MicrobeID™ Report

Project ID: OG142112

Prepared for:

Microbial Control Specialists
Syrinx Well Services

Monday, April 13, 2015

Sample Information

Sample Count: 8 Shipped Date: 11/01/14

Shipped From: 100100 River Don Lane Middletown, TX, 79002 Arrived Date: 11/02/14

Sample and Project Oerview: (Table 1)

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

- Archaeal and Bacterial populations of all samples were analyzed in parallel by 16s metagenome sequencing, using Ion PGM platform.
- Live, culturable cell enumeration was conducted using the MPN method, by culturing in indicator media for SRB, APB, IRB and NRB.

TABLE 1. Sample Overview

Sample ID	Sample Label	Ecolyse Tests Requested
OG142112-001	Source Water (river)	1. Metagenomics, 2. MPN*
OG142112-002	Tank 1 Waters	1. Metagenomics, 2. MPN*
OG142112-003	Tank 2 Waters	1. Metagenomics, 2. MPN*
OG142112-004	Tank 2 Post Biocide	1. Metagenomics, 2. MPN*
OG142112-005	Pipe1 Pig Envelope	1. Metagenomics, 2. MPN*
OG142112-006	Pipe2 Pig Envelope	1. Metagenomics, 2. MPN*
OG142112-007	Pipe3 Pig Envelope	1. Metagenomics, 2. MPN*
OG142112-008	Pipe4 Pig Envelope	1. Metagenomics, 2. MPN*
*MPN with these medias: PRD (detects APB), MPB (detects SRB), NRB (NRB), IRB		

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. Bacterial Quantification by Growth in Indicator Media.

Sample ID	Sample Label	SRB	IRB	APB	NRB
OG142112-001	Source Water (river)	4.20E+02	2.00E+01	4.20E+02	2.30E+02
OG142112-002	Tank 1 Waters	9.30E+06	2.00E+04	4.20E+05	2.30E+05
OG142112-003	Tank 2 Waters	2.30E+03	2.80E+03	9.20E+03	NG
OG142112-004	Test Tank Biocide	NG	NG	NG	NG
OG142112-005	Pipe1 Pig Envelope	2.30E+01	4.20E+02	9.20E+02	9.20E+04
OG142112-006	Pipe2 Pig Envelope	9.20E+04	9.20E+04	2.40E+07	2.30E+02
OG142112-007	Pipe3 Pig Envelope	4.20E+05	7.40E+05	7.40E+05	3.60E+07
OG142112-008	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).

Results of MPN Analysis 8 samples (Table 2)

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 (Tank 1 Waters) showed high levels of SRB, APB, and NRB and boarderline 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

Project Results Overview: Bacterial Diversity Analysis (Table 3)

Genetic – Based Diversity Analysis - Method

- Total DNA is isolated from the sample
- Bacteria and Archaea diversity determined by16s metagenomic analysis, Ion PGM
- Different from "bug bottles" because it does not depend on ability of bacteria to grow in artificial media
- Many novel and scientifically unknown bacteria are identified by genetic analysis
- Following traits assigned to identified bacteria and archaea where possible:
    - **Sulfidogen** (includes all bacteria that can make sulfide or H2S. This includes "true" SRB as well as TRB (thiosulfate reducing bacteria) and SuRB (sulfur reducing bacteria), 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 acids and/or inorganic acids, However, 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 NRB. Of particular relevance to O&G are the NRSOB (nitrate reducing sulfur oxidizing bacteria) promoted by nitrate injections
    - **BioDeg** - biodegrading bacteria (capable of breaking down unusual or atypical substrates such as O&G hydrocarbons, petrochemicals, cellulose, cell wall materials, toxic chemicals)
- Percent of population, and number of unique bacterial types (OTU) are provided as results

Genetic – Based Diversity Analysis – Overview Results

- DNA was isolated from 8 samples (Table 3)
- 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 types (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 3, Figure 1)
- The degree of similarity of each sample to every other sample is provided (Table 4)
- A list of the most abundant bacteria (greater than 1% of the population) is provided (Table 5)
- A complete list of all bacteria in the samples is available upon request

