even after decontamination efforts (e.g. biocide application) requires the need for understanding how these sorts of microbes impact engineered systems.

Interestingly, the spores of some *Bacillus* spp. are capable of catalyzing manganese oxidation. Rosson and Nealson (1982) isolated a *Bacillus* sp., strain SG-1, from marine sediments that produced spores capable of oxidizing Mn(II) to Mn(IV) oxides. Since that time, additional *Bacillus* spp. that produce spores that catalyze Mn(II) oxidation have been isolated from other marine samples including near surface marine sediments off the coast of California (Francis and Tebo 2002) and deep-sea hydrothermal plume water and sediment samples (Dick et al. 2006) as well as terrestrial subsurface samples at Henderson Molybdenum Mine in Colorado (Mayhew et al. 2008). These manganese oxidizing spores encrust themselves in Mn(IV) oxides. Why these microbes do so is unknown, but protection from environmental hazards such as dessication may be a possible explanation (Tebo et al. 2005; Phoenix and Konhauser 2008 and references therein).

Research regarding spore-induced Mn(II) oxidation has focused on strain SG-1 originally isolated by Rosson and Nealson (1982). Van Waasbergen et al. (1996) identified a cluster of

genes in strain SG-1 that were involved with manganese oxidation, and one of these genes (mnxG) coded for a putative multicopper oxidase (MCO) enzyme. These enzymes are located on the outer surface of the spore coat of these manganese oxidizing spores. Multicopper oxidases have also been linked to Mn(II) oxidation catalyzed by *Leptothrix discophora* strain SS-1 (Corstjens et al. 1997) and *Pseudomonas putida* strain GB-1 *(Brouwers et al. 1999)*. In the case of *Bacillus* sp. strain SG-1, evidence suggests that the multicopper oxidase present on the spore coats catalyzes the oxidation of Mn(II) to Mn(III) then to Mn(IV) (Webb et al. 2005; Soldatova et al. 2012).

Manganese is an abundant metal in the earth's crust and is a component of materials used to construct fuel system infrastructure (e.g. carbon steel). For example, carbon steels may contain over one percent manganese by weight, which is added to improve the mechanical properties of the steel, and manganese may be incorporated in some steels at even higher levels. Additionally, manganese can be present in water that may infiltrate pipelines, tanks or other infrastructure, and manganese oxide deposition on submerged materials has been observed in waters containing manganese concentrations of approximately 10-20 parts per billion (Dickinson et al. 1996; Dickinson et al. 1997; Lewandowski and Beyenal 2009). Manganese is important to microbes as this element is a cofactor in many enzymes and species of manganese can serve as both electron acceptors and electron donors for microbial metabolisms. Manganese can exist in oxidation states from 0 to +7, though oxidation states of +2, +3 and +4 are common in natural environments (Erhlich and Newman 2009). Microbes play an important role in manganese cycling as microbiologically catalyzed manganese oxidation can be up to five orders of magnitude faster than abiotic Mn(II) oxidation in the environment (Hastings and Emerson 1986). Manganese oxides are highly reactive species (Tebo et al. 2004), and microbially-produced manganese oxides have been linked to corrosion of stainless steel (Dickinson et al. 1997;

Linhardt 2010 and references therein). Thus, it is important to understand microbial manganese oxidation in engineered systems.

The goal of this study was to determine if metabolically active spores are present in automotive fuel environments. As described above, the manganese oxidizing ability of the spores of some *Bacillus* spp. have been well-studied. Additionally, metal-reduction by spores of a *Desulfotomaculum* sp. have been described by Junier et al. (2009). Since *Bacillus* spp. are commonly identified in fuel samples via both cultivation-dependent and cultivation-independent methods and the manganese-oxidizing abilities of the spores of some *Bacillus* spp. have been well-described, we hypothesized that manganese-oxidizing spores of *Bacillus* spp. may be present in these environments. We sampled automotive fuel environments (E10 and E85), used selective media to isolate putative manganese-oxidizing microbes, screened these microbes for manganese oxidation and used molecular techniques to identify these microbes and characterize a functional gene (mnxG) believed to be responsible for manganese oxidation.

## 4.3 Methods

Methods employed to cultivate and identify a *Bacillus* sp. that produces spores that host enzymes capable of promoting manganese oxidation are described below. These methods included both cultivation-based and molecular-based techniques.

### 4.3.1 Sample Collection

Six fuel samples (E10 and E85) were collected from fueling stations in sterilized 1-liter polycarbonate bottles. Bottles were field rinsed three times before collecting samples. Fuel samples were filtered through sterile, 0.2 micron polyethersulfone membranes (Pall Corporation)

using a vacuum filtration system. All equipment was sterilized before use. These filters were used for to inoculate media for cultivation experiments.

### 4.3.2 Isolation and identification of manganese-oxidizing microbes

Filter samples were streaked upon solid media intended to isolate manganese-oxidizing microbes. These solid media included M agar and K agar plates (Tebo et al. 2007) made with distilled water as well as with 75 percent (v/v) seawater with a balance of distilled water (see Table 4.1). After 1-2 week incubations, colonies were screened for putative manganese oxidation by visual inspection. Formation of brown crusts upon colonies was indicative of putative manganese oxidation, and this putative manganese oxidation was confirmed by the colorimetric leucoberbelin blue (LBB) spot test described by Krumbein and Altmann (1973). LBB (0.04 percent) in 45 mM acetic acid was dropped directly onto colonies on agar plates, and a reaction producing a blue color indicated the presence of manganese oxides (Figure 4.1). Single colonies of manganese-oxidizing microbes were picked and transferred to new agar plates. After several transfers (great than five) observation via light microscopy indicated uniform morphology and the presence of putative spores. Two manganese-oxidizing isolates (E10_4_A and E10_4_B) were inoculated into liquid media (M broth – 75 percent seawater, see Table 4.1), which is described by Tebo and colleagues (2007), for cultivation of larger quantities of cells for DNA extraction and spore-separation.

DNA was extracted from both of the cultures using the MoBio Powersoil DNA extraction kit (MoBio, Carlsbad, CA); the 10-minute vortexing step was replaced by one minute of bead beating. Small subunit ribosomal RNA (16S rRNA) gene amplification was carried out using primers 8F (5' – AGA GTT TGA TCC TGG CTC AF – 3') and 1492R (5' – GGT TAC CTT GTT ACG ACT T – 3') (Lane 1991). PCR, cloning, transformation and sequencing were carried out

Table 4.1: Composition of media used to isolate manganese oxidizing microbes from environmental samples. For media not containing seawater, seawater was replaced with distilled water. Liquid media did not include agar.

| Ingredient | K medium | M medium |
|---|---|---|
| Seawater | 750 ml | 750 ml |
| Distilled water | balance | balance |
| yeast extract | 0.5 g/l | 0.05 g/l |
| peptone | 2 g/l | 0.05 g/l |
| agar | 10 g/l | 15 g/L |
| HEPES buffer | 20 mM | 20 mM |
| $KHCO_3$ | - | 2 mM |
| $MnCl_2$ | 100 uM | 100 uM |

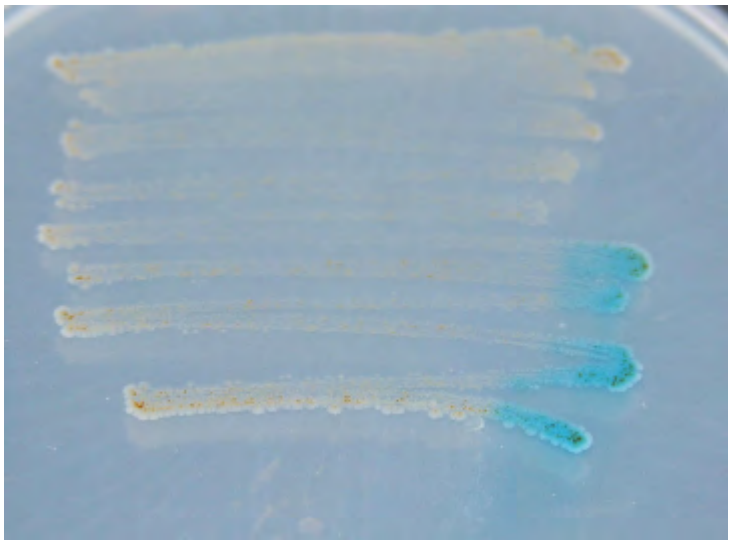

Figure 4.1: Leucoberbelin blue (LBB) spot test of Bacillus sp. strain E10_4_A on M agar containing 100 uM MnCl2. The blue color is indicative of manganese oxides. Also, manganese oxides (brown) can be seen on the colonies.

as described by Sahl et al. (2010). Sanger reads were called with PHRED (Ewing et al. 1998; Ewing and Green 1998) and assembled with PHRAP (www.phrap.org) via Xplorseq (Frank 2008). Initial identification of cultivar sequences was accomplished via the Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990) to compare sequences to the NCBI non-redundant nucleotide database (Benson et al. 2005). To more thoroughly establish phylogenetic relationships of cultivar sequences phylogenetic trees were created with MRBAYES (Ronquist and Huelsenbeck 2003). Sequences described by Francis and Tebo (2002), Dick et al. (2006), and Mayhew et al. (2008) were retrieved from Genbank, dereplicated (97 percent identity with UClust (Edgar et al. 2011)) in QIIME (Caporaso et al. 2010)) and aligned and masked with SSU-ALIGN (Nawrocki 2009). MRBAYES tree construction included one million generations of an MCMC run with the following parameters: nst = 6, rates = gamma, sample frequency = 1000, and nchains = 4. Phylogenetic trees were annotated using Figtree (http://tree.bio.ed.ac.uk/software/figtree/).

Attempts to amplify and sequence mnxG genes from both isolates were made. Gene amplification was carried out using primers mnxGIF (5' – ACG CAT GTC TTT CAC TAT CAT GTT CAT – 3') and mnxGIR (5' – AAA TAA GTG GTC ATG GAA GAA CCA TGC – 3') and PCR conditions described by Francis and Tebo (2002). Cloning, transformation and sequencing were carried out as described by Sahl et al. (2010). Bases were called with PHRED (Ewing et al. 1998; Ewing and Green 1998) via Xplorseq (Frank 2008). Though gene amplification occurred for both isolates, successful cloning and sequencing of the mnxG gene was only possible for isolate E10_4_A. The gene sequence from isolate E10_4_A was translated into a protein sequence via the web-based translation tool ExPASy (http://www.expasy.ch/tools/dna.html). The resulting protein sequence from isolate E10_4_A was compared to the Genbank non-redundant database via the Basic Local Alignment Search Tool (BLAST, Altschul et al. 1990). Protein

product sequences of mnxG genes from other studies (Waasbergen et al. 1996; Francis and Tebo 2002; Dick et al. 2006; Mayhew et al. 2008) were retrieved from Genbank for comparison purposes. Protein sequences were aligned with ClustalW (Thompson et al. 2002) and phylogenetic trees were created with MRBAYES (Ronquist and Huelsenbeck 2003) with the following parameters: number of generations = 100,000; rates = invgamma; Ngammacat = 4; aamodelpr = mixed, sample frequency = 100.

Additionally, primers were designed to facilitate the amplification of more diverse mnxG genes from environmental samples. All sequences labeled as mnxG genes in Genbank were downloaded and aligned with ClustalW (Thompson et al. 2002). Sequence alignments were visually inspected using ARB (Ludwig et al. 2004) to identify potential priming sites. The resulting primer sequences were mnxGcw_F (5' – CAY TGY CAY YTN TAY CCN C – 3') and mnxGcw_R (5' – TCR TAR TTC CAN CCN AC – 3'). The specificity of these primers was unacceptable and resulted in inappropriate products. As very few mnxG gene sequences are deposited in public databases, designing primers for amplifying these genes from environmental samples proves difficult. Therefore, screening for manganese-oxidizing microbes present in environmental samples requires targeted cultivation efforts to attempt to grow these types of microbes in the laboratory.

### 4.3.3 Spore separation and manganese oxidation tests

Spores were purified from one-liter, liquid cultures of isolate E10_4_A as described by Rosson and Nealson (1982) and modified by Dick and colleagues (2006). One-liter cultures that were greater than 90 percent sporulated (determined by phase-contrast microscopy) were concentrated by centrifugation (10000g, 5 minutes), then washed with distilled water containing 10 mM ethylenediaminetetraacetic acid (EDTA), five percent phenylmethylsulfonyl fluoride

(PMSF). Cells/spores were then suspended in 10 mM Tris buffer (pH 7.5), 10 mM EDTA, five percent PMSF and treated with lyzosyme (50 ug/ml) for 30 minutes at 37 $^{o}$C to lyse any remaining vegetative cells. Spores were then washed with 1 M sodium chloride, 10 mM EDTA, five percent PMSF followed by a wash with 0.15 M sodium chloride, 10 mM EDTA, five percent PMSF, 0.5 mM ascorbate (the ascorbate was added to remove manganese oxides formed during cultivation). The spores were then washed one time with 0.1 percent sodium dodecyl sulfate (SDS) followed by 5 washes with distilled water. The spores were suspended in distilled water and stored at 4 $^{o}$C.

Spore-induced manganese oxidation was investigated by suspending spores in 50-ul volumes of distilled water containing 100 uM manganese (II) chloride and 20 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) buffer (pH 7.5). The spores were incubated in the dark at room temperature. Manganese oxidation was tested at time intervals using the LBB method described by Krumbein and Altmann (1973) and Tebo et al. (2007). The spore suspensions were added to 250 ul of 0.04 percent LBB suspended in 45 mM acetic acid. After 15-minute incubations in the dark at room temperature, absorbance was measured at 620 nm on a spectrophotometer.

### 4.3.4 Spore-Induced Manganese Oxidation and Metal Surfaces

To investigate how microbes interact with metallic surfaces, a 20 nm layer of metallic iron was deposited onto glass microscope slides via vapor deposition. These slides are meant to simulate metal surfaces (e.g. steel) and are easy to inspect via light microscopy. These iron-coated slides were immersed in distilled water containing 100 uM manganese (II) chloride and 20 mM HEPES buffer (pH 7.5) and inoculated with purified spores of isolate E10_4_A. After 5

day incubations, slides were investigated with light microscopy, and the LBB spot test was used to determine if manganese oxides had been deposited on the iron slide surfaces.

## 4.4 Results

Attempts to cultivate *Bacillus* spp. that produce manganese-oxidizing spores from fuel tank samples resulted in two isolates that produced manganese oxides. These isolates were identified by 16S rRNA gene sequencing, and the ability of the purified spores to oxidize manganese was evaluated. Experimental results are described below.

### 4.4.1 Isolation and identification of manganese-oxidizing microbes

All six samples used for inoculation produced growth of multiple colonies on both K agar and M agar media made with both distilled water and 75 percent (v/v) seawater and distilled water after several days of incubation. Inspection of the colonies with phase-contrast microscopy indicated the presence of putative spore-forming microbes. Only one sample (an E10 fuel sample), which was plated onto M agar made with 75 percent seawater (see Table 4.1), produced isolates that showed evidence of manganese oxidation (colonies encrusted with brown solids) after 1-2 weeks. Manganese oxidation induced by these isolates was confirmed with the LBB spot test (Figure 4.1). The manganese oxidizing isolates are labeled E10_4_A and E10_4_B.

Nearly full-length (approximately 1500 base pair) sequences of 16S rRNA genes were obtained from both cultivars via Sanger sequencing. For initial identification of isolates E10_4_A and E10_4_B, these sequences were compared to the Genbank non-redundant nucleotide database via BLAST (Altschul et al. 1990). 16S rRNA gene sequences show greater than 99 percent sequence similarity (BLAST) to *Bacillus* spp. known to possess mnxG genes and

produce manganese-oxidizing spores isolated from marine sediments (accession numbers AF326366 and AF326363, Francis and Tebo 2002) and terrestrial subsurface samples collected at a mine (accession number EU004564, Mayhew et al. 2008). As molecular evidence has indicated that *Bacillus* spp. that possess mnxG genes are closely related phylogenetically (Mayhew et al. 2008), phylogenetic trees were created with MRBAYES to determine if the isolates produced in this study fell into phylogenetic clades with other *Bacillus* spp. that possess mnxG genes. As can be seen in Figure 4.2, the isolates examined in this study group with a cluster of *Bacillus* spp. known to possess mnxG genes. This lineage has been labeled as the PL-12 or HM06_02 cluster by previous researchers. The phylogenetic tree produced in this study shows similar topology to phylogenies created by Francis and Tebo (2002), Dick et al. (2006) and Mayhew et al. (2008).

Though PCR amplification of mnxG genes from both isolates was successful (based upon production of appropriately-sized PCR amplicons as judged by gel electrophoresis), only the mnxG gene sequence amplicon from isolate E10_4_A was successfully cloned and sequenced. The translated protein sequence of the mnxG gene sequence obtained from isolate E10_4_A shows greater than 97 percent identity (BLASTp) to mnxG protein sequences from the same *Bacillus* spp. that are closely related to the isolate via comparison of 16S rRNA genes. Figure 4.3 displays a phylogenetic tree of mnxG amino acid sequences from a variety of *Bacillus* spp. also evaluated in Francis and Tebo (2002); Dick et al. (2006) and Mayhew et al. (2008). The mnxG protein sequence from this study falls into the same clade associated with the PL-12/HM06-02 cluster identified by 16S rRNA gene analysis. The topology of this tree is also consistent with phylogenetic trees produced in previous studies (Francis and Tebo 2002; Dick et al. 2006; Mayhew et al. 2008).

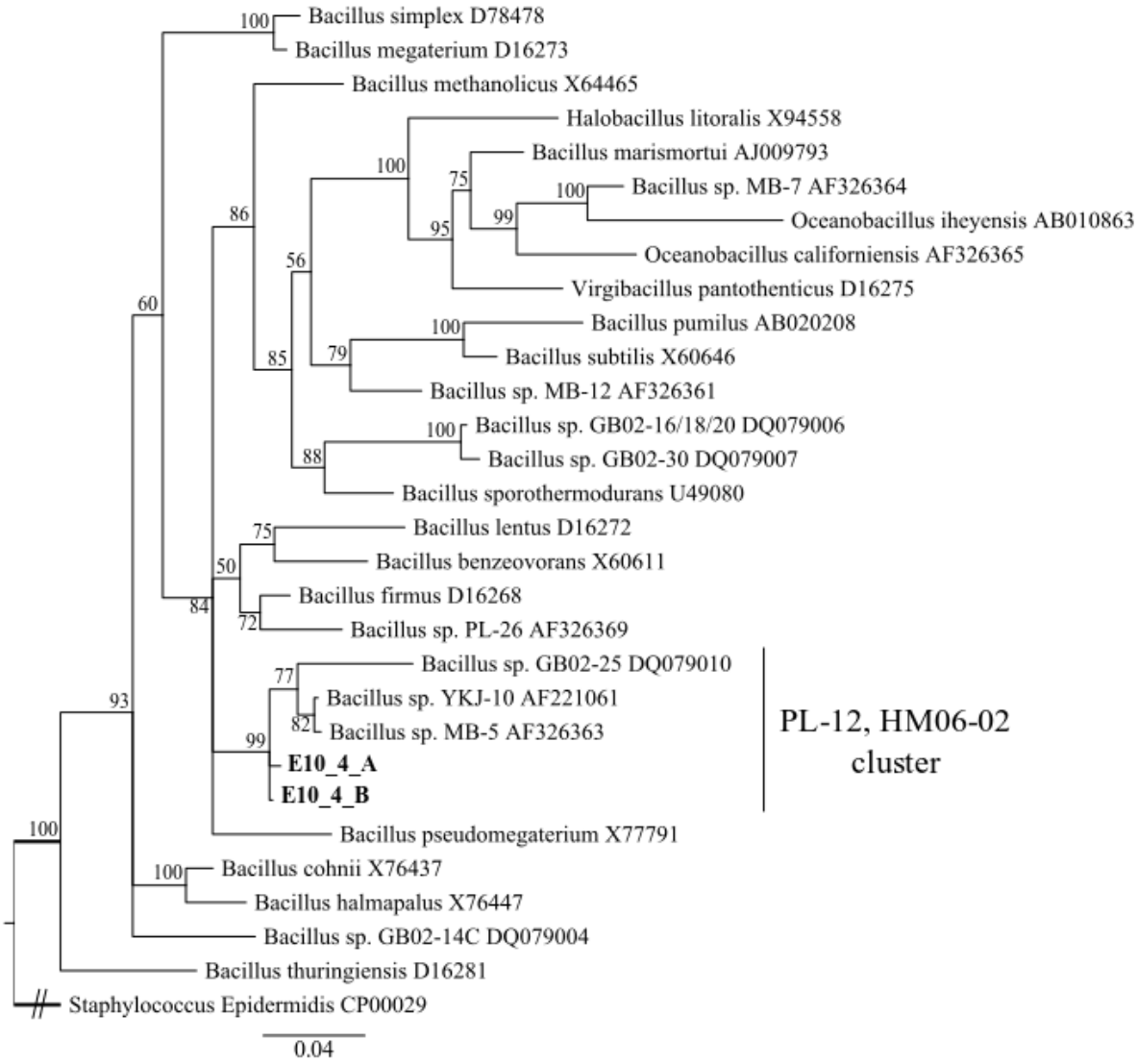


Figure 4.2: Phylogenetic tree of 16S rRNA gene sequences of Bacillus spp. created with MRBAYES. Numbers at the nodes of the tree represent posterior probabilities. Isolates E10_4_A and E10_4_B group within the PL-12/HM06-02 cluster desrcibed by Francis and Tebo (2002), Dick et al. (2006) and Mayhew et al. (2008).

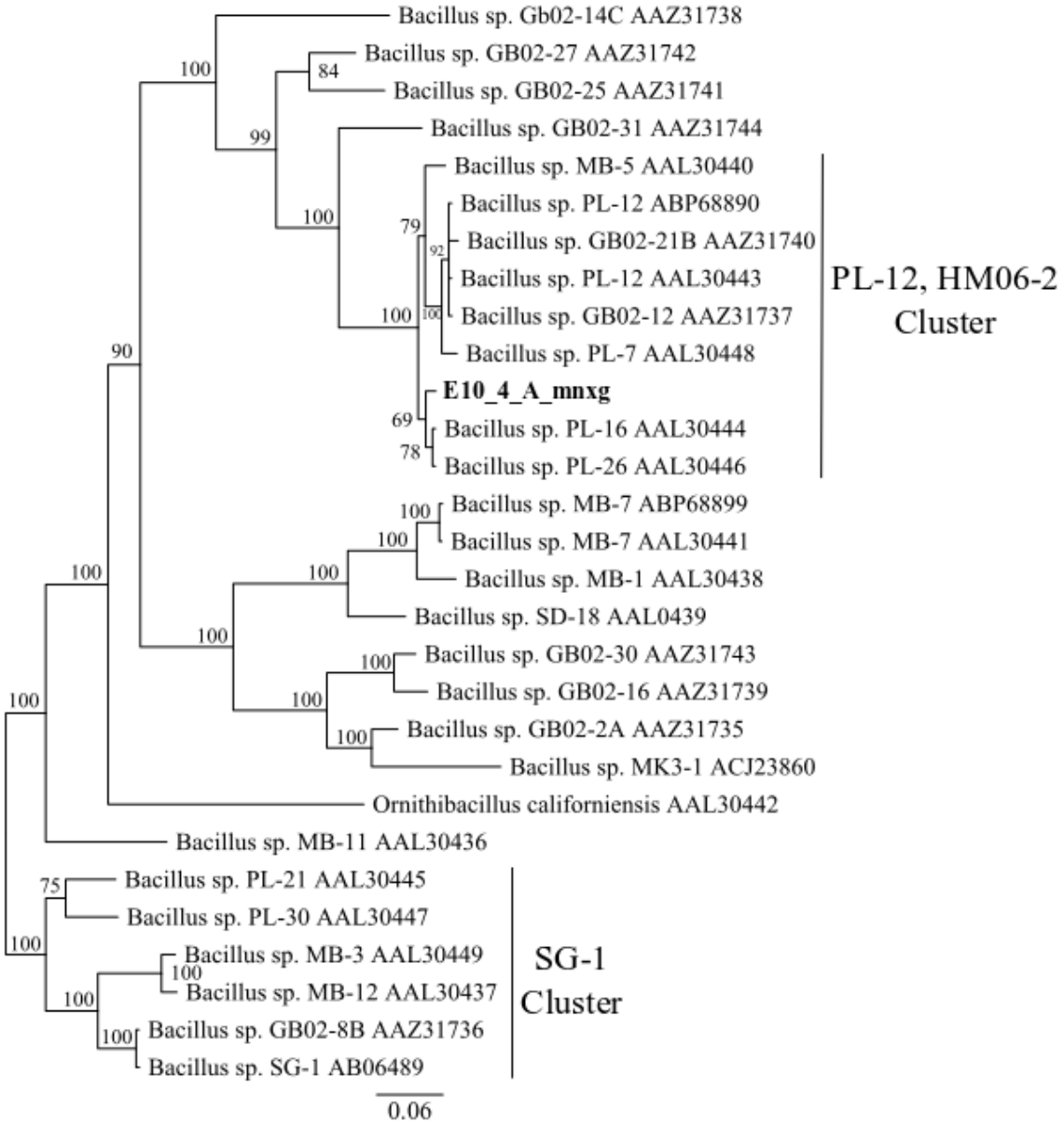


Figure 4.3: Unrooted phylogenetic tree of mnxG protein sequences from relevant Bacillus spp. as well as isolate E10_4_A. The mnxG sequence from isolate E10_4_A groups within the PL-12/HM06-02 cluster desrcibed by Francis and Tebo (2002), Dick et al. (2006) and Mayhew et al. (2008), which is consistent with 16S rRNA gene results.

### 4.4.2 Manganese Oxidation and Metal Surfaces

To verify that spores of isolate E10_4_A could promote the oxidation of Mn(II) and that manganese oxidation was not the result of vegetative cell metabolism, distilled water containing 100 uM $MnCl_2$ (and HEPES buffer) was inoculated with purified spores and incubated in the dark at room temperature. Manganese oxidation was determined by the LBB method. After 24 hours, approximately 30 percent of the soluble manganese present had been oxidized. After 72 hours, approximately 50 percent of the soluble manganese present in the experiment had been oxidized. Experiments were conducted in triplicate (range of approximately 40-55 percent of soluble manganese oxidized after 72 hours). Isolate E10_4_A does not seem to oxidize manganese as vigorously as *Bacillus* sp. strain SG-1, which has been studied extensively (Lee and Tebo 1994; Murray and Tebo 2007; Bargar et al. 2005; Bargar et al. 2000). *Bacillus* sp. strain SG-1 appears to produce greater amounts of manganese oxides on spore surfaces when grown on solid media containing manganese (II), and initial LBB tests indicate that *Bacillus* sp. strain SG-1 removes manganese (II) from solution at a greater rate than isolate E10_4_A.

