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Metagenomic Analyses of Three Samples from the MCU Process.

Overview

Three Modular Caustic-Side Solvent Extraction (CSSX) Unit (MCU) Strip Effluent Feed samples were sent from Savannah River National Laboratory (SRNL) to Microbial Insights (MI), Knoxville, TN, for molecular analysis. The objective of the analysis was to **profile and identify dominant members of the microbial community in these samples.** More effective control of microbial growth in the MCU depends on the fundamental understanding of the microorganisms responsible for system colonization that leads to biofilm formation and coalescer impairment. This analysis entailed Next-generation sequencing (NGS) of DNA, a collective term for advanced technologies that allow the determination of microbial species present. NGS, also commonly called metagenomics, provides for the identification of microorganisms down to the genus level. Although function, e.g. biofilm formation or corrosion, cannot always be predicted from identification, knowledge of the microorganisms present can offer insight into the potential microbial processes that may influence system function occurring in the MCU. Information on microbial identification can also influence control measures, e.g. operational/maintenance parameters or the choice of biocides to employ. This knowledge can help control undesired microbial activity as conventional cleaning and disinfection regimens may also contribute to inefficient biofilm control and dissemination of microbial resistance to remediation efforts.

Microorganisms are deceptively complicated in that they can survive in different environments due to their physiological adaptability (Whitman et al., 1998). When bacteria and/or fungi colonize on surfaces through attachment, plaques (biofilms) are formed that protect these microorganisms from either washing off or cleaning efforts. Once established, biofilms can produce acids and cause corrosion, which allows the microorganisms access to nutrients (e.g. iron) and makes them more resilient. In a liquid processing system like MCU, the microorganisms can further adapt metabolically to nutrient

availability. Biofilms can provide a constant supply of new microorganisms; as such, the affected system could become impacted, making biofouling or other complications more likely (Brigmon et al., 1997).

Two of the three samples analyzed were microbial enrichments from an MCU Strip Feed Tank sample previously described in SRNL-TR-2018-00254. These samples included an MCU Strip Feed Sample, an MCU Strip Feed Sample grown in low nutrient conditions, and an MCU Strip Feed Sample grown in anaerobic conditions. These samples were selected based on potential conditions in the MCU. Based on our earlier observations we requested both a fungal and separate bacteria analyses. The document 8_048QA-NGS-Report (Bacteria Report 048QA) refers to the bacteria analysis while the Report 76_048QA_NGS_Report (Fungal Report 048QA) refers to the fungal analysis (Microbial Insights A & B, 2019). We have attached the two MI reports and will refer to them separately in this overview as the Bacteria Report and the Fungal Report. In terms of discussion, we will only mention the dominant genera in each system.

1. Bacteria Report 048QA

These samples are listed in Table 1 of the report as well as the quality control (QC) data that is excellent. The range of 89.0-92.8 % (i.e. % Total Reads Classified to Taxonomic Level) is the percentage of the total reads that classified to each level of taxonomy. In other words, there was little damaged or unreadable DNA. Table 2 shows relative diversity between populations. All three samples had diverse microbial populations as measured by the Shannon Weave Diversity Index.

In an overview, a Principal Coordinate Analysis (PCoA) of the normalized relative abundance of all samples at the genus-level classifications is presented in Figure 1. Increasing distance between sample points on this plot indicate increasing different bacterial populations in the samples. These results indicate that while the source sample (Strip Feed Tank) was the same, the culture conditions (low nutrient or anaerobic) led to distinct microbial populations as demonstrated by the molecular analysis results (Microbial InsightsA, 2019). Figure 2 is a diagram that shows relatedness between samples based on genus-level identifications. While there is some commonality in genus identification in all three samples, there are major differences in relative abundance as observed by the ordering or hierarchical clustering correlated with growth conditions. We will discuss only those genera identified in the samples that are $\geq$1% abundance in the total microbial population.

