BIOFUELS WHITE PAPER

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1.0 Introduction

There is a renewed emphasis on the development of alternative fuels due to the global energy crisis and the effects of the environmental pollution produced by the use of fossil fuels. These alternative fuels would provide clean, renewable energy sources which could eventually replace fossil fuels as the primary source of energy. A list of alternative fuels that have been developed is provided in Table 1. Out of these alternative fuels there are four biofuels that are considered as the most likely replacements for fossil fuels. They are methane, hydrogen, bio-ethanol, and bio-butanol. For diesel fuels, biodiesel is also being considered as an alternative.

Alternative Fuels
Biodiesel
Electricity
Bio-butanol
Bio-ethanol
Hydrogen
Bio-methane
Propane
Dimethyl ether
Methanol

Table 1: Alternative Fuels

2.0 Important Types of Biofuels, Production Methods, and Concerns

2.1 Biodiesel

Biodiesel is fuel that has emerged as a potential replacement for fossil diesel because it is biodegradable, non-toxic, and renewable. It is also free of sulfur and benzene, can be blended with fossil diesel at any ratio, and does not require engine modifications in order to be used in common diesel engines. At present, biodiesel is mainly produced through a process that involves alkali catalyzed transesterification of vegetable oils or animal fats. However, new methods have been developed which utilize homogeneous acid catalysts to reduce the amount of free fatty acids produced or ionic liquid catalysts which are more thermally and chemically stable and less toxic[1, 2]. Biodiesel can still be toxic to humans in the pure or blended forms, therefore groundwater contamination remains a concern. Blended biodiesel can contain BTEX and methyl tert-butyl ether (MTBE) which are highly toxic. Bioremediation of pure and blended biodiesel is carried out by many of the same organisms that are involved in the biodegradation of petro-diesel such as *Methylibium petroleiphilum* PM1 which degrades MTBE and biosurfactant producers which help increase bioremediation3. One difference between the biodegradation of petro and biodiesel is that biodiesel is oxidized more easily than petro-diesel. The oxidation of biodiesel by microorganisms and other processes results in the production of peroxides, hyperoxides and various organic acids, aldehydes, ketones, and alcohols. This is an additional concern for biodiesel utilization given that the organic acids produced can lead to corrosion. Further, the biodiesel fuel itself can become rancid in a few weeks and lead to corrosion of pipes and storage tanks[4].

2.2 Bio-methane

Bio-methane is primarily produced by methanogens which are often found at livestock farms, landfills, wastewater treatment plants, and rice farms. Methanogens utilize fermentation products such as acetate, formate, methanol, methylamines, H2 and CO2 as carbon and energy sources. In turn, they produce high concentrations of methane, a greenhouse gas that contributes significantly to climate change. However, if this methane is harnessed it can serve as a renewable source of energy. This can be accomplished through the use of anaerobic digesters which consist of an anaerobic digestion chamber, a dome that allows for the biogas to accumulate, an inlet for feedstock, an outlet for the collection of the biogas, and an outlet for effluent waste. Electrical stimulation can also be employed to increase the yield of biogas from anaerobic digesters[5]. An advantage of bio-methane is that the infrastructure for transporting it is already in place because methane is the primary component of natural gas which comes from a non-renewable source. Bio-methane would only need to be cleaned to remove carbon dioxide, nitrogen, sulfur, and halogenated compounds so that purified methane could then be transported through the existing pipelines.

2.3 Bio-ethanol

Bio-ethanol is considered as one of the potential substitutes for conventional gasoline in order to reduce greenhouse gas emissions. It can be used directly or blended with conventional gasoline. Direct use of ethanol requires larger gas tanks and has been demonstrated to have difficulty starting engines at lower temperatures. Bio-ethanol is produced through the fermentation of simple sugars that are obtained from feedstock consisting of carbohydrate sources such as corn, soy beans, and sugar cane. The yeast *Saccharomyces cerevisiae* is the most commonly used microorganism for the generation of bio-ethanol, but other organisms including the bacteria *Zymomonas mobilis* can also be utilized[6, 7]. A major issue that hinders the use of ethanol is microbial influenced corrosion (MIC) by contaminating organisms such as acetic acid bacteria, sulfate reducing bacteria, and methanogens. Monitoring bio-ethanol systems for these organisms is important because acetic acid bacteria as well as some methanogens and some sulfate reducers can utilize ethanol and produce acetic acid8, 9. Acetic acid-producing microbes (acetogens) in particular have been highlighted as a cause of corrosion in storage tanks used for ethanol, gasoline-ethanol blends, and ultra-low sulfur diesel fuel tanks that have ethanol contamination[10, 11].

