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### **ABSTRACT**

Traditional ideology on the upper temperature limits of life, suggest that extreme temperatures like those observed in the Horn River Basin shale gas formation (i.e. up to 175°C), should alleviate microbiologically influenced corrosion and souring concerns associated with hydraulic fracturing procedures. The present case history investigates the accuracy of this theory. Horn River Basin wells currently in the flowback or production stage, and the source water used to fracture these wells were studied for the presence and identity of viable bacteria. The effectiveness of two biocides, a gluteraldehyde-based and a cocodiamine-based, in eradicating these bacteria in both the field and the laboratory, was also studied. The results show that source ponds are highly contaminated with bacteria. Furthermore, the high-temperature, high-pressure downhole conditions in this region are not sufficient for eradicating bacteria introduced during the fracturing process. These bacteria survive and establish viable, proliferating communities. Biocide, applied continuously “on-the-fly” during fracturing, was effective at mitigating the downhole bacteria concern. Laboratory study further showed that more established bacterial communities were much more resistant to biocidal treatment. Therefore, early mitigation strategies will likely be key in the prevention of microbiologically induced corrosion and souring.

## INTRODUCTION

Microbiologically influenced corrosion is a concern for the oil and gas industry, especially in applications which involve injection of external water sources (e.g. hydraulic fracturing). Hydraulic fracturing processes involve injection of enormous volumes of water, often gathered from surface ponds. Studies have shown that these sources are highly contaminated with bacteria and this contamination is subsequently transferred to the fractured well <sup>1</sup>. In addition, fracturing fluids often contain polyacrylamide or sugar-based polymers that can serve as an energy source for injected bacteria. These conditions put downhole and surface equipment at risk of microbiologically influenced corrosion.

Microbiologically influenced corrosion is the degradation of materials, as a result of the metabolic by-products of microorganisms, such as bacteria <sup>2</sup>. Free-floating (planktonic) bacteria attach themselves to surfaces, proliferate and grow into a biofilm (sessile) population. Planktonic bacteria are fairly benign until they attach themselves and develop into a biofilm. Biofilms involve a complex multi-layering of microorganisms, often forming in conjunction with other natural deposits (wax, scale, or asphaltene). The layering process creates microenvironments within the biofilm that provide suitable conditions for the growth and proliferation of a number of physiologically different bacteria. These consortia form complex, synergistic colonies that perpetuate the existence and growth of the biofilm.

The bacteria most often associated with microbiologically influenced corrosion are classified according to their metabolic processes:

*Sulfate-reducing bacteria* (SRB) are the most serious offender. These bacteria require an oxygen-free (anaerobic) environment for growth and proliferation. They circulate in a dormant state until they can associate with a developing biofilm. Deep within a biofilm exist microenvironments scrubbed free of oxygen due to the metabolic requirements of oxygen-requiring (aerobic) microorganisms, and in which SRB are able to become active. SRB are best known for reducing available sulfate (or thiosulfate) to sulphide, resulting in presence of hydrogen sulphide or ferrous sulphide, which can directly initiate corrosion cell development. However, SRB can also: 1) directly corrode iron metals through release of hydrogenase, an enzyme that reduces protective protons at the metal surface, thereby depolarizing the cathode and releasing hydrogen gas as a by-product, or 2) reduce organic acids, producing acetate, hydrogen gas and carbon dioxide as by-products <sup>2,3</sup>. The by-products of SRB activity can result in pitting, stress corrosion cracking, and blistering of carbon steel<sup>3</sup>.

*Acid-producing bacteria* (APB) strains can be either aerobic or anaerobic in nature. These bacteria produce organic acids as a by-product of their metabolism. Within the biofilm, these products accumulate at the metal surface, acidify the environment and accelerate corrosion <sup>2</sup>. Some aerobic APB produce extracellular polymers (i.e. slime). These bacteria provide the matrix that forms the biofilm and its layers. They produce concentration cells at the biofilm-metal surface, which can accelerate corrosion. Furthermore, their metabolic activities contribute to the oxygen differential in the various regions of the biofilm, creating anaerobic environments that promote SRB growth.