A. Results for MCU Strip Feed Sample

Table 3 indicates that 100% of the "reads" passed "quality filtering", which is the percent of the total reads that passed the QC measurement by the instrument. This is indicative of good sample integrity (i.e. negligible damaged DNA). Table 4 refers to the % reads or identification ranging from Kingdom (99.2%) to Species Level (77%). Figure 3 is a graphical interpretation of the data in Table 4. Table 5 lists the top phyla for the classification results for the MCU Strip Feed Sample. Interestingly 97.9 % of these are Proteobacteria, a major phylum of gram-negative bacteria. They include a wide variety of well-known bacteria found in soil and groundwater. Others are free-living (non-parasitic) or non-biofilm forming and include many of the bacteria responsible for nitrogen fixation. These free-living bacteria may still contribute to biofouling of MCU through accumulation and nutrient cycling. Again Figure 4 is a graph of the data shown in Table 5 demonstrating the predominance of Proteobacteria in the sample.

Table 6 lists the top genera and relative percent identified in the MCU Strip Feed Sample. The top three regarding relative abundance were *Enterobacter* (68.3%), *Comamonas* (12.3%), and *Erwinia* (10.2%). *Burkholderia*, *Clostridium*, and *Klebsiella* were all at 1%. *Enterobacter* are facultatively anaerobic fermenters meaning they can switch from oxygenated to anoxic environments. Facultatively anaerobic means if fluids stop circulating and the system goes anoxic, these microorganisms can thrive. Members of this genus have been found in biofilms of carbon steel exhibiting corrosion activity. *Comamonas* are aerobic chemoorganotrophs: organisms that obtain carbon and energy from the oxidation of reduced organic compounds. The list of compounds from which chemoorganotrophic organisms can generate energy and their sources of carbon is very long, making these microorganisms extremely versatile. *Erwinia* are facultatively anaerobic Proteobacteria that can use organic acids and carbohydrates as carbon and energy sources. Amino acids are used as nitrogen sources and acid may be produced fermentatively. *Burkholderia* are metabolically versatile bacteria capable of utilizing a broad spectrum of carbon and energy sources. Many are strict aerobes, but some species are also capable of nitrate reduction and have been shown to degrade aromatic hydrocarbons. *Clostridium* are spore forming obligate anaerobic bacteria possessing a fermentative type of metabolism. While some species are aerotolerant, growth requirements vary greatly between species, utilizing a range of compounds. Fermentation end products are usually a combination of alcohols and organic acids. Some species within Clostridium are acetogens. *Klebsiella* is a genus of facultative anaerobic chemoorganotrophs possessing both a respiratory and fermentative metabolism. A wide range of sugars are utilized for growth. Some species can fix nitrogen and degrade aromatic hydrocarbons. *Klebsiella* is ubiquitously found in soil, water, on plants and in mammals. One species, *K. oxytoca*, has been linked to steel corrosion (Chitra and Anand, 2018). Figure 5 is a graphical representation of Table 6.

B. Results for MCU Strip Feed Sample Low Nutrient

Table 7 states that 100% of the reads passed quality filtering, which means that there were no unreadable DNA. Table 8 and Figure 6 show the classification rate, which is satisfactory. Like the first sample, the MCU Strip Feed Sample Low Nutrient Table 9 and Figure 7 indicate the top phylum identified (99.3%) were Proteobacteria. This is not surprising since Proteobacteria constitute the largest and phenotypically most diverse division among prokaryotes and represent nearly a half of the partial and complete sequenced prokaryotic genomes (Itävaara et al., 2016). There were collectively more genera identified in this sample compared to the other samples. The top four genera identified regarding relative abundance were *Comamonas* (42.4%), *Pseudomonas* (32.7%), *Erwinia* (8.9%), *Enterobacter* (8.7%), and *Stenotrophomonas* (2.3%) (Table 10). Three of these, *Comamonas*, *Erwinia*, and *Enterobacter*, were a major proportion of the MCU Strip Feed Sample Proteobacteria population. The *Pseudomonas* bacteria is a genus of metabolically versatile, chemoorganotrophic aerobes where growth conditions vary greatly between species. Typically, oxygen is the terminal electron acceptor, though nitrate reduction or complete denitrification may also be used under anerobic conditions. *Pseudomonas* is ubiquitous in nature but may be less tolerant of acidic environments. *Stenotrophomonas* are also aerobic chemoorganotrophs, but metabolism is strictly aerobic. Biofilm production by *Stenotrophomonas* has been linked to corrosion of metal surfaces (Wallace et al., 1994). Some species have shown resistance to hexavalent chromium and being able to degrade xenobiotic compounds. *Stenotrophomonas* is ubiquitous in the environment. Figure 8 is a graphical illustration of the data in Table 10. Low nutrient environments cause selective pressure in microbial populations where dominant bacteria slowly assimilate while those deficient in primary energy source are in survival mode.

