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MicrobeID™ Report

Project ID: OG172112

Prepared for:

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Sample Information

Sample Count: 8
Shipped From: 100100 River Don Lane
Middletown, TX, 79002

Shipped Date:11/01/17
Arrived Date:11/02/17

Sample and Project Overview: (Table 1)

8 samples were received at Ecolyse Labs on Nov 02, 2014. These consisted of 4 water and 4 solids samples.

TABLE 1. Sample Overview

Sample ID	Sample Label	Tests Performed
OG172112-001	Source Water (river)	1. MPN*, 2. qPCR, 3. Metagenomics,
OG172112-002	Tank 1 Waters	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-003	Tank 2 Waters	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-004	Tank 2 Post Biocide	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-005	Pipe1 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-006	Pipe2 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-007	Pipe3 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-008	Pipe4 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
*MPN with these medias: PRD (detects APB), MPB (detects SRB), NRB (detects Nirgrate Reducing Bacteria), IRB (detects Iron Reducing Bacteria)		

Description of Analysis Methods:

The microbial population of each sample will be analyzed by a maximum of the 3 following methods, each of which provides a different perspective:

1. **MPN analysis** following NACE corrosion industry standard methods, is used to quantify viable and culturable bacteria able to grow in five different growth media preparations
    a. Quantifies cells/ml of SRB, IRB, NRB, APB, and GHB
    b. Dependent on bacteria being alive and able to grow in the media
    c. Viable cell count assay
2. **qPCR** analysis is a DNA based analysis that quantifies total microbes in a sample
    a. Quantifies cells/ml of all microbes in a sample
    b. Does not distinguish between living and dead cells
    c. Does not give information on types of cells
    d. Total cell count assay
3. **Amplicon Metagenomics** provides information on the types and relative abundance of bacteria and archaea in a sample
    a. Provides a list of all bacteria in the sample
    b. Does not distinguish between live and dead cells
    c. Does not provide cells/ml quantification
    d. Provides relative abundance
    e. Is not dependent on growth in media
    f. Detailed Population Structure analysis

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OG172112-003	Tank 2 Waters	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-004	Tank 2 Post Biocide	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-005	Pipe1 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-006	Pipe2 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-007	Pipe3 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
OG172112-008	Pipe4 Pig Envelope	1. MPN*, 2. qPCR, 3. Metagenomics
*MPN with these medias: PRD (detects APB), MPB (detects SRB), NRB (detects Nirgrate Reducing Bacteria), IRB (detects Iron Reducing Bacteria)		

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Project Results Overview: MPN Analysis Results (Table 2)

MPN Analysis Methods Overview

Samples were serial diluted and injected into indicator media, set up in triplicate
Cultures were read weekly for 30 days, following NACE Standard TMO 194-2004
Medias used were:

MPB (detects SRB)
PRD (detects APB and GHB)
IRB (detects iron reducing bacteria)
NRB (detects nitrate reducing bacteria)

TABLE 2. MPN Analysis - Bacterial Quantification by Growth in Indicator Media.

Sample ID	Sample Label	SRB	IRB	APB	NRB
OG172112-001	Source Water (river)	4.20E+02	2.00E+01	4.20E+02	2.30E+02
OG172112-002	Tank 1 Waters	9.30E+06	2.00E+04	4.20E+05	2.30E+05
OG172112-003	Tank 2 Waters	2.30E+03	2.80E+03	9.20E+03	NG
OG172112-004	Test Tank Biocide	NG	NG	NG	NG
OG172112-005	Pipe1 Pig Envelope	2.30E+01	4.20E+02	9.20E+02	9.20E+04
OG172112-006	Pipe2 Pig Envelope	9.20E+04	9.20E+04	2.40E+07	2.30E+02
OG172112-007	Pipe3 Pig Envelope	4.20E+05	7.40E+05	7.40E+05	3.60E+07
OG172112-008	Pipe4 Pig Envelope	7.40E+04	4.20E+04	2.30E+05	7.40E+05

Values are expressed in viable cells per mL. Values > E+05 are yellow. Values of E+04 are green, NG = no growth, grey, indicates bacterial levels are less than detection threshold (0.5E+00 cells per ml).