To investigate if spores of isolate E10_4_A promoted the deposition of manganese oxides on metallic surfaces, glass microscope slides that had a 20 nm layer of metallic iron deposited onto the surface via vapor deposition were exposed to purified spores in the presence of 100 uM Mn(II) (Figure 4.4). After 120 hours, the iron-coated glass slides were evaluated with light microscopy and the LBB spot test to determine if manganese oxides had formed on the metallic surface. Additionally, we looked for evidence of 'pits' or areas of the slide where the metallic iron had been removed. LBB spot tests indicated that manganese oxides had formed on the metallic iron surfaces. Though 'pits' did form on the metallic iron surfaces exposed to purified spores of isolate E10_4_A, these results were not consistently reproducible.

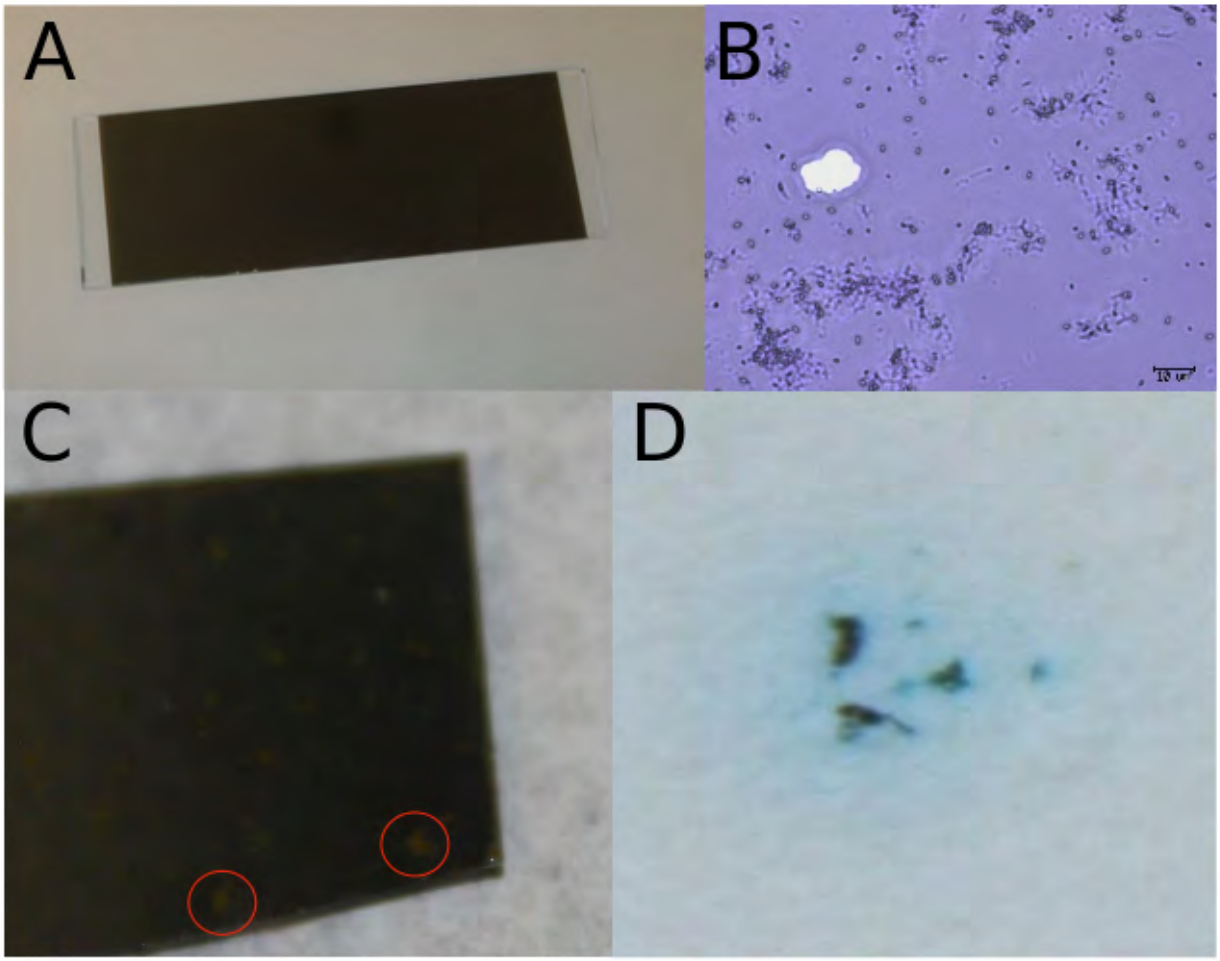


Figure 4.4: A) Glass microscope slide on which a 20 nm layer of iron has been deposited by vapor deposition. B) Light micrograph of spores of isolate E10_4_A on the surface of an iron-coated slide. A 'pit' is evidenced by a hole in the iron coating. C) Manganese oxides deposited on the surface of an irpn-coated slide by E10_4_A spores. D) Positive LBB test indicating that the metal oxides on the iron-coated slide test are manganese oxides.

## 4.5 Discussion

Though *Bacillus* spp. that produce manganese oxidizing spores have been isolated from environments such as marine sediments (Rosson and Nealson 1982; Francis and Tebo 2002; Dick et al. 2006) and terrestrial subsurface samples (Mayhew et al. 2008), to the authors' knowledge, this study is the first report of the isolation of these types of manganese-oxidizing spores from fuel environments. Phylogenetic analyses indicate that the isolates recovered in this study are closely-related to the microbes found in the marine and subsurface environments referenced above. As *Bacillus* spp. are frequently identified in fuel environments via cultivation-based as well as molecular methods and endospores are highly resistant structures capable of surviving harsh conditions such as dessication or exposure to toxic chemicals, these types of microbes could persist in fuel environments, potentially contributing to issues such as microbial corrosion or biofouling.

Our results clearly show that the *Bacillus* sp. isolated from E10 fuel in this study produces spores that catalyze the oxidation of Mn(II). The isolate possesses an mnxG gene that is known to code for a multicopper oxidase enzyme suspected to promote Mn(II) oxidation. Additionally, experiments involving iron-coated glass slides show that these microbes are capable of depositing manganese oxides onto metallic surfaces. Though the appearance of 'pits' on the iron-coated slides exposed to the manganese oxidizing spores suggests that these microbes may promote corrosion, these results were not reproducible and conclusions cannot be drawn regarding the corrosive nature of thee microbes from this data. However, the corrosive nature of microbial manganese oxidation and deposition of manganese oxides onto the surfaces of passive materials such as stainless steel has been described.

Pitting corrosion of stainless steels exposed to manganese oxidizing microbes has been well-documented (Lewandowski and Beyenal 2009; Linhardt 2010 and references therein). Manganese oxidizing microbes initiate pitting of stainless steel due to a shift in open circuit potential, which is termed ennoblement (Dickinson et al. 1996; Dickinson et al. 1997; Little et al. 1998). The process of ennoblement due to manganese oxidizing microbial activity begins when microbes catalyze the oxidation of manganese, and manganese oxides are deposited onto the stainless steel surface. The manganese oxides must be in electrical contact with the metal surface to impact corrosion processes. These manganese oxides that are in electrical contact with the metal surface are reduced via electrons generated at anodic sites on the metal surface which results in pitting corrosion. The reduced manganese is re-oxidized by the microbial consortia (or potentially spores) on the metal surface and the pitting process continues. This mechanism is based upon work with *Leptothrix discophora* (Dickinson et al. 1997; Olesen, et al. 2000; Geiser et al. 2002; Shi et al. 2002) as well as evaluating steels exposed to natural environments (Braughton et al. 2001; Shi et al. 2002).

Reports of carbon steel corrosion due to the activity of manganese oxidizing microbes are few, and this may be the result of protective effects of corrosion products that form on carbon steel surfaces (Olesen et al.2000). Interestingly, Linhardt (2010) observed degradation of a protective coating on carbon steel that was galvanically coupled to stainless steel exposed to manganese oxidizing microbes. Additionally, some laboratory work has suggested that manganese oxidizing microbes may increase corrosion rates of carbon steels (Rajasekar et al. 2007; Ashassi-Sorkhabi et al. 2012). Rajesekar observed increased corrosion of carbon steel in the presence of *Bacillus cereus* strain ACE4 that was shown to oxidize manganese and degrade hydrocarbons (diesel). Ashassi-Sorkhabi et al. (2012) examined the corrosive effects of a

manganese-oxidizing *Pseudoxanthomonas* sp. on carbon steel via electrochemical techniques and concluded that the microbes increased corrosion rates.

## 4.6 Summary

*Bacillus* spp. are ubiquitous microbes, likely due to the fact that these microbes produce highly resistant spores and may persist in many environments indefinitely, and *Bacillus* spp. are frequently identified in environments associated with fuel transportation and storage. Here we have isolated and identified *Bacillus* spp. that produce spores capable of catalyzing manganese oxidation from an E10 fuel sample. These types of microbes have been isolated from marine sediment samples (Rosson and Nealson 1982; Francis and Tebo 2002; Dick et al. 2006) as well as terrestrial subsurface samples (Mayhew et al. 2008), but to the authors' knowledge, metabolically active spores have not been investigated in the context of fuel system microbiology. These *Bacillus* spp. are likely to persist in fuel industry infrastructure and could theoretically contribute to issues such as microbial corrosion and biofouling due to deposition of manganese oxides (materials used for the construction of fuel system infrastructure such as carbon steels or stainless steels and water that infiltrates this infrastructure may be the source of manganese). Further research is needed to analyze the impact these microbes have on fuels and fuel system infrastructure.

# CHAPTER 5

## SUMMARY AND FUTURE DIRECTIONS

The focus of this research was characterizing the microbial diversity of automotive fuels and automotive fuel environments in the United States via both molecular-based techniques as well as cultivation-based methods in order to gain insight into how this diversity is impacting fuels and fuel system infrastructure. Molecular techniques included sequencing of 16S/18S rRNA genes (as well as 16S/18S rRNA transcribed into cDNA for some samples) via both Sanger sequencing and 454 pyrosequencing methodologies, which provided a survey of the microbial diversity associated with automotive fuel environments. Key microbes were cultivated in the laboratory to more thoroughly understand how these microbes impact microbial corrosion. Corrosion testing of carbon steels commonly used in fuel system infrastructure exposed to these microbes was conducted and reported elsewhere (Jain 2011, Sowards et al. in preparation, see Appendices B and C).

In Chapter 2, a pyrosequencing-based study indicated that fuel samples (biodiesel, diesel, E10, 85, ethanol and gasoline) are generally dominated by a few abundant phylotypes including *Halomonas* spp. and *Pseudomonas* spp.; though diverse phylotypes are present in these environments at lower relative abundances. Many of the prevalent phylotypes identified in pyrosequencing libraries have been reported to be associated with fuel environments by other researchers (Zobell 1946; Gaylarde et al. 1999; White et al. 2011). Microbes present in lower abundances may significantly impact fuel system infrastructure via such mechanisms as promoting localized corrosion. Beta diversity analyses indicated that fuel types did not seem to

select for unique microbial communities, which may be due to the fact that much of the infrastructure in fuel systems is exposed to many different fuel types and cross-contamination may be likely. Active and total microbial communities determined by sequencing both 16S/18S rRNA genes as well as 16S/18S rRNA (transcribed into cDNA) showed significant overlap, which may suggest that predominant phylotypes in these environments are well-suited to thrive in high-solvent environments and are active in fuel system infrastructure. However, some phylotypes such as *Halomonas* spp. had higher relative abundances in DNA-based pyrosequencing libraries compared to cDNA-based pyrosequencing libraries, which may suggest that some microbes may be less active members of the microbial communities in the fuel samples analyzed in this study. Additional efforts to characterize microbial diversity in fuel systems via both cultivation-based methods and molecular techniques is warranted to determine the best approaches for monitoring fuel system contamination. Additionally, as molecular-based techniques are constantly improving, the ability to monitor microbial diversity in many environments will likely improve.

As described in Chapter 3, environments exposed to fuel-grade ethanol and water were investigated via both molecular-based and cultivation-based methods. Molecular techniques indicated that these environments are dominated by *Acetobacter* spp. that oxidize ethanol to acetic acid. Interestingly, *Acetobacter* spp. are also identified in a portion of the fuel samples evaluated as part of the study described in Chapter 2. These microbes were also cultivated in the laboratory, and a suite of corrosion tests including electrochemical and mechanical testing techniques were used to evaluate the corrosive nature of these microbes (reported elsewhere – see Jain 2011, Sowards et al. in preparation, see Appendices B and C). Additionally, a sulfate-reducing bacterial consortium was cultivated in the laboratory and used for corrosion testing. Both of these types of microbes (acid producers and sulfate-reducers) enhanced corrosion of

steels commonly used for construction of fuel system infrastructure. As *Acetobacter* spp. are identified in several fuel types, we speculate that the addition of fuel-grade ethanol to automotive fuels (gasoline) may produce an environment that selects for microbes that oxidize ethanol to acetic acid, such as *Acetobacter* spp. Additionally, many microbes known to utilize by-products of ethanol oxidation (acetate) are found in these samples. Further research is warranted to explore potential solutions to the apparent microbial corrosion issues prevalent in environments exposed to fuel-grade ethanol and water.

Chapter 4 describes the cultivation and identification of *Bacillus* spp. that produce manganese-oxidizing spores. These microbes likely persist in fuel system environments and could potentially impact corrosion of materials such as stainless steels. This study increases the known environments in which these types of microbes are found. Further research is needed to understand the role these microbes play in fuel system environments.

To the author's knowledge, the research presented in this thesis provides one of the most in-depth molecular-based diversity surveys ever applied to fuel environments. The data generated in this research will provide information for industry personnel when considering monitoring, mitigation and prevention strategies for combating microbial contamination in fuel industry infrastructure. Future work could include continued molecular-based surveys to more thoroughly characterize the microbial communities in a variety of fuels and fuel environments. Additionally, continued microbial surveys could determine if the addition of ethanol to automotive fuels and fuel indusrty infrasctructure causes a shift in microbial communities that result in significant acetic acid production and related corrosion issues. Industry personnel have reported corrosion issues suspected to be microbial in nature impacting infrastructure exposed to fuel grade ethanol and water, and corrosion issues attibuted to acetic acid producing bacteria in infrastructure containting diesel fuel (suspected to also contain low concentrations of ethanol)

have also been reported (Battelle Final Report Study Number 10001550, Corrosion in Systems Storing and Dispensing Ultra Low Sulfur Diesel (ULSD), Hypotheses Investigation, September 2012). Additionally, cultivation-based methods should be applied to more thoroughly understand how key microbes present in fuel system infrastructure impact fuel degradation, biofouling and corrosion. The combination of molecular techniques as well as cultivation-based methods can be applied to determine the efficacy of strategies for mitigating or preventing microbial contamination or MIC such as the application of biocides. As molecular techniques such as DNA sequencing technology, bioinformatic tools and cultivation strategies continue to improve, a more thorough understanding of fuel system microbiology will be possible.

REFERENCES CITED

Agler, M. T., C. M. Spirito, J.. Usack, J. J. Werner, and L. T. Angenent. 2012. “Chain elongation with reactor microbiomes: upgrading dilute ethanol to medium-chain carboxylates.” *Energy & Environmental Science* 5 (8) (August): 8189–8192.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. “Basic Local Alignment Search Tool.” *Journal of Molecular Biology* 215 (3) (October 5): 403–410.

Amann, R. I., W. Ludwig, and K. H. Schleifer. 1995. “Phylogenetic Identification and in Situ Detection of Individual Microbial Cells Without Cultivation.” *Microbiological Reviews* 59 (1) (March 1): 143–169.

Ashassi-Sorkhabi, H., M. Moradi-Haghighi, G. Zarrini, and R. Javaherdashti. 2012. “Corrosion Behavior of Carbon Steel in the Presence of Two Novel Iron-oxidizing Bacteria Isolated from Sewage Treatment Plants.” *Biodegradation* 23 (1): 69–79. doi:10.1007/s10532-011-9487-8.

Atlas, R. M. 1981. “Microbial Degradation of Petroleum Hydrocarbons: An Environmental Perspective.” *Microbiological Reviews* 45 (1) (March): 180.

Badin, A., Tarfa Mustafa, C. Bertrand, A. Monier, C. Delolme, R. A. Geremia, and J. Bedell. 2012. “Microbial Communities of Urban Stormwater Sediments: The Phylogenetic Structure of Bacterial Communities Varies with Porosity.” *FEMS Microbiology Ecology* 81 (2): 324–338.

Bargar, J. R., B. M. Tebo, U. Bergmann, S. M. Webb, P. Glatzel, V. Q. Chiu, and M. Villalobos. 2005. “Biotic and Abiotic Products of Mn(II) Oxidation by Spores of the Marine Bacillus Sp. Strain SG-1.” *American Mineralogist* 90 (1) (January 1): 143–154.

Bargar, J.R, B.M Tebo, and J.E Villinski. 2000. “In Situ Characterization of Mn(II) Oxidation by Spores of the Marine Bacillus Sp. Strain SG-1.” *Geochimica Et Cosmochimica Acta* 64 (16) (August): 2775–2778.

Bartowsky, E. J., and P. A. Henschke. 2008. “Acetic Acid Bacteria Spoilage of Bottled Red wine —A Review.” *International Journal of Food Microbiology* 125 (1) (June 30): 60–70.

Beavers, J. A., F. Gui, and N. Sridhar. 2011. “Effects of Environmental and Metallurgical Factors on the Stress Corrosion Cracking of Carbon Steel in Fuel-Grade Ethanol.” *Corrosion* 67 (2) (February): 1–15.

Benson, D. A., I, Karsch-Mizrachi, D.J. Lipman, J. Ostell, and D. L. Wheeler. 2005. “GenBank.” *Nucleic Acids Research* 33 (Database Issue) (January 1): D34–D38.

Besemer, K., H. Peter, J. B. Logue, S. Langenheder, E. S. Lindström, L. J. Tranvik, and T. J. Battin. 2012. “Unraveling Assembly of Stream Biofilm Communities.” *The ISME Journal* 6 (8) (August): 1459–1468..

Biderre-Petit, C., D. Boucher, J. Kuever, P. Alberic, D. Jézéquel, B. Chebance, G. Borrel, G. Fonty, and P. Peyret. 2011. “Identification of Sulfur-Cycle Prokaryotes in a Low-Sulfate Lake (Lake Pavin) Using aprA and 16S rRNA Gene Markers.” *Microbial Ecology* 61 (2): 313–327.

Boucher, D., J. B. Laffaire, F. Jaziri, C. David, C. Biderre-Petit, P. Duquenne, E. Peyretaillade, and P. Peyret. 2011. “Bacterial Community Composition of Biological Degreasing Systems and Health Risk Assessment for Workers.” *Microbial Ecology* 62 (4) (November 1): 868–881.

Bowman, J. P., L. I. Sly, P. D. Nichols, and A. C. Hayward. 1993. “Revised Taxonomy of the Methanotrophs: Description of Methylobacter Gen. Nov., Emendation of Methylococcus, Validation of Methylosinus and Methylocystis Species, and a Proposal That the Family Methylococcaceae Includes Only the Group I Methanotrophs.” *International Journal of Systematic Bacteriology* 43 (4) (October 1): 735–753. doi:10.1099/00207713-43-4-735.

Bräuer, S. L., H. Cadillo-Quiroz, E. Yashiro, J. B. Yavitt, and S. H. Zinder. 2006. “Isolation of a Novel Acidiphilic Methanogen from an Acidic Peat Bog.” *Nature* 442 (7099) (May 14): 192–194.

Braughton, K. R., R. L. Lafond, and Z. Lewandowski. 2001. “The Influence of Environmental Factors on the Rate and Extent of Stainless Steel Ennoblement Mediated by Manganese-oxidizing Biofilms.” *Biofouling* 17 (3): 241–251.

Braun, M., F. Mayer, and G. Gottschalk. 1981. “<i>Clostridium Aceticum</i> (Wieringa), a Microorganism Producing Acetic Acid from Molecular Hydrogen and Carbon Dioxide.” *Archives of Microbiology* 128 (3): 288–293.

Brouwers, G.J., P. M. de Vrind, L. A. M. Corstjens, P. Cornelis, C. Baysse, and E. W. de Vrind-de Jong. 1999. “cumA, a Gene Encoding a Multicopper Oxidase, Is Involved in Mn2+ Oxidation in Pseudomonas Putida GB-1.” *Applied and Environmental Microbiology* 65 (4) (April 1): 1762–1768.

Brown, L.M., J.P. McComb, M.D. Vangsness, L.L. Bowen, S.S. Mueller, L.M. Balster, and C.A. Bleckmann. 2010. “Community Dynamics and Phylogenetics of Bacteria Fouling Jet A and JP-8 Aviation Fuel.” *International Biodeterioration & Biodegradation* 64 (3) (June): 253–261.

Bunge, J., L. Woodard, D. Böhning, J. A. Foster, S. Connolly, and H. K. Allen. 2012. “Estimating Population Diversity with CatchAll.” *Bioinformatics* 28 (7) (April 1): 1045–1047.

Busalmen, J.P, M Vázquez, and S.R de Sánchez. 2002. “New Evidences on the Catalase Mechanism of Microbial Corrosion.” *Electrochimica Acta* 47 (12) (May 7): 1857–1865.

Calvo, C., F. Martínez-Checa, F. Toledo, J. Porcel, and E. Quesada. 2002. “Characteristics of Bioemulsifiers Synthesised in Crude Oil Media by Halomonas Eurihalina and Their Effectiveness in the Isolation of Bacteria Able to Grow in the Presence of Hydrocarbons.” *Applied Microbiology and Biotechnology* 60 (3) (November 1): 347–351.

Canfield, D. E., F. J. Stewart, B. Thamdrup, L. De Brabandere, T. Dalsgaard, E. F. Delong, N. P. Revsbech, and O. Ulloa. 2010. “A Cryptic Sulfur Cycle in Oxygen-Minimum–Zone Waters Off the Chilean Coast.” *Science* 330 (6009) (December 3): 1375–1378.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena , J. K. Goodrich , J. I. Gordon , G. A. Huttley , S. .T Kelley , D. Knights , J. E. Koenig , R. E. Ley , C. A. Lozupone , D. McDonald , B. D. Muegge , M. Pirrung , J. Reeder , J. R. Sevinsky , P. J. Turnbaugh , W. A. Walters , J. Widmann , T. Yatsunenko , J. Zaneveld & R. Knight . 2010. “QIIME Allows Analysis of High-throughput Community Sequencing Data.” *Nature Methods* 7 (5) (May 1): 335–336.

Caporaso, J. G., K. Paszkiewicz, D. Field, R. Knight, and J. A. Gilbert. 2012. “The Western English Channel Contains a Persistent Microbial Seed Bank.” *The ISME Journal* 6 (6): 1089.

Cerrato, J. M., J. O. Falkinham III, A. M. Dietrich, W. R. Knocke, C. W. McKinney, and A. Pruden. 2010. “Manganese-oxidizing and -reducing Microorganisms Isolated from Biofilms in Chlorinated Drinking Water Systems.” *Water Research* 44 (13) (July): 3935–3945..

Chao, Y., N. Liu, T. Zhang, and S. Chen. 2010. “Isolation and Characterization of Bacteria from Engine Sludge Generated from Biodiesel-diesel Blends.” *Fuel* 89 (11) (November): 3358–3364.

Cleenwerck, I., K. Vandemeulebroecke, D. Janssens, and J. Swings. 2002. “Re-examination of the Genus Acetobacter, with Descriptions of Acetobacter Cerevisiae Sp. Nov. and Acetobacter Malorum Sp. Nov.” *International Journal of Systematic and Evolutionary Microbiology* 52 (5) (September 1): 1551–1558.

Cord-Ruwisch, R., W. Kleinitz, and F. Widdel. 1987. “Sulfate-reducing Bacteria and Their Activities in Oil Production.” *Journal of Petroleum Technology* 39 (1) (January).

Corstjens, P. L. A. M., J. P. M. de Vrind, T. Goosen, and E. W. de Vrind-de Jong. 1997. “Identification and Molecular Analysis of the Leptothrix Discophora SS-1 mofA Gene, a Gene Putatively Encoding a Manganese-oxidizing Protein with Copper Domains.” *Geomicrobiology Journal* 14 (2): 91–108.

Cuzin, N., A. S. Ouattara, M. Labat, and J. L. Garcia. 2001. “Methanobacterium Congolense Sp. Nov., from a Methanogenic Fermentation of Cassava Peel.” *International Journal of Systematic and Evolutionary Microbiology* 51 (2) (March 1): 489–493.

Dagbert, C., T. Meylheuc, and M. Bellon-Fontaine. 2006. “Corrosion Behaviour of AISI 304 Stainless Steel in Presence of a Biosurfactant Produced by Pseudomonas Fluorescens.” *Electrochimica Acta* 51 (24) (July 15): 5221–5227..

Davidova, I. A., K. E. Duncan, B. M. Perez-Ibarra, and J. M. Suflita. 2012. “Involvement of Thermophilic Archaea in the Biocorrosion of Oil Pipelines.” *Environmental Microbiology* 14 (7): 1762–1771.

DeGray, R. J., and L. N. Killian. 1960. “CORROSION—Bacterial Slime and Corrosion in Petroleum Product Storage.” *Industrial & Engineering Chemistry* 52 (12) (December 1): 74A–76A. doi:10.1021/ie50612a010.

DeLong, E. F., G. S. Wickham, and N. R. Pace. 1989. “Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells.” *Science* 243 (4896): 1360–1363.

Dick, G. J., Y. E. Lee, and B. M. Tebo. 2006. “Manganese(II)-Oxidizing Bacillus Spores in Guaymas Basin Hydrothermal Sediments and Plumes.” *Applied and Environmental Microbiology* 72 (5) (May 1): 3184–3190.

Dickinson, W. H., F. Caccavo, B. Olesen, and Z. Lewandowski. 1997. “Ennoblement of Stainless Steel by the Manganese-Depositing Bacterium Leptothrix Discophora.” *Applied and Environmental Microbiology* 63 (7) (July 1): 2502–2506.

Dickinson, W.H., F. Caccavo Jr, and Z. Lewandowski. 1996. “The Ennoblement of Stainless Steel by Manganic Oxide Biofouling.” *Corrosion Science* 38 (8) (August): 1407–1422.