C. Results for MCU Strip Feed Sample Anaerobic

Table 11 states that 100% of the reads passed quality filtering, meaning that no unreadable DNA were present in the sample. Table 12 and Figure 9 show the classification rate, which is satisfactory. Table 13 and Figure 10 demonstrate that again a high percentage (99.5%) of the population belongs to the phylum Proteobacteria. There were three genera that dominated this sample including *Enterobacter* (61.8%), *Comamonas* (32.9%), and *Klebsiella* (1.2%) (Table 14). While *Comamonas* species are aerobic, the fact they were found in the anaerobic enrichment indicates they were in large numbers initial inoculation as observed in the other sample results. Figure 11 is a graphical representation of Table 14.

2. Fungal Report 048QA

A fungal analysis was ordered based on microscopic observations and culture results of previous samples (SRNL-TR-2018-00254). Table 1 shows the 3 samples used for testing, which were split on reception by MI. The "Reads Passing Quality Filtering" and "% Reads Classified to Genus" were satisfactory. Tables 2 and 3 show that 100% of the reads passed quality filtering such that all the taxonomic levels could be determined.

A. Fungal Results for MCU Strip Feed Sample

Table 4 and Figure 2 show that the top phyla identified were Ascomycota (56.93%), Basidomycota (23.30%), Mucoromycota (17.48%), and Chytridiomycota (1.94%). The top genera identified were *Cladosporium* (48.81%), *Rhizophagus* (17.48%), *Trametes* (10.41%), *Malassezia* (10.24%), *Ramularia* (3.88%), *Ganoderma* (1.94%), *Anaeromyces* (2.65%), and *Aspergillus* (1.59%) (Table 5 and Figure 3). *Cladosporium* species are widely distributed in many environments, particularly around living and dead plant material as endophytes attached to pollen. This is relevant as pollen particles were observed in the Strip Feed Tank that can serve as a substrate for the growth of fungi (SRNL-TR-2018-00254). *Rhizophagus* species are essential to the cycling of environmental phosphorous. *Trametes* is a potent producer of lignin-degrading enzymes. *Malassezia* utilizes oils and lipids for metabolisms and is present as normal skin flora of humans and animals. *Ramularia* can survive in high nutrient, low oxygen environments. *Ganoderma* species produce several bioactive substances such as terpenoids and polysaccharides. *Anaeromyces* ferments cellulose as in pollen and other polysaccharides as well as carbohydrates. Due to its production of hydrolytic enzymes, this fungus can survive in many environments. *Aspergillus* species have also been linked to corrosion of magnesium and aluminum alloys (Qu et al., 2015; Jirón-Lazos et al, 2018).

B. Fungal Results MCU Strip Feed Sample Low Nutrient

Tables 6 and 7 and Figure 4 show the quality of the reads and classification rate, which are satisfactory. Table 8 and Figure 5 show that the top phyla identified were Ascomycota (66.97%), Mucoromycota (27.52%), and Basidomycota (3.67%). These three phyla were identified in the first sample but in different proportions (Table 4, Figure 2). Top genera classification demonstrates just four including *Kodamaea* (46.49%), *Rhizophagus* (27.52%), *Saccharomyces* (4.59%), and *Malassezia* (3.67%) (Table 9, Figure 6). *Kodamaea* is a non-spore producing genus of yeast of the Ascomycetes phylum found in a variety of environments. *Saccharomyces* is a genus of yeast used in a variety of food and alcohol

production processes as well as in the health industry due to its rapid growth and fermentation capabilities.