Samples with high bacterial activity (sample names highlighted in red)

- 1 sample (Pipe3 Pig Envelope) had high levels of growth in all medias (SRB, APB, IRB, NRB)
- 1 sample (Produced Waters 1 Waters) showed high levels of SRB, APB, and NRB and borderline high IRB
- 2 samples (Pipe2 Pig Envelope, Pipe4 Pig Envelope) were borderline SRB and IRB. Also had high APB and variable NRB.
- Combination of elevated and slightly elevated IRB and SRB is noted here

Samples with reduced bacterial activity (sample names highlighted in green)

- 1 samples (Test Tank Biocide) showed no bacterial growth
- 3 samples (Source Water, Tank 2 Waters, Pipe1 Pig Envelope) showed only low bacterial activity in all media

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Project Results: qPCR Analysis (Table 3)

qPCR Analysis Methods Overview

- DNA was isolated from each sample
- Samples were qPCR amplified using universal 16S (16S)
- Raw copy count was determined from qPCR results (CT Value).
- Microbes per ml results calculated from raw copy count and the volume of sample used for DNA isolation

Table 3. OG172112 Syrinx Microbial Cell Quantification by qPCR

Sample	Sample	Analyte	Ct Value	Microbes per ml Sample
OG172112-001	Source Water (river)	16S	15.14	1.30E+06
OG172112-002	Produced Waters 1	16S	26.3	1.42E+03
OG172112-003	Produced Waters 1	16S	22.85	1.57E+04
OG172112-004	PW 2 Post Biocide	16S	<LOD	<LOD
OG172112-005	Pipe1 Pig Envelope	16S	24.6	3.09E+05
OG172112-006	Pipe2 Pig Envelope	16S	26.27	2.88E+03
OG172112-007	Pipe3 Pig Envelope	16S	26.27	2.88E+03
OG172112-008	Pipe4 Pig Envelope	16S	23.51	1.90E+04
Analyte is primer pair (see methods). LOD is assay limit of detection CT Value is qPCR reaction threshold of detection CT Value is inversely related to cell concentration in sample				

Project Results Overview: Archaea and Bacterial Diversity Analysis

Genetic-Based Diversity Analysis-Method

- Total DNA is isolated from the samples.
- Bacterial and Archaeal diversity is determined by 16s Ion PGM metagenomics.
- Archaea diversity was separately determined using Archae specific Ion PGM metagenomics
- Following traits assigned to identified bacteria and archaea where possible:
  - **Sulfidogen**-includes all bacteria that can make sulfide or H_2S. This includes "true" SRB as well as TRB (thiosulfate-reducing bacteria) SuRB(sulfur-reducing bacteria) and peptide-fermenting bacteria (such as some Clostridia)
  - **SRB**-(sulfate-reducing bacteria) "true" SRB, utilize sulfate as respiratory electron acceptor
  - **APB**-(acid-producing bacteria) these make organic and/or inorganic acids. Not all APB result in a lowering of ambient pH.
  - **IRB**-(iron-reducing bacteria) many are strongly corrosive
  - **NRB**-(nitrate-reducing bacteria) many bacteria are nitrate reducers. Of particular relevance to the O&G industry are the NRSOB (nitrate-reducing sulfur-oxidizing bacteria) promoted by nitrate injections.
  - **Biodeg**-biodegrading bacteria. These bacteria are capable of breaking down unusual substrates such as O&G hydrocarbons, petrochemicals, cellulose, toxic chemicals etc.
  - **Methanogen** – Organisms that produce methane as a metabolic byproduct in anoxic conditions. These include organisms that live on the surface, as well as deep subsurface extremophiles.

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Genetic – Based Diversity Analysis – Overview Results

- DNA was isolated from 8 samples (Table 4)
- One sample (Tank 2 Post Biocide) had no DNA
  - Consistent with no growth in bug bottles, no bacteria in sample
- Over 156,994 microorganisms were analyzed genetically
- These were grouped into 86 different bacterial species (OTU)
- 10 Archaeal groups were present in the samples
- Metabolic assignments were provided for 64 of the 86 species identified.
- The distribution of SRB, IRB, APB, Biodeg, and NRB is provided (Table 4, Figure 1)
- Key SRB identified in the samples is provided (Table 5)
- The degree of similarity of each sample to every other sample is provided (Table 6)
- A list of the most abundant bacteria (greater than 1% of the population) is provided (Table 7)
- A complete list of all bacteria in the samples is available upon request

