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Microbial Recovery of Rare Earth Elements

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1.0 INTRODUCTION

Rare earth elements (REEs), which include yttrium, scandium, and the 15 lanthanides, occur in a variety of ores, especially monazite, bastnäsite, and apatite. Although these elements are relatively common in the Earth's crust, they are widely dispersed and typically present at low concentrations, making them difficult and costly to extract. Traditional extraction methods such as solvent extraction, ion exchange, coprecipitation, and crystallization require significant energy inputs and often generate large amounts of harmful waste.

Because REEs are essential to many modern technologies, there is a strong need for extraction methods that are more economical, less complex, and environmentally safer. Researchers are increasingly exploring microorganisms that naturally participate in REE cycling as potential alternatives to highly engineered, solvent-heavy extraction processes. Microbial mechanisms that influence the mobility of metals, including bioleaching, biosorption, and bioaccumulation, offer promising, eco friendly pathways for recovering REEs.

2.0 IMPORTANT METHODS FOR MICROBIAL RECOVERY OF RARE EARTH ELEMENTS

2.1 Bioleaching

Bioleaching is a targeted method for using microorganisms and their metabolites to solubilize desirable metals and enhance their extraction. Bioleaching involves first allowing microorganisms to attach and colonize on the mineral surfaces. Through their metabolic activity, these microorganisms release organic acids, enzymes, and other metabolites that leach out metals and dissolve minerals. These dissolved metals can then be recovered through precipitation, solvent extraction, ion exchange, or biological methods[1]. Biosurfactants produced by bacteria have also emerged as a potential way to aid in solubilizing REEs during bioleaching[2, 3]. The bioleaching process requires less energy and has less impact on the environment than traditional recovery methods, and it allows for processing of ore where it is normally not economically viable1. One genus that is commonly used for bioleaching is *Acidithiobacillus* because of their ability to thrive in acidic environments and produce organic acids. It has also been demonstrated that co-culture of *Acidithiobacillus* with other organisms such as *Klebsiella aerogenes* or *Acidiphilum* spp. improves the bioleaching efficiency[4]. Other bacterial genera that can be utilized in bioleaching include *Leptospirillum*, *Acidianus*, *Acidithrix*, *Acetobacter*, and *Gluconobacter*. The fungi *Aspergillus niger*, *Penicillium*, and *Paecilomyces* are also commonly utilized in bioleaching[5]. One drawback to the bioleaching process is that the characteristics of the ore and the environmental conditions play a major factor in determining what organism can be utilized.

2.2 Biosorption

Biosorption is the process by which dead or living biomass removes toxic metals from aqueous solutions—such as mining waste streams—through functional groups in bacterial cell membranes or in the cell walls of algae, plants, and fungi that serve as metal binding sites. Several organisms have been studied for their biosorption abilities, including the bacteria *Bacillus subtilis, Arthrobacter nicotianae, Leiseringia methylohalidivorans, Phaeobacter inhibens,* and *Micrococcus* species, as well as the fungi *Catenulostroma* and *Pichia.*[1,6]

Bacillus subtilis and *P. inhibens* show higher selectivity for heavier REEs like ytterbium and lutetium, while *L. methylohalidivorans* appears capable of binding all REEs. The fungus *Pichia* also binds all REEs and requires less biomass than the bacterial species studied, suggesting that eukaryotic organisms such as fungi and yeasts may be particularly well suited for biosorption.[7] Other eukaryotes (including the algae *Chlamydomonas reinhardtii, Cladophora fasicularis, Ulva lactuca, Chaetomorpha spp., Caulerpa sertularioides,* and *Valoniopsis pachynema*) have also demonstrated promising biosorption capabilities.[1,6]

Recent research on cyanobacteria shows that they produce negatively charged sugar moieties containing carbonyl and carboxyl groups that can bind REEs at levels of up to 10% of their dry mass. Nostoc species exhibited the highest efficiency, while *Synechococcus elongatus, Desmonostoc muscorum, Calothrix brevissima,* and an uncharacterized *Komarekiella* strain also performed well. Biosorption occurred rapidly, with most REEs binding within minutes, indicating strong potential for future industrial scale REE recovery.[8]

2.3 Bioaccumulation

In bioaccumulation, REEs or other metals first bind passively to the cell wall or cell membrane of living cells. They are then transported into the cell, where they are captured by chelating molecules produced by the organism and subsequently stored internally. Although this process typically serves as a detoxification mechanism, it also offers a potential strategy for recovering REEs.

