Comparison of Biocides Using New Microbial Detection Tool
Edward Corrin – Multichem Production Chemicals, Houston, Texas, USA
Stan Leong - OSP Microcheck Inc., Calgary, Alberta, Canada
Pat Whalen – LuminUltra Technologies Ltd., Fredericton, New Brunswick, Canada

ABSTRACT

Microbial contamination in the oil and gas industry is prevalent in both downstream and upstream activities. Fouling, corrosion and all the problems associated with respiration by-products contribute to lifting and production costs. Other problems include spoilage of drilling, well stimulation and production fluids. The impact of these problems can be managed by understanding where the microbes are and when they need to be controlled. Biocide programs are one effective way to control bio-burden in produced and surface waters. There are limited methods available that will quantitatively show the direct impact of a biocide program and offer an opportunity to optimize dosage rates and application points. The most common and least expensive methods involve inoculation of a nutrient medium specific to certain types of bacteria and incubation over a period of time. Detection of viable microbes in this way results in disadvantages, primarily the incubation time requirement which does not allow for timely adjustments to the biocide application. This paper summarizes results obtained using a novel technique for bio-burden detection via Adenosine Triphosphate (ATP) in surface and produced water in typical Southern USA operations. ATP is an excellent marker for all cells and commercial detection systems have been available for several decades. The advantage of ATP detection is that results are available in minutes, rather than days or weeks. However, traditional ATP detection systems had significant drawbacks, including assay interferences and poor sampling techniques, generating results that were difficult to interpret and apply. The current protocol has been refined to eliminate these deficiencies and produce repeatable results that are comparable with industry accepted culturing methods. In addition to examining bio-burdens from representative surface and produced waters, the technique was also used to compare the efficacy of several non-oxidizing biocides on the microbial populations.

INTRODUCTION

Microbes play a large role in the production and distribution of oil and natural gas. Microbially induced corrosion, formation souring, equipment fouling and treating problems are the focus of attention as they contribute to lifting and production costs for the producer. Traditionally, serial dilution methods have been used to identify populations of Acid Producing Bacteria (APB) and Sulphate Reducing Bacteria (SRB).
Company Field Service Representatives typically take a small kit with them into the field. Water samples are taken directly from the process vessel, piping, tank, pond, etc where microbes are suspected of causing some sort of problem. The media bottles are inoculated and a series of dilutions are created. Then all of the bottles are taken back to a central lab and incubated for anywhere from 14 days for detection of APB to 28 days for detection of SRB. The results are then used to recommend remediation treatments or further study.

What typically happens next is that during the time required for incubation, the problem has gotten worse, has spread to other processes or other changes are made to resolve the symptoms of the problem. One example is recycling wet, off spec oil back through a treater at an oil battery. There are many reasons why water is not sufficiently removed from oil during the treating process. A pad of material may form at the oil/water interface in the treater preventing the water from easily separating from the oil. These pads can be a result of biomass, paraffins /asphaltenes, corrosion by-products, etc from the field accumulating at the treater. By recycling off-spec oil back to the treater, some of the pad material may spread to the oil storage tanks. Now the problem has spread from the field to the treater at the oil battery to the oil storage tanks.

There are many examples of problems that are resolved by looking at the symptom (recycling the wet oil) instead of resolving the problem (reduce the solids coming in from the field). What is common is that all of this results in increased costs from downtime, lost production and higher material and manpower costs. The true cause of the problem may not have been addressed. In oil and gas production, problems are always in immediate need of a solution. Time is of the essence when operators make decisions on how they resolve problems and they use any information available at the time. So, the incubation period required for serial dilution testing may result in non or mis-diagnosing a problem associated with microbes.

Other testing procedures are available which may shorten the required incubation time and eliminate interferences sometimes associated with serial dilution testing. SRB Checkbottles are one example where a single vial is used per test. The proprietary media was designed to specifically support growth of obligate, anaerobic SRB. This is a very specific medium which does not react with H₂S to create a false positive result. No series of dilutions are required. A positive test is represented by a black film coating the entire inside surface of the vial under the full height of the liquid column. An estimate in the amount of microbes is determined by the amount of time required for a positive result to show. Table 1 is used to estimate the number of SRB in a water sample.

<table>
<thead>
<tr>
<th>Days to Turn Positive</th>
<th>SRB Range (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 to 2</td>
<td>10,000 +</td>
</tr>
<tr>
<td>2 to 4</td>
<td>1,000 to 10,000</td>
</tr>
<tr>
<td>4 to 6</td>
<td>100 to 1,000</td>
</tr>
<tr>
<td>6 to 8</td>
<td>10 to 100</td>
</tr>
<tr>
<td>8 to 10</td>
<td>1 to 10</td>
</tr>
</tbody>
</table>
As with the serial dilution method, incubation of the samples at >32C is required over the test period.