Table 4. Summary of Bacteria and Archaea Diversity using Genetic Analysis

Sample ID	Organisms Tested	Bacteria & Archaea OTU	Sulfidogens (TRB + SRB)	SRB	IRB	APB	Biodeg
OG172112-001 Source Water	26474	37	0.659% 4 otu	0.0233% 1 otu	0.091% 1 otu	0.089% 2 otu	8.87% 6 otu
OG172112-002 Produced Waters 1	22286	43	5.11% 6 otu	4.79% 2 otu	7.135% 2 otu	2.004% 4 otu	37.17% 6 otu
OG172112-003 Produced Waters 1	21757	42	1.91% 6 otu	0.9% 2 otu	2.78% 2 otu	0.618% 1 otu	6.27% 6 otu
OG172112-004 PW 2 Post Biocide	none	none	none	none	none	none	none
OG172112-005 Pipe1 Pig Envelope	19326	25	1.39% 5 otu	0.897% 1 otu	5.32% 2 otu	1.7% 2 otu	1.65% 4 otu
OG172112-006 Pipe2 Pig Envelope	23648	42	41.65% 2 otu	10.099% 1 otu	7.999% 2 otu	10.342% 6 otu	7.42% 3 otu
OG172112-007 Pipe3 Pig Envelope	21335	53	56.24% 5 otu	18.02% 2 otu	12.891% 2 otu	3.71% 5 otu	0% 0 otu
OG172112-008 Pipe4 Pig	22168	36	79.29% 1 otu	11.981% 2 otu	5.985% 2 otu	7.984% 4 otu	0% 0 otu
TOTAL	**156994**	**86**	***11 otu***	***5 otu***	***4 otu***	***13 otu***	***18 otu***

Key SRB and IRB found in these samples is provided in Table 4.

Table 5. Sulfidogens and Iron Reducing Bacteria

Bacterial types	Metabolisms	Notes
Desulfovibrio desulfuricans	Sulfidogen, SRB	Associated with oilfield H_2S
Desulfovibrio sp	Sulfidogen, SRB	Associated with oilfield H_2S
Desulfohalobium retbaense	Sulfidogen, SRB	Associated with oilfield H_2S
Dethiosulfatibacter aminovorans	Sulfidogen; TRB	Non-SRB Sulfidogen
Desulfonauticus autotrophicus	Sulfidogen, SRB	Associated with oilfield H_2S
Citrobacter sp	Sulfidogen, TRB	Probably not causing H_2S in the field
Enterobacter sp	Sulfidogen, TRB	Probably not causing H_2S in the field
Clostridium thiosulfatireducens	Sulfidogen, TRB	Generate H_2S from amino acids
Shewanella species	IRB Sulfidogen, TRB	Associated with corrosion
Pelobacter sp	IRB	Associated with metal
Rhodoferax sp	IRB	Corrosion associated

- *Desulfovibrio* species are well known sulfidogens in O&G formations and facilities
- *Shewanella* isolates are associated with metal corrosion

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Table 6. Project OG172112 Dominant Bacterial Species and Metabolic Trait. Most abundant bacteria (defined as present in at least 1% of one sample) are given, along with the percent abundance in that sample and a characteristic trait of relevance. Samples are highlighted by abundances (yellow, green, white, gray). A full list of all bacteria identified in these samples is available upon request.

SAMPLE	-001	-002	-003	-005	-006	-007	-008	Trait
Acidaminobacter sp	0	0	0	0	4.9	3.7	<1%	APB
Acidovorax defluvii	4.1	<1%	<1%	0	0	0	0	NRB
Acidovorax sp	1.4	10.4	<1%	<1%	0	0	0	NRB
Acinetobacter sp	<1%	<1%	<1%	<1%	3.1	0	0	BioDeg
Acinetobacter towneri	<1%	<1%	1.6	<1%	0	0	0	BioDeg
Aeromonas salmonicida	0	<1%	1.2	<1%	8.7	0	0	Biofilm
Alcaligenes sp	<1%	<1%	0	0	<1%	1.9	0	NRB
Alishewanella sp	0	0	<1%	1.6	0	0	0	TRB
Bacillus sp	4.8	<1%	<1%	0	0	0	0	Varies
Citrobacter freundii	<1%	<1%	1	1	0	0	0	TRB
Citrobacter sp	<1%	<1%	<1%	<1%	31.6	48.2	79.3	TRB
Clostridium botulinum	5.4	<1%	<1%	<1%	0	<1%	0	Ferm
Clostridium butyricum	1.1	1.3	<1%	0	2.6	<1%	2.6	APB
Comamonas testosteroni	0	0	0	0	3.4	0	0	BioDeg
Desulfonauticus autotrophicus	<1%	<1%	<1%	<1%	0	10%	10%	SRB
Desulfovibrio desulfuricans	0	4.8	<1%	0	0	<1%	0	SRB
Desulfovibrio sp	0	<1%	0	0	<1%	7.4	0	SRB
Dethiosulfatibacter aminovorans	<1%	<1%	0	0	3.3	2.8	3.7	TRB
Enterobacter asburiae	<1%	<1%	<1%	1.5	0	0	0	GHB
Enterobacter cloacae	<1%	<1%	<1%	3.5	0	0	0	GHB
Enterobacter sp	<1%	<1%	<1%	1	0	0	0	TRB
Enterococcus sp	0	<1%	0	1.7	1	0	3.7	APB
Klebsiella sp	<1%	<1%	<1%	<1%	3.3	0	<1%	Biofilm
Paenibacillus contaminans	7.4	33%	2.8	1	0	0	0	BioDeg
Pelobacter sp	<1%	5.5	<1%	1.5	<1%	<1%	1.60%	IRB
Pseudomonas putida	<1%	<1%	1.9	<1%	0	0	0	BioDeg
Pseudomonas sp	10.4	<1%	1.9	6.2	2.9	23.1	<1%	Varies
Rhodopseudomonas sp	56.7	42.1	70.5	73.3	36	12.4	20.6	GHB
Shewanella putrefaciens	<1%	1.4%	2.8	3.3	4.9	7.8	4.3	IRB