Bioaccumulation has been studied in several bacterial species, including *Methylorubrum extorquens* AM1, *Hansschlegelia quercus*, and *Bacillus cereus*. It has also been investigated in numerous algae known for their strong metal accumulating abilities, including *Euglena, Scenedesmus, Chlamydomonas, Cyclotella, Phaeodactylum, Porphyridium, Phormidium, Pseudochlorococcum, Chlorella, Thizoclonium*, and *Spirulina*.[1,9,10]

The methylotrophic bacteria *M. extorquens* AM1 and *H. quercus* have attracted particular interest because they uniquely depend on lanthanides for the activity of their methanol dehydrogenases. These organisms use a protein called lanmodulin (the first natural REE binding protein ever identified) to bind and take up lanthanides. Lanmodulin exhibits picomolar level affinity for lanthanides, and lanmodulin from these species shows a preference for binding lighter lanthanides over heavier ones.

Researchers have successfully isolated lanmodulin and demonstrated its effectiveness for small scale REE separation. Current work is focused on determining how lanmodulin could be leveraged for industrial scale REE isolation and purification.[11,12]

3.0 MOLECULAR BIOLOGY TOOLS FOR MONITORING MICROBIAL RECOVERY OF RARE EARTH ELEMENTS

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 qPCR assays that can be useful for monitoring organisms that play important roles in microbial recovery of rare earth elements.

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 involved in microbial recovery of rare earth elements has many important advantages over traditional culture-based methods.

CENSUS® can be used to quantify specific groups of organisms that are important for microbial recovery of rare earth elements (Table 1).

Target	Description
Biosurfactants	Quantifies microbial genes involved in the production of liposaccharide, lipopeptide, and glycolipid biosurfactants which can be utilized to aid in solubilizing REEs during bioleaching.
SHW	Quantifies *Shewanella*, including *Shewanella oneidensis* MR-1 which is capable of REE biosorption.

Table 1: Microbial Insights Census® Targets Available for Monitoring Microbial Recovery of Rare Earth Elements

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 microbial recovery of rare earth elements. 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[14]. 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.

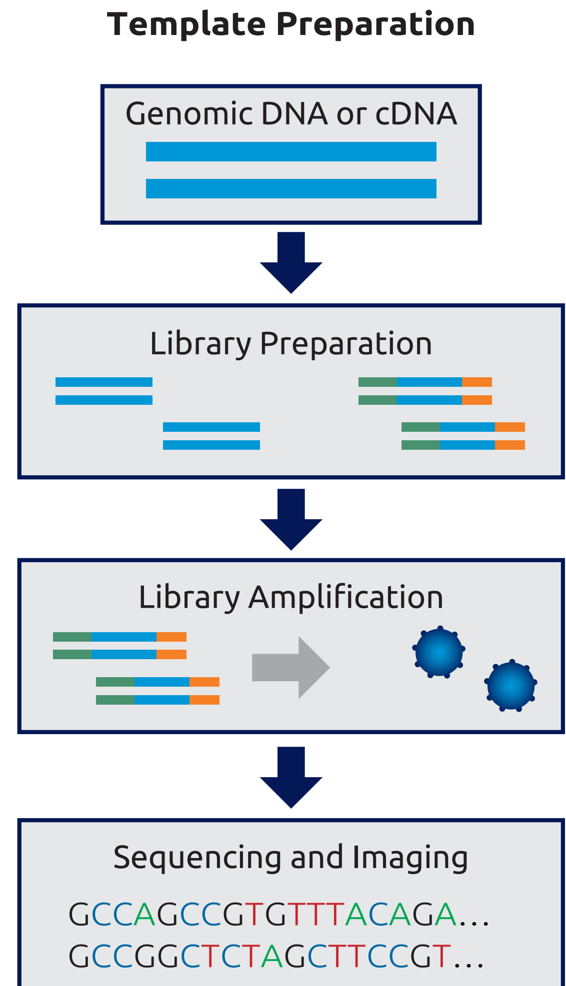

Figure 1: The NGS Approach – DNA Library Construction, Amplification, and Massively Parallel Sequencing

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)[15, 16]. 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[17]. 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.