Table 3. Summary of Bacteria and Archaea Diversity using Genetic Analysis

Sample ID	Org-anisms Tested	Bacteria Archaea OTU	Sulfidogens (TRB + SRB)	SRB	IRB	APB	Biodeg
OG142112-001 Source Water	26474	37	0.659% 4 otu	0.0233% 1 otu	0.091% 1 otu	0.089% 2 otu	8.87% 6 otu
OG142112-002 Tank 1 Waters	22286	43	5.11% 6 otu	4.79% 2 otu	7.135% 2 otu	2.004% 4 otu	37.17% 6 otu
OG142112-003 Tank 2 Waters	21757	42	1.91% 6 otu	0.9% 2 otu	2.78% 2 otu	0.618% 1 otu	6.27% 6 otu
OG142112-004 Tank 2 Post Biocide	none	none	none	none	none	none	none
OG142112-005 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
OG142112-006 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
OG142112-007 Pipe3 Pig Envelope	21335	53	56.24% 5 otu	18.02% 2 otu	12.891% 2 otu	3.71% 5 otu	0% 0 otu
OG142112-008 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 4. 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 minovorans	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 eld
Enterobacter sp	Sulfidogen, TRB	Probably not causing H_2S in the eld
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

- *Citrobacter* and *Enterobacter* are not typically associated with hydrogen sulfide generation in O&G facilities
- *Desulfovibrio* species are well known sulfidogens in O&G formations and facilities
- *Shewanella* isolates are associated with metal corrosion

Table 5. Project OG141003 Metabolic Assignments of Dominant Bacterial Species All bacterial species 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.

Comparisons of populations between samples

To get an overall view of a system, it is helpful to compare populations between different locations. Locations with similar bacterial populations reflect a combination of a common source of bacterial contamination along with a common physical environment.

Another way to present this information as a numerical value is to compare all samples to each other. This can be calculated as the number of bacteria that are present in both samples as a function of all bacteria present in both samples. This information is present in Table 4.

Table 4. Project OG141103 Population Similarities. Values indicate similarity of taxonomic profiles between samples. The value varies from 0 to 1, with 0 meaning that no bacteria are shared between the two samples and 1 being that all bacteria are shared between the two samples. Values greater than 0.5 are highlighted in yellow.

		-1	-2	-3	-5	-6	-7	-8
		Source Water	Tank 1 Waters	Tank 2 Waters	Pipe1 Pig Envelope	Pipe2 Pig Envelope	Pipe3 Pig Envelope	Pipe4 Pig
-1	Source Water	1						
-2	Tank 1 Waters	0.48	1					
-3	Tank 2 Waters	0.67	0.4	1				
-5	Pipe1 Pig Envelope	0.43	0.27	0.75	1			
-6	Pipe2 Pig Envelope	0.35	0.29	0.24	0.24	1		
-7	Pipe3 Pig Envelope	0.31	0.38	0.17	0.47	0.72	1	
-8	Pipe4 Pig	0.32	0.31	0.21	0.31	0.78	0.79	1

Results of population comparisons

- Three distinct populations were present among 7 samples.
- The Pig Envelope Samples from pipes 2, 3 and 4 were very similar to each other in terms of composition.
- Tank2 waters was found to be similar to Pipe1 envelope, suggesting a common origin. Source Water 1 was most similar to Tank1 Waters.