2.4 Bio-butanol

As an alternative fuel, bio-butanol has a longer carbon chain than ethanol as well as a higher volatility, polarity, combustion value, and octane rating. It is also less corrosive and has been found to have less ignition problems due to its lower heat of vaporization. Industrially most butanol is currently being generated from petroleum-based derivatives via the oxo process by using propylene, but it can also be generated as a renewable source through fermentation processes using organisms such as *Clostridium beijerickii* and *Clostridium acetobutylicum*. Difficulties associated with generating butanol through fermentation include the inhibition of microbial growth once butanol concentrations reach approximately 20g/L, and the need to establish and maintain the anaerobic conditions required before fermentation and throughout the process[12, 13].

2.5 Hydrogen

Biologically produced hydrogen is another potential alternative fuel and energy source. The advantage of using hydrogen as a fuel are that hydrogen leaks do not cause contamination in the environment or harm to humans or wildlife. Currently, the majority of hydrogen is produced by separating hydrogen atoms from methane, but this produces greenhouse gases that can contribute to global warming. An alternative, environmentally friendly method of hydrogen production is microbial hydrogen production via the fermentation of organic matter. This can be performed through two different methods: direct fermentation and microbial electrolysis cells[14].

2.5.1 Direct Fermentation

Direct fermentation utilizes microbes that breakdown organic matter, and the byproducts that are produced are combined by enzymes to produce hydrogen. Fermentation has already been used to generate biofuels and other products; therefore, many of the problems with scaling up systems have already been addressed. The main concerns of researchers are issues that are unique to hydrogen production. The research in this area is currently focused on making the fermentation systems produce hydrogen faster and increasing the product yield from the same amount of organic matter starting material[15].

2.5.2 Microbial Electrolysis Cells (MECs)

Microbial electrolysis cells (MECs) harness the energy and protons produced by microbes during the breakdown of organic matter. When these processes are combined with a small electric current, hydrogen can be produced[16]. This is a newer method, and researchers are still trying to identify lower-cost materials and the most effective microbes to use. The next step would then be to develop systems that can be scaled up to commercial sizes while keeping the same production rates and efficiencies that are observed at the bench scale.

2.5.3 Hydrogen Transport

An area of concern for hydrogen is its transport. In regions where hydrogen is in high demand; it is often transported via pipelines. In areas where there is small-scale or emerging demand, hydrogen is transported in cryogenic liquid tankers or gaseous tube trailers. There is also work being conducted on using barges as carriers. The cost of transporting hydrogen is high and is the cause of inefficiencies in using it as a fuel. Transporting it via pipeline is the more cost effective option in the long run, but the initial cost of constructing new pipelines is very high which creates a barrier. Unlike ethanol, microbes have not been shown to have an effect on the storage systems and pipelines that would be used for hydrogen. Therefore, the major concerns for hydrogen pipelines include the embrittlement of the steel pipes and welds due to the extreme cold of the hydrogen, potential leaks, and a need for a lower cost, more reliable and durable compression technology. Researchers are studying the use of reinforced polymers to improve the current pipelines while also studying how existing natural gas pipelines could be used to transport hydrogen[17, 18].

3.0 Molecular Biology Tools for Monitoring Biofuel Systems and Biodegradation

3.1 CENSUS®

CENSUS® is based on a molecular technique called quantitative polymerase chain reaction (qPCR), whereby many copies of a specific gene present in a total complement of DNA are generated. As each gene copy is made, a fluorescent marker is released, measured, and used to quantify the number of target genes present in the sample. The gene copied during the PCR process (the target gene) is determined by short segments of DNA called primers which are added to the reaction mixture.

Essentially, qPCR is analogous to a copy machine with a counter. The primers select which pages (the target gene) of the book (DNA) are copied, and the counter keeps a running total of how many pages were copied, i.e. the number of target genes in the sample. Because each target gene is characteristic of a specific organism or group of organisms, the number of copies generated with each PCR cycle indicates the abundance of these organisms in the environmental sample. Microbial Insights has developed and licensed qPCR assays that can be useful for monitoring organisms that play important roles in biofuel production and storage.