Bacteria are known to grow under a wide range of conditions. However, until recently, accepted belief was that the upper limit for their survival was 100°C. Part of the reason for this is that bacteria require a stable microbial environment in which water is present in liquid form. However, if an environment is under pressure, this stability can be achieved at much higher temperatures. Studies have shown that bacteria live within deep sea hydrothermal vents that develop along tectonic ridges and rifts <sup>4</sup>. These vents spew superheated waters in excess of 350°C. Bacteria collected from these vents grew at temperatures up to 100 to 121°C when cultured at atmospheric temperature <sup>5,6</sup>. However, when these bacteria samples were pressurized to *in situ* vent conditions, the temperature of optimal growth rate increased to 250 to 300°C <sup>5</sup>. Furthermore, these bacteria showed increases in protein and amino acid production, and were capable of utilizing oxidized and reduced metals to produce organic compounds. Thus, bacteria growth in high temperature, high pressure reservoirs such as found in the Horn River Basin may be possible.

In the present case history, a bacterial survey of typical Horn River Basin shale gas wells, along with their frac water sources, was completed using multiple testing methods. In addition, the effectiveness of two recommended biocides in eradicating bacteria isolated and cultured from the shale gas wells tested, was investigated under both standard and high temperature conditions.

## **EXPERIMENTAL PROCEDURE**

### **Field Water Sample Collection**

The Horn River Basin, located on Northern British Columbia, Canada, is a more novel region of shale gas production. The producing zones in this region are buried at great depths, and thus, under higher temperatures and pressures than observed in most other shale gas regions being produced. Bottomhole temperatures up to 175°C and pressures up to 44,000 kPa have been reported. A summary of the general Horn River Basin well characteristics is shown in Table 1. Wells targeting both the Evie and Muskwa zones of the Horn River formation were chosen for sampling. The Evie zone is buried at a greater depth than the Muskwa and thus, production from this zone is higher in temperature and pressure. However, this increase is further pronounced due the deeper burial of the Horn River formation as a whole, as it crosses to the west of the Bovie fault <sup>7</sup>. In the present study, the test wells that target the Evie formation lie to the west of the Bovie fault, while test wells targeting the Muskwa formation lie to the east of this fault. For the lower temperature and pressure Muskwa zone, two wells (one on flowback and one on production) and the source pond used to fracture these two wells were sampled and tested. None of these Muskwa samples were treated with biocide. For the higher temperature and pressure Evie zone, two wells (one well on flowback that had been biocide-treated during fracturing and one 'untreated' well on production) and the source pond used to fracture these two wells were sampled and tested. Test water sample characteristics are summarized in Table 2. All source pond and well samples were collected in sterile, amber-coloured 950ml glass bottles. Samples from wells were taken via stainless steel piping off the inlet separator or pipeline valves, following a 1 litre flush. Sample bottles were filled to overflowing and immediately capped off.

## **Serial Dilution Bacterial Enumeration**

Collected water samples from each of the locations were transported to local facilities in Fort Nelson, BC and underwent semi-quantitative enumerations of viable APB and SRB via serial dilution. Tests were run in accordance with the standards and guidelines set forth by The American Petroleum Institute (API) 'Methods for Detecting Bacteria Concentrations' (API-38)<sup>8</sup>. Culture media bottles (Dalynn Biologicals) for APB (ZPRA) and SRB (ZAPI and ZAPIS) were selected to match field water salinities. Media bottles for APB contained phenol red which, in the presence of metabolically-produced acid, turns from red-orange to yellow. Media bottles for SRB contained magnesium sulphate which is reduced by SRB in the presence of iron (i.e. a nail), releasing hydrogen sulphide gas which subsequently reacts with ferric ammonium sulphate to produce the insoluble, black precipitate, ferrous sulphide. In sour water samples, SRB tests are initiated from a SRB stripper media, incubated for 24 hours. The stripper media bottles (which are identical to the SRB media but do not contain a nail) strip the non-biogenic hydrogen sulphide gas out of the solution, to avoid the potential for false positive results. After the initial incubation period, serial dilutions into regular SRB media can be performed. Media were incubated for 7 days and 28 days for APB tests and SRB tests, respectively.

## **ATP Bacterial Enumeration**

Collected water samples underwent a second quantitative measurement of total viable microorganism concentration, which was conducted using the Kikkoman Lumintester C-110 Luminometer and Bacteria Quench-Gone Aqueous (QGA) Test Kit (OSP Microcheck Inc.). The Luminometer test method provides a direct measurement of Adenosine-triphosphate (ATP) levels present in water samples via Firefly Luciferase assay. When present in a water sample, ATP treated with the Luciferase enzyme will emit light which is subsequently detected and quantified by photomultiplier detection. The amount of emitted light is directly proportional to the concentration of microorganisms present. The Luminometer has a detection range of  $4 \times 10^{-12}$  to  $1 \times 10^{-6}$  M ATP. Results (measured in cATP) are then converted into a value of microbial equivalents per millilitre. Collected samples were tested according to the recommended Luminometer Test Method procedures provided with the kit. Although ATP is a direct indicator of total microorganism concentration, this method does not allow for the strain or species differentiation.