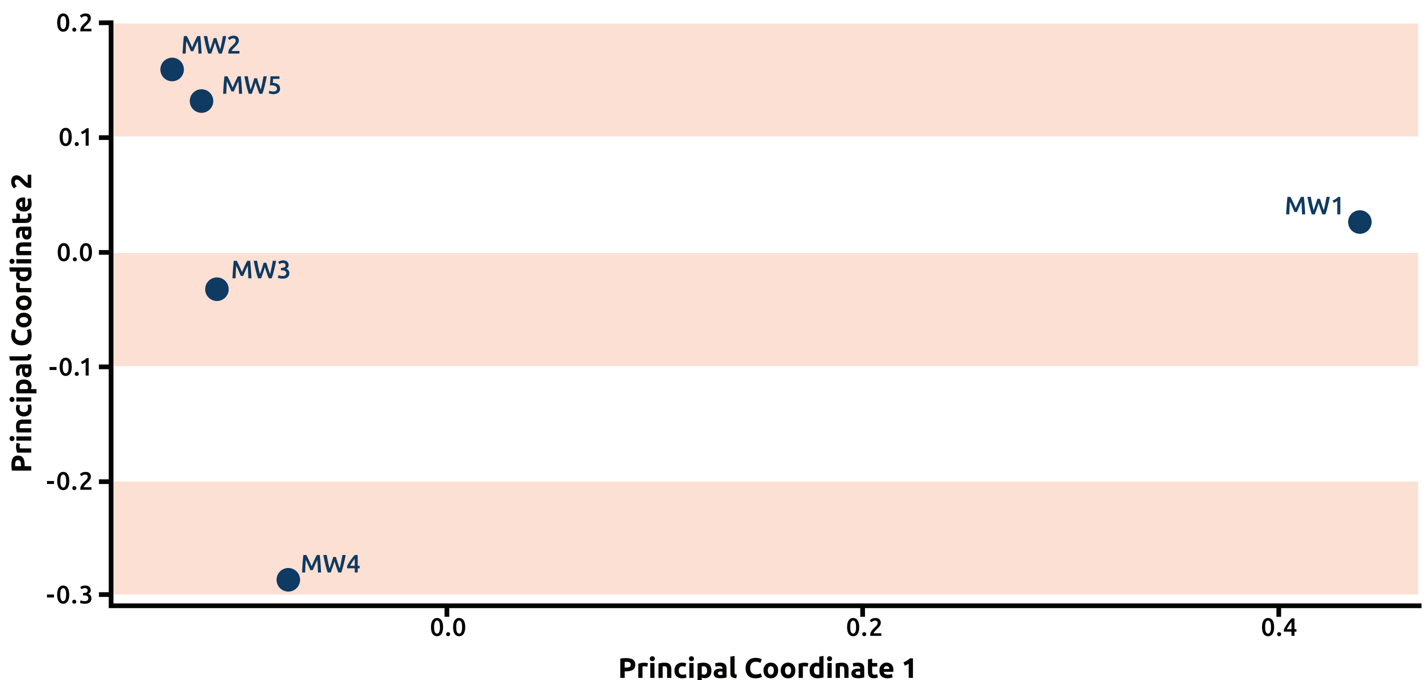

Figure 2: Principal Coordinate Analysis

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 2.