Trait abbreviations:
APB, Acid-Producing Bacteria, Biodeg, Biodegradation, IRB, Iron-Reducing Bacteria
Ferm, Fermenting Bacteria, GHB, General Heterotrophic Bacteria, MIC, Microbial-Influenced Corrosion
NRB, Nitrogen-Reducing Bacteria, SRB, Sulfate-Reducing Bacteria, TRB, Thiosulfate-Reducing Bacteria.

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Conclusions of Bacterial Population Testing

- 8 samples were received for testing
- These originated from source water, produced water (pre and post biocide treatment), and envelope samples from oil pipeline
- No bacteria were detected in the post-biocide treated samples
- Three distinct populations were present among 7 samples.
- Numerous sulfidogens, including SRB and non-SRB sulfidogens were identified
- Pig solids contained the higheset levels of corrosive organisms, including SRB and IRB.
- The Pig Envelope Samples from pipes 2, 3 and 4 were very similar to each other in terms of composition.
- Produced Waters 2 waters were found to be similar to Pipe 1 envelope, suggesting a common origin. Source Water 1 was most similar to Produced Waters 1.
- Data suggests pipe pigging solids contain organisms capable of influencing corrosion rates

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- **Notes on Taxonomic and Metabolic Assignment**

Organisms are referred to by the identity of the most closely matched organism in the database. However, this does not indicate 100% identity. In most cases, the most closely matched organisms are referred to as "uncultured organism" and as such there is no physiological or metabolic information for them. Organisms that fall below the cutoff for taxonomic assignment are listed as unclassified. Due to the unusual source of samples, a large number of organisms in the samples may unclassified. This indicates that they are novel organisms that have not been described in the scientific literature.

Metabolic assignments are inferred by the metabolic characteristics of the most closely related organism for which experimental data has been provided. Some metabolic groupings are overlapping and non-exclusive, e.g. many fermentative organisms generate organic acids or are capable of sulfidogenesis under some conditions. An overview of select metabolisms is provided in Appendix B.

APPENDIX B. Overview of Select Metabolic Processes

APB: Acid-Producing Bacteria

Acid-producing bacteria are of specific interest to the oilfield community as acid production directly and aggressively promotes corrosion. Several metabolic pathways result in the production of acids, including fermentation pathways that generate organic acids such as lactic acid and acetic acid, as well as those that generate inorganic acids such as sulfuric acid as a byproduct of the oxidation of inorganic sulfur compound. It should be noted that not all fermentative pathways result in acidification of the surrounding environment. The identification of bacteria as acid producing does not necessarily indicate acidification of bulk fluids.

Biodeg: Biodegradation

Some bacterial genera and species have the capacity to utilize "atypical" or "unusual" substrates as carbon sources. These bacteria are loosely referred to as Biodeg, for "Biodegradation". The definition used here for "atypical or unusual substrates" with reference to bacterial metabolism includes compounds that most bacteria cannot utilize as a food source. Unusual compounds Biodeg organisms might be able to "eat" include disinfectants, antibiotics, xenobiotics and detergents. Some degrade long chain polymers of sugars and carbohydrates, such as those found in cell wall materials. Others are able to degrade hydrocarbons. Hydrocarbons, including alkanes, alkenes, aromatic hydrocarbons, and waxes, are found naturally in great variety in crude oil and other petroleum compounds. Due to their structural diversity, most bacteria lack the capacity to utilize petroleum hydrocarbons as food sources. Each type of hydrocarbon-degrading microorganism is likely to be capable of metabolizing a few specific types of hydrocarbons.