## **Bacterial Isolation and Identification**

Water samples collected from the Muskwa wells (Well A and Well B) and their source pond (Source Pond 1), as well as the Evie wells (Well C and Well D) were also submitted for viable bacterial isolation and identification. Samples from the source pond used to fracture Wells C and D were not collected due to seasonal access restrictions, and thus were not included in this study. Upon arrival, the water samples were portioned and housed in either, aerobic or anaerobic environments. In their respective housing environments, aerobic and anaerobic bacteria were recovered from the water samples, inoculated into various media and incubated, as described in Champion Technologies Internal Documents (Innovotech 09-320-296, 09-320-

236 and 09-320-253) <sup>9-10</sup>. Following incubation, colonies were identified, scored for growth, enumeration and morphology. Individual aerobic bacterial strains were identified where possible; however, the anaerobic bacterium identification techniques applied did not permit individual strain identification. Three of the most prevalent isolated colonies were further purified and cryogenically preserved for future study.

### **Biocide Kill Analysis**

Effective biocide kill studies were performed on the highly contaminated water sample from Well D of the Evie formation. The biocides evaluated were cocodiamine-based and glutaraldehyde-based. For testing, labelled individual glass bottles containing 45ml of the 'Well D' water sample were dosed with either cocodiamine or glutaraldehyde, at one of three concentrations (i.e. 100, 400 or 750ppm) and exposed for one of two contact times (i.e. 1 or 4 hours). Following the pre-determined contact time, surviving bacteria were enumerated using the Luminometer test method according to the procedures described above. A non-biocide treated control was also prepared and tested to determine baseline bacterial contamination in the Well D sample.

### **Biocide Activity Evaluation**

Biocide activity evaluation studies were performed on the cryogenically preserved bacteria isolated from water samples of the Muskwa Wells A and B following the isolation and identification study described above. Individual test cultures (A-C1, A-C2 and B-C1) were grown into both planktonic and biofilm colonies from the cryopreserved stock, and tested against the cocodiamine- and glutaraldehyde-based biocides, as described in Champion Technologies Internal Document (Innovotech 09-320-237) <sup>12</sup>. Initial bacterial colony densities were  $10^4$  to  $10^6$  colony forming units per millilitre. To compare biocide efficacy under surface and bottomhole thermal conditions, one sample of each biocide was autoclaved in a pressure cooker to 130°C (maintained for 24 hours, then cooled to room temperature) prior to dilution and activity evaluation, and results were compared to non-autoclaved control samples. The four subsequent biocides (autoclaved cocodiamine, non-autoclaved cocodiamine, autoclaved glutaraldehyde and non-autoclaved glutaraldehyde) were evaluated at one of three specified contact times (30 minutes, 2 hours and 16 hours) and one of seven treatment levels (25, 50, 100, 200, 400, 500 and 750ppm). Minimum biocide concentrations required for the inhibition of planktonic bacterial growth (MIC), the eradication of planktonic bacteria (MBC), and the eradication of biofilms (MBEC) were determined by visual enumeration following exposure and a re-incubation period.

## **RESULTS**

### **Serial Dilution Bacterial Enumeration Results**

The results of the serial dilution analysis of the Muskwa zone wells (Well A and B) and their respective source pond (Source Pond 1) are shown in Figure 1 and summarized below.

*Well A* was positive for APB, but not SRB. Two of the APB bottles showed positive results (i.e. turned yellow), indicating a low bacterial contamination level (~100 bacteria per millilitre). Levels of APB less than 10,000 bacteria per millilitre are considered low; levels in the 10,000 to 100,000 bacteria per millilitre range are considered moderate; levels exceeding 100,000 bacteria per millilitre are considered high. However, a positive APB result in 2 or more bottles, although low on contamination scale, is typically considered significant enough to initiate a biocide treatment program.

*Well B* was positive for both APB and SRB. Again, two of the APB test bottles showed positive results, indicating a low APB contamination level. However, *Well B* also had 5 SRB bottles showing positive results (>100,000 bacteria per millilitre), indicating a very high SRB contamination level. Because most active SRB reside in sessile biofilms that cannot be measured by testing collected water samples, any positive SRB result found in water samples should be considered a significant concern.