3.1.1 Advantages of CENSUS® Compared to Culture-Dependent Methodologies

Culture-dependent methods such as plate counts or most probable number (MPN) analyses have been traditionally used to estimate bacterial populations in diverse environmental samples, with detection being, at best, semi-quantitative. However, plate counts and MPNs are dependent upon propagating the target microbial population on solid media (agar) plates or liquid growth media in the laboratory – an enrichment step which drastically changes the composition of the microbial community. Despite advances in the development of artificial media capable of supporting the growth of numerous diverse microbes, typically less than 10% and often less than 1% of the total population can be cultivated in the laboratory, resulting in “culture bias.”

CENSUS® analysis, on the other hand, is used to assay a genetic marker directly from the DNA of an environmental sample, completely eliminating the necessity to grow the target organisms for enumeration. Using CENSUS® to monitor organisms in biofuel systems has many important advantages over traditional culture-based methods as listed below.

CENSUS® can be used to quantify specific groups of organisms that are important for biofuel production, storage, and biodegradation (Table 2).

Target	Description
Methanogens	Quantifies total methanogens which are the primary source of biological methane production. They can be used in anaerobic digesters to produce methane that can be harnessed for energy. Methanogens are also a common contaminant in bio-ethanol where some methanogens can convert ethanol to acetate.
Sulfate Reducers	Quantifies sulfate reducing bacteria (SRB) which are a common contaminant in bio-ethanol where some SRB can utilize ethanol and produce acetate.
Acetic Acid Bacteria	Quantifies acetic acid bacteria which are a common contaminant in bio-ethanol. Acetic acid bacteria can convert ethanol into acetic acid which can cause corrosion in storage tanks with ethanol.
Fermenters	Quantifies fermenting bacteria which could be utilized for the production of hydrogen.
Biosurfactants	Quantifies microbial genes involved in the production of liposaccharide, lipopeptide, and glycolipid biosurfactants which can help increase the bioremediation of biodiesel.
QuantArray® Petro	Quantifies organisms and genes involved in the aerobic and anaerobic biodegradation of alkanes, BTEX, MTBE, napthalene, and other PAHs. It can provide a comprehensive evaluation of biodegradation potential for biodiesel fuels.

Table 2: Microbial Insights Census® Targets Available for Monitoring Alternative Fuel Systems and Biodegradation

3.2 Next Generation Sequencing (NGS)

3.2.1 What is NGS?

Next-generation DNA sequencing (NGS), or high-throughput sequencing, is a collection of advanced technologies for ascertaining the precise order of bases within a DNA molecule. In addition to its unprecedented throughput, NGS offers the advantages of scalability and speed in determining DNA sequences much less expensively than previous sequencing methods. With NGS, one can survey in a cost-effective manner the genomes of entire communities or microbiomes, including those of unculturable constituents. NGS provides identification of microorganisms present in a sample down to the taxonomic level of genus with no prior knowledge of the microbial community composition. Each sequenced segment of DNA is indicative of a specific microorganism. Although metabolic activity cannot always be predicted from phylogeny, comprehensive identification of the microorganisms present in an environment offers deep insight into the potential microbial processes impacting biofuel systems and bioremediation. No other microbial analysis provides more comprehensive characterization of the microbial community in a field sample or better answers the question: What microorganisms are present?

3.2.2 How Does NGS Work?

The various NGS platforms all provide massively parallel sequencing which allows millions of nucleic acid fragments to be sequenced simultaneously and rapidly[19]. While there are multiple unique NGS platforms, the overall steps and the underlying concepts of Next-Generation Sequencing are similar (Figure 1). The general methodology involves template or library preparation, nucleic acid sequencing, and data analysis. First, community genomic DNA (cgDNA) is extracted from an environmental sample and fragmented into a library of small nucleic acid segments. The ends of these DNA fragments are then joined (ligated) with a chemically synthesized adaptor molecule, which is a DNA molecule of known sequence. Second, the library is amplified and subsequently sequenced in millions of parallel reactions.