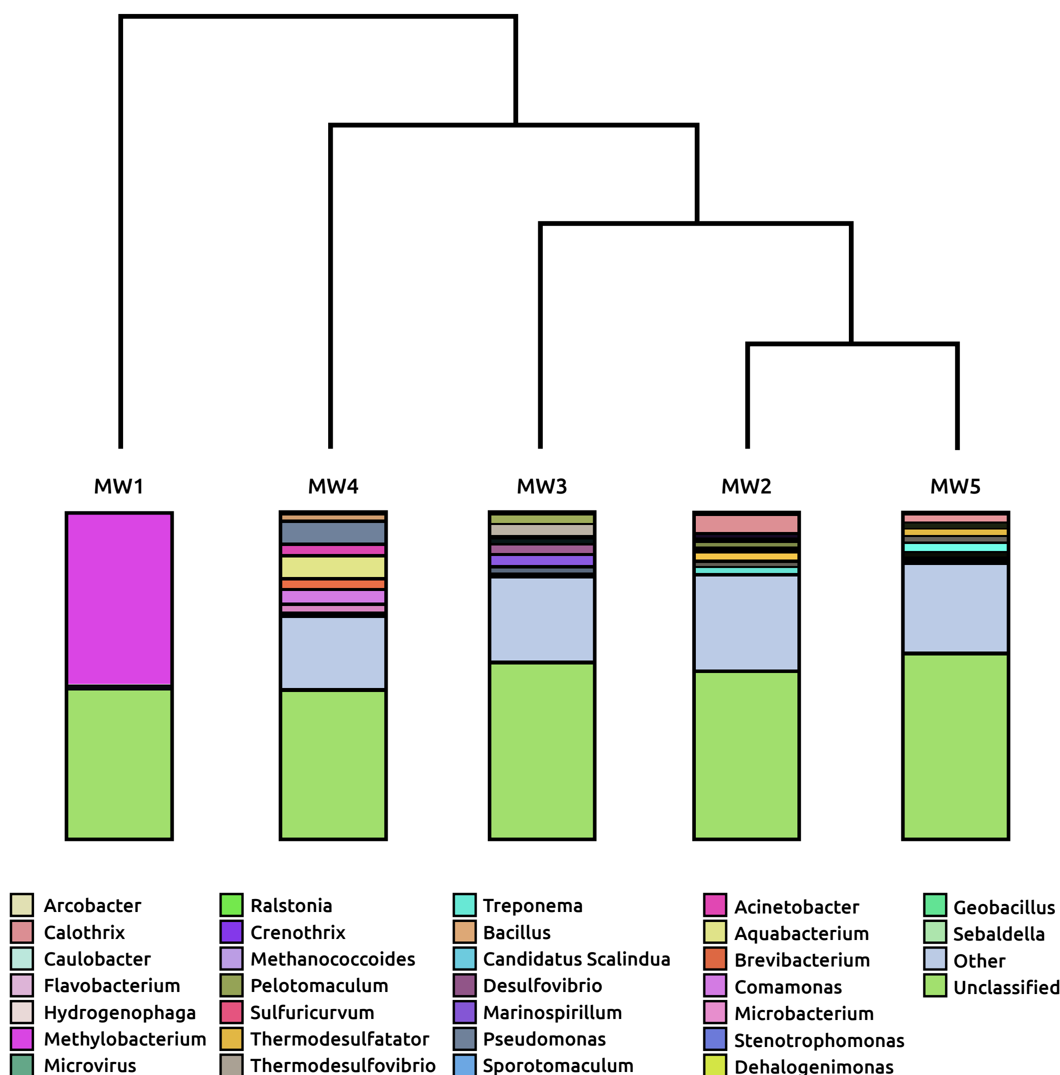

Figure 3: Hierarchical Clustering Dendrogram

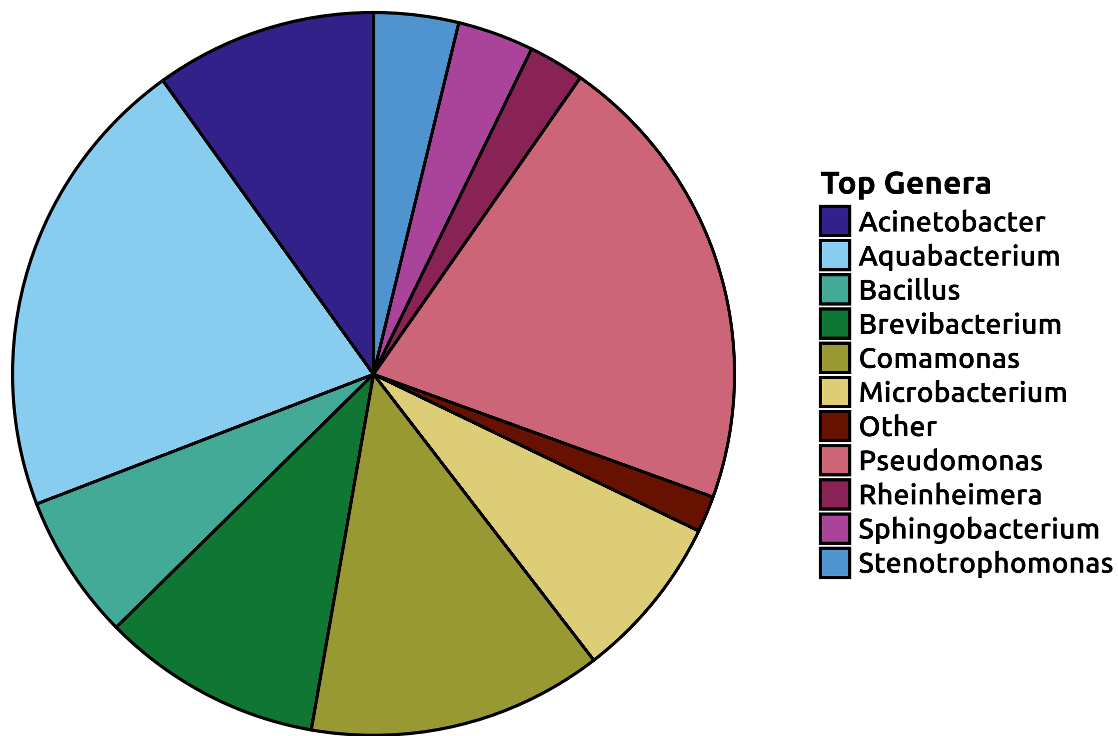

Figure 4: Pie Chart Displaying Top Genera Classifications

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 owygen as electron acceptors. They metabolize a broad range of organic acids but no carbonhydrates.
Pseudomonas	18,142	13.1%	Pseudomonas species can grow very rapidly to take advantage of carbon and oxygen availability. Members of this genus are gramnegative, 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, motil genus have ben 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-reduction of nitrite, nitrate, and nitrous oxide to nitrogen, while others have aresnite-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 2: Top Genera Classification Results

Including NGS analysis in routine monitoring can show how microbial populations shift over time, and trending the data with recovery rates can be used to determine how community succession affects bioleaching or biosorption efficiency. For example, NGS results indicating that *Acidithiobacillus* spp., a genus associated with bioleaching capabilities, comprises an increasing proportion of the microbial community over time would suggest that conditions are favorable for bioleaching. Table 3 provides a list of microorganisms associated with REE recovery that NGS can be used to identify.

Microorganism	**Role in REE Recovery**
Acidithiobacillus spp.	Bioleaching of REEs from phosphogypsum[4]
Acidiphilum spp.	Bioleaching of REEs from phosphogypsum[4]
Burkholderia thailandensis	Bioleaching of REEs from monazite[3]
Aspergillus niger	Bioleaching of REEs[5]
Penicillium spp.	Bioleaching of REEs[5]
Bacillus subtilis	Biosorption of La(III), Eu(III), and Tm(III)[1]
Arthrobacter nicotianae	Biosorption of Sm(III)[1]