*Source Pond 1* showed positive results for APB, but not SRB. The APB contamination level was, however, considered low (i.e. 2 positive bottles).

Results for the serial dilution analysis of the Evie zone wells (*Well C* and *D*) and their respective source pond (*Source Pond 2*) are shown in Figure 2 and summarized below.

*Well C* was treated with biocide 'on-the-fly' during fracturing. Serial dilution test results for this water sample were negative for both APB and SRB. Neither APB nor SRB test bottles showed any positive results.

*Well D* was positive for both APB and SRB. Four of the APB test bottles showed positive results, indicating a high APB contamination level (>10,000 bacteria per millilitre). In addition, 2 of the SRB test bottles showed positive results. Again, this indicates a low level of bacterial contamination, but can imply significant SRB contamination is present in the system within biofilms.

*Source Pond 2* showed positive results for both APB and SRB. Of the APB test bottles, 3 showed positive results, indicating APB contamination was relatively low (~1,000 bacteria per millilitre). Of the SRB bottles, 1 showed a positive result.

Serial dilution tests on the above wells were repeated on a monthly to quarterly basis. All above results were confirmed upon repeat analysis.

### **ATP Bacterial Enumeration Results**

The results of the ATP-based bacterial enumeration are shown in Table 3. Testing was performed on water samples from all wells. Source pond water sample testing, however, was not completed due to winter access restrictions on sample collection.

*Wells A and B* (Muskwa zone wells) results revealed contamination levels of 13,199 and 20,906 microbial equivalents per millilitre, respectively. These results, although slightly higher than the contamination levels revealed by serial dilution, likely better reflect actual bacterial levels due to the higher sophistication and accuracy of the ATP testing methodology. The results, however, still fall in the 'low' category, and thus confirm the previous serial dilution categorization results.

Well C, the Evie zone well that had been treated with biocide during fracturing, had extremely low levels of bacterial contamination (i.e. 1,286 microbial equivalents/ml). Due to the higher sensitivity of the Luminometer test method, some level of microbial contamination will always be reported since it is nearly impossible to completely sterilize an environment. The level of contamination observed in this sample would be considered acceptable for drinking water standards, according to the Luminometer interpretation guidelines (provided with the test kit). Thus, the results can be considered in agreement with the negative serial dilution results observed from these wells.

Well D, the biocide-untreated Evie zone well, had extremely high levels of bacterial contamination (i.e. 2,515,630 microbial equivalents/ml). This result confirms the high bacterial contamination results observed following the serial dilution tests.

### **Bacterial Isolation and Identification Results**

Results of the bacterial isolation and identification study are shown in Table 4. Source Pond 1 water samples contained 6 significant strains of aerobic bacteria that could be isolated, but no anaerobic. Two strains of aerobic bacteria were isolated from each of the Muskwa zone wells, Well A and Well B. However, anaerobic bacteria, specifically SRB, were isolated only from Well B. Interestingly, the isolated bacteria (namely, aerobic colonies) from the source pond sample had similar colony characteristics to the aerobic bacteria isolated from the two wells. These common bacteria may have originated in the source pond, but have adaptations that permit them to survive the fracturing process and downhole conditions. Unfortunately, confirmation of this relationship was not possible within the scope of this study.

Isolation and identification study results for the two Evie zone wells are also shown in Table 4. One aerobic bacterial strain and one anaerobic strain (specifically, SRB) were isolated from the untreated Evie zone well (Well D). However, growths cultured from the biocide-treated well of the Evie zone (Well C) were not identified as bacteria. Rather, the characteristics of these colonies were indicative of environmental yeasts.

### **Biocide Kill Analysis Results**

A summary of the effective biocide kill test results are shown in Table 5. In comparison to the non-biocide treated control, the biocides tested provided a minimum 78% (cocodiamine at 100 ppm and 1 hour contact time) and a maximum 99% (cocodiamine at 750 ppm and 4 hours contact time) kill of present bacteria. Minimum effective biocide kill (cocodiamine at 100 ppm) was increased to 91% when contact time was extended to 4 hours. Kill performance also improved with increasing concentration, although there was little difference in performance between the 400 and 750ppm concentrations. Glutaraldehyde performed slightly better at the low concentration level (i.e. 100 ppm), with a kill percentage of 83% at 1 hour contact and 95% at 4 hours contact, as compared to cocodiamine, which had 78% kill at 1 hour contact and 91% kill at 4 hours contact. Effective kill performance for the two biocides was nearly maximal at the higher concentration levels (i.e. 400 and 750 ppm).