Dinh, H. T., J. Kuever, M. Mußmann, A. W. Hassel, M. Stratmann, and F. Widdel. 2004. “Iron Corrosion by Novel Anaerobic Microorganisms.” *Nature* 427 (6977) (February 26): 829–832.

Dojka, M. A., P. Hugenholtz, S. K. Haack, and N. R. Pace. 1998. “Microbial Diversity in a Hydrocarbon- and Chlorinated-Solvent-Contaminated Aquifer Undergoing Intrinsic Bioremediation.” *Applied and Environmental Microbiology* 64 (10) (October): 3869–3877.

Dubiel, M., C. H. Hsu, C. C. Chien, F. Mansfeld, and D. K. Newman. 2002. “Microbial Iron Respiration Can Protect Steel from Corrosion.” *Applied and Environmental Microbiology* 68 (3) (March 1): 1440–1445. doi:10.1128/AEM.68.3.1440-1445.2002.

Duncan, K. E., L. M. Gieg, V. A. Parisi, R. S. Tanner, S. G. Tringe, J. Bristow, and J. M. Suflita. 2009. “Biocorrosive Thermophilic Microbial Communities in Alaskan North Slope Oil Facilities.” *Environmental Science & Technology* 43 (20) (October 15): 7977–7984.

Edgar, R. C., B. J. Haas, J. C. Clemente, C. Quince, and R. Knight. 2011. “UCHIME Improves Sensitivity and Speed of Chimera Detection.” *Bioinformatics* 27 (16) (August 15): 2194–2200.

Edmonds, P., and J. J. Cooney. 1967. “Identification of Microorganisms Isolated from Jet Fuel Systems.” *Applied Microbiology* 15 (2) (March 1): 411–416.

Enning, D., H. Venzlaff, J. Garrelfs, H. T. Dinh, V. Meyer, K. Mayrhofer, A. W. Hassel, M. Stratmann, and F. Widdel. 2012. “Marine Sulfate-reducing Bacteria Cause Serious Corrosion of Iron Under Electroconductive Biogenic Mineral Crust.” *Environmental Microbiology* 14 (7): 1772–1787.

Eppard, M.Martin, W. E. Krumbein, C. Koch, E. Rhiel, J. T. Staley, and E. Stackebrandt. 1996. “Morphological, Physiological, and Molecular Characterization of Actinomycetes Isolated from Dry Soil, Rocks, and Monument Surfaces.” *Archives of Microbiology* 166 (1): 12–22.

Erhlich, HL, and DK Newman. 2009. *Geomicrobiology, Fifth Edition*. CRC Press.

Ewing, B., and P. Green. 1998. “Base-Calling of Automated Sequencer Traces UsingPhred. II. Error Probabilities.” *Genome Research* 8 (3) (March 1): 186–194.

Ewing, B., L. Hillier, M. C. Wendl, and P. Green. 1998. “Base-Calling of Automated Sequencer Traces UsingPhred. I. Accuracy Assessment.” *Genome Research* 8 (3) (March 1): 175–185.

Flemming, H. C., “Economical and technical overview”, InHeitz, E., H.-C. Flemming, and W. Sand. 1996. *Microbially influenced corrosion of materials : scientific and engineering aspects*.

Francis, C. A., and B. M. Tebo. 2002. “Enzymatic Manganese(II) Oxidation by Metabolically Dormant Spores of Diverse Bacillus Species.” *Applied and Environmental Microbiology* 68 (2) (February 1): 874–880.

Frank, D. N. 2008. “XplorSeq: A Software Environment for Integrated Management and Phylogenetic Analysis of Metagenomic Sequence Data.” *BMC Bioinformatics* 9 (1) (October 7): 420.

Frankel, Gerald S. 2008. “Electrochemical Techniques in Corrosion: Status, Limitations and Needs.” *Journal of ASTM International* 5 (2).

Gaylarde, C. C., F. M. Bento, and J. Kelley. 1999. “Microbial Contamination of Stored Hydrocarbon Fuels and Its Control.” *Revista De Microbiologia* 30 (1).

Geiser, M., R. Avci, and Z. Lewandowski. 2002. “Microbially Initiated Pitting on 316L Stainless Steel.” *International Biodeterioration & Biodegradation* 49 (4) (June): 235–243.

Gevers, D., R. Knight, J. F. Petrosino, K. Huang, A. L. McGuire, B. W. Birren, K. E. Nelson, O. White, B. A. Methé, and C. Huttenhower. 2012. “The Human Microbiome Project: A Community Resource for the Healthy Human Microbiome.” *PLoS Biol* 10 (8) (August 14).

Gundlapally, S., and F. Garcia-Pichel. 2006. “The Community and Phylogenetic Diversity of Biological Soil Crusts in the Colorado Plateau Studied by Molecular Fingerprinting and Intensive Cultivation.” *Microbial Ecology* 52 (2): 345–357.

Gurtner, C., J. Heyrman, G. Piñar, W. Lubitz, J. Swings, and S. Rölleke. 2000. “Comparative Analyses of the Bacterial Diversity on Two Different Biodeteriorated Wall Paintings by DGGE and 16S rDNA Sequence Analysis.” *International Biodeterioration & Biodegradation* 46 (3) (October): 229–239.

Hamady, M., and R. Knight. 2009. “Microbial Community Profiling for Human Microbiome Projects: Tools, Techniques, and Challenges.” *Genome Research* 19 (7) (July 1): 1141–1152.

Hastings, D., and S. Emerson. 1986. “Oxidation of Manganese by Spores of a Marine Bacillus: Kinetic and Thermodynamic Considerations.” *Geochimica Et Cosmochimica Acta* 50 (8) (August): 1819–1824.

Heist, P. 2009. “Ethanol Producer Magazine | EthanolProducer.com.” http://www.ethanolproducer.com/articles/5464/identifying-controlling-the-most-common-microbial-contaminants.

Hugenholtz, P., C. Pitulle, K. L. Hershberger, and N. R. Pace. 1998. “Novel Division Level Bacterial Diversity in a Yellowstone Hot Spring.” *Journal of Bacteriology* 180 (2) (January 15): 366–376.

Huse, S., J. Huber, H. Morrison, M. Sogin, and D. M. Welch. 2007. “Accuracy and Quality of Massively Parallel DNA Pyrosequencing.” *Genome Biology* 8 (7): 1–9.

Inskeep, W. P., D. B. Rusch, Z. J. Jay, M. J. Herrgard, M. A. Kozubal, T. H. Richardson, R. E. Macur, et al. 2010. “Metagenomes from High-Temperature Chemotrophic Systems Reveal Geochemical Controls on Microbial Community Structure and Function.” *PLoS ONE* 5 (3) (March 19): e9773.

Ishii, S., T. Shimoyama, Y. Hotta, and K. Watanabe. 2008. “Characterization of a Filamentous Biofilm Community Established in a Cellulose-fed Microbial Fuel Cell.” *BMC Microbiology* 8 (January 10): 6.

Jain, L. A. “Evaluation of the Propensity for Microbiologically Influenced Corrosion of Steels in Fuel Grade Ethanol Environments.” Ph.D. Dis. Colorado School of Mines. 2011.

Jan-Roblero, J., A. Posadas, J. Z. Díaz de la Serna, R. García, and C. Hernández-Rodríguez. 2008. “Phylogenetic Characterization of Bacterial Consortia Obtained of Corroding Gas Pipelines in Mexico.” *World Journal of Microbiology and Biotechnology* 24 (9): 1775–1784.

Jan-Roblero, J., J.M. Romero, M. Amaya, and S. Le Borgne. 2004. “Phylogenetic Characterization of a Corrosive Consortium Isolated from a Sour Gas Pipeline.” *Applied Microbiology and Biotechnology* 64 (6): 862–867.

Javaherdashti, R.. 2008. *Microbiologically Influenced Corrosion: An Engineering Insight*. Springer.

Javaherdashti, R.. 2011. “Impact of Sulphate-reducing Bacteria on the Performance of Engineering Materials.” *Applied Microbiology and Biotechnology* 91 (6) (September 1): 1507–1517.

Javaherdashti, R., R.K. Singh Raman, C. Panter, and E.V. Pereloma. 2006. “Microbiologically Assisted Stress Corrosion Cracking of Carbon Steel in Mixed and Pure Cultures of Sulfate Reducing Bacteria.” *International Biodeterioration & Biodegradation* 58 (1) (July): 27–35.

Jayaraman, A., A.k. Sun, and T.k. Wood. 1998. “Characterization of Axenic Pseudomonas Fragi and Escherichia Coli Biofilms That Inhibit Corrosion of SAE 1018 Steel.” *Journal of Applied Microbiology* 84 (4): 485–492..

Jones, D. A. 1996. *Principles and Prevention of Corrosion*. Prentice Hall.

Jones, S. E., and J. T. Lennon. 2010. “Dormancy Contributes to the Maintenance of Microbial Diversity.” *Proceedings of the National Academy of Sciences* 107 (13) (March 30): 5881–5886.

Junier, P., M. Frutschi, N. S. Wigginton, E. J. Schofield, J. R. Bargar, and R. Bernier-Latmani. 2009. “Metal Reduction by Spores of Desulfotomaculum Reducens.” *Environmental Microbiology* 11 (12): 3007–3017.

Kamke, J., M. W. Taylor, and S. Schmitt. 2010. “Activity Profiles for Marine Sponge-associated Bacteria Obtained by 16S rRNA Vs 16S rRNA Gene Comparisons.” *The ISME Journal* 4 (4): 498.

Kane, R. D. 2007. *Stress corrosion cracking of carbon steel in fuel grade ethanol : review, experience, survey, field monitoring, and laboratory testing : downstream segment.* 2nd ed., May 2007. Washington, DC: American Petroleum Institute.

Kane, R. D., N. Sridhar, M. P. Brongers, J. A. Bea, A. K. Agrawal, and L. J. Klein. 2005. “Stress corrosion cracking in fuel ethanol : A recently recognized phenomenon.” *Materials performance* 44 (12): 50–55.

Kersters, Karel, Puspita Lisdiyanti, Kazuo Komagata, and Jean Swings. 2006. “The Family Acetobacteraceae: The Genera Acetobacter, Acidomonas, Asaia, Gluconacetobacter, Gluconobacter, and Kozakia.” In *The Prokaryotes*, ed. Martin Dworkin, Stanley Falkow, Eugene Rosenberg, Karl-Heinz Schleifer, and Erko Stackebrandt, 163–200. Springer New York.

King, R. A., J. D. A. Miller, and D. S. Wakerley. 1973. “Corrosion of Mild Steel in Cultures of Sulphate-reducing Bacteria: Effect of Changing the Soluble Iron Concentration During Growth.” *British Corrosion Journal* 8 (2): 89–93.

Klappenbach, Joel A., Paul R. Saxman, James R. Cole, and Thomas M. Schmidt. 2001. “Rrndb: The Ribosomal RNA Operon Copy Number Database.” *Nucleic Acids Research* 29 (1) (January 1): 181–184.

Konhauser, Kurt O. 2012. *Introduction to Geomicrobiology*. Wiley.

Kotsyurbenko, O. R., M. W. Friedrich, M. V. Simankova, A. N. Nozhevnikova, P. N. Golyshin, K. N. Timmis, and R. Conrad. 2007. “Shift from Acetoclastic to H2-Dependent Methanogenesis in a West Siberian Peat Bog at Low pH Values and Isolation of an Acidophilic Methanobacterium Strain.” *Applied and Environmental Microbiology* 73 (7) (April 1): 2344–2348.

Krumbein, W. E., and H. J. Altmann. 1973. “A New Method for the Detection and Enumeration of Manganese Oxidizing and Reducing Microorganisms.” *Helgoland Marine Research* 25 (2): 347–356.

Lane, D. J. 1991. *Nucleic Acid Techniques in Bacterial Systematics*.

Lee, A. K., and D. K. Newman. 2003. “Microbial Iron Respiration: Impacts on Corrosion Processes.” *Applied Microbiology and Biotechnology* 62 (2): 134–139.

Lee, Jason S., Richard I. Ray, and Brenda J. Little. 2010. “An Assessment of Alternative Diesel Fuels: Microbiological Contamination and Corrosion Under Storage Conditions.” *Biofouling* 26 (6): 623–635.

Leek, Jeffrey T., Robert B. Scharpf, Héctor Corrada Bravo, David Simcha, Benjamin Langmead, W. Evan Johnson, Donald Geman, Keith Baggerly, and Rafael A. Irizarry. 2010. “Tackling the Widespread and Critical Impact of Batch Effects in High-throughput Data.” *Nature Reviews Genetics* 11 (10) (October 1): 733–739.

Lee, Patrick K. H., F. Warnecke, Eoin L. Brodie, Tamzen W. Macbeth, Mark E. Conrad, Gary L. Andersen, and Lisa Alvarez-Cohen. 2012. “Phylogenetic Microarray Analysis of a Microbial Community Performing Reductive Dechlorination at a TCE-Contaminated Site.” *Environmental Science & Technology* 46 (2) (January 17): 1044–1054.

Lee, Whonchee, Zbigniew Lewandowski, Per H Nielsen, and W Allan Hamilton. 1995. “Role of Sulfate-reducing Bacteria in Corrosion of Mild Steel: A Review.” *Biofouling* 8 (3): 165–194.

Lee, Yoon, and Bradley M. Tebo. 1994. “Cobalt(II) Oxidation by the Marine Manganese(II)-Oxidizing Bacillus Sp. Strain SG-1.” *Applied and Environmental Microbiology* 60 (8) (August 1): 2949–2957.

Lennon, Jay T., and Stuart E. Jones. 2011. “Microbial Seed Banks: The Ecological and Evolutionary Implications of Dormancy.” *Nature Reviews Microbiology* 9 (2) (February 1): 119–130. .

Lewandowski, Z., and H. Beyenal. 2009. “Mechanisms of Microbially Influenced Corrosion.” In *Marine and Industrial Biofouling*, ed. Hans-Curt Flemming, P. Sriyutha Murthy, R. Venkatesan, and Keith Cooksey, 4:35–64. Springer Series on Biofilms. Springer Berlin Heidelberg.

Ley, Ruth E., J. Kirk Harris, Joshua Wilcox, John R. Spear, Scott R. Miller, Brad M. Bebout, Julia A. Maresca, Donald A. Bryant, Mitchell L. Sogin, and Norman R. Pace. 2006. “Unexpected Diversity and Complexity of the Guerrero Negro Hypersaline Microbial Mat.” *Applied and Environmental Microbiology* 72 (5) (May 1): 3685–3695.

Lin, Bin, Christelle Hyacinthe, Steeve Bonneville, Martin Braster, Philippe Van Cappellen, and Wilfred F. M. Röling. 2007. “Phylogenetic and Physiological Diversity of Dissimilatory Ferric Iron Reducers in Sediments of the Polluted Scheldt Estuary, Northwest Europe.” *Environmental Microbiology* 9 (8): 1956–1968.

Lin, Bin, Carlos M Monreal, James T Tambong, Carlos B Miguez, and Lorna Carrasco-Medina. 2009. “Phylogenetic Analysis of Methanotrophic Communities in Cover Soils of a Landfill in Ontario.” *Canadian Journal of Microbiology* 55 (9) (September): 1103–1112.

Linhardt, P. 2010. “Twenty Years of Experience with Corrosion Failures Caused by Manganese Oxidizing Microorganisms.” *Materials and Corrosion* 61 (12): 1034–1039.

Lin, Xueju, David Kennedy, Jim Fredrickson, Bruce Bjornstad, and Allan Konopka. 2012. “Vertical Stratification of Subsurface Microbial Community Composition Across Geological Formations at the Hanford Site.” *Environmental Microbiology* 14 (2): 414–425.

Lisdiyanti, P, K Katsura, W Potacharoen, R Navarro, Y Yamada, T Uchimura, and K Komagata. 2003. “Diversity of Acetic Acid Bacteria in Indonesia, Thailand, and the Philippines.” *Microbiology and Culture Collections* 19 (2): 91–99.

Little, BJ, and R Ray. 2002. “A Perspective on Corrosion Inhibition by Biofilms.” *Corrosion* 58 (5) (May): 424–428.

Little, B., R. Ray, K. Hart, and P. Wagner. 1995. “Fungal-induced corrosion of wire rope.” *Materials performance* 34 (10): 55–58.

Little, Brenda J., and Jason S. Lee. 2007. *Microbiologically Influenced Corrosion*. John Wiley & Sons.

Little, Brenda, Patricia Wagner, Kevin Hart, Richard Ray, Dennis Lavoie, Kenneth Nealson, and Carmen Aguilar. 1998. “The Role of Biomineralization in Microbiologically Influenced Corrosion.” *Biodegradation* 9 (1): 1–10.

Little, Brenda, Patricia Wagner, and Florian Mansfeld. 1992. “An Overview of Microbiologically Influenced Corrosion.” *Electrochimica Acta* 37 (12) (September): 2185–2194.

Little, B., P. Wagner, and D. Duquette. 1988. “Technical Note: Microbiologically Induced Increase in Corrosion Current Density of Stainless Steel Under Cathodic Protection.” *Corrosion* 44 (5) (May): 270–274.

Liu, Ruyin, Zhisheng Yu, Hongguang Guo, Miaomiao Liu, Hongxun Zhang, and Min Yang. 2012. “Pyrosequencing Analysis of Eukaryotic and Bacterial Communities in Faucet Biofilms.” *Science of The Total Environment* 435–436 (0) (October 1): 124–131.

Lodders, Nicole, and Peter Kämpfer. 2012. “A Combined Cultivation and Cultivation-independent Approach Shows High Bacterial Diversity in Water-miscible Metalworking Fluids.” *Systematic and Applied Microbiology* 35 (4) (June): 246–252.

Logue, Jürg B., and Eva S. Lindström. 2010. “Species Sorting Affects Bacterioplankton Community Composition as Determined by 16S rDNA and 16S rRNA Fingerprints.” *The ISME Journal* 4 (6): 729.

López, Miguel A., F. Javier Zavala Díaz de la Serna, Janet Jan-Roblero, Juan M. Romero, and César Hernández-Rodríguez. 2006. “Phylogenetic Analysis of a Biofilm Bacterial Population in a Water Pipeline in the Gulf of Mexico.” *FEMS Microbiology Ecology* 58 (1): 145–154.

Lou, Xiaoyuan, and Preet M. Singh. 2010. “Role of Water, Acetic Acid and Chloride on Corrosion and Pitting Behaviour of Carbon Steel in Fuel-grade Ethanol.” *Corrosion Science* 52 (7) (July): 2303–2315.

Lou, Xiaoyuan, Di Yang, and Preet M. Singh. 2010. “Film Breakdown and Anodic Dissolution During Stress Corrosion Cracking of Carbon Steel in Bioethanol.” *Journal of The Electrochemical Society* 157 (2) (February 1): C86–C94.

Lou, X., D. Yang, and P. M. Singh. 2009. “Effect of Ethanol Chemistry on Stress Corrosion Cracking of Carbon Steel in Fuel-Grade Ethanol.” *Corrosion* 65 (12): 785–797.

Lovley, D. R., and Lovley DR. 1991. “Dissimilatory Fe(III) and Mn(IV) Reduction., Dissimilatory Fe(III) and Mn(IV) Reduction.” *Microbiological Reviews, Microbiological Reviews* 55, 55 (2, 2) (June): 259, 259.

Lozupone, Catherine, and Rob Knight. 2005. “UniFrac: a New Phylogenetic Method for Comparing Microbial Communities.” *Applied and Environmental Microbiology* 71 (12) (December 1): 8228–8235.

Ludwig, Wolfgang, Oliver Strunk, Ralf Westram, Lothar Richter, Harald Meier, Yadhukumar, Arno Buchner, et al. 2004. “ARB: a Software Environment for Sequence Data.” *Nucleic Acids Research* 32 (4) (March 15): 1363–1371.

Madigan, Michael T., John M. Martinko, David Stahl, and David P. Clark. 2010. *Brock Biology of Microorganisms*. 13th ed. Benjamin Cummings.

Magot, Michel, Bernard Ollivier, and Bharat K.C. Patel. 2000. "Microbiology of Petroleum Reservoirs." *Antonie Van Leeuwenhoek* 77 (2): 103–116.

Mansfeld, F. 2003. "The Use of Electrochemical Techniques for the Investigation and Monitoring of Microbiologically Influenced Corrosion and Its Inhibition – a Review." *Materials and Corrosion* 54 (7): 489–502.

Martínez-Checa, F., F. Toledo, R. Vilchez, E. Quesada, and C. Calvo. 2002. "Yield Production, Chemical Composition, and Functional Properties of Emulsifier H28 Synthesized by Halomonas Eurihalina Strain H-28 in Media Containing Various Hydrocarbons." *Applied Microbiology and Biotechnology* 58 (3) (March 1): 358–363.

Mayhew, Lisa E., Elizabeth D. Swanner, Andy P. Martin, and Alexis S. Templeton. 2008. "Phylogenetic Relationships and Functional Genes: Distribution of a Gene (mnxG) Encoding a Putative Manganese-Oxidizing Enzyme in Bacillus Species." *Applied and Environmental Microbiology* 74 (23) (December): 7265–7271.

McGowan, Laura, Rodney Herbert, and Gerard Muyzer. 2004. "A Comparative Study of Hydrocarbon Degradation by Marinobacter Sp., Rhodococcus Sp. and Corynebacterium Sp. Isolated from Different Mat Systems." *Ophelia* 58 (3): 271–281.

McNamara, Christopher J., Thomas D. Perry, Ryan Leard, Ktisten Bearce, James Dante, and Ralph Mitchell. 2005. "Corrosion of Aluminum Alloy 2024 by Microorganisms Isolated from Aircraft Fuel Tanks." *Biofouling* 21 (5-6): 257–265.

Mehanna, Maha, Regine Basseguy, Marie-Line Delia, and Alain Bergel. 2009. "Role of Direct Microbial Electron Transfer in Corrosion of Steels." *Electrochemistry Communications* 11 (3) (March): 568–571.

Mehdi, Hasanshahian, and Emtiazi Giti. 2008. "Investigation of Alkane Biodegradation Using the Microtiter Plate Method and Correlation Between Biofilm Formation, Biosurfactant Production and Crude Oil Biodegradation." *International Biodeterioration & Biodegradation* 62 (2) (September): 170–178.

Mnif, S., M. Chamkha, M. Labat, and S. Sayadi. 2011. "Simultaneous Hydrocarbon Biodegradation and Biosurfactant Production by Oilfield-selected Bacteria." *Journal of Applied Microbiology* 111 (3): 525–536.

Mnif, S., M. Chamkha, and S. Sayadi. 2009. "Isolation and Characterization of Halomonas Sp. Strain C2SS100, a Hydrocarbon-degrading Bacterium Under Hypersaline Conditions." *Journal of Applied Microbiology* 107 (3): 785–794.

Monroy, O. A. Ramos, M. J. Hernández Gayosso, N. Ruiz Ordaz, G. Zavala Olivares, and C. Juárez Ramírez. 2011. “Corrosion of API XL 52 Steel in Presence of Clostridium Celerecrescens.” *Materials and Corrosion* 62 (9): 878–883.

Morales, J., P. Esparza, S. González, R. Salvarezza, and M.P. Arévalo. 1993. “The Role of Pseudomonas Aeruginosa on the Localized Corrosion of 304 Stainless Steel.” *Corrosion Science* 34 (9) (September): 1531–1540.

Mori, Koji, Hirohito Tsurumaru, and Shigeaki Harayama. 2010. “Iron Corrosion Activity of Anaerobic Hydrogen-consuming Microorganisms Isolated from Oil Facilities.” *Journal of Bioscience and Bioengineering* 110 (4) (October): 426–430.

Mullis, K., F. Faloona, S. Scharf, R. Saiki, G. Horn, and H. Erlich. 1986. “Specific Enzymatic Amplification of DNA In Vitro: The Polymerase Chain Reaction.” *Cold Spring Harbor Symposia on Quantitative Biology* 51 (January 1): 263–273.

Murray, Karen J., and Bradley M. Tebo. 2007. “Cr(III) Is Indirectly Oxidized by the Mn(II)-Oxidizing Bacterium Bacillus Sp. Strain SG-1.” *Environmental Science & Technology* 41 (2) (January 1): 528–533.

Nawrocki, Eric Paul, and Washington University (Saint Louis, Mo.). 2009. “Structural RNA Homology Search and Alignment Using Covariance Models”. Saint Louis, Mo: Washington University. http://openscholarship.wustl.edu/etd.

Neihof, RA. 1988. “Microbes in Fuel: An Overview with a Naval Perspective.” In *Distillate Fuel: Contamination, Storage and Handling*. Philadelphia: American Society for Testing and Materials.

Nelson, Denice K., Timothy M. LaPara, and Paige J. Novak. 2010. “Effects of Ethanol-Based Fuel Contamination: Microbial Community Changes, Production of Regulated Compounds, and Methane Generation.” *Environmental Science & Technology* 44 (12) (June 15): 4525–4530.

Nicholson, Wayne L., Nobuo Munakata, Gerda Horneck, Henry J. Melosh, and Peter Setlow. 2000. “Resistance of Bacillus Endospores to Extreme Terrestrial and Extraterrestrial Environments.” *Microbiology and Molecular Biology Reviews* 64 (3) (September): 548.

Norman, R. Sean, Roberto Frontera-Suau, and Pamela J. Morris. 2002. “Variability in Pseudomonas Aeruginosa Lipopolysaccharide Expression During Crude Oil Degradation.” *Applied and Environmental Microbiology* 68 (10) (October 1): 5096–5103.

Obuekwe, Christian O., Donald W. S. Westlake, Fred D. Cook, and J. William Costerton. 1981. “Surface Changes in Mild Steel Coupons from the Action of Corrosion-Causing Bacteria.” *Applied and Environmental Microbiology* 41 (3) (March 1): 766–774.

Olesen, B. H., P. H. Nielsen, and Z. Lewandowski. 2000. “Effect of Biomineralized Manganese on the Corrosion Behavior of C1008 Mild Steel.” *Corrosion* 56 (1) (January): 80–89.

Olsen, G J, D J Lane, S J Giovannoni, N R Pace, and D A Stahl. 1986. “Microbial Ecology and Evolution: A Ribosomal RNA Approach.” *Annual Review of Microbiology* 40 (1): 337–365.

Osburn, Magdalena R., Alex L. Sessions, Charles Pepe-Ranney, and John R. Spear. 2011. “Hydrogen-isotopic Variability in Fatty Acids from Yellowstone National Park Hot Spring Microbial Communities.” *Geochimica Et Cosmochimica Acta* 75 (September 1): 4830–4845.