The measurement of Adenosine Triphosphate (ATP) has long been used as a means to rapidly quantify total microorganisms in a variety of food hygiene and industrial applications. In early protocols, ATP was extracted from cells by boiling the sample in a buffer. The extracted ATP was reacted with the enzyme-substrate complex Luciferin-Luciferase. In this reaction, the Luciferase enzyme catalyzes the ATP-mediated oxidation of Luciferin. In the process, ATP is cleaved to yield Adenosine Monophosphate (AMP) and pyrophosphate, concurrently releasing a photon of light. The amount of light generated is proportional to the ATP concentration, which is proportional to the biomass.

Unfortunately, these early adaptations were unsuitable for use in oilfield environments because of a variety of interferences, including dissolved and suspended solids, hydrocarbon contamination, and even residual biocides. All of these interferences can contribute to one or both of the inhibition of the ATP-Luciferase assay, or the ability for light to penetrate to the photodetector of the luminometer. Since these early days, numerous improvements have been made to the ATP test protocol and it has gained widespread commercial and industrial use. However, many of the interferences characteristic of oilfield samples had remained a problem. Recently, LuminUltra Technologies (Fredericton, New Brunswick, Canada) has released a line of test kits designed specifically to overcome these interferences to provide an accurate indication of biomass-related (i.e. Intra-cellular, or simply Cellular) ATP in oilfield samples.

MATERIALS AND METHODS

Materials

Biocides: Bronopol was supplied by OSP Microcheck Inc. as 2K7 Water Soluble Paks. Bronopol W-M is a winterized, liquid biocide that is manufactured by OSP Microcheck Inc. Gluteraldehyde was supplied by BASF as Myacide GA25. Bromochlorodimethylhydantoin (BCDMH) is an oxidizing biocide supplied by BWA Water Additives. The DDAC quaternary amine biocide is a proprietary biocide of MultiChem Production Chemicals.

Analytical Tools: ATP tests were performed using the QuenchGone Aqueous (QGA) test kit manufactured by Luminultra Technologies and a Kikkoman Lumitester C-110 luminometer, supplied by OSP Microcheck Inc. The Modified Postgate’s B and Phenol Red Dextrose was supplied in 12 mL capacity glass vials and sealed with elastomer septa from VK Enterprises. SRB Checkbottles were supplied by OSP Microcheck Inc.
**Method – APB & SRB Serial Dilution**

All types of media were supplied using a total dissolved solids concentration of 5%. The vials were filled with 9 mL of media to facilitate subsequent 10:1 dilution (media:sample). Dilution series of 6 vials series were used. Prior to inoculation, the septum of each media vial in the series was wiped thoroughly with an alcohol swab to sterilize the septa. The 100 mL sample bottles were mixed gently prior to withdrawal of inoculums. Two Sterile 3 mL capacity syringes with 21 gauge needles were used to withdraw 2 mL of each sample from the 100 mL sample bottles. One mL of sample from the syringe was inoculated into the first vial in the series of PRD media and 1 mL in the other syringe was inoculated into the first vial in the SRB media series. These syringes were discarded. Two new syringes were used to process the remainder of both media series – one for the PRD series and one for the MPB series. After the first vial was inoculated with sample, the new syringe was inserted through the septa and the vial contents were mixed by withdrawing 1.5 mL of media/inoculums into the syringe and rapidly expelling the contents back into the vial. This mixing step was repeated 3 times for each vial in the series. After mixing, 1 mL of solution was drawn into the syringe and was transferred into the next vial in the series and was mixed as before. This procedure was followed for each vial in the series.

**Method – SRB Checkbottle**

SRB Checkbottles were supplied by OSP Microcheck Inc. The media supplied was compatible with total dissolved solids concentrations of between 10,000 mg /L and 100,000 mg /L. Prior to inoculation, the septum of each media vial was wiped thoroughly with an alcohol swab to sterilize. The 100 mL sample bottles were mixed gently prior to withdrawal of inoculums. A sterile 3 mL capacity syringe with 21 gauge needle was used to withdraw 2 mL of each sample from the 100 mL sample bottles. The SRB Checkbottle was inoculated with 2 mL of each water sample. The syringe was withdrawn and disposed appropriately. The SRB Checkbottles were then incubated at 35°C and checked daily for a positive reaction.

**Method – ATP Measurement**

The test method for ATP in this experiment was the QuenchGone Aqueous (QGA) method by LuminUltra Technologies. This method was selected on the basis that it includes a process and reagent system designed to overcome interferences that traditionally limit the effectiveness of ATP measurement in samples typical of produced oilfield waters. Such interferences include dissolved solids concentration, free biocide residuals, and oil contamination.