### **Biocide Activity Evaluation**

A summary table of both, the autoclaved and non-autoclaved biocide (cocodiamine and glutaraldehyde) sample activity on the inhibition and eradication of two strains of bacteria cultured from Well A (A-C1 and A-C2) and one strain of bacteria from Well B (B-C1) is shown in Table 6.

*Organism A-C1* experienced not only complete inhibition of growth (MIC), but also complete eradication of planktonic colonies by cocodiamine samples (both autoclaved and non-autoclaved) at all concentrations and contact times tested. The more resistant biofilms, however, required a cocodiamine (autoclaved or non-autoclaved) minimum dose of 500ppm at 30 minutes, 50ppm at 2 hours or less than 25 ppm at 16 hours for complete eradication. The organism A-C1 was much more resistant to glutaraldehyde (autoclaved and non-autoclaved). Inhibition and eradication of A-C1 planktonic colonies, and eradication of A-C1 biofilms was incomplete at all glutaraldehyde concentrations and contact times tested.

*Organism A-C2* showed similar susceptibility to cocodiamine. Planktonic colonies were completely inhibited and eradicated at all concentrations and contact times tested. Non-autoclaved samples of cocodiamine were able to further eradicate biofilm colonies at 750ppm and 30 minutes, 25ppm and 2 hours, or less than 25ppm and 16 hours. Autoclaved samples performed similarly, and required 500ppm and 30 minutes, 100ppm and 2 hours or less than 25ppm and 16 hours to completely eradicate biofilms. Again, organism A-C2 was more resistant to glutaraldehyde (autoclaved and non-autoclaved) activity. Inhibition and eradication of planktonic and biofilm colonies by glutaraldehyde was incomplete at all concentrations and contact times tested.

*Organism B-C1* was more resistant to cocodiamine activity than were the previous two colonies. Complete inhibition of planktonic growth was still achieved by both autoclaved and non-autoclaved samples of cocodiamine at all concentrations and contact times tested. However, complete B-C1 planktonic bacterial colony eradication was only achieved at higher concentrations for the contact times tested. Non-autoclaved samples of cocodiamine completely eradicated planktonic bacteria at 25ppm and both, 30 minutes and 2 hours, and 100ppm and 16 hours. Autoclaved samples were effective at less than 25ppm and 30 minutes, 50ppm and both, 2 and 16 hours. Biofilms required much higher concentrations. A minimum of 500ppm and 30 minutes was required for complete biofilm eradication by the non-autoclaved cocodiamine samples. However, only incomplete eradication was achieved by the non-autoclaved cocodiamine sample at the other concentrations and contact times tested, as well as by the autoclaved cocodiamine sample at all concentrations and contact times tested. Glutaraldehyde was able to achieve complete inhibition of planktonic growth at all concentrations and contact times tested, but was unable to completely eradicate planktonic and biofilm colonies at any of the concentrations and contact times.

## **DISCUSSION**

Shale gas fracturing fluids, due to the high volume requirements, are often collected from various, natural surface water sources. These sources are highly contaminated with bacteria (both APB and SRB). Despite the harsh downhole environmental conditions of the Horn River Basin, if water sources remain untreated, viable bacteria can become established downhole following injection. Furthermore, these bacteria will be carried with flowback return water and

can potentially contaminate surface flowlines and equipment, as well. The present study provided strong evidence for the survival and adaptation of bacteria in these high-temperature, high-pressure wells. The reservoir conditions alone, were insufficient for eradicating injected bacteria. Rather, bacteria survived and proliferated regardless of downhole temperature and pressure, as evidenced by the equivalent, and in some cases, elevated contamination levels observed in water samples from wells as compared to their source water. Source water samples contained a higher variety of isolatable bacterial strains, as compared to well samples, but it was clear that several strains of bacteria were able to survive and adapt to the downhole conditions. Levels of contamination also increased with time downhole, as evidenced by the higher bacterial concentrations in the wells several years old as compared to their younger equivalents. Following downhole establishment, APB communities appeared to give rise to SRB communities, as evidenced by the subsequent development of SRB contamination in wells fractured with source pond water negative for viable SRB. Source waters may indeed, provide a dormant SRB supply that is not detectable during sampling, but that can become incorporated in downhole biofilms and develop into viable, proliferating communities. Alternatively, these bacterial communities may also have been indigenous to the formation, itself. Despite bacterial origin, the presence of downhole and surface contamination is evident in these untreated wells.