The sequencing step is similar to previous methods: the bases of each DNA fragment are sequentially identified from light signals emitted as the complement to each fragment strand is resynthesized. The net result is a set of newly identified 'strings' of nucleotides called 'reads' that represent specific members of the microbial community present in the original sample. Comparisons of next-generation sequencing results between samples can reveal important differences or shifts in the microbial community by location, over time, or in response to site activities.

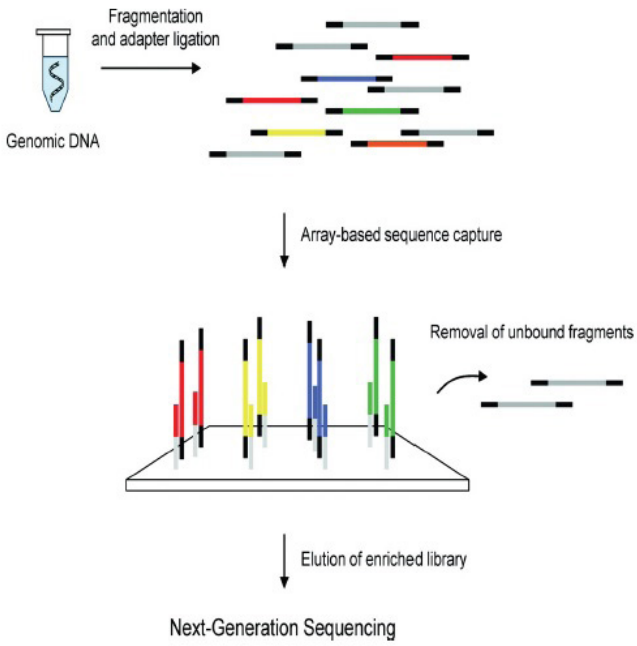

Figure 1: The NGS Approach – DNA Library Construction, Amplification, and Massively Parallel Sequencing

3.2.3 NGS Data Analysis and Interpretation

NGS is not quantitative like quantitative polymerase chain reaction (qPCR). Sequencing results obtained from NGS technology are reported as relative abundances with units of "percent of hits"—the percent of total sequences that have been identified as belonging to a particular microbial genus. Because NGS generates massive sequencing datasets, it is necessary to apply a suite of bioinformatic tools to extract meaningful biological information and to make valid inferences and predictions. These analytic and statistical techniques are described in more detail as follows.

3.2.4 Diversity Indices

The Shannon diversity index is a quantitative measurement that characterizes how many different genera are present in the sample and takes into account the distribution of the number of organisms classified to each genus present in the sample (commonly referred to as species evenness)[20, 21]. The Shannon diversity index increases in value as the number of genera increases and as the number of organisms present per genera becomes even. Simpson's index measures the probability that two individuals selected randomly from the sample would belong to different genera: the greater the value, the greater the sample diversity. The Chao1 index is an excellent indicator of species richness and is based on the number of reads when one (singleton) or two (doubleton) operational taxonomic units (OTUs) are observed. This value is the predicted number of genera based on the number of singletons and doubletons. The total genera observed is presented here, but does not include reads unclassified at genus species.

3.2.5 Principal Coordinate Analysis

Principal coordinate analysis (PCoA) is used to visualize differences in microbial communities between samples[22]. Unlike more traditional methods such as principal component analysis (PCA), PCoA calculates complex functions for the axes rather than dimensional scaling used in PCA. Therefore, PCoA is able to better demonstrate dissimilarities that may be nuanced in PCA tests. PCoA accomplishes this by using a dissimilarity matrix to assign each sample a location in dimensional space, then changes the coordinate system to display the data in two dimensions. This analysis allows the user to visualize multidimensional data in two dimensions. The scatterplot in Figure 2 shows a PCoA of the normalized relative abundance of all samples at the genus-level classifications. Increasing distance between sample points on this plot indicates increasing dissimilarity between bacterial populations in the samples. From the opposite perspective, the microbial community compositions of samples that group near each other in the PCoA plot are more similar. For example, the bacterial community of MW2 is highly similar to that of MW5 (Figure 2, upper left corner). Conversely, the microbial community of MW1 is not particularly similar to those of any other sample collected.