C. Fungal Results for MCU Strip Feed Sample Anaerobic

Tables 10 and 11 and Figure 7 show the quality of the reads and classification rate, which are satisfactory. Table 12 and Figure 8 show that the top phyla identified were Basidomycota (57.60%), Mucoromycota (30.40%), and Ascomycota (9.60%). Again, these three phyla were identified in the other two samples but in different proportions. Top genera classification demonstrates just five including *Malassezia* (38.205), *Rhizophagus* (30.40%), *Trametes* (17.60%), *Candida* (4.80%), and *Colletotrichum* (2.40%) (Table 13, Figure 9). *Candida* is often used in biofuel production due to its fermentation abilities and degrades n-alkanes making it useful in bioremediation of hydrocarbons. *Colletotrichum* life styles are varied and change with the host or environment.

Summary

One of the samples was just liquid (i.e. the MCU feed tank). The MCU Strip Feed Tank samples were exposed to different growth conditions (i.e. anaerobic and low nutrient) that the microbial population could encounter in the tanks. Low nutrient and aerobic conditions are very possible as the biofilm develops. While we have not received a chemical analysis of the MCU Strip Feed Tank liquid sample it appears low nutrient. Anaerobic conditions form gradually inside the biofilm as metabolic activity increases on the surface of the biofilm. If the MCU system is shut down for any period of time, anaerobic conditions can occur as available oxygen is consumed. The results demonstrate that all three samples had versatility and robustness of bacterial and fungal populations. Taking a sample like this should be likened to a snapshot in time of a dynamic process. It is apparent from the results shown here that the sample was aerobic and low nutrient at the time of analysis based on the molecular signatures. These populations can change with activity as well as other factors. The genera identified include those known to cause biofouling and corrosion in industrial systems. These organisms can survive on trace chemicals and carbon and build up over time in a variety of environments. The occurrence of microbial proliferation in industrial systems depends on a complex interaction of chemical, physical, operational, and engineering parameters. No single factor could account for all these microbial occurrences, so personnel must consider all the above parameters in devising a solution to this microbial problem. We need to more closely examine biofilm and/or biofouling control strategies as well as means of limiting the *occurrence* of opportunistic microorganisms such as those identified in the MCU. These control strategies include maintenance issues, biocide applications, or operational parameters for this process as well as processes throughout SRS.

Recommendations

Microorganisms are ubiquitous in the environment, including air (Whitman et al., 1998). In air, microorganisms vary considerably in concentration, ranging from approximately $3.9x10^2 – 1.2x10^3$ cells m^{-3} in forests, to as high as $1.9x10^7 – 1.0x10^9$ cells m^{-3} during agricultural activities (Lighthart, 1997). With this general information as a reference to high and low microbial densities, the microbial content of air in contact with MCU processes can be estimated. For most microbes, organic carbon is required to drive metabolic activity and serves as both carbon and energy source. Organic carbon in the air in buildings has been estimated at 14.8 μgm^{-3} but could vary considerably (Na, et al. 2005). Below are

recommended approaches for minimizing biofouling problems related to the MCU and similar processes.

1. Develop monitoring system for the MCU to determine microbial status. This would include either periodic direct (microbial sampling) or in-situ (electrochemistry) testing (Turick et al. 2019). Testing would be preferred over waiting for operating pressure changes to indicate that a shutdown is necessary. Dissolved and/or suspended solids could also be used as a monitoring parameter.
2. Apply a system compatible biocide routinely to minimize microbial activity. The biocide would have to be compatible to those exposed surface materials in the MCU.
3. Develop a maintenance plan for the MCU in case of future "down time". This plan would include necessary draining, flushing, and biocide application to prevent future biofouling conditions.
4. Utilize filtration systems where possible to minimize microbial and other organic contamination of the MCU systems.
5. Analyze chemicals used in the MCU for biodegradation potential to assess nutrient availability for microorganisms and how that can be managed in the future to minimize biofouling.

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