The QGA method was undertaken using test kits obtained from LuminUltra via OSP Microcheck as well as a Kikkoman Lumimeter C-110 luminometer and other standard laboratory equipment including both 12mm and 17mm test tube racks and two adjustable volume micropipettors (100 to 1000 µL, and 1 to 5 mL).
The procedure used was as follows (NOTE: A more extensive procedure can be found on the QGA test kit instruction sheet provided by LuminUltra):

1. **Preparation (at the start of each day of testing):** Turn on power to luminometer and ensure that all reagents have equilibrated to ambient temperature before running any tests. Ensure that all laboratory equipment is clean and properly calibrated.

2. **Calibration (At the start of each day of testing):** Add 2 drops (100µL) of Luminase (Luciferin-Luciferase reagent) and 2 drops (100µL) of UltraCheck 1 (1 ng/mL ATP Standard) to an unused 12x55mm culture tube. Swirl gently, place into the luminometer, and hit ‘READ’. Record machine output in relative Light Units (RLU) as RLU_\text{UC1}.

3. **Sample Preparation (Run each step for each sample in sequence):**
   a. **Acquire Sample:** Ensure that the sample contained used for collection is clean – in many cases is it sufficient to simply rinse the sample container using the sample to be collected multiple times prior to taking the actual sample for analysis. Ensure that the sample volume acquired is sufficient to be representative of the system from which it came – typically 500mL samples or larger are recommended. If possible, attempt to prepare the sample within 4 hours of collection. If this is not possible, store the sample in a cool location (a refrigerator is recommended) for up to 24 hours prior to following the remaining steps in this procedure. If samples have been stored cool prior to preparation and analysis, allow them to come to ambient temperature prior to analysis.
   b. **Filtration:** Mix the sample via inversion (~5 times) to ensure homogeneity. Acquire an unused 60mL syringe and draw a 20mL volume of the sample into the syringe. Attach a Quench-Gone Glass-microfiber depth filter to the syringe via luer-lok, and filter the sample into a waste receptacle.
   c. **Extraction:** Disassemble the syringe and filter arrangement; reattach the filter to the syringe barrel and hold over an unused 17x100mm culture tube. Use a micropipettor to add 1mL of UltraLyse 7 (ATP extraction reagent) to the syringe barrel, replace the plunger, and collect the UltraLyse 7 reagent in the culture tube. The contents of the culture tube are referred to as the ‘ATP Extract’.

   **NOTE:** At this point, the ATP Extract is stable for up to 1 day at room temperature (i.e. 20 ± 4ºC) and up to 7 days when stored refrigerated (i.e. 5 ± 3ºC).

4. **Sample Analysis (Run each step for each prepared sample in sequence):**
   a. **Dilution:** Obtain the ATP extract prepared in 3(c). If it was stored refrigerated, allow to warm to ambient temperature. Using a micropipettor,
add 9mL of UltraLute (ATP Extract Dilution Buffer) to the ATP Extract tube. Cap and mix this ‘Diluted ATP Extract’.

b. **Assay:** Using a micropipettor, transfer 100µL of Diluted ATP Extract to an unused 12x55mm culture tube. Add 2 drops (100µL) of Luminase (Luciferin-Luciferase reagent). Swirl gently, place into the luminometer, and hit ‘READ’. Record machine output in relative Light Units (RLU) as RLU<sub>Sample</sub>.

c. **Calculation:** To calculate ATP concentration, the following formula is used:

\[
ATP_{Sample} (\text{pg} / \text{mL}) = \frac{RLU_{Sample}}{RLU_{UC1}} \times \frac{10,000}{V_{Sample} (\text{mL})}
\]  

Where:
- \( RLU_{Sample} \) is the RLU reading for the sample (4b);
- \( RLU_{UC1} \) is the RLU for UltraCheck 1 Calibration (2);
- \( V_{sample} \) is the volume of sample used in the test (3b); and
- 10,000 is a factor that accounts for the 1/10 dilution factor in the test plus the factor of 1,000 to convert ng ATP to pg ATP.

**Method – Biocide Evaluation Procedure**

On April 28<sup>th</sup>, 2009, 4 liters of produced water was collected from a separator which was located approximately 25 feet from its corresponding wellhead in the Barnett Shale Formation region (Texas, USA). This water collection was performed under a nitrogen gas blanket in an attempt to mimic the anaerobic environment in the well and separator system. The water was tested in the field and was determined to have a TDS concentration of 7.2% and a pH of 5.9. This water was refrigerated until the date of the experiment (approximately 5 days).