Micrococcus spp.	Biosorption of heavy REEs[18]
Shewanella oneidensis MR-1	Biosorption of La, Eu, Yb[13]
Agrobacterium sp. HN1	Biosorption of La(III) and Ce(III)[1]
Methylorubrum extorquens AM1	Bioaccumulation of gadolinium[10]
Bacillus cereus	Bioaccumulation of cerium and neodymium[9]

Table 3. Applying NGS to REE Recovery

NGS can identify microorganisms even if they currently can't be detected by CENSUS® qPCR. If a microorganism is detected by NGS and is highly correlated with successful bioleaching, biosorption, or bioaccumulation processes in a system, Microbial Insights can use this information to design a custom CENSUS® qPCR assay for more targeted monitoring.

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. analyses can be performed on nearly any sample type including water, soil, solids, Bio-Traps®, and swabs of biofilms. As a point of comparison, collecting a background sample or a series of samples over time is also recommended to aid in interpretation. For more detailed information on sample collection, complete protocols are available on the sampling page of the Microbial Insights website (*https://microbe.com/us-sampling-protocols/*).

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. If the sampling location is remote and overnight shipping to the lab isn't possible, on-site DNA extraction using Bio-Extract™ kits can be beneficial. Extracted DNA is more stable compared to a standard sample, and this increased preservation eliminates the need for daily overnight shipments.

5.0 QUALITY ASSURANCE/QUALITY CONTROL (QA/QC) PARAMETERS

For more than 34 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. QA/QC procedures included standard with every analysis include:

- 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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