Pace, Norman R. 1997. “A Molecular View of Microbial Diversity and the Biosphere.” *Science* 276 (5313) (May 2): 734–740. doi:10.1126/science.276.5313.734.

Pace, N. 2009. “Mapping the Tree of Life: Progress and Prospects.” *Microbiology and Molecular Biology Reviews* 73 (4) (December 1): 565–576.

Passman, F.J, B.L McFarland, and M.J Hillyer. 2001. “Oxygenated Gasoline Biodeterioration and Its Control in Laboratory Microcosms.” *International Biodeterioration & Biodegradation* 47 (2) (March): 95–106.

Passman, Frederick J. 2003. *Fuel and Fuel System Microbiology-- Fundamentals, Diagnosis, and Contamination Control*. ASTM International.

Pavissich, J.p., I.t. Vargas, B. González, P.a. Pastén, and G.e. Pizarro. 2010. “Culture Dependent and Independent Analyses of Bacterial Communities Involved in Copper Plumbing Corrosion.” *Journal of Applied Microbiology* 109 (3): 771–782.

Pepe-Ranney, Charles, William M. Berelson, Frank A. Corsetti, Merika Treants, and John R. Spear. 2012. “Cyanobacterial Construction of Hot Spring Siliceous Stromatolites in Yellowstone National Park.” *Environmental Microbiology* 14 (5): 1182–1197.

Phoenix, V. R., and K. O. Konhauser. 2008. “Benefits of Bacterial Biomineralization.” *Geobiology* 6 (3): 303–308.

Pope, DH. 1988. “Organic Acid Corrosion of Carbon Steel: A Mechanism of Microbiologically Influenced Corrosion.” In *Corrosion*. Houston.

Postgate, J. R. 1979. *The Sulphate-Reducing Bacteria*. CUP Archive.

Potekhina, J. S., N. G. Sherisheva, L. P. Povetkina, A. P. Pospelov, T. A. Rakitina, F. Warnecke, and G. Gottschalk. 1999. “Role of Microorganisms in Corrosion Inhibition of Metals in Aquatic Habitats.” *Applied Microbiology and Biotechnology* 52 (5) (November 1): 639–646.

Pruesse, Elmar, Jörg Peplies, and Frank Oliver Glöckner. 2012. “SINA: Accurate High-throughput Multiple Sequence Alignment of Ribosomal RNA Genes.” *Bioinformatics* 28 (14) (July 15): 1823–1829.

Pruesse, Elmar, Christian Quast, Katrin Knittel, Bernhard M. Fuchs, Wolfgang Ludwig, Jörg Peplies, and Frank Oliver Glöckner. 2007. "SILVA: a Comprehensive Online Resource for Quality Checked and Aligned Ribosomal RNA Sequence Data Compatible with ARB." *Nucleic Acids Research* 35 (21) (December 1): 7188–7196.

Ragon, Marie, Michaël C. Fontaine, David Moreira, and Purificación López-García. 2012. "Different Biogeographic Patterns of Prokaryotes and Microbial Eukaryotes in Epilithic Biofilms." *Molecular Ecology* 21 (15): 3852–3868.

Ragon, Marie, Gwendal Restoux, David Moreira, Anders Pape Møller, and Purificación López-García. 2011. "Sunlight-exposed Biofilm Microbial Communities Are Naturally Resistant to Chernobyl Ionizing-radiation Levels." *PloS One* 6 (7): e21764.

Rahman, KAJA SHEIK MUJIBUR, Thahira Rarhman, Perumalsami Lakshmanaperumalsamy, and Ibrahim M. Banat. 2002. "Occurrence of crude oil degrading bacteria in gasoline and diesel station soils." *Journal of basic microbiology* 42 (4): 284–291.

Rajasekar, A., T. Ganesh Babu, S. Karutha Pandian, S. Maruthamuthu, N. Palaniswamy, and A. Rajendran. 2007. "Biodegradation and Corrosion Behavior of Manganese Oxidizer Bacillus Cereus ACE4 in Diesel Transporting Pipeline." *Corrosion Science* 49 (6) (June): 2694–2710.

Rajasekar, A., S. Maruthamuthu, Y. P. Ting, R. Balasubramanian, and Pattanathu K. S. M. Rahman. 2012. "Bacterial Degradation of Petroleum Hydrocarbons." In *Microbial Degradation of Xenobiotics*, ed. Shree Nath Singh, 339–369. Environmental Science and Engineering. Springer Berlin Heidelberg.

Rajasekar, Aruliah, Balakrishnan Anandkumar, Sundaram Maruthamuthu, Yen-Peng Ting, and Pattanathu Rahman. 2010. "Characterization of Corrosive Bacterial Consortia Isolated from Petroleum-product-transporting Pipelines." *Applied Microbiology and Biotechnology* 85 (4): 1175–1188.

Ray, Richard I., Jason S. Lee, and Brenda J. Little. 2010. *Iron-Oxidizing Bacteria: A Review of Corrosion Mechanisms in Fresh Water and Marine Environments*.

Reddy, Gundlapally S. N., Ruth M. Potrafka, and Ferran Garcia-Pichel. 2007. "Modestobacter Versicolor Sp. Nov., an Actinobacterium from Biological Soil Crusts That Produces Melanins Under Oligotrophy, with Emended Descriptions of the Genus Modestobacter and Modestobacter Multiseptatus Mevs Et Al. 2000." *International Journal of Systematic and Evolutionary Microbiology* 57 (9) (September 1): 2014–2020.

Reeder, Jens, and Rob Knight. 2010. "Rapid Denoising of Pyrosequencing Amplicon Data: Exploiting the Rank-abundance Distribution." *Nature Methods* 7 (9) (September): 668–669.

Revetta, Randy P., Adin Pemberton, Regina Lamendella, Brandon Iker, and Jorge W. Santo Domingo. 2010. “Identification of Bacterial Populations in Drinking Water Using 16S rRNA-based Sequence Analyses.” *Water Research* 44 (5) (March): 1353–1360.

Revie, R. Winston. 2011. *Uhlig's Corrosion Handbook*. John Wiley & Sons.

Rodríguez-Rodríguez, Carlos E., Evelyn Rodríguez-Cavallini, and Rigoberto Blanco. 2009. “Bacterial Contamination of Automotive Fuels in a Tropical Region: The Case of Costa Rica.” *Revista De Biología Tropical* 57 (3) (September): 489–504.

Romano, Ida, Assunta Giordano, Licia Lama, Barbara Nicolaus, and Agata Gambacorta. 2005. “Halomonas Campaniensis Sp. Nov., a Haloalkaliphilic Bacterium Isolated from a Mineral Pool of Campania Region, Italy.” *Systematic and Applied Microbiology* 28 (7) (August 24): 610–618.

Ronquist, Fredrik, and John P. Huelsenbeck. 2003. “MrBayes 3: Bayesian Phylogenetic Inference Under Mixed Models.” *Bioinformatics* 19 (12) (August 12): 1572–1574.

Rosson, R. A., and K. H. Nealson. 1982. “Manganese Binding and Oxidation by Spores of a Marine Bacillus.” *Journal of Bacteriology* 151 (2) (August 1): 1027–1034.

Sahl, Jason W., Nathaniel Fairfield, J. Kirk Harris, David Wettergreen, William C. Stone, and John R. Spear. 2010. “Novel Microbial Diversity Retrieved by Autonomous Robotic Exploration of the World’s Deepest Vertical Phreatic Sinkhole.” *Astrobiology* 10 (2) (March): 201–213..

Salvarezza, R. C., M. F. L. de Mele, and H. A. Videla. 1983. “Mechanisms of the Microbial Corrosion of Aluminum Alloys.” *Corrosion* 39 (1) (January): 26–32.

Sánchez-Porro, Cristina, Bhavleen Kaur, Henrietta Mann, and Antonio Ventosa. 2010. “Halomonas Titanicae Sp. Nov., a Halophilic Bacterium Isolated from the RMS Titanic.” *International Journal of Systematic and Evolutionary Microbiology* 60 (12) (December 1): 2768–2774.

Schloss, Patrick D., Dirk Gevers, and Sarah L. Westcott. 2011. “Reducing the Effects of PCR Amplification and Sequencing Artifacts on 16S rRNA-Based Studies.” *PLoS ONE* 6 (12) (December 14): e27310.

Schloss, Patrick D., Sarah L. Westcott, Thomas Ryabin, Justine R. Hall, Martin Hartmann, Emily B. Hollister, Ryan A. Lesniewski, et al. 2009. “Introducing Mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities.” *Applied and Environmental Microbiology* 75 (23) (December 1): 7537–7541.

Shi, X., R. Avci, and Z. Lewandowski. 2002. “Microbially Deposited Manganese and Iron Oxides on Passive Metals—Their Chemistry and Consequences for Material Performance.” *Corrosion* 58 (9) (September): 728–738.

Shi, Xianming, Recep Avci, and Zbigniew Lewandowski. 2002. “Electrochemistry of Passive Metals Modified by Manganese Oxides Deposited by Leptothrix Discophora: Two-step Model Verified by ToF-SIMS.” *Corrosion Science* 44 (5) (May): 1027–1045.

Sievers, Martin, Wolfgang Ludwig, and Michael Teuber. 1994. “Phylogenetic Positioning of Acetobacter, Gluconobacter, Rhodopila and Acidiphilium Species as a Branch of Acidophilic Bacteria in the A-subclass of Proteobacteria Based on 16S Ribosomal DNA Sequences.” *Systematic and Applied Microbiology* 17 (2) (August): 189–196.

Singleton, David R., Liza Guzmán Ramirez, and Michael D. Aitken. 2009. “Characterization of a Polycyclic Aromatic Hydrocarbon Degradation Gene Cluster in a Phenanthrene-Degrading Acidovorax Strain.” *Applied and Environmental Microbiology* 75 (9) (May): 2613–2620.

Šmejkalová, Hana, Tobias J. Erb, and Georg Fuchs. 2010. “Methanol Assimilation in Methylobacterium Extorquens AM1: Demonstration of All Enzymes and Their Regulation.” *PLoS ONE* 5 (10) (October 1): e13001.

Soldatova, Alexandra, Cristina Butterfield, Oyeyemi Oyerinde, Bradley Tebo, and Thomas Spiro. 2012. “Multicopper Oxidase Involvement in Both Mn(II) and Mn(III) Oxidation During Bacterial Formation of MnO<sub>2</sub>.” *Journal of Biological Inorganic Chemistry*: 1–8.

Sridhar, N., K. Price, J. Buckingham, and J. Dante. 2006. “Stress corrosion cracking of carbon steel in ethanol.” *Corrosion* 62 (8): 687–702.

Stackebrandt, Erko, Peter Schumann, Esther Schüler, and Hans Hippe. 2003. “Reclassification of Desulfotomaculum Auripigmentum as Desulfosporosinus Auripigmenti Corrig., Comb. Nov.” *International Journal of Systematic and Evolutionary Microbiology* 53 (Pt 5) (September): 1439–1443.

Stepanauskas, Ramunas. 2012. “Single Cell Genomics: An Individual Look at Microbes.” *Current Opinion in Microbiology* 15 (5) (October): 613–620.

Stevenson, Bradley S., Heather S. Drilling, Paul A. Lawson, Kathleen E. Duncan, Victoria A. Parisi, and Joseph M. Suflita. 2011. “Microbial Communities in Bulk Fluids and Biofilms of an Oil Facility Have Similar Composition but Different Structure.” *Environmental Microbiology* 13 (4): 1078–1090.

Suflita, Joseph M., Deniz F. Aktas, Athenia L. Oldham, Beatriz Monica Perez-Ibarra, and Kathleen Duncan. 2012. “Molecular Tools to Track Bacteria Responsible for Fuel Deterioration and Microbiologically Influenced Corrosion.” *Biofouling* 28 (9): 1003–1010.

Szabo, Jeffrey G., Nur Muhammad, Benjamin Packard, Greg Meiners, Paul Kefauver, and John Hall. 2009. “Bacillus Spore Uptake onto Heavily Corroded Iron Pipe in a Drinking Water Distribution System simulatorA Paper Submitted to the Journal of Environmental Engineering and Science.” *Canadian Journal of Civil Engineering* 36 (11) (November): 1867–1871.

Taylor, Mark, Jean-Baptiste Ramond, Marla Tuffin, Stephanie Burton, Kirsten Eley, and Don Cowan. 2012. “Mechanisms and Applications of Microbial Solvent Tolerance.” In *Microbial Stress Tolerance for Biofuels*, ed. Zonglin Lewis Liu, 22:177–208. Microbiology Monographs. Springer Berlin / Heidelberg.

Tebo, B. M., B. G. Clement, G. J. Dick, C. J. Hurst, R. L. Crawford, J. L. Garland, D. A. Lipson, A. L. Mills, and L. D. Stetzenbach. 2007. “Biotransformations of manganese.” (Ed.3): 1223–1238.

Tebo, Bradley M., John R. Bargar, Brian G. Clement, Gregory J. Dick, Karen J. Murray, Dorothy Parker, Rebecca Verity, and Samuel M. Webb. 2004. “Biogenic Manganese Oxides: Properties and Mechanisms of Formation.” *Annual Review of Earth and Planetary Sciences* 32 (1): 287–328.

Tebo, Bradley M., Hope A. Johnson, James K. McCarthy, and Alexis S. Templeton. 2005. “Geomicrobiology of manganese(II) Oxidation.” *Trends in Microbiology* 13 (9) (September): 421–428.

Thompson, Julie D., Toby. J. Gibson, and Des G. Higgins. 2002. “Multiple Sequence Alignment Using ClustalW and ClustalX.” In *Current Protocols in Bioinformatics*. John Wiley & Sons, Inc.

Du Toit, W. J., and I. S. Pretorius. 2002. “The occurrence, control and esoteric effect of acetic acid bacteria in winemaking.” *Annals of microbiology* 52 (2): 155–179.

Tokajian, Sima T., Fuad A. Hashwa, Ian C. Hancock, and Pierre A. Zalloua. 2005. “Phylogenetic Assessment of Heterotrophic Bacteria from a Water Distribution System Using 16S rDNA Sequencing.” *Canadian Journal of Microbiology* 51 (4): 325–335.

Uchiyama, Taku, Kimio Ito, Koji Mori, Hirohito Tsurumaru, and Shigeaki Harayama. 2010. “Iron-Corroding Methanogen Isolated from a Crude-Oil Storage Tank.” *Applied and Environmental Microbiology* 76 (6) (March 15): 1783–1788.

Urzì, Clara, Lorenzo Brusetti, Paola Salamone, Claudia Sorlini, Erko Stackebrandt, and Daniele Daffonchio. 2001. “Biodiversity of Geodermatophilaceae Isolated from Altered Stones and Monuments in the Mediterranean Basin.” *Environmental Microbiology* 3 (7): 471–479.

US DOE. 2012. “Alternative Fuels Data Center: Ethanol Fuel Basics.” *Alternative Fuels Data Center*. http://www.afdc.energy.gov/fuels/ethanol_fuel_basics.html.

US EPA, ORD. 2012. “Science Inventory | Record Report | US EPA”. Data & Tools,. Accessed October 29. http://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=241931.

Venzlaff, Hendrik, Dennis Enning, Jayendran Srinivasan, Karl J.J. Mayrhofer, Achim Walter Hassel, Friedrich Widdel, and Martin Stratmann. 2013. “Accelerated Cathodic Reaction in Microbial Corrosion of Iron Due to Direct Electron Uptake by Sulfate-reducing Bacteria.” *Corrosion Science* 66 (0) (January): 88–96. .

Vos, Paul, George Garrity, Dorothy Jones, Noel R. Krieg, Wolfgang Ludwig, Fred A. Rainey, Karl-Heinz Schleifer, and William B. Whitman. 2009. *Bergey’s Manual of Systematic Bacteriology: Volume 3: The Firmicutes*. Springer.

Waasbergen, L. G. van, M. Hildebrand, and B. M. Tebo. 1996. “Identification and Characterization of a Gene Cluster Involved in Manganese Oxidation by Spores of the Marine Bacillus Sp. Strain SG-1.” *Journal of Bacteriology* 178 (12) (June 1): 3517–3530.

Wang, Qiong, George M. Garrity, James M. Tiedje, and James R. Cole. 2007. “Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy.” *Applied and Environmental Microbiology* 73 (16) (August 15): 5261–5267.

Wang, Yong, and Pei-Yuan Qian. 2009. “Conservative Fragments in Bacterial 16S rRNA Genes and Primer Design for 16S Ribosomal DNA Amplicons in Metagenomic Studies.” *PLoS ONE* 4 (10) (October 9): e7401.

Webb, Samuel M., Gregory J. Dick, John R. Bargar, and Bradley M. Tebo. 2005. “Evidence for the Presence of Mn(III) Intermediates in the Bacterial Oxidation of Mn(II).” *Proceedings of the National Academy of Sciences of the United States of America* 102 (15) (April 12): 5558–5563.

Wei, Na, and Kevin T. Finneran. 2011. “Influence of Ferric Iron on Complete Dechlorination of Trichloroethylene (TCE) to Ethene: Fe(III) Reduction Does Not Always Inhibit Complete Dechlorination.” *Environmental Science & Technology* 45 (17) (September 1): 7422–7430.

White, Colin, Matthew Tancos, and Darren A. Lytle. 2011. “Microbial Community Profile of a Lead Service Line Removed from a Drinking Water Distribution System.” *Applied and Environmental Microbiology* 77 (15) (August 1): 5557–5561.

Whitehead, Terence R, and Michael A Cotta. 2004. “Isolation and Identification of Hyper-ammonia Producing Bacteria from Swine Manure Storage Pits.” *Current Microbiology* 48 (1) (January): 20–26.

White, Judith, Jack Gilbert, Graham Hill, Edward Hill, Susan M. Huse, Andrew J. Weightman, and Eshwar Mahenthiralingam. 2011. “Culture-Independent Analysis of Bacterial Fuel Contamination Provides Insight into the Level of Concordance with the Standard Industry Practice of Aerobic Cultivation.” *Applied and Environmental Microbiology* 77 (13) (July 1): 4527–4538.

Whyte, L. G., L. Bourbonniére, and C. W. Greer. 1997. “Biodegradation of Petroleum Hydrocarbons by Psychrotrophic Pseudomonas Strains Possessing Both Alkane (alk) and Naphthalene (nah) Catabolic Pathways.” *Applied and Environmental Microbiology* 63 (9) (September 1): 3719–3723.

Woese, Carl R., and George E. Fox. 1977. “Phylogenetic Structure of the Prokaryotic Domain: The Primary Kingdoms.” *Proceedings of the National Academy of Sciences* 74 (11) (November 1): 5088–5090.

Woese, C R. 1987. “Bacterial Evolution.” *Microbiological Reviews* 51 (2) (June): 221–271.

Woese, C. R., O. Kandler, and M. L. Wheelis. 1990. “Towards a Natural System of Organisms: Proposal for the Domains Archaea, Bacteria, and Eucarya.” *Proceedings of the National Academy of Sciences* 87 (12) (June 1): 4576–4579.

Von Wolzogen Kuehr, C. A. H., and L. S. van der Vlugt. 1934. “Graphitization of Cast Iron as an Electrobiochemical Process in Anaerobic Soils.” *Water* 18 (16): 147–165.

Xing, Peng, Liang Guo, Wei Tian, and Qinglong L Wu. 2011. “Novel Clostridium Populations Involved in the Anaerobic Degradation of Microcystis Blooms.” *The ISME Journal* 5 (5) (May): 792–800.

Yemashova, Natalia A., Valentina P. Murygina, Dmitry V. Zhukov, Arpenik A. Zakharyantz, Marina A. Gladchenko, Vasu Appanna, and Sergey V. Kalyuzhnyi. 2007. “Biodeterioration of Crude Oil and Oil Derived Products: a Review.” *Reviews in Environmental Science and Bio/Technology* 6 (4) (October 1): 315–337.

Yuan, S.J., and S.O. Pehkonen. 2007. “Microbiologically Influenced Corrosion of 304 Stainless Steel by Aerobic Pseudomonas NCIMB 2021 Bacteria: AFM and XPS Study.” *Colloids and Surfaces B: Biointerfaces* 59 (1) (September 1): 87–99.

Zhang, T., H.H.P. Fang, and B.C.B. Ko. 2003. “Methanogen Population in a Marine Biofilm Corrosive to Mild Steel.” *Applied Microbiology and Biotechnology* 63 (1): 101–106.

Zhao, He-Ping, Lei Wang, Jiao-Rong Ren, Zhuo Li, Min Li, and Hong-Wen Gao. 2008. “Isolation and Characterization of Phenanthrene-degrading Strains Sphingomonas Sp. ZP1 and Tistrella Sp. ZP5.” *Journal of Hazardous Materials* 152 (3) (April 15): 1293–1300.

Zhu, Xiang Y., John Lubeck, and John J. Kilbane. 2003. “Characterization of Microbial Communities in Gas Industry Pipelines.” *Applied and Environmental Microbiology* 69 (9) (September 1): 5354–5363.

Zobell, Claude E. 1946. “Action of Microorganisms on Hydrocarbons.” *Bacteriological Reviews* 10 (1-2) (March): 1–49.

# APPENDIX A

## PYROSEQUENCE BLAST INFORMATION

Chapter 2 describes a microbial diversity survey of automotive fuels in the United States. Table A-1 displays the top BLAST hit for representative sequences for each OTU. OTUs were created with the average neighbor clustering method in mothur (Schloss et al. 2009). Each representative sequence was BLASTed against the Silva SSURef111_NR database (Pruesse et al. 2007). The Silva SSURef111_NR database (Pruesse et al. 2007) was quality filtered as described by Pepe-Ranney, et al. (2012) and trimmed to the primer-targeted region of the 16S rRNA gene (515F-927R). Information regarding the distrubution of OTUs from each sample can be found in Chapter 2. A phylum-level distribution of OTUs in each sample can be found in Figure 2.1. A bar chart displaying the distribution of OTUs for each sample is displayed in Figure 2.3, and Figure 2.4 displays a heat map of the 50 most abundant taxa identified in the fuel samples via 16S rRNA gene pyrosequencing.