Microbiologically induced corrosion can be responsible for costly and hazardous equipment failures. Industry best practice recommends application of a biocide-treatment program whenever bacteria are found to be present within a production system, since a single identified planktonic bacterium is a strong indication that large numbers already exist in sessile colonies. Due to the high initial contamination of fracture source water, biocide treatment is often incorporated into the fracture process. The present field study showed that treatment of fracture water continuously “on-the-fly” with 150ppm of glutaraldehyde resulted in: 1) elimination of APB and SRB contamination, as evidenced by serial dilution bacterial enumeration methods; and 2) reduction in microorganism levels to potable water equivalents, as evidenced by ATP microbial enumeration methods. Isolation and identification study results suggest that the microorganisms detected in the latter study may be environmental yeasts, rather than bacteria. All serial dilution enumeration tests were repeated on a quarterly basis to ensure consistency of results. The biocidal effect of the one-time treatment was sustainable up to the current time (i.e. six months). Laboratory analysis of biocide activity revealed much more biocide-resistant bacterial colonies, particularly in established biofilms. These results stress the importance of biocide treatment during the fracturing process to eliminate bacteria before they can attach to equipment surfaces and establish more resistant biofilm colonies that are much more difficult and costly to eradicate. The two biocides investigated in the present study, cocodiamine and glutaraldehyde, effectively eradicate bacteria at various treatment levels and contact times, depending on the resistance and development level of the colony. These results were consistent with previous studies on fracture water biocide-treatment effectiveness<sup>1</sup>. All biocide treatment programs, however, should be supplemented with a continuous monitoring program to ensure treatment effectiveness and sustainment.

## **CONCLUSIONS**

1. Frac water source ponds are an excellent and likely source of bacterial contamination for wells
2. The harsh downhole conditions of the Horn River formation are not sufficient for eradicating bacteria
3. Both APB and SRB can survive and grow in the formation, even at the most extreme conditions observed in the Horn River Basin
4. Bacterial resistance and survival lasts years beyond the initial flowback period
5. Downhole establishment of APB communities, may lead to the eventual development of SRB communities
6. Communities of bacteria may be intrinsic to this formation
7. Both cocodiamine- and glutaraldehyde-based biocides are effective in eradicating bacteria in these high temperature, high pressure wells
8. High temperatures had very little effect on biocide performance

**TABLE 1:**

**HORN RIVER BASIN WELL PRODUCTION: GENERAL CHARACTERISTICS**

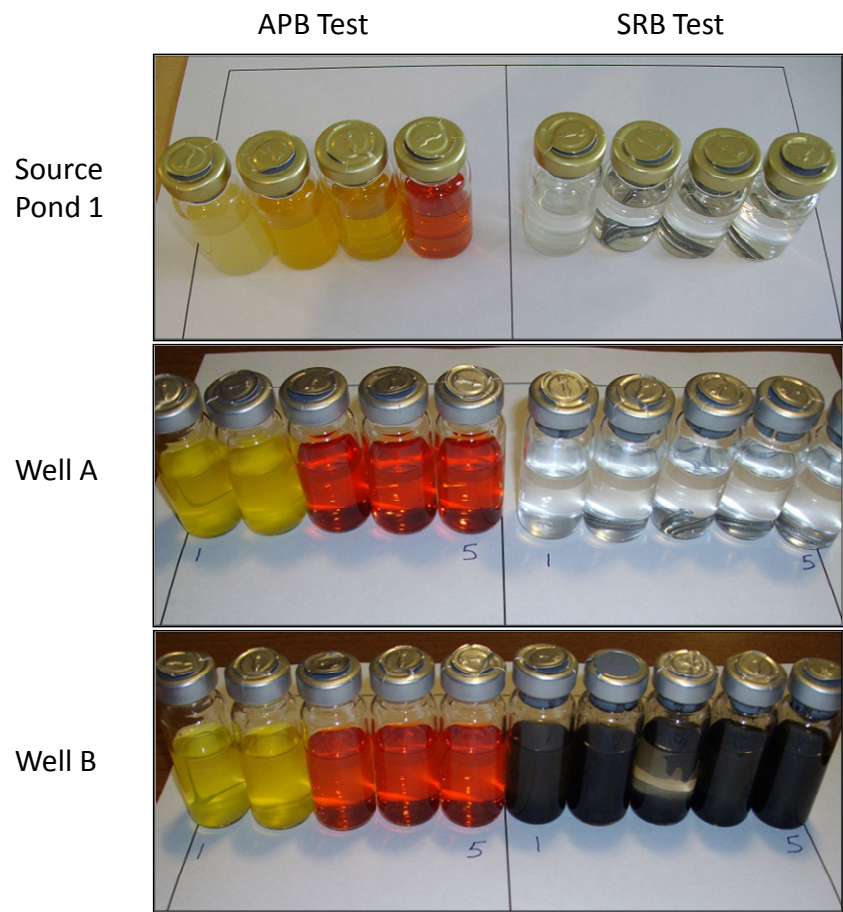
Gas (e <sup>3</sup> m <sup>3</sup> /day)	Water (m <sup>3</sup> /day)	Condensate (m <sup>3</sup> /day)	H <sub>2</sub> S (ppm)	CO <sub>2</sub> (%)	Chloride (ppm)	Total Dissolved Solids (ppm)	Temperature (°C)	Pressure (kPa)
40 - 226	0.3 - 103	0	≤ 128	≤ 21	10 – 21,000	24,000 – 36,000	35 – 175	2,000 - 44,000