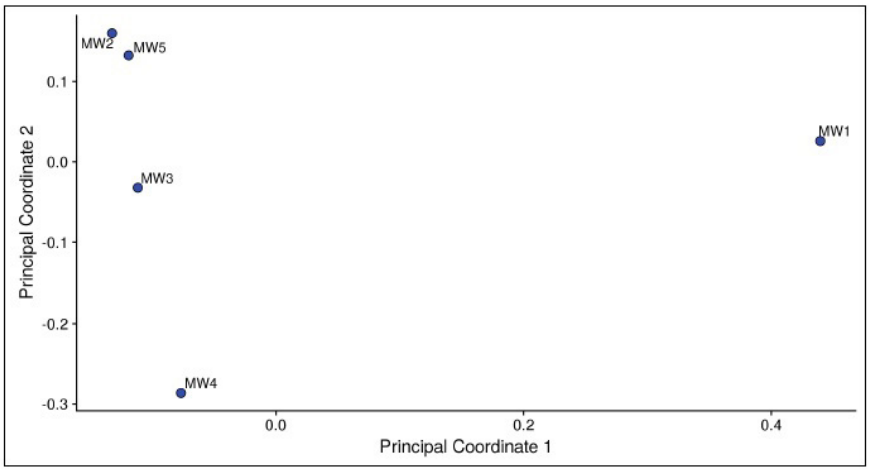

Figure 2: Principal Coordinate Analysis

3.2.6 Hierarchical Clustering Dendrogram

Hierarchical clustering is accomplished by comparing dissimilarities between the samples using complete agglomeration of the Bray-Curtis dissimilarity. This groups together samples which are the least dissimilar. The length of the branches indicate the amount of dissimilarity between samples. Therefore, shorter branches are more similar. An example of a Hierarchical Clustering Dendrogram is shown in Figure 3. The bar chart beneath each sample shows the relative abundance of the top 8 of genus-level classifications, along with all other classified and unclassified genera. Notice that samples MW2 and MW5 cluster together in Figure 3 while MW1 is an outlying branch.

NGS is most appropriate for identifying members of the microbial community present in a sample when little is known about the process in question. NGS data are presented graphically using pie charts showing the relative proportion of the top phylum classification results (see Figure 4 below) and top genus classification results. The top genus classification results are further elaborated in tables providing the specific genus, the corresponding number of reads and percent total reads, and a brief description of the primary metabolic activities exhibited by members comprising the particular genus. A partial example of top genus classification results is shown in Table 3.