Table A-1: Top BLAST hits for OTUs described in Chapter 2.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 1 | 100 | Z76666 | P.pseudoalcaligenes (strain LMG 1225T) 16S rRNA gene |
| 2 | 99.47 | HE575932 | Corynebacterium sp. 20.8 KSS partial 16S rRNA gene |
| 3 | 98.13 | AB614557 | Uncultured bacterium gene for 16S rRNA |
| 4 | 100 | JQ350845 | Pseudomonas putida strain SNIST 16S ribosomal RNA gene |
| 5 | 100 | AJ811961 | Flavobacterium fryxellicola 16S rRNA gene |
| 6 | 98.93 | FJ436724 | Salinicoccus sp. ZXM223 16S ribosomal RNA gene |
| 7 | 99.2 | JN368471 | Lactobacillus brevis strain ABRIINW-K 16S ribosomal RNA gene |
| 8 | 99.2 | JN854162 | Pinus roxburghii isolate ROXB04 chloroplast |
| 9 | 100 | FN556578 | Enterobacteriaceae bacterium JW72.7a partial 16S rRNA gene |
| 10 | 99.46 | JF733789 | Bacterium 1/3/6 16S ribosomal RNA gene |
| 11 | 99.2 | GQ246607 | Corallococcus exiguus strain HBUM94202 16S ribosomal RNA gene |
| 12 | 99.47 | Y11330 | K.erythromyxa 16S rRNA gene |
| 13 | 97.59 | AY741146 | Azospirillum amazonense strain 21R 16S ribosomal RNA gene |
| 14 | 98.66 | DQ426701 | Bacterium GNCr-2 16S ribosomal RNA gene |
| 15 | 100 | EU370418 | Pseudomonas sp. 108Z1 16S ribosomal RNA gene |
| 16 | 98.93 | HE575976 | uncultured bacterium partial 16S rRNA gene |
| 17 | 99.73 | AY219713 | Petrobacter succinimandens BON4 16S ribosomal RNA gene |
| 18 | 99.73 | FJ679899 | Uncultured bacterium clone 4-3F18 16S ribosomal RNA gene |
| 19 | 100 | AY922995 | Halomonas phoceae strain CCUG 5096 16S ribosomal RNA gene |
| 20 | 99.73 | JQ033866 | Uncultured Peptostreptococcaceae bacterium clone SRO176C10c 16S ribosomal RNA gene |
| 21 | 100 | EU449560 | Uncultured Rhodanobacter sp. clone Plot4-2F01 16S ribosomal RNA gene |
| 22 | 92.49 | BA000039 | Thermosynechococcus elongatus BP-1 DNA |
| 23 | 98.93 | JN032380 | Uncultured Bacillus sp. clone WeeA_F02 16S ribosomal RNA gene |
| 24 | 99.73 | JN618337 | Phenylobacterium sp. 1.11185 16S ribosomal RNA gene |
| 25 | 99.73 | GU179843 | Uncultured Firmicutes bacterium clone D022041H05 16S ribosomal RNA gene |
| 26 | 99.47 | JF228179 | Uncultured bacterium clone ncd2607d08c1 16S ribosomal RNA gene |
| 27 | 100 | JN644603 | Delftia lacustris strain HG7_3A 16S ribosomal RNA gene |
| 28 | 98.93 | GQ416919 | Uncultured Carnobacteriaceae bacterium clone F1aug.1 16S ribosomal RNA gene |
| 29 | 99.46 | EU740417 | Salinisphaera sp. EPR72 16S ribosomal RNA gene |
| 30 | 98.11 | DQ196634 | Desulfovibrio sp. BL-157 16S ribosomal RNA gene |
| 31 | 100 | JF706521 | Sphingomonas sp. PDD-35b-8 16S ribosomal RNA gene |
| 32 | 99.73 | HQ753315 | Uncultured organism clone ELU0034-T174-S-NIPCRAMgANb_000570 small subunit ribosomal RNA gene |
| 33 | 98.93 | AB294176 | Alkalibacterium psychrotolerans gene for 16S rRNA |
| 34 | 98.93 | EU266828 | Uncultured Rhodocyclaceae bacterium clone D12_22 small subunit ribosomal RNA gene |
| 35 | 99.73 | JN367254 | Uncultured Lysobacter sp. clone SeqSEEZ234 16S ribosomal RNA gene |
| 36 | 99.73 | FR682740 | Hymenobacter sp. R-36553 partial 16S rRNA gene |
| 37 | 100 | HM224494 | Paracoccus sp. TPD47 16S ribosomal RNA gene |
| 38 | 100 | FR682758 | Deinococcus sp. R-36713 partial 16S rRNA gene |
| 39 | 99.47 | DQ985059 | Ornithinimicrobium sp. JL1084 16S ribosomal RNA gene |
| 40 | 99.2 | FM874283 | Uncultured bacterium partial 16S rRNA gene |
| 41 | 98.93 | HM334212 | Uncultured bacterium clone ncd981g09c1 16S ribosomal RNA gene |
| 42 | 99.47 | JF198244 | Uncultured bacterium clone ncd2310f09c1 16S ribosomal RNA gene |
| 43 | 100 | X81665 | A.lwoffii 16S rRNA gene (DSM2403) |
| 44 | 99.2 | HM126856 | Uncultured bacterium clone SINI451 16S ribosomal RNA gene |
| 45 | 99.47 | DQ121391 | Nocardioides lentus strain KSL-19 16S ribosomal RNA gene |
| 46 | 100 | HM276817 | Uncultured bacterium clone ncd529b10c1 16S ribosomal RNA gene |
| 47 | 100 | GQ240639 | Acetobacter pasteurianus strain DSM 2347 16S ribosomal RNA gene |
| 48 | 98.66 | HM322821 | Uncultured bacterium clone ncd403h09c1 16S ribosomal RNA gene |
| 49 | 98.93 | HE575970 | uncultured bacterium partial 16S rRNA gene |
| 50 | 98.94 | AY996854 | Beutenbergia sp. 91196 16S ribosomal RNA gene |
| 51 | 100 | EU868854 | Chromohalobacter sp. C3-1 16S ribosomal RNA gene |
| 52 | 100 | AB220104 | Methylobacterium sp. PB282 gene for 16S rRNA |
| 53 | 99.73 | AY279980 | Spirosoma escalantus strain HDK201 16S ribosomal RNA gene |
| 54 | 100 | M59064 | Pseudomonas diminuta 16S ribosomal RNA. |
| 55 | 100 | DQ532236 | Uncultured bacterium clone JSC8-D10 16S ribosomal RNA gene |
| 56 | 99.47 | EU888580 | Arsenicicoccus bolidensis strain FR2_MS13c 16S ribosomal RNA gene |
| 57 | 97.07 | JF121474 | Uncultured bacterium clone ncd1391e06c2 16S ribosomal RNA gene |
| 58 | 99.46 | HM263290 | Uncultured bacterium clone ncd263c08c1 16S ribosomal RNA gene |
| 59 | 99.46 | DQ222224 | Bradyrhizobium sp. CCBAU 71283012 16S ribosomal RNA gene |
| 60 | 100 | DQ672568 | Skermanella aerolata strain 5416T-32 16S ribosomal RNA gene |
| 61 | 98.91 | FN421489 | Uncultured bacterium partial 16S rRNA gene |
| 62 | 97.57 | AJ630296 | Unidentified Cytophagales/green sulfur bacterium OPB56 partial 16S rRNA gene |
| 63 | 99.2 | JF417728 | Uncultured bacterium clone QQSB09 16S ribosomal RNA gene |
| 64 | 99.73 | X90830 | Nocardioides sp. 16S rRNA gene |
| 65 | 99.2 | FJ595102 | Rathayibacter tritici strain ICPB70004 (FH-5) 16S ribosomal RNA gene |
| 66 | 100 | FN421540 | Uncultured bacterium partial 16S rRNA gene |
| 67 | 99.46 | DQ129634 | Uncultured bacterium clone AKIW412 16S ribosomal RNA gene |
| 68 | 99.73 | HQ641379 | Belnapia sp. CPCC 100156 16S ribosomal RNA gene |
| 69 | 100 | EF661571 | Roseomonas aerilata strain 5420S-30 16S ribosomal RNA gene |
| 70 | 100 | HQ012835 | Acetobacterium sp. enrichment culture clone DhR^2/LM-A07 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 71 | 100 | EU647258 | Methylocystis sp. B14 16S ribosomal RNA gene |
| 72 | 99.73 | JN090860 | Hymenobacter sp. DCY57 16S ribosomal RNA gene |
| 73 | 98.92 | HM565030 | Uncultured Niastella sp. clone H-133 16S ribosomal RNA gene |
| 74 | 99.2 | HQ433569 | Uncultured bacterium clone GOP_R 16S ribosomal RNA gene |
| 75 | 99.46 | EF559168 | Uncultured bacterium clone G35_D8_H_B_A08 16S ribosomal RNA gene |
| 76 | 100 | JN021530 | Pandoraea pnomenusa strain LX-1 16S ribosomal RNA gene |
| 77 | 99.19 | HM366478 | Uncultured bacterium clone ADB-18 16S ribosomal RNA gene |
| 78 | 99.19 | EF090267 | Sphingobacterium spiritivorum strain NCTC 11386 16S ribosomal RNA gene |
| 79 | 96.54 | GU594676 | Uncultured bacterium clone P2-DW53-100 16S ribosomal RNA gene |
| 80 | 97.62 | U41563 | Geothrix fermentans 16S rRNA gene |
| 81 | 99.73 | Y18836 | Hymenobacter gelipurpurascens 16S rRNA gene |
| 82 | 99.46 | GQ448629 | Uncultured bacterium clone calf784_6wks_grp2_C03 16S ribosomal RNA gene |
| 83 | 100 | HM835438 | Uncultured bacterium clone nby564b09c1 16S ribosomal RNA gene |
| 84 | 97.55 | FM209353 | Uncultured bacterium partial 16S rRNA gene |
| 85 | 99.19 | EU522663 | Uncultured actinobacterium clone Nap1-8C 16S ribosomal RNA gene |
| 86 | 99.47 | HQ256839 | Microbacteriaceae bacterium PDD-32b-35 16S ribosomal RNA gene |
| 87 | 97.04 | JN038271 | Uncultured Bacteroidetes bacterium clone EK_An348 16S ribosomal RNA gene |
| 88 | 98.92 | JF417834 | Uncultured bacterium clone ZQMB08 16S ribosomal RNA gene |
| 89 | 99.2 | JF182978 | Uncultured bacterium clone ncd2119f12c1 16S ribosomal RNA gene |
| 90 | 99.2 | JN793954 | Planifilum sp. P8 16S ribosomal RNA gene |
| 91 | 100 | JF706512 | Methylobacterium sp. PDD-35b-27 16S ribosomal RNA gene |
| 92 | 98.93 | HQ246262 | Bacillus sp. 7A9S1 16S ribosomal RNA gene |
| 93 | 100 | AJ519622 | Uncultured alpha proteobacterium partial 16S rRNA gene |
| 94 | 99.73 | AY133079 | Uncultured spirochete clone ccslm226 16S ribosomal RNA gene |
| 95 | 100 | JF156769 | Uncultured bacterium clone ncd1815b08c1 16S ribosomal RNA gene |
| 96 | 100 | JN989297 | Achromobacter sp. A4067 16S ribosomal RNA gene |
| 97 | 98.93 | HM238122 | Uncultured Firmicutes bacterium clone FF_34 16S ribosomal RNA gene |
| 98 | 99.73 | FJ625375 | Uncultured bacterium clone HF_NC_36 16S ribosomal RNA gene gene |
| 99 | 99.73 | GQ396950 | Uncultured bacterium clone AK4AB2_12B 16S ribosomal RNA gene |
| 100 | 99.46 | FJ593846 | Rhizobium sp. GB_080331_06 16S ribosomal RNA gene |
| 101 | 99.2 | GU355644 | Clostridiaceae bacterium OCF 9 16S ribosomal RNA gene |
| 102 | 100 | JF496488 | Massilia dura strain WA5-4 16S ribosomal RNA gene |
| 103 | 99.47 | X84446 | Corynebacterium xerosis partial 16S rRNA gene |
| 104 | 99.46 | JF970583 | Brevibacillus centrosporus 16S ribosomal RNA gene |
| 105 | 97.32 | EU861889 | Uncultured soil bacterium clone bacnit32 16S ribosomal RNA gene |
| 106 | 100 | EF157133 | Uncultured bacterium clone 91-78 16S ribosomal RNA gene |
| 107 | 98.65 | AF445698 | Uncultured CFB group bacterium clone SM1G04 16S ribosomal RNA gene |
| 108 | 99.73 | JF178437 | Uncultured bacterium clone ncd2075f08c1 16S ribosomal RNA gene |
| 109 | 99.73 | JF505948 | Dietzia psychralcaliphila strain KNUC9014 16S ribosomal RNA gene |
| 110 | 96.69 | GQ135277 | Uncultured bacterium clone 3g03 16S ribosomal RNA gene |
| 111 | 99.46 | FR669217 | Uncultured bacterium partial 16S rRNA gene |
| 112 | 99.2 | JN020221 | Uncultured Acidobacteria bacterium clone cher6_2B_78 small subunit ribosomal RNA gene |
| 113 | 97.84 | Y13043 | D.murrayi 16S rRNA gene |
| 114 | 95.16 | JF177366 | Uncultured bacterium clone ncd2059b03c1 16S ribosomal RNA gene |
| 115 | 97.06 | HM274427 | Uncultured bacterium clone ncd555b05c1 16S ribosomal RNA gene |
| 116 | 98.93 | AY831385 | Quadrisphaera granulorum strain AG019 16S ribosomal RNA gene |
| 117 | 99.19 | EF612405 | Uncultured alpha proteobacterium clone OS-C99 16S ribosomal RNA gene |
| 118 | 100 | JN866565 | Uncultured bacterium clone HLC6 16S ribosomal RNA gene |
| 119 | 99.73 | HM445934 | Uncultured bacterium clone W-16S-3 16S ribosomal RNA gene |
| 120 | 99.46 | HM278213 | Uncultured bacterium clone ncd552a05c1 16S ribosomal RNA gene |
| 121 | 100 | HQ010830 | Uncultured bacterium clone F_SBR_55 16S ribosomal RNA gene |
| 122 | 98.65 | JQ342929 | Paenibacillus agaridevorans strain GYB23 16S ribosomal RNA gene |
| 123 | 100 | HQ716356 | Uncultured bacterium clone T1WK059 16S ribosomal RNA gene |
| 124 | 100 | JN038240 | Uncultured Moraxellaceae bacterium clone EK_CK524 16S ribosomal RNA gene |
| 125 | 97.59 | X71858 | C.polysaccharolyticum gene for 16S ribosomal RNA |
| 126 | 98.93 | JN366734 | Bacillus coagulans strain 55N1-6 16S ribosomal RNA gene |
| 127 | 100 | JQ085635 | Uncultured bacterium clone VHW_F_L9 16S ribosomal RNA gene |
| 128 | 98.93 | FJ948107 | Luteimonas sp. BZ92r 16S ribosomal RNA gene |
| 129 | 100 | EF133555 | Chelatococcus sp. 'enrichment culture MARBPYR' 16S ribosomal RNA gene |
| 130 | 98.65 | EU469117 | Uncultured bacterium clone SP3_g03 16S ribosomal RNA gene |
| 131 | 99.2 | X77958 | K.aurantiacus (IFO 15268) 16S rRNA gene. |
| 132 | 99.73 | FN908504 | Sphingobacterium sp. WS 4556 partial 16S rRNA gene |
| 133 | 100 | JN685473 | Uncultured bacterium clone NK-Q16 16S ribosomal RNA gene |
| 134 | 95.43 | AM981378 | Uncultured Salinibacter sp. partial 16S rRNA |
| 135 | 98.93 | JF193804 | Uncultured bacterium clone ncd2240g12c1 16S ribosomal RNA gene |
| 136 | 99.73 | JN217198 | Uncultured bacterium clone TERI-KL72 16S ribosomal RNA gene |
| 137 | 99.47 | HM366521 | Uncultured bacterium clone ADB-61 16S ribosomal RNA gene |
| 138 | 98.39 | U88044 | Amaricoccus tamworthensis 16S ribosomal RNA gene |
| 139 | 97.29 | FN436088 | Uncultured bacterium partial 16S rRNA gene |
| 140 | 99.73 | AB374370 | Uncultured endolithic bacterium gene for 16S rRNA |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 141 | 97.3 | EF157089 | Uncultured bacterium clone 91-112 16S ribosomal RNA gene |
| 142 | 99.19 | Y10109 | M.gryphiswaldense SSU rRNA gene |
| 143 | 99.47 | FJ671906 | Uncultured bacterium clone Ll142-2G11 16S ribosomal RNA gene |
| 144 | 99.47 | JN013936 | Uncultured bacterium clone X1-15 16S ribosomal RNA gene |
| 145 | 100 | AY363245 | Hydrocarboniphaga effusa strain AP103 16S ribosomal RNA gene |
| 146 | 100 | EU864470 | Uncultured bacterium clone E46 16S ribosomal RNA gene |
| 147 | 99.46 | FM872958 | Uncultured bacterium partial 16S rRNA gene |
| 148 | 100 | AF517773 | Hyperthermophilic bacterium OGL-7B 16S ribosomal RNA gene |
| 149 | 99.73 | HM214546 | Deinococcus reticulitermitis strain TM-1 16S ribosomal RNA gene |
| 150 | 98.11 | JF181758 | Uncultured bacterium clone ncd2099h11c1 16S ribosomal RNA gene |
| 151 | 99.47 | HM173633 | Thiobacillus thioparus strain Pankhurst T4 16S ribosomal RNA (rrs) gene |
| 152 | 99.46 | GU295968 | Flavobacterium sp. ARSA-108 16S ribosomal RNA gene |
| 153 | 96.47 | HM298801 | Uncultured bacterium clone ncd899b02c1 16S ribosomal RNA gene |
| 154 | 97.3 | FJ152959 | Uncultured bacterium clone TX4CB_90 16S ribosomal RNA gene |
| 155 | 100 | JN381552 | Providencia sp. SRS82 16S ribosomal RNA gene |
| 156 | 99.2 | JF417822 | Uncultured bacterium clone QQSB103 16S ribosomal RNA gene |
| 157 | 99.73 | AM934735 | Uncultured Chromatiales bacterium partial 16S rRNA gene |
| 158 | 100 | JN020187 | Uncultured alpha proteobacterium clone cher4_1B_11 small subunit ribosomal RNA gene |
| 159 | 98.93 | FM873018 | Uncultured bacterium partial 16S rRNA gene |
| 160 | 99.73 | HQ674839 | Uncultured Steroidobacter sp. clone MWM3-4 16S ribosomal RNA gene |
| 161 | 97.07 | DQ129588 | Uncultured bacterium clone AKIW600 16S ribosomal RNA gene |
| 162 | 99.2 | HQ595216 | Uncultured Acidobacteria bacterium clone IC3076 16S ribosomal RNA gene |
| 163 | 97.59 | HM264029 | Uncultured bacterium clone ncd297g07c1 16S ribosomal RNA gene |
| 164 | 99.19 | DQ386220 | Halanaerobiaceae bacterium Benz1 16S ribosomal RNA gene |
| 165 | 94.37 | JF776915 | Uncultured alpha proteobacterium clone QZ-J4 16S ribosomal RNA gene |
| 166 | 99.46 | JF148534 | Uncultured bacterium clone ncd1689a10c1 16S ribosomal RNA gene |
| 167 | 100 | EF126993 | Flavobacterium cucumis strain R2A45-3 16S ribosomal RNA gene |
| 168 | 100 | HQ834240 | Pseudacidovorax sp. NH-1 16S ribosomal RNA gene |
| 169 | 99.2 | Z94012 | S.macedonicus DNA for 16S ribosomal RNA |
| 170 | 94.93 | JF177332 | Uncultured bacterium clone ncd2058h11c1 16S ribosomal RNA gene |
| 171 | 98.93 | EU794246 | Uncultured Firmicutes bacterium clone EMP_M38 16S ribosomal RNA gene |
| 172 | 99.73 | JF417757 | Uncultured bacterium clone QQSB38 16S ribosomal RNA gene |
| 173 | 100 | AY133091 | Uncultured low G+C Gram-positive bacterium clone ccslm238 16S ribosomal RNA gene |
| 174 | 99.73 | DQ125826 | Uncultured bacterium clone AKAU4049 16S ribosomal RNA gene |
| 175 | 99.46 | EU794298 | Uncultured Bacteroides sp. clone EMP_Z12 16S ribosomal RNA gene |
| 176 | 99.73 | FR749985 | Eubacterium tenue partial 16S rRNA gene |
| 177 | 99.73 | AB636985 | Uncultured bacterium gene for 16S rRNA |
| 178 | 98.93 | AB066346 | Bacillus sp. TH64 gene for 16S rRNA |
| 179 | 100 | HM041946 | Uncultured Synergistetes bacterium clone NRB29 16S ribosomal RNA gene |
| 180 | 100 | JF223534 | Uncultured bacterium clone ncd2786c04c1 16S ribosomal RNA gene |
| 181 | 99.47 | JN378723 | Curtobacterium flaccumfaciens strain 2 |
| 182 | 100 | HQ260323 | Pseudomonas sp. PDD-24b-12 16S ribosomal RNA gene |
| 183 | 100 | GQ358825 | Uncultured bacterium clone 1530-P-8D 16S ribosomal RNA gene |
| 184 | 96.71 | FJ672328 | Uncultured bacterium clone Ll142-3N1 16S ribosomal RNA gene |
| 185 | 99.2 | JN660076 | Lactobacillus sp. TRF8 16S ribosomal RNA gene |
| 186 | 97.86 | JF227118 | Uncultured bacterium clone ncd2590h08c1 16S ribosomal RNA gene |
| 187 | 100 | HM131832 | Nitrospira sp. enrichment culture clone LPTV-S11 16S ribosomal RNA gene |
| 188 | 100 | AM696989 | Uncultured bacterium partial 16S rRNA gene |
| 189 | 100 | JF129310 | Uncultured bacterium clone ncd1577f03c1 16S ribosomal RNA gene |
| 190 | 96.26 | FJ825494 | Uncultured bacterium clone 189_BE5_14 16S ribosomal RNA gene |
| 191 | 98.93 | AY093470 | Uncultured bacterium clone MB-B2-105 16S ribosomal RNA |
| 192 | 99.47 | JF178438 | Uncultured bacterium clone ncd2075f12c1 16S ribosomal RNA gene |
| 193 | 94.1 | HM362532 | Uncultured bacterium clone BG123 16S ribosomal RNA gene |
| 194 | 100 | X74674 | A.caviae (ATCC 15468T) gene for 16S ribosomal RNA |
| 195 | 100 | FN794237 | Uncultured bacterium partial 16S rRNA gene |
| 196 | 98.38 | JF165260 | Uncultured bacterium clone ncd1923d10c1 16S ribosomal RNA gene |
| 197 | 98.93 | EU135614 | Salinimicrobium terrae strain YIM-C338 16S ribosomal RNA gene |
| 198 | 99.73 | HM111817 | Uncultured alpha proteobacterium clone SHNZ686 16S ribosomal RNA gene |
| 199 | 95.15 | AJ888562 | Uncultured Bacteroidetes partial 16S rRNA gene |
| 200 | 97.59 | HM008785 | Uncultured bacterium clone Camel_AAR_079 16S ribosomal RNA gene |
| 201 | 98.66 | EU776336 | Uncultured bacterium clone KO2_aai18h10 16S ribosomal RNA gene |
| 202 | 97.03 | JF176890 | Uncultured bacterium clone ncd2051e04c2 16S ribosomal RNA gene |
| 203 | 99.73 | HQ183746 | Uncultured Thermoflavimicrobium sp. clone De384 16S ribosomal RNA gene |
| 204 | 97.25 | JN680639 | Uncultured Erysipelotrichi bacterium clone SL127 16S ribosomal RNA gene |
| 205 | 99.46 | HM269537 | Uncultured bacterium clone ncd251a06c1 16S ribosomal RNA gene |
| 206 | 99.73 | AB175385 | Uncultured bacterium gene for 16S rRNA |
| 207 | 94.4 | JF175679 | Uncultured bacterium clone ncd2031e10c1 16S ribosomal RNA gene |
| 208 | 98.66 | DQ129366 | Uncultured bacterium clone AKIW942 16S ribosomal RNA gene |
| 209 | 100 | JF220905 | Uncultured bacterium clone ncd2657a09c1 16S ribosomal RNA gene |
| 210 | 99.46 | EU459880 | Uncultured bacterium clone SQ_aah80f05 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 211 | 100 | JF496359 | Massilia niastensis strain A6-12 16S ribosomal RNA gene |
| 212 | 100 | HQ697733 | Uncultured bacterium clone Bms_MS122 16S ribosomal RNA gene |
| 213 | 96.27 | HM269861 | Uncultured bacterium clone ncd256c04c1 16S ribosomal RNA gene |
| 214 | 99.46 | FN667048 | Uncultured compost bacterium partial 16S rRNA gene |
| 215 | 100 | JN411342 | Gulbenkiania mobilis strain IARI-MB-18 16S ribosomal RNA gene |
| 216 | 95.15 | AY192276 | Uncultured candidate division OP10 bacterium clone W1-4H 16S ribosomal RNA gene |
| 217 | 98.93 | HQ190391 | Uncultured bacterium clone BR14 16S ribosomal RNA gene |
| 218 | 98.93 | AB294346 | Uncultured bacterium gene for 16S rRNA |
| 219 | 99.47 | HM748600 | Brevibacterium luteolum strain BS 1-6 16S ribosomal RNA gene |
| 220 | 97.57 | JF215085 | Uncultured bacterium clone ncd2492d04c1 16S ribosomal RNA gene |
| 221 | 95.19 | JN038858 | Uncultured delta proteobacterium clone P-R71 16S ribosomal RNA gene |
| 222 | 98.93 | HM319084 | Uncultured bacterium clone ncd347e04c1 16S ribosomal RNA gene |
| 223 | 99.73 | X80618 | Rhodococcus erythropolis 16S rRNA gene |
| 224 | 97.29 | EU888000 | Uncultured Clostridia bacterium clone L47 16S ribosomal RNA gene |
| 225 | 97.34 | EF157216 | Uncultured bacterium clone 101-50 16S ribosomal RNA gene |
| 226 | 99.46 | DQ177475 | Hymenobacter psychrotolerans strain Tibet-IIU11 16S ribosomal RNA gene |
| 227 | 99.73 | FJ152768 | Uncultured bacterium clone TX5A_60 16S ribosomal RNA gene |
| 228 | 98.92 | EU835464 | Uncultured bacterium clone 3M02 16S ribosomal RNA gene |
| 229 | 99.19 | JF496493 | Hymenobacter xinjiangensis strain WA6-1 16S ribosomal RNA gene |
| 230 | 96.48 | EF559008 | Uncultured bacterium clone C55_D6_L_B_B09 16S ribosomal RNA gene |
| 231 | 98.65 | FJ716071 | Uncultured bacterium clone LC108 16S ribosomal RNA gene |
| 232 | 98.93 | FN428677 | Geobacillus thermoglucosidasius partial 16S rRNA gene |
| 233 | 98.12 | HM323381 | Uncultured bacterium clone ncd411g11c1 16S ribosomal RNA gene |
| 234 | 97.33 | GU454881 | Uncultured bacterium clone ambient_uncontrolled-20 16S ribosomal RNA gene |
| 235 | 95.45 | Y12373 | Thiocapsa pfennigii 16S rRNA gene |
| 236 | 99.46 | FR749893 | Clostridium malenominatum partial 16S rRNA gene |
| 237 | 97.87 | JQ337487 | Uncultured bacterium clone EE-2-A8 16S ribosomal RNA gene |
| 238 | 99.2 | HM828724 | Uncultured bacterium clone nby479g01c1 16S ribosomal RNA gene |
| 239 | 99.73 | L09187 | Clostridium fervidus (ATCC 43204) 16S ribosomal RNA (16S rRNA) gene. |
| 240 | 96.79 | HQ119202 | Uncultured bacterium isolate 1112865250914 16S ribosomal RNA gene |
| 241 | 99.2 | HQ743286 | Uncultured organism clone ELU0014-T176-S-NIPCRAMgANa_000239 small subunit ribosomal RNA gene |
| 242 | 96.8 | AB487112 | Uncultured bacterium gene for 16S ribosomal RNA |
| 243 | 95.14 | FJ891053 | Uncultured bacterium clone AY6_23 16S ribosomal RNA gene |
| 244 | 98.13 | AB487787 | Uncultured bacterium gene for 16S ribosomal RNA |
| 245 | 97.03 | AB491722 | Uncultured bacterium gene for 16S ribosomal RNA |
| 246 | 95.97 | JQ303016 | Cytophagaceae bacterium R9-9 16S ribosomal RNA gene |
| 247 | 98.12 | AB195873 | Uncultured bacterium gene for 16S rRNA |
| 248 | 99.46 | DQ001644 | Uncultured bacterium clone E101-45 16S ribosomal RNA gene |
| 249 | 99.46 | FN667436 | Uncultured compost bacterium partial 16S rRNA gene |
| 250 | 98.12 | HQ121112 | Uncultured bacterium isolate 1112863845165 16S ribosomal RNA gene |
| 251 | 99.46 | AEPD01000042 | Prevotella buccae ATCC 33574 contig00047 |
| 252 | 99.46 | GU179801 | Uncultured bacterium clone D004024G11 16S ribosomal RNA gene |
| 253 | 99.47 | HQ598991 | Uncultured Acidobacteria bacterium clone SEW_08_283 16S ribosomal RNA gene |
| 254 | 99.2 | JN516012 | Uncultured organism clone SBZF_9205 16S ribosomal RNA gene |
| 255 | 95.95 | HQ242576 | Uncultured Bacteroidetes/Chlorobi group bacterium clone M7LBP3_10C05 16S ribosomal RNA gene |
| 256 | 97.57 | HM450026 | Uncultured bacterium clone WM06x_B2D 16S ribosomal RNA gene |
| 257 | 96.61 | EU794202 | Uncultured Lachnospiraceae bacterium clone EMP_L40 16S ribosomal RNA gene |
| 258 | 94.03 | HQ190444 | Uncultured bacterium clone BR91 16S ribosomal RNA gene |
| 259 | 98.38 | AJ133796 | Syntrophus sp. 16S rRNA gene |
| 260 | 95.72 | EU132874 | Uncultured bacterium clone FFCH14732 16S ribosomal RNA gene |
| 261 | 98.93 | HQ405607 | Bacterium enrichment culture clone AOM-SR-B16 16S ribosomal RNA gene |
| 262 | 99.46 | JF834124 | Uncultured bacterium clone BYND-5-6 16S ribosomal RNA gene |
| 263 | 95.97 | EF018660 | Uncultured planctomycete clone Amb_16S_946 16S ribosomal RNA gene |
| 264 | 92.88 | FJ478545 | Uncultured bacterium clone p9b24ok 16S ribosomal RNA gene |
| 265 | 97.59 | FR853407 | Uncultured bacterium partial 16S rRNA gene |
| 266 | 97.32 | AF524858 | Bacterium K-4b6 16S ribosomal RNA gene |
| 267 | 99.19 | JF262044 | Thermoanaerobacterales bacterium SGL43 16S ribosomal RNA gene |
| 268 | 94.89 | AB307643 | Uncultured bacterium gene for 16S rRNA |
| 269 | 93.03 | GQ406147 | Uncultured alpha proteobacterium clone AS-45-1 16S ribosomal RNA gene |
| 270 | 96.44 | GQ138450 | Uncultured bacterium clone 01f11 16S ribosomal RNA gene |
| 271 | 98.67 | JN825448 | Uncultured Gemmatimonadetes bacterium clone Alchichica_AQ2_2_1B_50 small subunit ribosomal RNA gene |
| 272 | 100 | AM420075 | Uncultured Fusobacterium sp. partial 16S rRNA gene |
| 273 | 100 | HQ754367 | Uncultured organism clone ELU0036-T191-S-N1_000091 small subunit ribosomal RNA gene |
| 274 | 94.89 | JF175112 | Uncultured bacterium clone ncd2021e07c1 16S ribosomal RNA gene |
| 275 | 99.47 | JF235469 | Uncultured bacterium clone ncd2724a10c1 16S ribosomal RNA gene |
| 276 | 98.66 | X70954 | P.propionicus 16S rRNA |
| 277 | 98.11 | HM268966 | Uncultured bacterium clone ncd241b12c1 16S ribosomal RNA gene |
| 278 | 95.76 | HQ740302 | Uncultured organism clone ELU0008-T58-S-NI_000395 small subunit ribosomal RNA gene |
| 279 | 98.92 | EU474827 | Uncultured bacterium clone TAK_aaa03d01.Contig2 16S ribosomal RNA gene |
| 280 | 96.79 | JF116337 | Uncultured bacterium clone ncd1350h08c1 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 281 | 95.72 | HM186231 | Uncultured bacterium clone HDB_SIOO1047 16S ribosomal RNA gene |
| 282 | 99.2 | HE576794 | Megasphaera elsdenii strain DSM 20460 draft genome |
| 283 | 94.92 | FJ437927 | Uncultured bacterium clone FGL7S_B139 16S ribosomal RNA gene |
| 284 | 100 | FN421900 | Uncultured bacterium partial 16S rRNA gene |
| 285 | 97.06 | FM177580 | Rhodocista pekingensis partial 16S rRNA gene |
| 286 | 99.46 | GU214129 | Uncultured Deinococcus sp. clone IAFpp7118 16S ribosomal RNA gene |
| 287 | 91.22 | HM565054 | Uncultured Chloroflexi bacterium clone N-229 16S ribosomal RNA gene |
| 288 | 91.47 | HQ640569 | Uncultured bacterium clone B91 16S ribosomal RNA gene |
| 289 | 88.14 | JN387499 | Uncultured microorganism clone Group54_e 16S ribosomal RNA gene |
| 290 | 99.47 | JN387530 | Uncultured microorganism clone Group61_c 16S ribosomal RNA gene |
| 291 | 98.67 | EF516309 | Uncultured bacterium clone FCPT556 16S ribosomal RNA gene |
| 292 | 100 | HQ788263 | Uncultured organism clone ELU0112-T277-S-NI_000247 small subunit ribosomal RNA gene |
| 293 | 97.34 | FJ479212 | Uncultured bacterium clone p11i01ok 16S ribosomal RNA gene |
| 294 | 93.09 | EU134116 | Uncultured bacterium clone FFCH14012 16S ribosomal RNA gene |
| 295 | 96.79 | FJ769440 | Uncultured bacterium clone FP_B12 16S ribosomal RNA gene |
| 296 | 98.92 | GQ356960 | Uncultured bacterium clone Fe_B_143 16S ribosomal RNA gene |
| 297 | 97.33 | HQ121228 | Uncultured bacterium isolate 1112863845264 16S ribosomal RNA gene |
| 298 | 96.26 | JF176663 | Uncultured bacterium clone ncd2046g02c1 16S ribosomal RNA gene |
| 299 | 97.86 | EU134456 | Uncultured bacterium clone FFCH12847 16S ribosomal RNA gene |
| 300 | 97.32 | GQ889475 | Gemmata sp. SD2-5 16S ribosomal RNA gene |
| 301 | 96 | FJ466076 | Uncultured bacterium clone B208 16S ribosomal RNA gene |
| 302 | 93.5 | JN854198 | Pinus greggii isolate GREG02 chloroplast |
| 303 | 96.28 | HM333963 | Uncultured bacterium clone ncd1129e02c1 16S ribosomal RNA gene |
| 304 | 93.32 | AB630921 | Uncultured bacterium gene for 16S ribosomal RNA |
| 305 | 99.73 | JN084209 | Bacteroides vulgatus strain NMBE-6 16S ribosomal RNA gene |
| 306 | 97.06 | HM278693 | Uncultured bacterium clone ncd559b07c1 16S ribosomal RNA gene |
| 307 | 98.66 | Y13671 | Sulfurospirillum deleyianum 16S rRNA gene |
| 308 | 99.19 | EU463264 | Uncultured bacterium clone molerat_aai72e10 16S ribosomal RNA gene |
| 309 | 98.93 | JF429007 | Uncultured bacterium clone G73 16S ribosomal RNA gene |
| 310 | 96.81 | JF267661 | Uncultured bacterium clone V6_1 16S ribosomal RNA gene |
| 311 | 99.19 | HQ762329 | Uncultured organism clone ELU0049-T299-S-NIPCRAMgANa_000495 small subunit ribosomal RNA gene |
| 312 | 97.06 | AB603831 | Uncultured Firmicutes bacterium gene for 16S rRNA |
| 313 | 98.66 | GQ082178 | Uncultured bacterium clone nbw1201c01c1 16S ribosomal RNA gene |
| 314 | 91.6 | AB079645 | Green non-sulfur bacterium B1-5 gene for 16S ribosomal RNA |
| 315 | 97.86 | EU887965 | Uncultured Clostridia bacterium clone S21 16S ribosomal RNA gene |
| 316 | 93.58 | EF063624 | Uncultured bacterium clone Eb85 16S ribosomal RNA gene |
| 317 | 99.46 | AB637329 | Uncultured bacterium gene for 16S rRNA |
| 318 | 99.2 | AY349404 | Selenomonas sp. oral clone GI064 16S ribosomal RNA gene |
| 319 | 98.4 | HQ910326 | Uncultured bacterium clone P-11_B19 16S ribosomal RNA gene |
| 320 | 98.91 | JF171142 | Uncultured bacterium clone ncd1960f07c1 16S ribosomal RNA gene |
| 321 | 98.13 | HQ730084 | Phormidium sp. CENA135 16S ribosomal RNA gene |
| 322 | 98.13 | EF540416 | Uncultured soil bacterium clone MK5a 16S ribosomal RNA gene |
| 323 | 99.73 | JN713457 | Porphyromonas sp. canine oral taxon 290 clone ZR043 16S ribosomal RNA gene |
| 324 | 99.46 | EU133920 | Uncultured bacterium clone FFCH18791 16S ribosomal RNA gene |
| 325 | 98.4 | HQ540311 | Sandaracinus amylolyticus strain NOSO 4 16S ribosomal RNA gene |
| 326 | 94.96 | EF157190 | Uncultured bacterium clone 101-104 16S ribosomal RNA gene |
| 327 | 98.59 | EU134827 | Uncultured bacterium clone FFCH5852 16S ribosomal RNA gene |
| 328 | 89.37 | AM997595 | Uncultured deep-sea bacterium 16S rRNA gene |
| 329 | 99.2 | FJ468381 | Uncultured bacterium clone C074 16S ribosomal RNA gene |
| 330 | 94.65 | JF135724 | Uncultured bacterium clone ncd1563h11c1 16S ribosomal RNA gene |
| 331 | 100 | HQ534365 | Sporanaerobacter sp. C5BEL 16S ribosomal RNA gene |
| 332 | 99.2 | JF830199 | Bacterium enrichment culture clone B6(2011) 16S ribosomal RNA gene |
| 333 | 98.39 | HQ403234 | Uncultured bacterium clone AGS-HL2-5 16S ribosomal RNA gene |
| 334 | 96.8 | EU369154 | Uncultured bacterium clone MBHOS-05 16S ribosomal RNA gene |
| 335 | 97.36 | EU777318 | Uncultured bacterium clone OK1_a12_1 16S ribosomal RNA gene |
| 336 | 97.08 | JF128994 | Uncultured bacterium clone ncd1563d02c1 16S ribosomal RNA gene |
| 337 | 100 | HM445193 | Uncultured bacterium clone BL1288hO9 16S ribosomal RNA gene |
| 338 | 95.44 | HM186713 | Uncultured bacterium clone HDB_SIOU532 16S ribosomal RNA gene |
| 339 | 96.51 | JF834124 | Uncultured bacterium clone BYND-5-6 16S ribosomal RNA gene |
| 340 | 100 | AB600138 | Aurantimonas sp. AU22 gene for 16S rRNA |
| 341 | 94.62 | AM935477 | Uncultured Actinomycetales bacterium partial 16S rRNA gene |
| 342 | 98.66 | AY225640 | Uncultured Acidobacteriaceae bacterium clone AT-s2 16S ribosomal RNA gene |
| 343 | 98.4 | HQ121095 | Uncultured bacterium isolate 1112863845149 16S ribosomal RNA gene |
| 344 | 95.76 | HM438025 | Uncultured Acidimicrobiales bacterium clone T502G 16S ribosomal RNA gene |
| 345 | 98.39 | CR933063 | Uncultured bacterium partial 16S rRNA gene from clone 056G09_P_DI_P58. |
| 346 | 98.39 | AB486629 | Uncultured bacterium gene for 16S ribosomal RNA |
| 347 | 99.73 | FJ557738 | Uncultured bacterium clone ET_F_1c06 16S ribosomal RNA gene |
| 348 | 95.98 | JF344020 | Uncultured planctomycete clone RODAS-042 16S ribosomal RNA gene |
| 349 | 97.07 | AM935706 | Uncultured Planctomycetales bacterium partial 16S rRNA gene |
| 350 | 98.38 | EU466501 | Uncultured bacterium clone BH2_aao24g01 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 351 | 97.12 | EF016806 | Uncultured actinobacterium clone E1B-B3-114 16S ribosomal RNA gene |
| 352 | 97.06 | JN869158 | Uncultured bacterium clone MS39 16S ribosomal RNA gene |
| 353 | 98.38 | GQ354947 | Uncultured bacterium clone 4-103 16S ribosomal RNA gene |
| 354 | 99.73 | GQ018958 | Uncultured bacterium clone nbu192g04c1 16S ribosomal RNA gene |
| 355 | 98.92 | JF180716 | Uncultured bacterium clone ncd2149e12c1 16S ribosomal RNA gene |
| 356 | 99.46 | EU704750 | Uncultured bacterium clone 1P-1-D13 16S ribosomal RNA gene |
| 357 | 99.2 | DQ125547 | Uncultured bacterium clone AKAU3523 16S ribosomal RNA gene |
| 358 | 97.07 | HM333963 | Uncultured bacterium clone ncd1129e02c1 16S ribosomal RNA gene |
| 359 | 98.64 | DQ664246 | Emticicia sp. IMCC1731 16S ribosomal RNA gene |
| 360 | 98.66 | AB187508 | Nostoc sp. KK-01 gene for 16S ribosomal RNA |
| 361 | 98.93 | GQ114789 | Uncultured bacterium clone nbw683d10c1 16S ribosomal RNA gene |
| 362 | 97.87 | EU474450 | Uncultured bacterium clone BHSD_aaa04a10 16S ribosomal RNA gene |
| 363 | 96.45 | FJ028774 | Uncultured rumen bacterium clone TWBRB59 16S ribosomal RNA gene |
| 364 | 95.99 | X81942 | Pirellula sp. partial 16S rRNA gene (Schlesner 302) |
| 365 | 98.38 | FP565814 | Salinibacter ruber M8 chromosome |
| 366 | 93.82 | HQ190393 | Uncultured bacterium clone BR16 16S ribosomal RNA gene |
| 367 | 98.93 | FJ671519 | Uncultured bacterium clone LL143-8F11 16S ribosomal RNA gene |
| 368 | 98.92 | FJ825496 | Uncultured bacterium clone 99_2_BE4_10 16S ribosomal RNA gene |
| 369 | 100 | JF234426 | Uncultured bacterium clone ncd2706g01c1 16S ribosomal RNA gene |
| 370 | 93.7 | DQ532133 | Uncultured bacterium clone JPL1_33 16S ribosomal RNA gene |
| 371 | 99.73 | GU269545 | Meniscus glaucopis strain ATCC 29398 16S ribosomal RNA gene |
| 372 | 98.66 | HM315381 | Uncultured bacterium clone ncd463b10c1 16S ribosomal RNA gene |
| 373 | 98.36 | FN667309 | Uncultured compost bacterium partial 16S rRNA gene |
| 374 | 97.35 | HM262897 | Uncultured bacterium clone ncd244f10c1 16S ribosomal RNA gene |
| 375 | 98.67 | HM315996 | Uncultured bacterium clone ncd489b08c1 16S ribosomal RNA gene |
| 376 | 97.57 | CU927155 | Uncultured Spirochaetes bacterium 16S rRNA gene from clone QEDN6BA11. |
| 377 | 99.19 | JF223682 | Uncultured bacterium clone ncd2793b06c1 16S ribosomal RNA gene |
| 378 | 98.4 | HQ183777 | Uncultured Clostridium sp. clone De316 16S ribosomal RNA gene |
| 379 | 96.8 | GU056052 | Uncultured bacterium clone DGS1-13 16S ribosomal RNA gene |
| 380 | 100 | FM996433 | Uncultured bacterium partial 16S rRNA gene |
| 381 | 99.73 | GQ138465 | Uncultured bacterium clone 01h04 16S ribosomal RNA gene |
| 382 | 96.55 | FJ799140 | Bacterium enrichment culture clone EtOH-24 16S ribosomal RNA gene |
| 383 | 98.39 | JQ085668 | Uncultured bacterium clone VHW_F_M28 16S ribosomal RNA gene |
| 384 | 96.21 | EU458567 | Uncultured bacterium clone HY2_g05_2 16S ribosomal RNA gene |
| 385 | 95.71 | JF176808 | Uncultured bacterium clone ncd2050h03c2 16S ribosomal RNA gene |
| 386 | 96.27 | HQ397461 | Uncultured bacterium clone HSS164 16S ribosomal RNA gene |
| 387 | 97.25 | JN178820 | Uncultured bacterium clone TX2_8I24 16S ribosomal RNA gene |
| 388 | 97.05 | EU327343 | Halanaerobaculum tunisiense strain 6SANG 16S ribosomal RNA gene |
| 389 | 99.46 | JN873208 | Uncultured Clostridiales bacterium clone JXS2-6 16S ribosomal RNA gene |
| 390 | 96.79 | FJ479355 | Uncultured bacterium clone p7h15ok 16S ribosomal RNA gene |
| 391 | 99.47 | GQ025214 | Uncultured bacterium clone nbw132e08c1 16S ribosomal RNA gene |
| 392 | 99.73 | HQ395219 | Bacterium enrichment culture clone ecb25 16S ribosomal RNA gene |
| 393 | 99.73 | HQ864192 | Uncultured bacterium clone TP-SL-B-249 16S ribosomal RNA gene |
| 394 | 99.73 | EU234149 | Uncultured bacterium clone A02 16S ribosomal RNA gene |
| 395 | 94.68 | GU941080 | Uncultured bacterium clone N701B_92 16S ribosomal RNA gene |
| 396 | 91.06 | DQ396875 | Scenedesmus obliquus strain UTEX 393 chloroplast |
| 397 | 94.92 | JN367230 | Uncultured delta proteobacterium clone SeqSEEZ210 16S ribosomal RNA gene |
| 398 | 97.58 | HQ397362 | Uncultured Flexibacter sp. clone RS130 16S ribosomal RNA gene |
| 399 | 96.8 | AB630620 | Uncultured bacterium gene for 16S ribosomal RNA |
| 400 | 99.45 | Y08956 | T.rossianum 16S rRNA gene (strain NR-27T) |
| 401 | 98.39 | HM186489 | Uncultured bacterium clone HDB_SIOP831 16S ribosomal RNA gene |
| 402 | 94.77 | HQ716557 | Uncultured bacterium clone T2WK15F29 16S ribosomal RNA gene |
| 403 | 98.67 | JN398120 | Uncultured bacterium clone SWB0402-10 16S ribosomal RNA gene |
| 404 | 99.47 | GQ495224 | Delta proteobacterium BABL1 16S ribosomal RNA gene |
| 405 | 98.67 | HQ697824 | Uncultured bacterium clone Bms_CK373 16S ribosomal RNA gene |
| 406 | 97.33 | GQ134220 | Uncultured bacterium clone 02b08 16S ribosomal RNA gene |
| 407 | 95.99 | GQ263197 | Uncultured bacterium clone F3_162X 16S ribosomal RNA gene |
| 408 | 99.46 | EU794075 | Uncultured Alistipes sp. clone EMP_AA14 16S ribosomal RNA gene |
| 409 | 92.8 | GU174143 | Uncultured bacterium clone XJC62 16S ribosomal RNA gene |
| 410 | 93.05 | GQ134404 | Uncultured bacterium clone 04e09 16S ribosomal RNA gene |
| 411 | 96.26 | JN409254 | Uncultured actinobacterium clone HG-B02201 16S ribosomal RNA gene |
| 412 | 99.2 | HQ864151 | Uncultured bacterium clone TP-SL-B-157 16S ribosomal RNA gene |
| 413 | 98.4 | JF228252 | Uncultured bacterium clone ncd2609a10c1 16S ribosomal RNA gene |
| 414 | 98.93 | JF198592 | Uncultured bacterium clone ncd2316d04c1 16S ribosomal RNA gene |
| 415 | 98.4 | JF168907 | Uncultured bacterium clone ncd2081h06c1 16S ribosomal RNA gene |
| 416 | 98.93 | EU703214 | Uncultured cyanobacterium clone XZNMC45 16S ribosomal RNA gene |
| 417 | 96.52 | AM774207 | Uncultured planctomycete partial 16S rRNA gene |
| 418 | 92.2 | AB237705 | Uncultured bacterium gene for 16S rRNA |
| 419 | 99.46 | ABXP01000185 | Carboxydibrachium pacificum DSM 12653 ctg_1106511212256 |
| 420 | 97.34 | EU755074 | Uncultured Chloroflexi bacterium clone HM-1 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 421 | 98.91 | FJ951901 | Uncultured bacterium clone H21 16S ribosomal RNA gene |
| 422 | 98.12 | JF226664 | Uncultured bacterium clone ncd2583e02c1 16S ribosomal RNA gene |
| 423 | 98.93 | FJ375448 | Uncultured bacterium clone ACS35 16S ribosomal RNA gene |