On site, 5% TDS Modified Postgate’s B (MPB) and 5% TDS Phenol Red Dextrose (PRD) bacterial growth media was inoculated to provide a stock culture of the native bacteria from the water source. Incubation was performed until the experiment began (approximately 5 days).

On May 4<sup>th</sup>, 2009, two liters of the collected field water was taken out of refrigeration, measured and dispensed into a sterile 3L flask under a nitrogen blanket to serve as the master sample. This water was allowed to warm to room temperature. Once at room temperature, one hundred mL of positive MPB and one hundred mL of positive PRD growth media that was inoculated in the field was added to the master sample. The bacteria were allowed to acclimate to the water for two hours, after which, the master sample was then divided and dispensed into 10 separate labeled glass bottles, each containing 300mL of the master sample.

A ‘time zero’ sample was taken from the control sample and inoculated into MPB and PRD media, inoculated into OSP Microcheck SRB Check Bottles, and tested with the
LuminUltra QGA test kit in order to provide a baseline bacterial population number. Biocides were then administered to the samples and the bottles were placed onto a shaker to prevent the samples from becoming static.

The dosages were administered according to Table 2 (all values in mg/L as active ingredient):

<table>
<thead>
<tr>
<th>Sample</th>
<th>Bronopol</th>
<th>Gluteraldehyde</th>
<th>DDAC Quaternary Amine</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 (Control)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#2</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#3</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#4</td>
<td>200</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#5</td>
<td>500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>#6</td>
<td>-</td>
<td>50</td>
<td>-</td>
</tr>
<tr>
<td>#7</td>
<td>-</td>
<td>100</td>
<td>-</td>
</tr>
<tr>
<td>#8</td>
<td>-</td>
<td>200</td>
<td>-</td>
</tr>
<tr>
<td>#9</td>
<td>-</td>
<td>500</td>
<td>-</td>
</tr>
<tr>
<td>#10</td>
<td>-</td>
<td>-</td>
<td>50</td>
</tr>
<tr>
<td>#11</td>
<td>-</td>
<td>-</td>
<td>100</td>
</tr>
<tr>
<td>#12</td>
<td>-</td>
<td>-</td>
<td>200</td>
</tr>
<tr>
<td>#13</td>
<td>-</td>
<td>-</td>
<td>500</td>
</tr>
</tbody>
</table>

After one hour of contact time with the biocides, samples were taken from each of the 10 sample bottles and tested with the same analytical tools as done at time zero; The bottles were then placed back on the shaker overnight. After 24 hours of contact time with the biocides, another round of tests was performed on all ten samples. All sampling and testing of the bottles was done under a nitrogen blanket.

RESULTS

Table 3 shows the raw data collected at 1 hour and 24 hours following set-up of the biocide evaluation.

<table>
<thead>
<tr>
<th>BIOCIDE DOSAGE</th>
<th>APB Dilution (log CFU/mL) @ Time</th>
<th>SRB Dilution (log CFU/mL) @ Time</th>
<th>SRB Checkbottle (log CFU/mL) @ Time</th>
<th>ATP (pg/mL) @ Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2 24</td>
<td>1 24</td>
<td>1 24</td>
<td>1772.2 2634.2</td>
</tr>
<tr>
<td>50 mg/L Bronopol</td>
<td>2 0 6 6 4 3 ND</td>
<td>518.5 71.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 mg/L Bronopol</td>
<td>2 0 6 5 2 ND</td>
<td>141.5 35.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>200 mg/L Bronopol</td>
<td>0 0 6 4 0 ND</td>
<td>38.7 52.2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The following sub-sections discuss the results from each analytical method individually.

**Acid Producing Bacteria (APB) Serial Dilution Data**

The following table summarizes the results found using serial dilution to estimate the microbial Acid Producing Bacteria (APB) population. The control sample was found to have $10^2$ CFU/mL at time zero, after 1 hour and after overnight incubation.

<table>
<thead>
<tr>
<th>Biocide Dosage</th>
<th>% Inhibition Relative to Control</th>
<th>@ 1 hour</th>
<th>@ 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/L Bronopol</td>
<td>0%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>100 mg/L Bronopol</td>
<td>0%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>200 mg/L Bronopol</td>
<td>99%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>500 mg/L Bronopol</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>50 mg/L Gluteraldehyde</td>
<td>99%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>100 mg/L Gluteraldehyde</td>
<td>99%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>200 mg/L Gluteraldehyde</td>
<td>99%</td>
<td>99%</td>
<td></td>
</tr>
<tr>
<td>500 mg/L Gluteraldehyde</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>50 mg/L DDAC</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>100 mg/L DDAC</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>200 mg/L DDAC</td>
<td>0%</td>
<td>0%</td>
<td></td>
</tr>
<tr>
<td>500 mg/L DDAC</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
</tbody>
</table>

Bronopol treated samples were found to have 0% microbial inhibition at the lower dosages after 1 hour incubation and 99% inhibition after overnight incubation. Gluteraldehyde treated samples showed 99% inhibition at all dosages in both the 1 hour and overnight incubation samples. DDAC treated samples were found to have 0% microbial inhibition at all dosages after both 1 hour and overnight incubation periods.