**TABLE 2:**

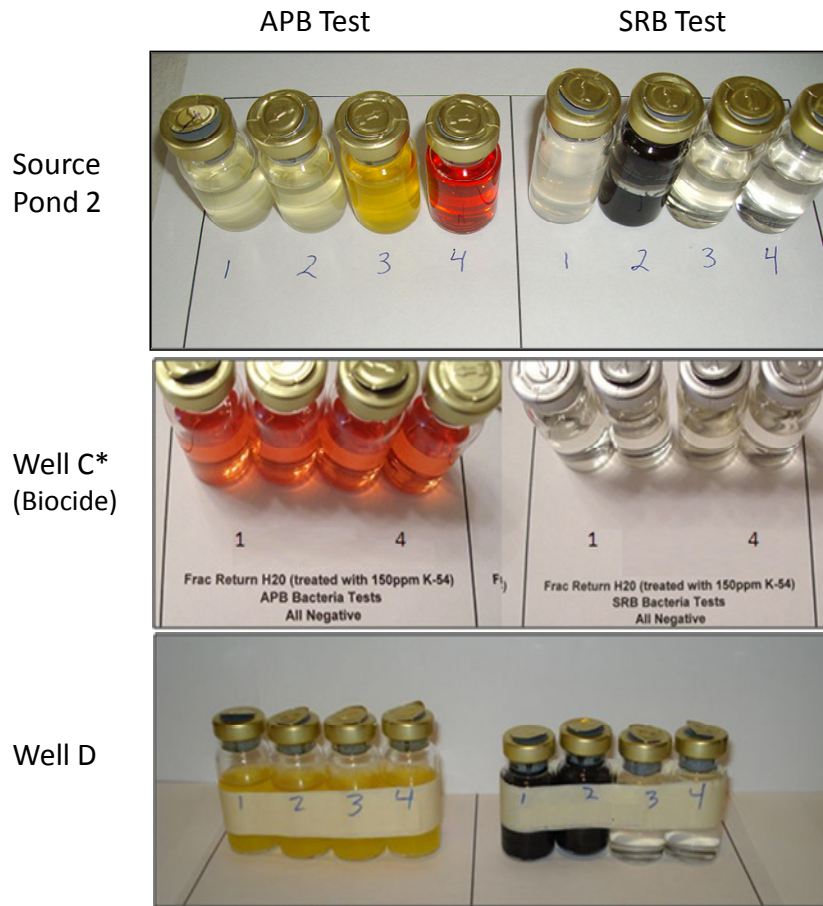
**SUMMARY OF WATER SAMPLE CHARACTERISTICS**

Sample ID	Temperature (°C)	Pressure (kPa)	Stage	Treatment
<b>Muskwa Zone</b>				
Source Pond 1	n/a	n/a	n/a	None
Well A	140	2,700	Flowback	None
Well B	140	2,700	Production	None
<b>Evie Zone</b>				
Source Pond 2	n/a	n/a	n/a	None
Well C	175	44,000	Flowback	Biocide**
Well D	175	44,000	Production	None

\*\*Biocide: Bactron™ K-54 applied at 150ppm during fracturing



**FIGURE 1** – Serial Dilution Results for Muskwa Zone Wells (A and B) and Source Pond 1



**FIGURE 2** – Serial Dilution Results for Evie Zone Wells (C and D) and Source Pond 2

**TABLE 3:**  
SUMMARY OF LUMINOMETER (ATP) BACTERIAL ENUMERATION RESULTS

Sample ID	ATP Bacterial Enumeration (microbial equivalents/ml)
<b>Muskwa Zone</b>	
Source Pond 1	n/a
Well A	13,199
Well B	20,906
<b>Evie Zone</b>	
Source Pond 2	n/a
Well C**	1,286
Well D	2,515,630