| 424 | 97.33 | FJ190073 | Uncultured bacterium clone 3-oxy 16S ribosomal RNA gene |
| 425 | 100 | JF920316 | Uncultured bacterium clone Giantp OTU9 16S ribosomal RNA gene |
| 426 | 95.98 | EU939412 | Uncultured bacterium clone WF16S_137 16S ribosomal RNA gene |
| 427 | 94.59 | EU771902 | Uncultured bacterium clone arma_f07_2 16S ribosomal RNA gene |
| 428 | 97.33 | GQ133314 | Uncultured bacterium clone 03d07 16S ribosomal RNA gene |
| 429 | 99.2 | HQ716716 | Uncultured bacterium clone T2WK15B96 16S ribosomal RNA gene |
| 430 | 98.4 | FJ478921 | Uncultured bacterium clone p9e20ok 16S ribosomal RNA gene |
| 431 | 97.33 | GQ402548 | Uncultured bacterium clone PW003 16S ribosomal RNA gene |
| 432 | 96.52 | FJ894331 | Uncultured bacterium clone nbt38h04 16S ribosomal RNA gene |
| 433 | 97.89 | JN872548 | Xanthomonadaceae bacterium SAP40_3 16S ribosomal RNA gene |
| 434 | 94.89 | JF809725 | Uncultured bacterium clone RS-B9 16S ribosomal RNA gene |
| 435 | 97.6 | FJ848420 | Uncultured bacterium clone DLN-120 small subunit ribosomal RNA gene |
| 436 | 99.46 | EU407204 | Uncultured bacterium clone GW-19 16S ribosomal RNA gene |
| 437 | 97.07 | AY493924 | Uncultured soil bacterium clone 355 small subunit ribosomal RNA gene |
| 438 | 100 | HM315029 | Uncultured bacterium clone ncd443f10c1 16S ribosomal RNA gene |
| 439 | 95.15 | GQ009418 | Uncultured bacterium clone nbw776c12c1 16S ribosomal RNA gene |
| 440 | 98.93 | EU680819 | Alicyclobacillus sp. AYTae-7 16S ribosomal RNA gene |
| 441 | 98.64 | FJ801214 | Uncultured bacterium clone ZWB4-17 16S ribosomal RNA gene |
| 442 | 99.45 | AM950255 | Uncultured bacterium partial 16S rRNA gene |
| 443 | 91.44 | HM269106 | Uncultured bacterium clone ncd243a08c1 16S ribosomal RNA gene |
| 444 | 98.66 | JF217190 | Uncultured bacterium clone ncd2527c12c1 16S ribosomal RNA gene |
| 445 | 98.12 | FJ946574 | Uncultured Rhizobiales bacterium clone ESS-C3n 16S ribosomal RNA gene |
| 446 | 97.6 | GQ094015 | Uncultured bacterium clone nbw428a03c1 16S ribosomal RNA gene |
| 447 | 99.2 | HQ910311 | Uncultured bacterium clone P-13_B6 16S ribosomal RNA gene |
| 448 | 91.4 | HQ163658 | Uncultured Bacteroidetes bacterium clone SIAC459 16S ribosomal RNA gene |
| 449 | 98.93 | GU559805 | Uncultured bacterium clone B21.4 16S ribosomal RNA gene |
| 450 | 95.72 | U81649 | Unidentified eubacterium clone vadinBA26 16S ribosomal RNA gene |
| 451 | 98.67 | JF421161 | Uncultured Actinobacteridae bacterium clone Ppss_Ma113 16S ribosomal RNA gene |
| 452 | 98.13 | DQ532166 | Uncultured bacterium clone JSC2-A4 16S ribosomal RNA gene |
| 453 | 99.46 | GQ448016 | Uncultured bacterium clone calf32_2wks_grp1_A10 16S ribosomal RNA gene |
| 454 | 98.66 | AM420230 | Uncultured Tannerella sp. partial 16S rRNA gene |
| 455 | 95.87 | HQ697754 | Uncultured bacterium clone Bms_MS191 16S ribosomal RNA gene |
| 456 | 97.33 | EF516279 | Uncultured bacterium clone FCPS556 16S ribosomal RNA gene |
| 457 | 95.21 | EU786123 | Uncultured bacterium clone 3H-24 16S ribosomal RNA gene |
| 458 | 96.54 | HQ609714 | Uncultured bacterium clone AS-119 16S ribosomal RNA gene |
| 459 | 98.38 | EU794218 | Uncultured Ruminococcaceae bacterium clone EMP_V45 16S ribosomal RNA gene |
| 460 | 99.73 | JF224715 | Uncultured bacterium clone ncd2552a02c1 16S ribosomal RNA gene |
| 461 | 99.46 | JF319191 | Uncultured Acidobacteria bacterium clone LJ-B110 16S ribosomal RNA gene |
| 462 | 95.34 | HQ222269 | Magnetospirillum sp. enrichment culture clone Van25 16S ribosomal RNA gene |
| 463 | 97.59 | AB630550 | Uncultured bacterium gene for 16S ribosomal RNA |
| 464 | 99.73 | FN421783 | Uncultured bacterium partial 16S rRNA gene |
| 465 | 95.58 | GQ249600 | Uncultured delta proteobacterium clone D58 16S ribosomal RNA gene |
| 466 | 98.93 | FN822744 | Leuconostoc gasicomitatum LMG 18811 complete genome |
| 467 | 98.67 | HQ697754 | Uncultured bacterium clone Bms_MS191 16S ribosomal RNA gene |
| 468 | 97.87 | JN367190 | Uncultured actinobacterium clone SeqSEEZ169 16S ribosomal RNA gene |
| 469 | 98.92 | HM992535 | Uncultured Synergistaceae bacterium clone Alk1-11C 16S ribosomal RNA gene |
| 470 | 99.73 | HE583128 | Uncultured bacterium partial 16S rRNA gene |
| 471 | 99.64 | HQ395115 | Uncultured bacterium clone OTUb3 16S ribosomal RNA gene |
| 472 | 98.92 | AY212715 | Uncultured bacterium clone 265ds10 16S ribosomal RNA gene |
| 473 | 99.46 | JF412420 | Cyclobacterium sp. 20035 16S ribosomal RNA gene |
| 474 | 91.2 | JN559677 | Uncultured bacterium clone CA_247 16S ribosomal RNA gene |
| 475 | 88.44 | EU135220 | Uncultured bacterium clone FFCH14129 16S ribosomal RNA gene |
| 476 | 92.21 | DQ532133 | Uncultured bacterium clone JPL1_33 16S ribosomal RNA gene |
| 477 | 99.19 | FP885871 | Beta vulgaris subsp. maritima genotype male-sterile G mitochondrion |
| 478 | 93.85 | HQ910253 | Uncultured bacterium clone P-8_B3 16S ribosomal RNA gene |
| 479 | 97.84 | HM468032 | Uncultured bacterium clone b25 16S ribosomal RNA gene |
| 480 | 96.26 | EF516491 | Uncultured bacterium clone FCPP673 16S ribosomal RNA gene |
| 481 | 92.33 | AB630939 | Uncultured bacterium gene for 16S ribosomal RNA |
| 482 | 100 | X82146 | A.nigrifaciens 16S rRNA gene (NCIMB 8614T) |
| 483 | 98.66 | HQ740302 | Uncultured organism clone ELU0008-T58-S-NI_000395 small subunit ribosomal RNA gene |
| 484 | 99.19 | EU474561 | Uncultured bacterium clone BHSD_aaa02g07 16S ribosomal RNA gene |
| 485 | 96.5 | EU245295 | Uncultured organism clone MAT-CR-H5-E08 16S ribosomal RNA gene |
| 486 | 92.66 | HM240957 | Uncultured bacterium clone 092 16S ribosomal RNA gene |
| 487 | 98.11 | JF182317 | Uncultured bacterium clone ncd2109e04c1 16S ribosomal RNA gene |
| 488 | 98.93 | FP565814 | Salinibacter ruber M8 chromosome |
| 489 | 95.19 | EU134569 | Uncultured bacterium clone FFCH4177 16S ribosomal RNA gene |
| 490 | 91.22 | EU861840 | Uncultured soil bacterium clone C09_bac_con 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 491 | 91.41 | JN091907 | Uncultured bacterium clone Z151 16S ribosomal RNA gene |
| 492 | 92.78 | EU786123 | Uncultured bacterium clone 3H-24 16S ribosomal RNA gene |
| 493 | 100 | FJ976339 | Uncultured Leptotrichia sp. clone 12B523 16S ribosomal RNA gene |
| 494 | 98.93 | FM873606 | Uncultured bacterium partial 16S rRNA gene |
| 495 | 96.79 | EF516491 | Uncultured bacterium clone FCPP673 16S ribosomal RNA gene |
| 496 | 91.04 | EF602805 | Uncultured bacterium clone 16saw30-1a07.p1k 16S ribosomal RNA gene |
| 497 | 91.01 | GQ360025 | Uncultured bacterium clone IM29 16S ribosomal RNA gene |
| 498 | 97.87 | FJ670641 | Uncultured bacterium clone LL143-1B1 16S ribosomal RNA gene |
| 499 | 98.93 | HM099520 | Bacterium FRC-RU4 16S ribosomal RNA gene |
| 500 | 98.66 | JF128994 | Uncultured bacterium clone ncd1563d02c1 16S ribosomal RNA gene |
| 501 | 97.84 | HQ118574 | Uncultured bacterium isolate 1112851586960 16S ribosomal RNA gene |
| 502 | 96.51 | DQ404719 | Uncultured bacterium clone 655933 16S ribosomal RNA gene |
| 503 | 98.92 | JF181172 | Uncultured bacterium clone ncd2029d01c1 16S ribosomal RNA gene |
| 504 | 98.13 | FJ672119 | Uncultured bacterium clone Ll142-3C11 16S ribosomal RNA gene |
| 505 | 95.72 | JF905994 | Uncultured Sphaerobacter sp. clone ASC193 16S ribosomal RNA gene |
| 506 | 99.73 | HM992557 | Uncultured anaerobic bacterium clone Alk1-10H 16S ribosomal RNA gene |
| 507 | 100 | HQ395211 | Bacterium enrichment culture clone ecb17 16S ribosomal RNA gene |
| 508 | 99.2 | EU133045 | Uncultured bacterium clone FFCH2529 16S ribosomal RNA gene |
| 509 | 97.57 | HQ766916 | Uncultured organism clone ELU0060-T398-S-NIPCRAMgANa_000526 small subunit ribosomal RNA gene |
| 510 | 99.2 | FJ710760 | Uncultured bacterium clone Dok41 16S ribosomal RNA gene |
| 511 | 99.2 | FR682689 | Patulibacter minatonensis partial 16S rRNA gene |
| 512 | 98.66 | AB630834 | Uncultured bacterium gene for 16S ribosomal RNA |
| 513 | 98.92 | EU773364 | Uncultured bacterium clone BH1_aao27a03 16S ribosomal RNA gene |
| 514 | 95.68 | FQ659331 | Uncultured bacterium 16S ribosomal RNA gene clone I1DF081 |
| 515 | 95.43 | EU133663 | Uncultured bacterium clone FFCH12849 16S ribosomal RNA gene |
| 516 | 97.84 | AB197852 | Chimaereicella boritolerans gene for 16S ribosomal RNA |
| 517 | 93.88 | FN667465 | Uncultured compost bacterium partial 16S rRNA gene |
| 518 | 98.39 | HQ190542 | Uncultured bacterium clone BP73 16S ribosomal RNA gene |
| 519 | 95.68 | DQ532354 | Uncultured bacterium clone KSC6-80 16S ribosomal RNA gene |
| 520 | 98.66 | HM263370 | Uncultured bacterium clone ncd267h03c1 16S ribosomal RNA gene |
| 521 | 96.77 | DQ129376 | Uncultured bacterium clone AKIW1012 16S ribosomal RNA gene |
| 522 | 99.2 | HQ761212 | Uncultured organism clone ELU0047-T268-S-NIPCRAMgANa_000394 small subunit ribosomal RNA gene |
| 523 | 90.93 | FN687457 | Uncultured bacteria partial 16S rRNA gene |
| 524 | 98.66 | AB668499 | Uncultured bacterium gene for 16S rRNA |
| 525 | 98.67 | JF167685 | Uncultured bacterium clone ncd2019b08c1 16S ribosomal RNA gene |
| 526 | 98.14 | JF225469 | Uncultured bacterium clone ncd2562g07c1 16S ribosomal RNA gene |
| 527 | 91.96 | EU473616 | Uncultured bacterium clone BG_aaa01d03.Contig1 16S ribosomal RNA gene |
| 528 | 99.73 | FM996545 | Uncultured bacterium partial 16S rRNA gene |
| 529 | 98.92 | EU794241 | Uncultured Bacteroidales bacterium clone EMP_I6 16S ribosomal RNA gene |
| 530 | 95.53 | JN409061 | Uncultured Bacteroidetes bacterium clone HG-J02178 16S ribosomal RNA gene |
| 531 | 98.11 | GU117703 | Bacteroidetes bacterium 10AO 16S ribosomal RNA gene |
| 532 | 91.96 | GU455109 | Uncultured bacterium clone ambient_alkaline-126 16S ribosomal RNA gene |
| 533 | 99.46 | EU777318 | Uncultured bacterium clone OK1_a12_1 16S ribosomal RNA gene |
| 534 | 99.19 | FJ680668 | Uncultured bacterium clone 5-3O23 16S ribosomal RNA gene |
| 535 | 98.66 | GQ923768 | Legionella sp. 21718 16S ribosomal RNA gene |
| 536 | 97.3 | AF507701 | Uncultured soil bacterium clone C0108 16S ribosomal RNA gene |
| 537 | 97.58 | EF111094 | Uncultured Bacteroidetes bacterium clone RBE1CI-139 16S ribosomal RNA gene |
| 538 | 99.2 | FN436028 | Uncultured bacterium partial 16S rRNA gene |
| 539 | 85.12 | DQ404837 | Uncultured bacterium clone 655966 16S ribosomal RNA gene |
| 540 | 95.47 | EF157190 | Uncultured bacterium clone 101-104 16S ribosomal RNA gene |
| 541 | 98.16 | AF013971 | Thiomicrospira sp. SL-1 16S ribosomal RNA gene |
| 542 | 98.4 | JN409030 | Uncultured planctomycete clone HG-J02127 16S ribosomal RNA gene |
| 543 | 97.04 | EU704879 | Uncultured bacterium clone 1P-1-L16 16S ribosomal RNA gene |
| 544 | 96.25 | X81955 | Planctomyces sp. partial 16S rRNA gene (Schlesner 664) |
| 545 | 99.2 | EU542486 | Uncultured bacterium clone Er-MLAYS-95 16S ribosomal RNA gene |
| 546 | 99.73 | JF428970 | Uncultured bacterium clone E88 16S ribosomal RNA gene |
| 547 | 97.3 | EU794314 | Uncultured Treponema sp. clone EMP_J31 16S ribosomal RNA gene |
| 548 | 98.41 | DQ532167 | Uncultured bacterium clone JSC2-A6 16S ribosomal RNA gene |
| 549 | 98.66 | HM366506 | Uncultured bacterium clone ADB-46 16S ribosomal RNA gene |
| 550 | 99.2 | HM186163 | Uncultured bacterium clone HDB_SION791 16S ribosomal RNA gene |
| 551 | 97.86 | EU104217 | Uncultured bacterium clone N1512_48 16S ribosomal RNA gene |
| 552 | 94.35 | EU132276 | Uncultured bacterium clone FFCH2719 16S ribosomal RNA gene |
| 553 | 96.26 | JF210530 | Uncultured bacterium clone ncd2424c02c1 16S ribosomal RNA gene |
| 554 | 98.13 | EF157190 | Uncultured bacterium clone 101-104 16S ribosomal RNA gene |
| 555 | 98.93 | JQ088434 | Uncultured bacterium clone Ba19W-B10 16S ribosomal RNA gene |
| 556 | 100 | FJ876262 | Acholeplasma oculi strain Panangala 59 16S ribosomal RNA gene and 16S-23S ribosomal RNA intergenic spacer |
| 557 | 98.67 | Y08935 | Coprothermobacter platensis 16S rRNA gene |
| 558 | 99.73 | HQ727630 | Uncultured Bacteroidetes bacterium clone BC_CK676 16S ribosomal RNA gene |
| 559 | 91.3 | U81670 | Unidentified eubacterium clone vadinBB60 16S ribosomal RNA gene |
| 560 | 98.93 | AM935498 | Uncultured Chloroflexi bacterium partial 16S rRNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 561 | 93.87 | HQ863967 | Uncultured bacterium clone TP-DE-B53 16S ribosomal RNA gene |
| 562 | 95.2 | EU219931 | Uncultured bacterium clone C6B 16S ribosomal RNA gene |
| 563 | 96.25 | HQ397147 | Uncultured bacterium clone NLS2.22 16S ribosomal RNA gene |
| 564 | 93.85 | HM238172 | Uncultured Chloroflexi bacterium clone BF_77 16S ribosomal RNA gene |
| 565 | 97.85 | FJ517632 | Rhodococcus sp. ER4 16S ribosomal RNA gene |
| 566 | 97.59 | HQ118941 | Uncultured bacterium isolate 1112865250735 16S ribosomal RNA gene |
| 567 | 94.13 | AB534005 | Uncultured bacterium gene for 16S rRNA |
| 568 | 95.2 | JN825448 | Uncultured Gemmatimonadetes bacterium clone Alchichica_AQ2_2_1B_50 small subunit ribosomal RNA gene |
| 569 | 96.77 | JF429312 | Uncultured bacterium clone DR305 16S ribosomal RNA gene |
| 570 | 96.24 | HQ119174 | Uncultured bacterium isolate 1112865250894 16S ribosomal RNA gene |
| 571 | 99.73 | FQ659487 | Uncultured bacterium 16S ribosomal RNA gene clone I1BH071 |
| 572 | 100 | L09164 | Thermoanaerobacter pseudethanolicus ATCC 33223 16S ribosomal RNA gene |
| 573 | 98.66 | AF407708 | Uncultured bacterium clone G58 16S ribosomal RNA gene |
| 574 | 92.78 | AM176838 | Uncultured bacterium partial 16S rRNA gene |
| 575 | 97.88 | AM935477 | Uncultured Actinomycetales bacterium partial 16S rRNA gene |
| 576 | 99.19 | CP001685 | Leptotrichia buccalis DSM 1135 |
| 577 | 99.46 | GQ060463 | Uncultured bacterium clone nbw01g03c1 16S ribosomal RNA gene |
| 578 | 98.13 | FJ543060 | Uncultured Verrucomicrobia bacterium clone B09-02G 16S ribosomal RNA gene |
| 579 | 98.4 | AB023839 | Weissella thailandensis gene for 16S rRNA |
| 580 | 94.89 | AY193286 | Uncultured candidate division OP11 bacterium clone WSA68 16S ribosomal RNA gene |
| 581 | 98.93 | EU133266 | Uncultured bacterium clone FFCH12560 16S ribosomal RNA gene |
| 582 | 96.79 | JN409018 | Uncultured cyanobacterium clone HG-J0292 16S ribosomal RNA gene |
| 583 | 95.35 | HM565028 | Uncultured Carnobacterium sp. clone H-147 16S ribosomal RNA gene |
| 584 | 98.93 | AY921851 | Uncultured Chloroflexi bacterium clone AKYH412 16S ribosomal RNA gene |
| 585 | 93.32 | JF210530 | Uncultured bacterium clone ncd2424c02c1 16S ribosomal RNA gene |
| 586 | 96.83 | JN038228 | Uncultured alpha proteobacterium clone EK_CK579 16S ribosomal RNA gene |
| 587 | 94.92 | FN393488 | Uncultured delta proteobacterium partial 16S rRNA gene |
| 588 | 96.51 | JF727734 | Uncultured bacterium clone bac491 16S ribosomal RNA gene |
| 589 | 99.19 | FJ658290 | Uncultured bacterium clone Winter_MachineA&B_HEPAoff_2d01 16S ribosomal RNA gene |
| 590 | 92.82 | GQ138450 | Uncultured bacterium clone 01f11 16S ribosomal RNA gene |
| 591 | 95.44 | FN667431 | Uncultured compost bacterium partial 16S rRNA gene |
| 592 | 99.45 | FJ681777 | Uncultured bacterium clone 5-8G19 16S ribosomal RNA gene |
| 593 | 98.66 | FJ679561 | Uncultured bacterium clone 4-2D7 16S ribosomal RNA gene |
| 594 | 96.52 | JN367190 | Uncultured actinobacterium clone SeqSEEZ169 16S ribosomal RNA gene |
| 595 | 99.73 | JF714888 | Psychrobacter sp. DAB_AL62B 16S ribosomal RNA gene |
| 596 | 95.98 | HQ119024 | Uncultured bacterium isolate 1112865250796 16S ribosomal RNA gene |
| 597 | 84.64 | AB015522 | Uncultured alpha proteobacterium gene for 16S ribosomal RNA |
| 598 | 99.73 | GQ259595 | Uncultured bacterium clone GR0108_74 16S ribosomal RNA gene |
| 599 | 99.19 | JF136669 | Uncultured bacterium clone ncd1577h12c1 16S ribosomal RNA gene |
| 600 | 96.52 | EF612368 | Uncultured actinobacterium clone OS-C108 16S ribosomal RNA gene |
| 601 | 98.13 | DQ469574 | Halothiobacillus sp. HL27 16S ribosomal RNA gene |
| 602 | 94.59 | DQ676339 | Uncultured Chlorobi bacterium clone MVP-4 16S ribosomal RNA gene |
| 603 | 97.24 | EF636834 | Uncultured Flexibacteraceae bacterium clone 6 16S ribosomal RNA gene |
| 604 | 97.33 | AB237708 | Uncultured bacterium gene for 16S rRNA |
| 605 | 99.19 | GU455231 | Uncultured bacterium clone mesophilic_alkaline-116 16S ribosomal RNA gene |
| 606 | 97.6 | JN038255 | Uncultured Bacteroidetes bacterium clone EK_CK570 16S ribosomal RNA gene |
| 607 | 99.46 | AB286550 | Uncultured bacterium gene for 16S rRNA |
| 608 | 98.39 | EU135157 | Uncultured bacterium clone FFCH5374 16S ribosomal RNA gene |
| 609 | 94.65 | JN087902 | Uncultured bacterium clone NIT_55 16S ribosomal RNA gene |
| 610 | 98.37 | EU775282 | Uncultured bacterium clone gir_aah96e09 16S ribosomal RNA gene |
| 611 | 99.19 | FM179649 | Bacteroidetes bacterium AKB-K1-255 partial 16S rRNA gene |
| 612 | 96.01 | JN409188 | Uncultured bacterium clone HG-B02102 16S ribosomal RNA gene |
| 613 | 98.66 | FJ671223 | Uncultured bacterium clone LL143-5P11 16S ribosomal RNA gene |
| 614 | 96.33 | JF198592 | Uncultured bacterium clone ncd2316d04c1 16S ribosomal RNA gene |
| 615 | 99.2 | CP002364 | Desulfobulbus propionicus DSM 2032 |
| 616 | 100 | HM186037 | Uncultured bacterium clone HDB_SION1019 16S ribosomal RNA gene |
| 617 | 97.08 | ACZK01000043 | Prevotella sp. oral taxon 302 str. F0323 cont1.43 |
| 618 | 98.4 | EU490279 | Uncultured bacterium clone ERB-F7 16S ribosomal RNA gene |
| 619 | 99.46 | EF515298 | Uncultured bacterium clone 22e09 16S ribosomal RNA gene |
| 620 | 89.76 | FJ894959 | Uncultured bacterium clone nbt188b09 16S ribosomal RNA gene |
| 621 | 95.98 | AY571796 | Uncultured Acidobacteria bacterium clone 352G 16S ribosomal RNA gene |
| 622 | 98.92 | AM000022 | Larkinella insperata 16S rRNA gene |
| 623 | 98.39 | JN409077 | Uncultured actinobacterium clone HG-B0157 16S ribosomal RNA gene |
| 624 | 97.59 | JQ311888 | Uncultured bacterium clone OTU80-98 16S ribosomal RNA gene |
| 625 | 97.6 | AM935790 | Uncultured Chloroflexi bacterium partial 16S rRNA gene |
| 626 | 98.66 | EF157268 | Uncultured bacterium clone 101-124 16S ribosomal RNA gene |
| 627 | 100 | FJ983040 | Uncultured bacterium clone OPEN_PLAQUE_16 16S ribosomal RNA gene |
| 628 | 98.93 | GU266444 | Uncultured soil bacterium clone SBPYR03 16S ribosomal RNA gene |
| 629 | 97.81 | GQ461624 | Uncultured bacterium clone BR16 16S ribosomal RNA gene |
| 630 | 99.2 | EU861898 | Uncultured soil bacterium clone bacnit45 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 631 | 94.92 | HQ864192 | Uncultured bacterium clone TP-SL-B-249 16S ribosomal RNA gene |
| 632 | 100 | FJ978564 | Uncultured bacterium clone CL_F_124 16S ribosomal RNA gene |
| 633 | 99.46 | AY570582 | Uncultured bacterium clone PL-11B8 16S ribosomal RNA gene |
| 634 | 98.92 | FJ672138 | Uncultured bacterium clone Ll142-3D1 16S ribosomal RNA gene |
| 635 | 98.93 | DQ223729 | Megasphaera sueciensis strain VTT E-97791 16S ribosomal RNA gene |
| 636 | 97.84 | GQ472354 | Uncultured bacterium clone 3S4-6 16S ribosomal RNA gene |
| 637 | 99.73 | HM445960 | Uncultured bacterium clone W-16S-33 16S ribosomal RNA gene |
| 638 | 97.59 | FJ672138 | Uncultured bacterium clone Ll142-3D1 16S ribosomal RNA gene |
| 639 | 96.01 | EF018760 | Uncultured bacterium clone Amb_16S_1221 16S ribosomal RNA gene |
| 640 | 99.19 | JQ352762 | Flavobacteriaceae bacterium SCSIO 03483 16S ribosomal RNA gene |
| 641 | 97.33 | JF103728 | Uncultured bacterium clone ncd1158e12c1 16S ribosomal RNA gene |
| 642 | 99.2 | GQ448539 | Uncultured bacterium clone calf784_6wks_grp1_H11 16S ribosomal RNA gene |
| 643 | 99.46 | EU458672 | Uncultured bacterium clone RA_f06_2 16S ribosomal RNA gene |
| 644 | 99.46 | EF540455 | Pelagibacterium luteolum strain 1_C16_27 16S ribosomal RNA gene |
| 645 | 97.06 | EU786123 | Uncultured bacterium clone 3H-24 16S ribosomal RNA gene |
| 646 | 98.4 | DQ130040 | Uncultured Thermomicrobium sp. clone GR108 16S ribosomal RNA gene |
| 647 | 89.92 | HQ156044 | Uncultured prokaryote clone 08031003-Z7EU_2TH_2_2_F07 16S ribosomal RNA gene |
| 648 | 99.46 | AF445745 | Uncultured firmicute clone SM2H09 16S ribosomal RNA gene |
| 649 | 95.03 | AY571796 | Uncultured Acidobacteria bacterium clone 352G 16S ribosomal RNA gene |
| 650 | 95.92 | HQ119005 | Uncultured bacterium isolate 1112865250786 16S ribosomal RNA gene |
| 651 | 96.51 | EU772236 | Uncultured bacterium clone AS2_aao35e07 16S ribosomal RNA gene |
| 652 | 98.39 | HQ183792 | Uncultured Clostridiales bacterium clone De122 16S ribosomal RNA gene |
| 653 | 98.13 | GU367198 | Glycomyces sp. YIM 65802 16S ribosomal RNA gene |
| 654 | 97.33 | HM332632 | Uncultured bacterium clone ncd996b04c1 16S ribosomal RNA gene |
| 655 | 95.98 | HM319234 | Uncultured bacterium clone ncd350g10c1 16S ribosomal RNA gene |
| 656 | 95.68 | HQ681995 | Uncultured bacterium clone ZBAF1-105 16S ribosomal RNA gene |
| 657 | 96.8 | Y10819 | P. freudenreichii subsp. shermanii partial 16S rRNA |
| 658 | 94.39 | JN023717 | Uncultured bacterium clone bar-a56 16S ribosomal RNA gene |
| 659 | 99.47 | DQ647171 | Uncultured Anaerophaga sp. clone TCB200x 16S ribosomal RNA gene |
| 660 | 95.19 | FM209159 | Uncultured Chloroflexi bacterium partial 16S rRNA gene |
| 661 | 97.06 | EU255702 | Uncultured cyanobacterium clone Mat-CYANO-S3 16S ribosomal RNA gene |
| 662 | 96.79 | JN428208 | Uncultured organism clone SBXY_2437 16S ribosomal RNA gene |
| 663 | 99.2 | JF733795 | Bacterium 2/2/5/24 16S ribosomal RNA gene |
| 664 | 97.85 | HQ910257 | Uncultured bacterium clone P-11_B17 16S ribosomal RNA gene |
| 665 | 97.03 | EU468734 | Uncultured bacterium clone OK3_b09 16S ribosomal RNA gene |
| 666 | 100 | JN867416 | Uncultured bacterium clone 83 16S ribosomal RNA gene |
| 667 | 99.46 | JF417866 | Uncultured bacterium clone HC18-11B31 16S ribosomal RNA gene |
| 668 | 99.2 | JF240724 | Uncultured bacterium clone ncd2806f06c1 16S ribosomal RNA gene |
| 669 | 98.4 | FJ152635 | Uncultured bacterium clone TX1A_83 16S ribosomal RNA gene |
| 670 | 95.72 | AB369179 | Uncultured bacterium gene for 16S rRNA |
| 671 | 97.59 | GQ396948 | Uncultured bacterium clone AK4AB2_10H 16S ribosomal RNA gene |
| 672 | 98.93 | HM269992 | Uncultured bacterium clone ncd258h07c1 16S ribosomal RNA gene |
| 673 | 98.93 | AY921935 | Uncultured Chloroflexi bacterium clone AKYG1722 16S ribosomal RNA gene |
| 674 | 98.39 | HM124375 | Methylosinus sp. 24-21 16S ribosomal RNA gene |
| 675 | 98.4 | HQ864169 | Uncultured bacterium clone TP-SL-B-204 16S ribosomal RNA gene |
| 676 | 98.39 | GQ108731 | Uncultured bacterium clone nbw692h01c1 16S ribosomal RNA gene |
| 677 | 99.73 | JF506651 | Haemophilus parainfluenzae strain NCTC 10672 16S ribosomal RNA gene |
| 678 | 95.99 | HQ119844 | Uncultured bacterium isolate 1112842460661 16S ribosomal RNA gene |
| 679 | 94.32 | JF028528 | Uncultured bacterium clone ncd267a11c1 16S ribosomal RNA gene |
| 680 | 92.29 | HQ183999 | Uncultured bacterium clone De228 16S ribosomal RNA gene |
| 681 | 100 | HM298813 | Uncultured bacterium clone ncd898b07c1 16S ribosomal RNA gene |
| 682 | 98.12 | EU101232 | Uncultured bacterium clone RS06101_B33 16S ribosomal RNA gene |
| 683 | 98.4 | FJ671445 | Uncultured bacterium clone LL143-7K3 16S ribosomal RNA gene |
| 684 | 95.26 | JF800773 | Uncultured bacterium clone BT1112 16S ribosomal RNA gene |
| 685 | 99.73 | EU794214 | Uncultured Treponema sp. clone EMP_S39 16S ribosomal RNA gene |
| 686 | 98.66 | JN873190 | Uncultured bacterium clone JXS1-33 16S ribosomal RNA gene |
| 687 | 97.29 | DQ805606 | Uncultured bacterium clone RL203_aai64h03 16S ribosomal RNA gene |
| 688 | 96.02 | HQ910280 | Uncultured bacterium clone P-8_B4 16S ribosomal RNA gene |
| 689 | 99.46 | FJ152792 | Uncultured bacterium clone TX5A_84 16S ribosomal RNA gene |
| 690 | 98.15 | JF223997 | Uncultured bacterium clone ncd2807g01c1 16S ribosomal RNA gene |
| 691 | 96 | JN380995 | Legionella sp. Edu-2 16S ribosomal RNA gene |
| 692 | 100 | AJ617861 | Uncultured bacterium 16S rRNA gene |
| 693 | 98.09 | FJ711204 | Actinomadura sp. KC-IT-H5 16S ribosomal RNA gene |
| 694 | 98.64 | EU794162 | Uncultured Porphyromonadaceae bacterium clone EMP_E32 16S ribosomal RNA gene |
| 695 | 100 | U91544 | Shewanella alga 16S ribosomal RNA (rrs) gene |
| 696 | 99.47 | JN175334 | Halothiobacillus neapolitanus strain CIP 104769 16S ribosomal RNA gene |
| 697 | 92.25 | AB488224 | Uncultured bacterium gene for 16S ribosomal RNA |
| 698 | 91.44 | EF368008 | Cohnella yongneupensis strain 5YN10-14 16S ribosomal RNA gene |
| 699 | 98.07 | EU289423 | Uncultured bacterium clone 1-2B 16S ribosomal RNA gene |
| 700 | 97.33 | JN899152 | Uncultured bacterium clone WP3-60-16S2 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 701 | 98.13 | FN436095 | Uncultured bacterium partial 16S rRNA gene |
| 702 | 96.53 | HM187442 | Uncultured bacterium clone HDB_SISU675 16S ribosomal RNA gene |
| 703 | 100 | HQ218593 | Uncultured bacterium clone N-151 16S ribosomal RNA gene |
| 704 | 99.73 | CP003093 | Pseudoxanthomonas spadix BD-a59 |
| 705 | 100 | FJ889323 | Uncultured Sphingomonas sp. clone Plot18-2G06 16S ribosomal RNA gene |
| 706 | 98.67 | Y12593 | S.capprae 16S rRNA gene |
| 707 | 100 | HQ860588 | Uncultured bacterium clone C-87 16S ribosomal RNA gene |
| 708 | 96.49 | EU335222 | Uncultured bacterium clone BacA_057 16S ribosomal RNA gene |
| 709 | 100 | Y18171 | Clostridium roseum 16S rRNA gene |
| 710 | 96.27 | JF198827 | Uncultured bacterium clone ncd2320d06c1 16S ribosomal RNA gene |
| 711 | 99.73 | EU104040 | Uncultured bacterium clone M0111_41 16S ribosomal RNA gene |
| 712 | 100 | HM480263 | Uncultured alpha proteobacterium clone Kir51gry B2.c39 16S ribosomal RNA gene |
| 713 | 99.73 | HM186919 | Uncultured bacterium clone HDB_SIPF540 16S ribosomal RNA gene |
| 714 | 97.33 | HQ119844 | Uncultured bacterium isolate 1112842460661 16S ribosomal RNA gene |
| 715 | 98.12 | AJ630296 | Unidentified Cytophagales/green sulfur bacterium OPB56 partial 16S rRNA gene |
| 716 | 98.93 | CU925767 | Uncultured Chloroflexi bacterium 16S rRNA gene from clone QEDN9DG07. |
| 717 | 95.97 | AM935486 | Uncultured Acidobacteriaceae bacterium partial 16S rRNA gene |
| 718 | 99.46 | AY746987 | Desulfovibrio aerotolerans strain DvO5 16S ribosomal RNA gene |
| 719 | 98.92 | JN656876 | Uncultured Bacteroidetes bacterium clone KWK23F.05 16S ribosomal RNA gene |
| 720 | 98.96 | DQ404599 | Uncultured bacterium clone 656043 16S ribosomal RNA gene |
| 721 | 99.19 | DQ816661 | Uncultured bacterium clone aab20h10 16S ribosomal RNA gene |
| 722 | 98.93 | JF429411 | Uncultured bacterium CSL142 |
| 723 | 100 | HM104378 | Halomonas sp. AMP-12 16S ribosomal RNA gene |
| 724 | 99.46 | HQ698306 | Bradyrhizobium sp. 75L2DEPT 16S ribosomal RNA gene |
| 725 | 98.93 | FN689723 | Sporotalea propionica partial 16S rRNA gene |
| 726 | 99.46 | CU920136 | Uncultured Bacteroidetes bacterium 16S rRNA gene from clone QEDT3DA02. |
| 727 | 98.39 | JN635729 | Burkholderia sp. GPB24 16S ribosomal RNA gene |
| 728 | 93.79 | JF429020 | Uncultured bacterium clone G99 16S ribosomal RNA gene |
| 729 | 100 | JN860405 | Uncultured beta proteobacterium clone T13M-B11 16S ribosomal RNA gene |
| 730 | 99.2 | AB539938 | Uncultured bacterium gene for 16S rRNA |
| 731 | 98.93 | JN713500 | Aerococcus viridans canine oral taxon 331 clone 1D024 16S ribosomal RNA gene |
| 732 | 100 | Z49828 | Y.enterocolitica gene for 16S ribosomal RNA (strain ER-3206-92) |
| 733 | 100 | JN700137 | Brevundimonas vesicularis strain L17 16S ribosomal RNA gene |
| 734 | 97.33 | HM445394 | Uncultured bacterium clone GP28374d1O 16S ribosomal RNA gene |
| 735 | 100 | JN613484 | Novosphingobium sp. O-MR18 16S ribosomal RNA gene |
| 736 | 99.2 | EU156147 | Uncultured delta proteobacterium clone pCOF_65.7_D12 16S ribosomal RNA gene |
| 737 | 99.73 | AJ009495 | uncultured bacterium SJA-149 16S rRNA gene |
| 738 | 99.47 | GQ369135 | Dyella sp. Z3-YC6867 16S ribosomal RNA gene |
| 739 | 99.73 | Y13364 | Gemella sanguinis 16S rRNA gene (strain 2045-94) |
| 740 | 99.46 | JN846928 | Moraxellaceae bacterium 14b-m12-2 16S ribosomal RNA gene |
| 741 | 98.93 | GQ264250 | Uncultured bacterium clone WW1_b52 16S ribosomal RNA gene |
| 742 | 98.89 | AY703459 | Uncultured bacterium isolate ALT24 16S ribosomal RNA gene |
| 743 | 97.06 | JQ088436 | Uncultured bacterium clone Ba19W-B12 16S ribosomal RNA gene |
| 744 | 93.01 | EU475540 | Uncultured bacterium clone SBSD_aaa02a10 16S ribosomal RNA gene |
| 745 | 98.93 | FN401323 | Bacteroides ureolyticus partial 16S rRNA gene |
| 746 | 99.73 | HQ120263 | Uncultured bacterium isolate 1112864242202 16S ribosomal RNA gene |
| 747 | 99.47 | L40620 | Geodermatophilus obscurus obscurus 16S ribosomal RNA (16S rRNA) gene. |
| 748 | 99.47 | JN530317 | Uncultured organism clone SBZO_1731 16S ribosomal RNA gene |
| 749 | 100 | JF042864 | Uncultured bacterium clone ncd487h01c1 16S ribosomal RNA gene |
| 750 | 93.62 | DQ125579 | Uncultured bacterium clone AKAU3574 16S ribosomal RNA gene |
| 751 | 97.57 | FJ674389 | Uncultured bacterium clone LL141-1G11 16S ribosomal RNA gene |
| 752 | 96.31 | JN443250 | Uncultured organism clone SBYB_6783 16S ribosomal RNA gene |
| 753 | 97.57 | DQ905206 | Uncultured bacterium clone 013-b3 16S ribosomal RNA gene |
| 754 | 99.2 | HQ588841 | Roseomonas sp. BZ31r 16S ribosomal RNA gene |
| 755 | 93.59 | JN443250 | Uncultured organism clone SBYB_6783 16S ribosomal RNA gene |
| 756 | 100 | GU562448 | Acetoanaerobium noterae strain ATCC 35199 16S ribosomal RNA gene |
| 757 | 99.73 | CP002541 | Spirochaeta sp. Buddy |
| 758 | 99.73 | AY835922 | Algoriphagus locisalis strain MSS-170 16S ribosomal RNA gene |
| 759 | 99.46 | JN511345 | Uncultured organism clone SBZF_819 16S ribosomal RNA gene |
| 760 | 98.93 | GQ866113 | Salinimicrobium sp. KMM 6320 16S ribosomal RNA gene |
| 761 | 99.46 | JF905978 | Uncultured Olivibacter sp. clone ASC145 16S ribosomal RNA gene |
| 762 | 99.73 | JN995250 | Stenotrophomonas sp. enrichment culture clone CW-4Y 16S ribosomal RNA gene |
| 763 | 98.92 | HQ857727 | Uncultured Flavobacteriaceae bacterium clone BPS_L271 16S ribosomal RNA gene |
| 764 | 97.32 | AF371948 | Uncultured bacterium clone p-5460-2Wb5 16S ribosomal RNA gene |
| 765 | 99.13 | JN217203 | Uncultured bacterium clone TERI-KL77 16S ribosomal RNA gene |
| 766 | 97.08 | AB487354 | Uncultured bacterium gene for 16S ribosomal RNA |
| 767 | 98.66 | AM419042 | Azospirillum rugosum partial 16S rRNA gene |
| 768 | 99.72 | JF137450 | Uncultured bacterium clone ncd1589e07c1 16S ribosomal RNA gene |
| 769 | 98.4 | HM299855 | Uncultured bacterium clone ncd765h02c1 16S ribosomal RNA gene |
| 770 | 99.47 | EU266839 | Uncultured Actinomycetaceae bacterium clone D12_40 small subunit ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 771 | 99.46 | JN662526 | Pedobacter sp. A16.2 16S ribosomal RNA gene |
| 772 | 99.73 | JN862928 | Acinetobacter sp. Max N.07 16S ribosomal RNA gene |
| 773 | 99.47 | JN885186 | Streptomyces atrovirens strain S3RS9 16S ribosomal RNA gene |
| 774 | 99.73 | JF131310 | Uncultured bacterium clone ncd1496h02c1 16S ribosomal RNA gene |
| 775 | 97.28 | HM445411 | Uncultured bacterium clone GP28374hO9 16S ribosomal RNA gene |
| 776 | 99.47 | X79450 | Sanguibacter keddieii ST74 16S rDNA |
| 777 | 98.41 | U28233 | Burkholderia solanacearum strain R142 16S ribosomal RNA gene |
| 778 | 98.66 | JF505962 | Georgenia muralis strain KNUC9028 16S ribosomal RNA gene |
| 779 | 96.01 | AB233210 | Legionella yabuuchiae gene for 16S rRNA |
| 780 | 98.91 | JF800718 | Uncultured bacterium clone BT420 16S ribosomal RNA gene |
| 781 | 97.87 | HQ220040 | Pseudonocardia sp. AAI2 16S ribosomal RNA gene |
| 782 | 97.06 | HQ184012 | Uncultured bacterium clone De1870 16S ribosomal RNA gene |
| 783 | 97.81 | HM992533 | Uncultured Peptococcaceae bacterium clone BTEX1-2H 16S ribosomal RNA gene |
| 784 | 98.92 | JQ231157 | Citrobacter freundii strain UMS5/10 16S ribosomal RNA gene |
| 785 | 95.99 | EU542497 | Uncultured bacterium clone Er-LLAYS-19 16S ribosomal RNA gene |
| 786 | 99.73 | FN547417 | Flectobacillus sp. WG3 partial 16S rRNA gene |
| 787 | 94.24 | EU134681 | Uncultured bacterium clone FFCH2290 16S ribosomal RNA gene |
| 788 | 98.93 | HQ121102 | Uncultured bacterium isolate 1112863845158 16S ribosomal RNA gene |
| 789 | 98.42 | JN433325 | Uncultured organism clone SBXZ_4082 16S ribosomal RNA gene |
| 790 | 99.46 | FJ823892 | Uncultured Magnetospirillum sp. clone MFC63C09 16S ribosomal RNA gene |
| 791 | 98.1 | HM445156 | Uncultured bacterium clone GBL17O79 16S ribosomal RNA gene |
| 792 | 96.72 | EU234149 | Uncultured bacterium clone A02 16S ribosomal RNA gene |
| 793 | 98.12 | EF204460 | Epilithonimonas lactis strain H1 16S ribosomal RNA gene |
| 794 | 97.53 | JN090824 | Uncultured bacterium clone d_145_AG_Acet_117 16S ribosomal RNA gene |
| 795 | 97.34 | AB488308 | Uncultured bacterium gene for 16S ribosomal RNA |
| 796 | 96.51 | HQ183885 | Uncultured Sphaerobacter sp. clone De182 16S ribosomal RNA gene |
| 797 | 98.93 | EU134205 | Uncultured bacterium clone FFCH13578 16S ribosomal RNA gene |
| 798 | 99.19 | JN038707 | Uncultured Bacteroidetes bacterium clone MP-R33 16S ribosomal RNA gene |
| 799 | 99.47 | HQ327121 | Okibacterium sp. TP-Snow-C12 16S ribosomal RNA gene |
| 800 | 98.93 | HE582841 | Uncultured bacterium partial 16S rRNA gene |
| 801 | 99.47 | JF096723 | Uncultured bacterium clone ncd1313h06c1 16S ribosomal RNA gene |
| 802 | 94.15 | EU335394 | Uncultured bacterium clone BacC-u_075 16S ribosomal RNA gene |
| 803 | 100 | Y12703 | Paracoccus marcusii 16S rRNA gene |
| 804 | 94.41 | JF135912 | Uncultured bacterium clone ncd1566g08c1 16S ribosomal RNA gene |
| 805 | 97.86 | FR691503 | Uncultured bacterium partial 16S rRNA gene |
| 806 | 98.13 | EU132646 | Uncultured bacterium clone FFCH10726 16S ribosomal RNA gene |
| 807 | 95.5 | FJ366223 | Uncultured bacterium clone TS25_a04b08 16S ribosomal RNA gene |
| 808 | 97.86 | JF828748 | Uncultured bacterium clone 46 16S ribosomal RNA gene |
| 809 | 98.66 | HQ218462 | Uncultured bacterium clone N-18 16S ribosomal RNA gene |
| 810 | 97.86 | AY728067 | Uncultured low G+C Gram-positive bacterium clone S1-6-CL2 16S ribosomal RNA gene |
| 811 | 99.2 | JF274912 | Aeromicrobium sp. PL34a1_S1 16S ribosomal RNA gene |
| 812 | 93.84 | AY703459 | Uncultured bacterium isolate ALT24 16S ribosomal RNA gene |
| 813 | 97.59 | JQ309638 | Paenibacillus sp. YIM 110206 16S ribosomal RNA gene |
| 814 | 98.92 | DQ450752 | Uncultured Bacteroidetes bacterium clone E08_WMSP1 16S ribosomal RNA gene |
| 815 | 100 | EF510502 | Uncultured bacterium clone P2D1-720 16S ribosomal RNA gene |
| 816 | 97.07 | HM445156 | Uncultured bacterium clone GBL17O79 16S ribosomal RNA gene |
| 817 | 98.67 | HQ896304 | Bacterium enrichment culture clone T12RRH100B12 clone T12RRH100B12 16S ribosomal RNA gene |
| 818 | 95.38 | DQ129304 | Uncultured bacterium clone AKIW813 16S ribosomal RNA gene |
| 819 | 98.67 | Z78211 | N.plantarum 16S rRNA gene |
| 820 | 99.2 | JF239458 | Uncultured bacterium clone ncd2786h12c1 16S ribosomal RNA gene |
| 821 | 99.46 | AF127413 | Gluconacetobacter sacchari isolate IF9645 16S ribosomal RNA gene |
| 822 | 99.2 | JF229350 | Uncultured bacterium clone ncd2625f02c1 16S ribosomal RNA gene |
| 823 | 92.43 | HQ156044 | Uncultured prokaryote clone 08031003-Z7EU_2TH_2_2_F07 16S ribosomal RNA gene |
| 824 | 99.47 | JF233959 | Uncultured bacterium clone ncd2699d06c1 16S ribosomal RNA gene |
| 825 | 98.66 | HQ597264 | Uncultured Acidobacteria bacterium clone AEG_08_337 16S ribosomal RNA gene |
| 826 | 98.13 | EU245118 | Uncultured organism clone MAT-CR-H2-D11 16S ribosomal RNA gene |
| 827 | 99.46 | HQ910320 | Uncultured bacterium clone P-8_B21 16S ribosomal RNA gene |
| 828 | 96.99 | JF727691 | Uncultured alpha proteobacterium clone bac722 16S ribosomal RNA gene |
| 829 | 99.47 | JF828748 | Uncultured bacterium clone 46 16S ribosomal RNA gene |
| 830 | 97.86 | JN471837 | Uncultured organism clone SBYS_698 16S ribosomal RNA gene |
| 831 | 99.2 | FJ671445 | Uncultured bacterium clone LL143-7K3 16S ribosomal RNA gene |
| 832 | 97.33 | FJ366223 | Uncultured bacterium clone TS25_a04b08 16S ribosomal RNA gene |
| 833 | 99.2 | AY571806 | Nocardioides sp. 43/50 16S ribosomal RNA gene |
| 834 | 98.4 | JF238812 | Uncultured bacterium clone ncd2775h06c1 16S ribosomal RNA gene |
| 835 | 99.19 | GQ058027 | Uncultured bacterium clone nbw1069b06c1 16S ribosomal RNA gene |
| 836 | 95.42 | FN668192 | Uncultured Flexibacter sp. partial 16S rRNA gene |
| 837 | 98.66 | AF273081 | Bosea minatitlanensis 16S ribosomal RNA gene |
| 838 | 98.4 | EU888823 | Uncultured Clostridium sp. clone B03 16S ribosomal RNA gene |
| 839 | 97.33 | DQ378174 | Uncultured Actinomycetales bacterium clone F09_Pitesti 16S ribosomal RNA gene |
| 840 | 99.47 | JF703618 | Uncultured Marmoricola sp. clone HaG1 16S ribosomal RNA gene |