**Sulfate Reducing Bacteria (SRB) Serial Dilution Data**

Similarly, results were obtained using modified post gate B media to isolate Sulfate Reducing Bacteria (SRB) populations in the produced water sample. Control samples all showed $10^6$ SRB/mL at time zero, after 1 hour and overnight incubation.
Table 5 – SRB Serial Dilution Results

<table>
<thead>
<tr>
<th>Biocide Dosage</th>
<th>% Inhibition Relative to Control @ 1 hour</th>
<th>@ 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/L Bronopol</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>100 mg/L Bronopol</td>
<td>0%</td>
<td>90%</td>
</tr>
<tr>
<td>200 mg/L Bronopol</td>
<td>0%</td>
<td>99%</td>
</tr>
<tr>
<td>500 mg/L Bronopol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50 mg/L Gluteraldehyde</td>
<td>&gt;99.99%</td>
<td>&gt;99.99%</td>
</tr>
<tr>
<td>100 mg/L Gluteraldehyde</td>
<td>&gt;99.99%</td>
<td>&gt;99.99%</td>
</tr>
<tr>
<td>200 mg/L Gluteraldehyde</td>
<td>&gt;99.99%</td>
<td>&gt;99.99%</td>
</tr>
<tr>
<td>500 mg/L Gluteraldehyde</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>50 mg/L DDAC</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>100 mg/L DDAC</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>200 mg/L DDAC</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>500 mg/L DDAC</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Bronopol treated samples showed 90% microbial inhibition and 99% inhibition at 100 mg /L and 200 mg /L dosage respectively after overnight incubation. No changes were seen, compared to the control, at the lower dosage or after only 1 hour incubation. DDAC treated samples all showed no SRB activity reduction at any dosage or after any incubation period.

**Sulfate Reducing Bacteria (SRB) Checkbottle Data**

Using the SRB Checkbottle procedure, the produced water sample resulted in $10^4$+ SRB /mL.

Table 6 – SRB Checkbottle Results

<table>
<thead>
<tr>
<th>Biocide Dosage</th>
<th>% Inhibition Relative to Control @ 1 hour</th>
<th>@ 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/L Bronopol</td>
<td>90%</td>
<td>ND</td>
</tr>
<tr>
<td>100 mg/L Bronopol</td>
<td>99%</td>
<td>ND</td>
</tr>
<tr>
<td>200 mg/L Bronopol</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>500 mg/L Bronopol</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>50 mg/L Gluteraldehyde</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>100 mg/L Gluteraldehyde</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>200 mg/L Gluteraldehyde</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>500 mg/L Gluteraldehyde</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>50 mg/L DDAC</td>
<td>99.9%</td>
<td>ND</td>
</tr>
<tr>
<td>100 mg/L DDAC</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>200 mg/L DDAC</td>
<td>99.99%</td>
<td>ND</td>
</tr>
<tr>
<td>500 mg/L DDAC</td>
<td>99.99%</td>
<td>ND</td>
</tr>
</tbody>
</table>

Samples treated with DDAC showed no SRB growth, except at 50 mg /L, where 99.9% inhibition was found. Bronopol treated samples at 50 mg /L and 100 mg /L showed 90%
and 99% inhibition respectively. Samples treated at 200 mg /L and 500 mg /L dosages showed no SRB activity. All the gluteraldehyde treated samples showed no SRB activity at any dosage.