IRB: Iron-Reducing Bacteria, Fe(III)RB

In the absence of oxygen, many microbes can use Fe(III) as an electron acceptor, reducing it to Fe(II). Iron reduction has been observed under both acidophilic and neutrophilic conditions. Two common iron-reducing genera are Shewanella and Geobacter. In addition to IRB activity, Shewanella species produce chelators that solubilize Fe(III) oxides (Lovley et al, 2004). Shewanella are capable of growing in corrosive biofilms where they have been shown to remove the protective H2 film layer that normally protects iron surfaces from corrosion under anoxic conditions. IRB should not be confused with iron oxidizing bacteria, which are aerobes responsible for a rust brown staining and slimy growth in surface waters.

NRB: Nitrate Reducing Bacteria

NRB are able to reduce nitrates to nitrites, nitrous oxide, or nitrogen under anaerobic conditions in a process termed denitrification. Most are heterotrophic facultative anaerobic bacteria including such common bacteria as *Paracoccus, Pseudomonas, Alcaligenes,* and *Bradyrhizobioium*. A few bacteria use

such reduction processes as hydrogen acceptor reactions and hence as a source of energy; in this case the end product is ammonia. Denitrification is a normal part of nitrogen cycling and not all NRB are of concern to O&G infrastructure.

A subcategory of NRB is the **NRSOB**: Nitrate-Reducing Sulfur-Oxidizing Bacteria are a specific subgroup of NRB whose levels are increased in reservoirs following nitrate injections (Gittel et al 2009; Grigoryan et al, 2008; Hubert and Voordouw, 2007). Growth of NRSOB suppresses the activity of SRB, and thus reducing sulfidogenisis. Some Epsilonproteobacteria can also oxidize petroleum sulfur compounds and utilize nitrate as an electron acceptor for growth, and thus may be considered hydrocarbon degrading. Massive dominance of related Epsilonproteobacteria has been observed in other petroleum samples, for example in formation waters from a Canadian oil sands reservoir containing severely biodegraded oil. (Kodama, Y and Kazuya Watanabe, 2003; Hubert et al, 2011). Sulfurospirillum are nitrate-reducing, sulfur oxidizing bacteria (NRSOB) members of the class Epsilonproteobacteria and are sometimes referred to as “Campylobacter” in older publications. The way in which nitrate addition can affect the SRB population involves several pathways. First, nitrate is a thermodynamically more favorable electron acceptor than sulfate, thus NRB have a competitive advantage. To emphasize the complexity of the metabolism in oilfield samples, it should be noted that under some conditions, these bacteria are also sulfidogens capable of reducing sulfur and thus producing H_2S (Finster K et al, 1997).

Sulfidogenesis: (e.g. SRB, TRB, SuRB)
The metabolic pathways of most interest to the oilfield community are those that generate significant levels of hydrogen sulfide (H_2S). In addition to inorganic processes, biogenic processes can generate significant levels of hydrogen sulfide, primarily through the action of sulfidogenic bacteria. Bacteria that evolve hydrogen sulfide are commonly referred to as “sulfidogens”. Sulfate-reducing bacteria (SRB) are particularly aggressive at sulfide production and are the group of bacteria most commonly implicated oil filed biogenic sulfide production (Barton et al, 2009). Hydrogen sulfide formation by sulfate-reducing bacteria (SRB) under strict anaerobic circumstances is a common problem in sediments, sewer systems, oil reservoirs and anaerobic effluents (Holmer & Storkholm, 2001; McComas et al., 2001). The emission of H_2S into the atmosphere of sewer systems does not only imply odor nuisances and possible health risks. It also induces the biological production of sulfuric acid in the aerobic zones, causing severe corrosion of the inner surface of concrete sewer structures (Sand, 1987; Vincke et al., 2002). Hence, preventive or curative actions are needed.

While SRB are traditionally associated with O&G system sulfide generation, sulfur- and thiosulfate-reducing bacteria (SuRB and TRB, respectively) can also generate significant levels of H_2S and contribute to corrosion and souring (Hulecki JC et al, 2009, Magot et al 1997, Agrawal et al, 2010). Compared to SRB, the TRB are harder to classify taxonomically, as they are members of bacterial genera that can include non-tSRB members. Examples of sulfidogenic TRB commonly found in oilfield samples include *Halanaerobium congolense*, as well as some *Thermoanaerobacter*, and *Spirochaeta*. Additionally, many common enteric bacteria are sulfidogenic, including *Citrobacter* and *Salmonella*.

Thermophiles:
A thermophile is an organism that can survive and often thrives in environments having relatively high temperatures ranging between 45 and 122 °C.

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