Table A-1: continued.

| OTU | BLAST % ID to Best Hit | Accession # of Best BLAST Hit | Full Name of Best BLAST Hit |
|---|---|---|---|
| 841 | 95.99 | HQ121127 | Uncultured bacterium isolate 1112863845180 16S ribosomal RNA gene |
| 842 | 99.47 | GQ246683 | Microbacterium sp. M1T8B9 16S ribosomal RNA gene |
| 843 | 97.87 | GQ167189 | Uncultured bacterium clone SP2-0 16S ribosomal RNA gene |
| 844 | 99.2 | JF145628 | Uncultured bacterium clone ncd1641a09c1 16S ribosomal RNA gene |
| 845 | 98.66 | JN685484 | Uncultured bacterium clone NK-M22 16S ribosomal RNA gene |
| 846 | 99.2 | AP008956 | Populus alba chloroplast DNA |
| 847 | 94.41 | EU335298 | Uncultured bacterium clone BacB_041 16S ribosomal RNA gene |
| 848 | 95 | AF050606 | Uncultured eubacterium WCHB1-64 16S ribosomal RNA gene |
| 849 | 95.94 | EU468481 | Uncultured bacterium clone OK1_f08 16S ribosomal RNA gene |
| 10a | 90.21 | GU361138 | Haloarcula sp. MGG3 16S ribosomal RNA gene |
| 10e | 97.54 | Y16814 | Zygosaccharomyces lentus 18S rRNA gene |
| 11a | 93.19 | AJ969888 | uncultured archaeon 16S rRNA gene |
| 12a | 98.41 | AB266915 | Uncultured bacterium gene for 16S rRNA |
| 13a | 97.88 | AB477231 | Natrinema pellirubrum gene for 16S ribosomal RNA |
| 14a | 98.15 | AF033672 | Methanocalculus halotolerans 16S ribosomal RNA gene |
| 15a | 98.41 | X51423 | Methanothrix soehngenii DNA for a ribosomal RNA gene cluster |
| 16a | 97.86 | HQ157641 | Uncultured archaeon clone TSHNAA4 16S ribosomal RNA gene |
| 17a | 96.45 | FN391256 | Uncultured euryarchaeote partial 16S rRNA gene |
| 18a | 96.56 | AM947496 | Uncultured Haloarcula sp. partial 16S rRNA |
| 19a | 98.15 | AY570664 | Uncultured archaeon clone PL-9A3 16S ribosomal RNA gene |
| 1a | 98.41 | EF468473 | Halorubrum tebenquichense strain JCM12290 16S ribosomal RNA gene |
| 1e | 94.55 | X75080 | Pinus wallichiana 18S rRNA gene. |
| 20a | 90.19 | GU361138 | Haloarcula sp. MGG3 16S ribosomal RNA gene |
| 21a | 98.41 | HQ157635 | Uncultured archaeon clone TSHNAA14 16S ribosomal RNA gene |
| 22a | 94.44 | AF050612 | Uncultured archaeon WCHD3-30 16S ribosomal RNA gene |
| 23a | 98.15 | HI592318 | Sequence 7 from Patent WO2010115424. |
| 24a | 97.36 | HM159606 | Halobacteriaceae archaeon R44 16S ribosomal RNA gene |
| 25a | 92.72 | EU869371 | Uncultured haloarchaeon clone ARDARCSS13 16S ribosomal RNA gene |
| 26a | 97.62 | FN391234 | Uncultured haloarchaeon partial 16S rRNA gene |
| 27a | 95.28 | GQ374968 | Uncultured haloarchaeon clone Cry7_clone27 16S ribosomal RNA gene |
| 28a | 98.14 | EU635904 | Uncultured archaeon clone SSW_L4_E01 16S ribosomal RNA gene |
| 2a | 98.15 | JN714436 | Uncultured haloarchaeon clone XKL39 16S ribosomal RNA gene |
| 2e | 91.35 | X51576 | Tomato 17S rRNA gene |
| 3a | 98.41 | CU466680 | Environmental 16s rDNA sequence from Evry wastewater treatment plant anoxic basin. |
| 3e | 91.38 | X75080 | Pinus wallichiana 18S rRNA gene. |
| 4a | 97.62 | JF802164 | Haloarchaeon 2ANA_DGR 16S ribosomal RNA gene |
| 4e | 97.84 | Z75582 | S.unisporus 18S rRNA gene |
| 5a | 98.41 | AY454559 | Uncultured crenarchaeote clone D_B11 16S ribosomal RNA gene |
| 5e | 96.24 | X75080 | Pinus wallichiana 18S rRNA gene. |
| 6a | 98.15 | AJ270234 | Haloarchaeon str. T3.1 partial 16S rRNA gene |
| 6e | 97.01 | X67238 | S.tuberosum gene for 18S rRNA |
| 7a | 98.41 | FJ793266 | Halorubrum sp. GS1 16S ribosomal RNA gene |
| 7e | 94.64 | FJ153124 | Yarrowia sp. TFM01 18S ribosomal RNA gene |
| 8a | 96.83 | HQ425155 | Uncultured archaeon clone GA41 16S ribosomal RNA gene |
| 8e | 97.1 | X79407 | A.lasiocarpa 18S rRNA gene |
| 9a | 97.09 | GU363383 | Uncultured haloarchaeon clone LP4_4A_2 16S ribosomal RNA gene |
| 9e | 94.96 | X79407 | A.lasiocarpa 18S rRNA gene |