**ATP Measurement Data**

<table>
<thead>
<tr>
<th>Biocide Dosage</th>
<th>% Inhibition Relative to Control</th>
<th>@ 1 hour</th>
<th>@ 24 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 mg/L Bronopol</td>
<td>70.7%</td>
<td>97.3%</td>
<td></td>
</tr>
<tr>
<td>100 mg/L Bronopol</td>
<td>92.0%</td>
<td>98.7%</td>
<td></td>
</tr>
<tr>
<td>200 mg/L Bronopol</td>
<td>97.8%</td>
<td>98.0%</td>
<td></td>
</tr>
<tr>
<td>500 mg/L Bronopol</td>
<td>ND</td>
<td>98.7%</td>
<td></td>
</tr>
<tr>
<td>50 mg/L Gluteraldehyde</td>
<td>65.8%</td>
<td>97.3%</td>
<td></td>
</tr>
<tr>
<td>100 mg/L Gluteraldehyde</td>
<td>73.0%</td>
<td>97.8%</td>
<td></td>
</tr>
<tr>
<td>200 mg/L Gluteraldehyde</td>
<td>63.5%</td>
<td>97.8%</td>
<td></td>
</tr>
<tr>
<td>500 mg/L Gluteraldehyde</td>
<td>ND</td>
<td>97.1%</td>
<td></td>
</tr>
<tr>
<td>50 mg/L DDAC</td>
<td>28.5%</td>
<td>20.7%</td>
<td></td>
</tr>
<tr>
<td>100 mg/L DDAC</td>
<td>43.5%</td>
<td>53.2%</td>
<td></td>
</tr>
<tr>
<td>200 mg/L DDAC</td>
<td>54.6%</td>
<td>86.0%</td>
<td></td>
</tr>
<tr>
<td>500 mg/L DDAC</td>
<td>ND</td>
<td>82.2%</td>
<td></td>
</tr>
</tbody>
</table>

**Bronopol:** After 1 hour incubation with Bronopol, measurement of ATP showed 70.7% inhibition of microbes at 50 mg /L, up to 97.8% inhibition at 200 mg /L dosage. After overnight incubation, the maximum of 98.7% inhibition was observed at 100 mg /L dosage.

**Gluteraldehyde:** Incubation of samples for 1 hour after treatment with gluteraldehyde resulted in 65.8% microbial inhibition at 50 mg /L, up to 73% inhibition at 100 mg /L dosage. Overnight incubation with gluteraldehyde produced a maximum of 97.8% inhibition at 100 mg /L dosage.

**DDAC Quaternary Amine:** DDAC treated samples resulted in 28.5% inhibition at 50 mg /L, up to 54.6% inhibition at 200 mg /L after 1 hour incubation. Overnight incubation produced a maximum of 86% inhibition at 200 mg /L. At the lowest dosage of 50 mg /L, overnight inhibition was actually less than the comparable one hour incubation sample and may have been indicative of the microbial population starting to re-grow or the microbes became more metabolically active.

**Overall Results**

Initially, the results of the different test methods appear to provide some conflicting information. However, a closer examination of the results, in combination with reflection on what each analytical tool is showing, suggests some interesting relationships in the results.
The results from this study suggest that the specificity of the media and any residual biocide will impact what results are seen. The SRB Checkbottles are specific for anaerobic SRB and showed changes in the viability of this portion of the population. The Modified Postgate’s B and Phenol Red Dextrose media build more on results from SRB Checkbottles by showing the viability of both SRB and APB in the water sample. The ATP measurements showed the response in metabolic activity to different types and dosages of biocide and biocide contact times. ATP results did not show the immediate fluctuations in microbial viability as picked up by the other media. However, the results did show more minute differences, and hence is more precise.

Assuming that the SRB Checkbottles are very specific to anaerobic, SRB species, then Bronopol proved to be a very good biocide at dosages greater than 100 mg/L with 1 hour or more of contact time. The Bronopol treated sample at 200 mg/L with an hour or more of contact time or 50 mg/L with over 24 hour contact time was required before we saw any significant microbial inhibition with the serial dilution method. Thus, we were able to culture more microbes with the serial dilution method than with the SRB Checkbottle method from the same water sample. Further to this, the ATP testing indicates that we were able to achieve reasonable metabolic inhibition with Bronopol at 200 mg/L with one hour contact time. We were still able to see some inhibition within an hour at 50 mg/L and 100 mg/L. Excellent inhibition was achieved at dosages of 50 mg/L and greater after 24 hour contact time. The ATP testing was able to differentiate effectiveness between the different biocide dosages and incubation times that the serial dilution and SRB Checkbottle methods were not able to do.

Results from the DDAC treated samples illustrate the same trends. Only some obligate, anaerobic SRB growth was found after 1 hour treatment at 50 mg/L. Using the less selective serial dilution media, we found significantly more microbes both in the phenol red dextrose broth and the modified Post Gate’s B medium. The ATP testing identified differences in metabolic activity at 1 hour and 24 hours of incubation; however, it also differentiated between performances of the biocide at low dosage versus higher dosages.

Gluteraldehyde treated samples were found to have no microbial growth using both the SRB Checkbottles and serial dilution media. Viable microbes appeared to be inhibited by the gluteraldehyde. This information would have made it difficult to understand how this biocide worked at various dosages. However, ATP testing revealed that although some biocidal activity was seen at 50 mg/L dosage after one hour, good microbial suppression was only achieved after 24 hours of contact time with this biocide. So although not viable, there were still some metabolically active microbes still present.