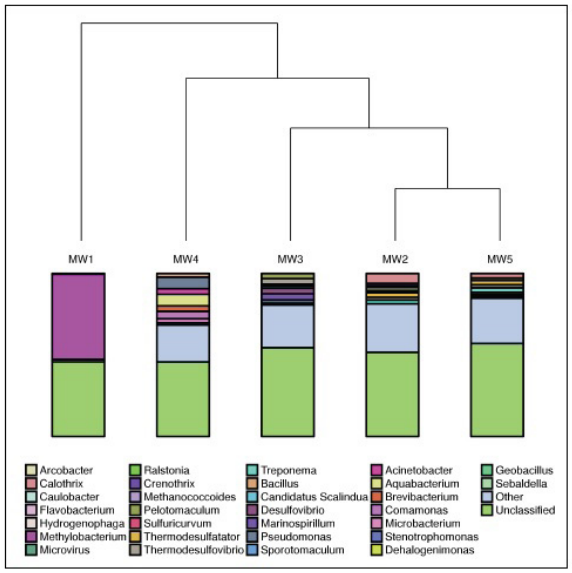

Figure 3: Hierarchical Clustering Dendrogram

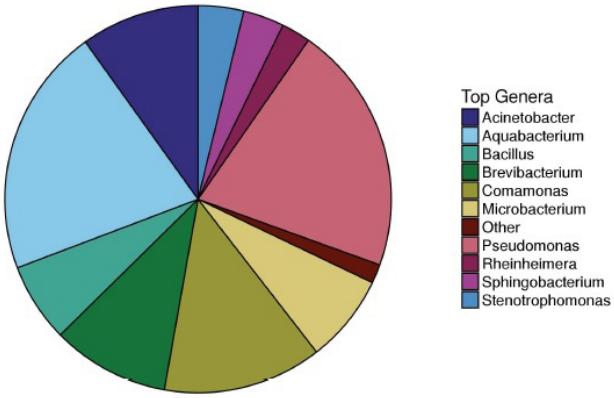

Figure 4: Pie Chart Displaying Top Genera Classifications

Genus	Reads	Percent	Description
Aquabacterium	18,154	13.1%	This genus was isolated from biofilms in Berlin drinking water. They are capable of microaerophilic growth and use nitrate and oxygen as electron acceptors. They metabolize a broad range of organic acids but no carbohydrates.
Pseudomonas	18,142	13.1%	Pseudomonas species can grow very rapidly to take advantage of carbon and oxygen availability. Members of this genus are gram-negative, chemoorganotrophic, and aerobic. Pseudomonas are frequently involved in the early stages of biofilm formation. Biofilms can be detrimental to the underlying surface, leading to biodeterioration of the metal surface.
Comamonas	11,585	8.4%	Members of this aerobic, motile genus have been associated with natural biodegradation and can occur in soil, water, activate sludge, food waste compost, subterranean forest sediment, wetlands, and landfills. Some members have the ability to perform anoxic-reduciton of nitrite, nitrate, and nitrous oxide to nitrogen, while others have arsenite-oxidizing abilities. At least one species has the ability to degrade phenols while another one can degrade 3-chloroaniline. One member can oxidize thiosulfate.
Acinetobacter	8,639	6.2%	These aerobic bacteria can be found in soil and water. Acinetobacter are pioneering species in biofilm formation, and they have been associated with the corrosion of copper plumbing, carbon steel, and stainless steel.
Brevibacterium	8,570	6.2%	These aerobic actinomycetes have a respiratory metabolism.

Table 3: Top Genera Classification Results

In summary, the analysis of NGS data can provide broad insights into microbial community dynamics, such as differences in microbial diversity between a background well and a contaminated well, or overall temporal shifts in microbial community composition. Information on potential microbial activities occurring at a contaminated site can then be used to develop tools or select quantitative PCR targets for routine monitoring and ultimately controlling a complex microbial process.

4.0 Sample Collection Procedures

4.1 Sample Collection and Preservation

Collecting samples for CENSUS® and NGS analysis is no more difficult than collecting groundwater or soil samples for common chemical analyses and can be readily incorporated into a routine sampling event. Below are guidelines to follow when collecting samples for any DNA-based analysis.

1. Use clean latex (or similar) gloves when collecting and handling samples.
2. Keep samples cold (~4°C) to minimize changes in the microbial community.
    a. Place samples on ice or freezer packs in a cooler immediately after collection.
    b. As soon as possible (preferably overnight), ship samples to the laboratory.
    c. Include enough ice/freezer packs to ensure that samples remain cold during shipment.

Microbial Insights (MI) has been receiving field samples for DNA-based analyses for over 25 years and has performed extensive in-house testing of sample preservation and shipping requirements. Overnight shipment at 4°C combined with immediate DNA extraction upon sample receipt at the laboratory minimizes changes to the microbial community.

NGS analysis can be performed on nearly any sample type including groundwater, soil, sediments, and Bio-Traps®. Groundwater samples can be submitted using 1 L poly bottles or using Bio-Flo filters (Figure 3). Bio-Flo filters can be readily attached to ¼ inch tubing and are compatible with low-flow purging/sampling pumps. For more detailed information on sample collection, complete protocols are available on the sampling page of the MI website (http://www.microbe.com/samplingcensus/).

5.0 Quality Assurance/Quality Control (QA/QC) Parameters

For more than 25 years, the primary mission at Microbial Insights (MI) has been to provide the most accurate and precise data in the industry to ensure that our clients can use our results as an integral part of site management decisions.

The accuracy of MI's data is attributed not only to the quality of our assays and continued investment in instrumentation but also the experience of our staff and rigorous QA/QC procedures that are second to none.

- **Date of Extraction:** DNA and RNA extractions are performed the day that the samples are received by MI to minimize the possibility of any changes to the microbial community prior to analysis.
- **Extraction Blanks:** An extraction blank (no sample added) is processed alongside each set of field samples from DNA extraction through analysis to ensure that cross contamination has not occurred.
- **Negative Controls:** A negative control (no DNA) is included to ensure that cross contamination has not occurred.

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