# APPENDIX B

## INVESTIGATION OF MICROBIOLOGICALLY INFLUENCED CORROSION OF LINEPIPE STEELS IN ETHANOL AND ACETIC ACID SOLUTIONS

As described in Chapter 3 of this thesis, acetic-acid-producing bacteria (*Acetobacter* spp.) and a sulfate-reducing bacterial consortium that included *Desulfosporosinus* spp. were cultivated from fuel storage tanks containing fuel-grade ethanol and water. A manuscript describing electrochemical corrosion testing of these microbes on linepipe steels has been submitted to NACE 2013. Luke Jain is the first author of this paper. I am a co-author and contributed to the research by maintaining and monitoring bacterial cultures, aiding with corrosion test setup and conducting molecular microbiology work. Citation information for the paper is displayed at the end of Appendix B. Pyrosequencing analyses described in Chapter 3 as well as Sanger sequencing of 16S rRNA genes from environmental DNA indicated that *Acetobacter* spp. and *Desulfosporosinus* spp. were present in the fuel storage tanks, and the microbes cultured from these samples showed the ability to grow with ethanol as a carbon source, which suggests that these sorts of microbes could be problematic in environments exposed to fuel-grade ethanol and water. Electrochemical corrosion testing indicated that the sulfate-reducing bacterial consortium increased general corrosion rates of linepipe steels (API X52 and API X70) and that both the sulfate-reducing consortium and the *Acetobacter* spp. increased pitting corrosion of the steels.

**Citation Information**

Jain, L A.,. C. **Williamson**, J. R. Spear, D. L. Olson, R. D. Kane and B. Mishra.

"Microbiologically Influenced Corrosion of Linepipe Steels in Ethanol and Acetic Acid Solutions." Submitted to NACE 2013.

# APPENDIX C

## INVESTIGATION OF MICROBIOLOBICALLY INFLUENCED CORROSION AND FATIGUE CRACK GROWTH BEHAVIOR OF PIPELINE STEELS

As described in Chapter 3 of this thesis, acetic-acid-producing bacteria (*Acetobacter* spp.) and a sulfate-reducing bacterial consortium that included *Desulfosporosinus* spp. were cultivated from fuel storage tanks containing fuel-grade ethanol and water. Experiments to determine how these microbes impact fatigue crack growth behavior of pipeline materials have been conducted at the National Institute of Standards and Technology in Boulder, Colorado. Citation information for a manuscript presented at the DOD Corrosion Conference is included at the end of Appendix C. The first author of this paper is Jeff Sowards, and I contributed to the work through maintaining and monitoring bacterial cultures and aiding with corrosion test setup. This paper describes the crack growth behavior of API X52 and API X70 steels in the presence of simulated fuel-grade ethanol as well as cultures of the *Acetobacter* sp. isolated from samples ECT.1 (Chapter 3). Fatigue crack growth rates were increased due to the presence of acetic-acid-producing bacteria. This research has continued to evaluate the impact of the sulfate-reducing consortium described in Chapter 3 on the crack growth behavior of pipeline and tank steels. A manuscript describing this work is currently in preparation.

**Citation Information**

J. W. Sowards, T. D. Weeks, J. D. McColskey, **C. Williamson**, L. Jain and J. R. Fekete. "Effect of ethanol fuel and microbiologically influenced corrosion on the fatigue crack growth behavior of pipeline steels." Presented at DOD Corrosion Conference 2011, La Quinta, Calif., August 1, 2011.

# APPENDIX D

## INVESTIGATION OF SULFUR BIOMINERALIZATION AT A SPRING SYSTEM IN THE CANADIAN HIGH ARCTIC

A supraglacial sulfur spring on Ellesmere Island, Canada provides a unique environment for the study of microbial sulfur metabolism. Citation information for a manuscript regarding sulfur biommineralization at this site is described at the end of Appendix D. The paper is published in *Geobiology*, and Damhnait Gleeson is the first author of the paper. I am a co-author of the paper and contributed to the work by conducting molecular lab work and bioinformatic work. Sanger sequencing of 16S rRNA genes indicated the presence of microbes such as *Ralstonia* spp, *Sulfuricurvales* spp., *Sulfurovumales* spp. and *Thiomicrospira* spp. Cultivation experiments indicated the presence of sulfur-oxidizing bacteria that produced elemental sulfur.

**Citation Information**

Gleeson, D. F., **C. Williamson**, S. E. Grasby, R. T. Pappalardo, J. R. Spear, and A. S. Templeton. 2011. "Low Temperature S0 Biomineralization at a Supraglacial Spring System in the Canadian High Arctic." *Geobiology* 9 (4): 360–375.

# APPENDIX E

## INVESTIGATION OF SULFUR LITHOTROPHY AT A SPRING SYSTEM IN THE CANADIAN HIGH ARCTIC

Microbial sulfur metabolism was studied at a supraglacial spring at Borup Fiord Pass on Ellesmere Island in the Canadian High Arctic. Citation information of a manuscript regarding this work is described at the end of Appendix E. Katherine Wright is the first author of this paper. I am a co-author of the manuscript and contributed to the research through aiding with molecular lab work and bioinformatic analyses. The paper combines bioenergetic and metagenomic anlayses to investigate microbial sulfur metabolism.

**Citation Information**

Wright, K. E., **C. Williamson**, S. E. Grasby, J. R. Spear and A. S. Templeton. "Metagenomic evidence for sulfur lithotrophy by Epsilonproteobacteria as the major energy source for primary productivity in a sub-aerial arctic glacial deposit, Borup Fiord Pass." Submitted to Frontiers in Extreme Microbiology.