Figure 1. Chart Showing Distribution of Select Traits Between Samples

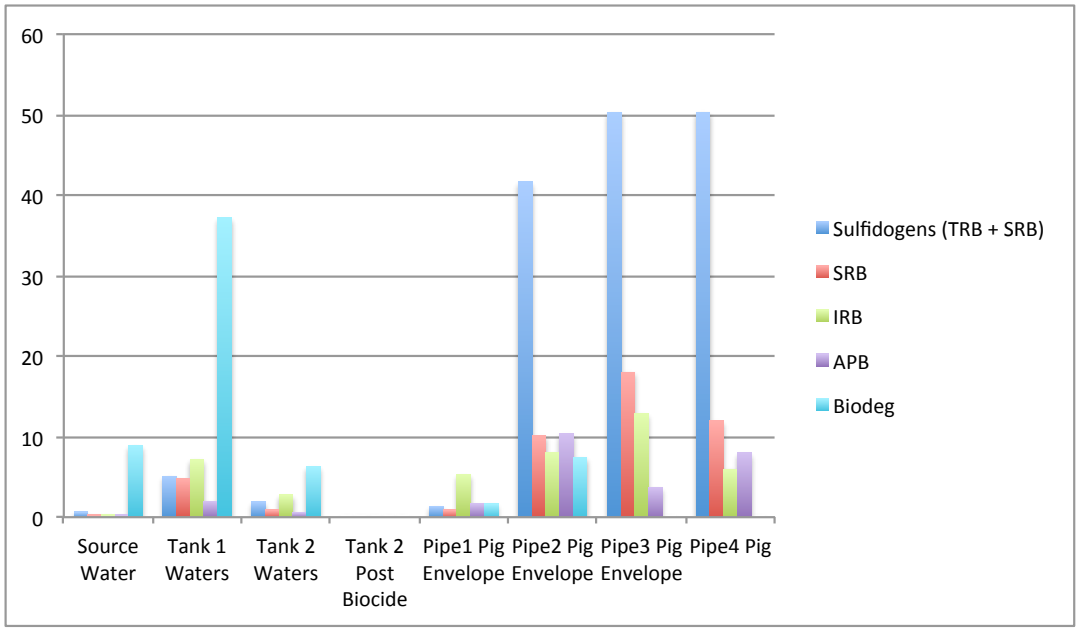

Figure 1 presents a graph with the percent abundance of key metabolic traits in each sample.

APPENDIX A. Methods

For microbial analysis, DNA was subject to bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) using primers 515F- GTGCCAGCMGCCGCGGTAA and 806R- TAATCTWTGGGVHCATCAGG.

Samples were amplified for pyrosequencing using a forward and reverse fusion primer. The forward primer was constructed with (5'-3') linker (CCATCTCATCCCTGCGTGTCTCCGACTCAG), an 8-10bp barcode, and the XXXXXXXX primer (see above primer). The reverse fusion primer was constructed with (5'-3') a biotin molecule, the linker (CCTATCCCCTGTGTGCCTTGGCAGTCTCAG), and the XXXXXXX primer (see above primer). Amplifications were performed in 25 ul reactions with Qiagen HotStar Taq master mix (Qiagen Inc, Valencia, California), 1ul of each 5uM primer, and 1ul of template. Reactions were performed on ABI Veriti thermocyclers (Applied Biosytems, Carlsbad, California) under the following thermal profile: 95○C for 5 min, then 35 cycles of 94○C for 30 sec, 54○C for 40 sec, 72○C for 1 min, followed by one cycle of 72○C for 10 min and 4○C hold.

Amplification products were visualized with eGels (Life Technologies, Grand Island, New York). Products were then pooled equimolar and each pool was cleaned with Diffinity RapidTip (Diffinity Genomics, West Henrietta, New York), and size selected using Agencourt AMPure XP (BeckmanCoulter, Indianapolis, Indiana). Size selected pools were then quantified and 150 ng of DNA were hybridized to OT2-400 Ion Sphere beads (Life Technologies) to create single stranded DNA following Ion PGM Protocols (Life Technologies). Single stranded DNA was diluted and used in emPCR reactions, which were performed and subsequently enriched. Sequencing following established manufacture protocols (Life Technologies).

MPN analysis was carried out by serially diluting 1 mL of sample eightfold in selective media chosen by the customer. The media's used are Modified Postgate's B Broth (MPB) for the growth of Sulfate Reducing Bacteria, Phenol Dextrose Red Broth (PRD) for the enumeration of acid-producing bacteria, Iron-Reducing Broth (IRB) for the enumeration of iron-reducing bacteria, and Nitrogen-Reducing Broth (NRB) for the enumeration of nitrogen-reducing bacteria. Dilutions were carried out in triplicate and growth was compared to the FDA Bacterial Analytical Manual appendix 2 to determine the most probable number. It should be noted that the MPN is an estimate of growth units or colony-forming units and not individual bacterial cells.

Overview of Select Metabolic Processes and Traits

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. 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 sulfidogenisis under some conditions.

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 a 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 are 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 suppress 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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