From a purely biocidal performance perspective, our testing suggested the use of either gluteraldehyde or Bronopol based biocides for this application. Dosages of 50 mg/L – 100 mg/L with continuous contact for a minimum of 24 hours would be required to get maximum kill. Both acid producing bacteria and sulphate reducing bacteria would be susceptible to these dosages of biocides. Of course selection of a biocide for a particular
DISCUSSION

There are several inherent drawbacks to relying solely on traditional nutrient media to culture microbes for population measurement. It has been suggested that plate counts, serial dilution methods and other culture-based tests are incomplete in detecting the total population. Culturability is affected primarily by the ability of captured microbes to proliferate on the growth medium provided, under specific growth conditions. It has been estimated that less than 1% of the species present in an environmental sample will form colonies under any given set of growth conditions. Sampling methods introduce stressors not present in the native environment. These stressors may include, but are not limited to, introduction of oxygen into the sample, disruption of biofilms and varying water chemistries (for example, salinity in the culture environment). Factors such as temperature and nutrient availability are also different as a result of culturing media and techniques when compared to the natural environment. These stressors will put significant selective pressure on the sample population and influence its viability.

There are situations where traditional bacterial growth media lacks nutrients required for the growth of field bacteria cultures. For example, many wells in the west Texas, Midland and San Angelo area have very unique water chemistries with respect to their organic acid contents. Conventional formulations of MPB and API containing lactate and or acetate as the primary carbon source for the sulfate reducing bacteria have been known to be inadequate for culturing field samples in that area. Water from the well systems show the presence of a variety of organic acids that are not normally found in gas well water samples. Without these organic acids present in the growth media, the sulfate reducing bacteria from these wells will not culture with a high degree of success. This case illustrates one example where an alternative bacterial detection method would be highly beneficial. When the field personnel consistently see no SRB growth over a large number of wells with the conventional media and are at the same time seeing evidence of MIC and souring, a field device, such as the new ATP-based method described in this paper, could provide a quick field analysis of the total bacterial population. If large bacterial populations were detected by ATP, much higher than what was shown with the growth media alone, then operators would know to investigate further into the situation.

Biocides themselves are a significant stressor to a microbial population. In SRB Checkbottles, the residual biocide in the sample may still be above the microbially inhibitory concentration (MIC) required to control growth. This would have resulted in a false negative test. Biocide interference is not as pronounced in serial dilution culture methods because the biocides are effectively removed through dilution. The SRB Checkbottle results may have been more consistent with serial dilution bottles if the biocides were in-activated appropriately in the sample before being inoculated into the medium. In the ATP test, the biocide interferences are removed through filtration so are not of concern.
Microbes exposed to these stressors are not only killed, but enter into various states of metabolism. The term “Viable but Nonculturable” state (VBNC) has been coined to describe microbes that are still viable, but will not reproduce in media cultures. Cells that enter into a VBNC state are metabolically very different from active, reproducing cells. The ability of certain biocides to act on VBNC cells may be altered based on the route of biocide toxicity. Biocides such as Bronopol, which must be integrated into transmembrane proteins, must first be metabolized by the microbe, which may affect a vegetative microbe differently than a microbe in a VBNC state. Biocides with shorter half lives, like Bronopol, may not be persistent enough to be biocidal or biostatic if microbes in a VBNC state return to normal metabolism after the biocide degrades below its MIC. This would be particularly important in serial dilution tests that require long periods of incubation such as for SRB and iron reducing bacteria (IRB). These tests require incubation periods of 28 days and more while the half life of Bronopol can be 8 days depending on pH, temperature and photoperiods.

Further, both the serial dilution and SRB Checkbottle methods only provide an estimate of population based on logarithmic values. Changes in these values can result from changes in microbial population. They can also be as a result of the small sample volume used for inoculation. Microbes are particles that exist in aggregate. They tend to form flocs and clumps. A small sample may contain more flocs and clumps than another small sample taken from the same water. Thus it is normal to see results from serial dilution testing of the same water that differs by $10^1$ or $10^2$ /mL. At times, this phenomenon will also cause a positive result in one dilution vial, while the next most and next least dilution vials show a negative result. In solid media, aggregates of microbes will form a colony instead of a single cell. This is why it is normal to run a triplicate series (or more) with these types of media to get a consensus result. With this amount of background variation, it would be difficult to discern biocide efficacy of <90-99% inhibition. And biocide efficacy results of >99.9% may not be indicative of a good kill, rather that in combination with many factors involved with the media and incubation procedures. These effects would be amplified in traditional field testing environments encountered in day to day oilfield operations. In these situations, water chemistry, temperature, sampling techniques, incubation periods, light are all influenced by Mother Nature and technician skill.