\*\*Well treated with biocide during fracturing

**TABLE 4:**  
SUMMARY OF BACTERIAL ISOLATION AND IDENTIFICATION RESULTS

Sample ID	Aerobic Bacteria	Anaerobic Bacteria	Other
<b>Muskwa Zone</b>			
Source Pond 1	Gram negative Bacillus, possibly <i>Sphingomonas paucimobilis</i>	None	None
	Gram negative Bacillus, possibly <i>Brevundimonas vesicularis</i>		
	Gram negative Bacillus, possibly <i>Comamonas testosteroni</i> or <i>Psuedomonas alcaligenes</i>		
	Gram positive coccoid chains, strain unidentifiable		
	Gram positive coccoid clusters, strain unidentifiable		
	Gram positive coccoid solitary cells, strain unidentifiable		
Well A	Gram negative diplococci, possibly <i>Aeromonas hydrophila/caviae</i>	None	None
	Gram positive cocci, strain unidentifiable		
Well B	Gram positive Bacillus, strain unidentifiable	Sulfate-Reducing Bacteria (SRB), strain unidentifiable	None
	Gram negative Bacillus, strain unidentifiable		
<b>Evie Zone</b>			
Well C	None	None	Gram positive and negative rod shaped clusters, possibly Environmental Yeast
Well D	Gram positive coccoid identified as <i>Staphylococcus epidermidis</i> ,	Sulfate-Reducing Bacteria (SRB), strain unidentifiable	None

**TABLE 5:**  
SUMMARY OF BACTRON™ K-48 AND K-54 KILL STUDY RESULTS

Biocide	Biocide Concentration	Microbial Equivalents	Percent Kill
	(ppm)	(# per ml)	(%)

Untreated	0	2,515,630	0
1 Hour Contact Time			
Cocodiamine	100	559,516	78
	400	40,220	98
	750	47,346	98
Glutaraldehyde	100	430,935	83
	400	179,514	93
	750	110,663	96
4 Hour Contact Time			
Cocodiamine	100	226,708	91
	400	37,913	98
	750	32,094	99
Glutaraldehyde	100	123,454	95
	400	73,109	97
	750	54,242	98

**TABLE 6:**

**SUMMARY OF AUTOCLAVED AND NON-AUTOCLAVED BIOCIDES ACTIVITY ON CULTURED SHALE GAS WELL BACTERIA**

Organism A-C1							
Contact Time	Biocide	Minimum Kill Concentration (ppm)					
		Standard Biocide Conditions			Autoclaved Biocide Conditions		
		MIC	MBC	MBEC	MIC	MBC	MBEC
30 min	Cocodiamine	<25	<25	500	<25	<25	500
2 hr		<25	<25	50	<25	<25	50
16 hr		<25	<25	<25	<25	<25	<25
30 min	Glutaraldehyde	>750	>750	>750	>750	>750	>750
2 hr		>750	>750	>750	>750	>750	>750
16 hr		>750	>750	>750	>750	>750	>750
Organism A-C2							
Contact Time	Biocide	Minimum Kill Concentration (ppm)					
		Standard Biocide Conditions			Autoclaved Biocide Conditions		
		MIC	MBC	MBEC	MIC	MBC	MBEC
30 min	Cocodiamine	<25	<25	>750	<25	<25	500
2 hr		<25	<25	25	<25	<25	100
16 hr		<25	<25	<25	<25	<25	<25
30 min	Glutaraldehyde	>750	>750	>750	>750	>750	>750
2 hr		>750	>750	>750	>750	>750	>750
16 hr		>750	>750	>750	>750	>750	>750
Organism B-C1							
Contact	Biocide	Minimum Kill Concentration (ppm)					

Time		Standard Biocide Conditions			Autoclaved Biocide Conditions		
		MIC	MBC	MBEC	MIC	MBC	MBEC
30 min	Cocodiamine	<25	25	500	<25	<25	>750
2 hr		<25	25	>750	<25	50	750
16 hr		<25	100	>750	<25	50	750
30 min	Glutaraldehyde	>750	>750	>750	>750	>750	>750
2 hr		>750	>750	>750	>750	>750	>750
16 hr		>750	>750	>750	500	750	>750

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