Metabolic marker testing, such as the ATP protocol used here, are promising to address some of the major issues inherent with traditional culturing methods. This new ATP technique is proving to be an excellent biomarker of microbial life in waters containing interferences such as brine, biocides, suspended solids and hydrocarbons. Our results are suggesting that, although not specific to respiration, ATP testing reveals detail in population dynamics not possible with media based culturing methods. The testing provided a “snap shot” of the microbial population at the time of sampling. Rather than the logarithmic differences in results seen with either the serial dilution or SRB Checkbottles results, resolution of the ATP testing was available down to pg/mL levels.
This may cause some problems with data interpretation. With traditional serial dilution testing, it was possible to see very high levels of kill (>99.99%) from a biocide treatment, as we saw in our testing. ATP testing throughout several different industries shows that complete microbial inhibition is very difficult to achieve. It was demonstrated that ATP reductions on the order of 90-99% (1-2 log) were demonstrative of a maximum kill situation. This represents a significant advancement in ATP testing capabilities, as previous ATP test methods were unable to overcome interferences relating to water chemistry and from extra-cellular ATP (i.e. dead cells), resulting in an inability to detect greater than 50% kill in general. Normal drinking water can have anywhere from 2 pg/mL of ATP to 15 pg/mL. Assuming that one E. Coli sized microbe contains 0.001 pg of ATP, this would be the equivalent of $10^{3.3}$ to $10^{4.7}$ microbes /mL. Previous testing with ATP in produced and surface waters shows that there are at least these many microbes remaining in a sample, even after what is considered a very effective batch treatment. Evaluation of ATP data will need to be different than with other incubation methods, especially if trying to explain the results of a biocide treatment to a customer.

ATP concentration is dependent on the metabolic state of a microbial population. The more active, and growing, a microbial population is, the more ATP is generated. Impacts of VBNC state microbes are important considerations of this test. If the testing is done on the sample immediately after capturing it from the source, impacts of VBNC state microbes are minimized. However, results may not be as representative if samples are collected and shipped to a distant lab for testing. Again, stressors such as temperature, nutrient availability and water chemistry will put artificial selection pressures on the population and affect the end results of the test.

Having demonstrated and discussed the differences between traditional culture-based analytical tools and new metabolic tools such as ATP measurement, Table 8 presents a comparison between the different tools:

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Serial Dilution and SRB Checkbottle</th>
<th>2\textsuperscript{nd} Generation ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detected in Method</td>
<td>On the order of 1% total microorganisms</td>
<td>Total microorganisms – all species including VBNC</td>
</tr>
<tr>
<td>Time to Obtain Results</td>
<td>14 days for APB, 28 days for SRBs</td>
<td>&lt;10 minutes</td>
</tr>
<tr>
<td>What do Results Show</td>
<td>Differentiate SRB/APB/Others</td>
<td>Total biological activity (no differentiation)</td>
</tr>
</tbody>
</table>

### CONCLUSIONS

All the tests evaluated for this paper appear to reveal different but related information about microbial activity in an oilfield environment. Different factors impact the
interpretation required for each test. These factors need to be carefully considered when evaluating microbial activity and selecting methods of control. Data from each of the different test methods are important in providing a more complete picture of what a microbial population looks like in a water sample. This highlights the importance of having multiple of these tools available for use as diagnostic measurements to ensure that the right conclusions are arrived at in field applications. Furthermore, there are a great many situation where rapid feedback is required when auditing or monitoring oilfield processes. ATP monitoring can be used as a tool to gain rapid understanding of a particular situation, for example differentiating between abiotic and biotic process impacts.

ATP testing promises less specificity and higher resolution of population dynamics in a water sample. Future testing could include comparison of microscope results with ATP/SRB Checkbottle results to explore relationship of biocides dosage, SRB spores and vegetative cells. ATP results could also be compared with tests and assays for other biomarkers, for example APS reductase. In specific field testing, it may be useful to compare ATP and SRB Checkbottle results against serial dilution results in a slickwater fracture application. Formation souring is a major consideration for use of biocides in fracture fluids and so this would be an economically important application to evaluate. Fluids mixed on the fly can be compared to fluids flowed back from the formation for biocide residual and bioactivity. ATP testing can also be useful in situations where there is not enough time to allow cultures to fully develop before a water source is used in a fracturing operation. ATP testing, in conjunction with other detection methods such as the SRB Checkbottles can be used to get the best possible bacterial analysis in order to make a cost effective biocide treatment plan.

---