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Flow cytometry as a tool for oilfield biocide efficacy testing and monitoring

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ABSTRACT

Currently, oilfield biocide performance testing relies on classical microbiological culture techniques and culture-independent based methods such as ATP measurement. Although each method has its merits, there are significant drawbacks for each. For example, results from culturing require a two to four week incubation, which delays optimization of the biocide application. It also assumes that the microbes are able to grow in oilfield culture media. ATP photometry has reduced the time necessary for system optimization to one day, but there can be challenges with quantification of stressed and dying – yet intact – microbes. Flow cytometry is a technique that allows a real-time, absolute measurement of individual cells and gives insight into microbial kill mechanisms by distinguishing physiologic states other than alive or dead. Based on these advantages we show that flow cytometry may be a better alternative for biocide selection compared to culture- and ATP-based methods. Here we show that kill studies done with flow cytometry can give more meaningful and significant results compared to culture-based methods and commercially available ATP kits on pure-culture preparations and oilfield water samples.

Introduction

Quantification of microbial cells in industrial settings is a critical step to avoid problems that can potentially affect production. In oilfield systems, diligent microbial monitoring should be exercised routinely to identify microbial contaminations. A number of methods exist to this end, most of which fail to identify the complete population present or fail to provide any insight into the metabolic state of the cells. The selection and application of biocides in oilfield production systems is necessary to reduce the risk of microbial corrosion, bio-fouling and the biogenic production of toxic hydrogen sulfide. Despite recent advances in molecular methods, oilfield biocide efficacy testing still relies heavily on classical methodologies, such as serial dilution, that have disadvantages, such as the inability to culture all genera of Bacteria and Archaea and the amount of time needed to draw conclusions from results. Several commercially available ATP quantification methods, known as second-generation ATP kits, have improved microbial monitoring in the field, but for biocide evaluations they have a limited ability to infer viability changes in a heterogeneous microbial population, mostly because they do not account for dormant (e.g. viable, but not culturable) organisms. Specifically, previous studies have shown that measurement of ATP concentration alone cannot determine if half of the cells died or if all the cells reduced their ATP by half (Kell et al., 1991; Davey and Kell, 1996). Recently, new improvements on the conventional second-generation ATP test and the development a third-generation method have been achieved with the addition of AMP measurements to detect dormant organisms (Keasler et al., 2013). While the third-generation ATP method has significantly advanced routine monitoring and aided the optimization of biocide application in the field, it cannot provide details about biocide mechanisms or determine how a biocide will affect various segments of a complex microbial population. Thus, a laboratory method, such as flow cytometry, that can make viability determinations on a microscopic scale, should be able to report the status of a population by providing a means to more accurately determine viability on a cell-by-cell basis (Kell, 1988; Kell et al., 1991; Davey and Kell, 1996).

Flow cytometry has historically been used for clinical diagnostics, the evaluation of antimicrobials, the study of microparticles found in the bloodstream, the study of the cell cycle, and to measure protein expression for the biomedical industry, but this work is the first to demonstrate its use for petroleum microbiology (Novo and Shapiro, 2000; Carey et al., 2007; Orozco and Lewis,

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Table 1
Physiological definitions pertaining to flow cytometry studies.

Viable	Cell is anionically charged with an intact membrane
Membrane damage	Mechanical damage to cytoplasmic membrane which allows cell-impermeant molecules to enter and exit
Depolarized	Loss of proton motive force and trans-membrane-electrical potential by arrest of oxidative phosphorylation

2010; Rosner et al., 2013). The ability of flow cytometry to detect and count individual cells together with its capacity to indicate specific physiologic states beyond alive or dead make this technology particularly useful for studies with complex microbial populations, such as those encountered in the oilfield. In this paper, we discuss the advantages of using flow cytometry during the early stages of the biocide qualification process and show that this methodology provides more accurate data compared to any of the current field methods available. However, despite its many advantages, currently flow cytometry is not available outside the laboratory and should be combined with other detection methods such as third-generation ATP quantification for routine field monitoring.

Flow cytometry background

The most beneficial aspect of flow cytometry is the ability to analyze hundreds of thousands of cells one-by-one in a few seconds. The enhancement in microbial detection using flow cytometry over traditional methods has been well documented (Davey and Kell, 1996; Mortimer et al., 1998; Nebe-von Caron et al., 1998; Joux and Lebaron, 2000; Novo and Shapiro, 2000). Previous work by Caron et al. described protocols that take advantage of microbial cell membrane polarization to determine viability (Nebe-von Caron et al., 1998). Those studies demonstrated how classical culturing methods compare to flow cytometry methods using the model organism *Salmonella*. Other studies by Shapiro et al. have shown how similar flow cytometry methods coupled with different staining protocols, can be used to measure bacterial viability in *Staphylococcus aureus* and *Micrococcus luteus* (Novo and Shapiro, 2000).

In this work, two key indicators of microbial viability, membrane damage and cellular polarization, were utilized. To determine membrane damage, the most common class of compounds used today includes impermeant stains that intercalate into the DNA. Cell impermeant compounds, such as propidium iodide are excluded from the interior of a microbial cell by an intact membrane. Specifically, any compounds that possess at least two positive charges and most negatively charged organic compounds cannot cross the cell membrane. Although exceptions are noted, the uptake of these membrane impermeant molecules by microbes is an indication of membrane damage and is extrapolated to indicate if a cell is either viable or dead (Mortimer et al., 1998; Novo and Shapiro, 2000). Additionally, it is possible to determine microbial viability from measurement of the (*trans*)-membrane potential (Deer et al., 1995; Nebe-von Caron et al., 1998). Typically, the electrical potential across the membrane of a healthy microbial cell is between 100 and 200 mV. This energy potential is directly responsible for activities such as ATP synthesis, uptake of nutrients, locomotion, and protein localization (Strahl and Hamoen, 2010). A reduction of the membrane potential (MP) is referred to as cellular depolarization. Measurement of the membrane potential can be used to further differentiate biocide treated microbes between four physiologic states: viable, stressed, injured, and dead (Nebe-von Caron et al., 1998; Joux and Lebaron, 2000). To measure the membrane potential we used a Bis-oxanol stain. Bis-oxanol possesses a negative charge and is mostly excluded from cells with negatively-charged MP. Conversely, Bis-oxanol is readily taken up by cells that have a slightly negative or neutral charge.

A description of the possible physiological states seen with these protocols is shown in Table 1. The combined use of cell stains with flow cytometry provides a new way to detect, count, and analyze microbial cells in oilfield fluids and expands the tool box of methodologies available for microbial monitoring and qualification of antimicrobial chemistries.

Materials and methods

Flow cytometry

To quantify microbial cells with flow cytometry, 500 μL of each sample was added to 1500 μL of double 0.22 μm filtered phosphate-buffered-saline (*df*PBS) in a 2 mL microfuge tube, vortexed for 3 s, then pelleted for 4 min at 10,000 × *g* in an Eppendorf 5415D Centrifuge. The supernatant was discarded and the pellet was resuspended in 400 μL *df*PBS, vortexed for 5 s, and then added to a 5 mL flow tube with a standardized concentration (300/μL) of 6 μm

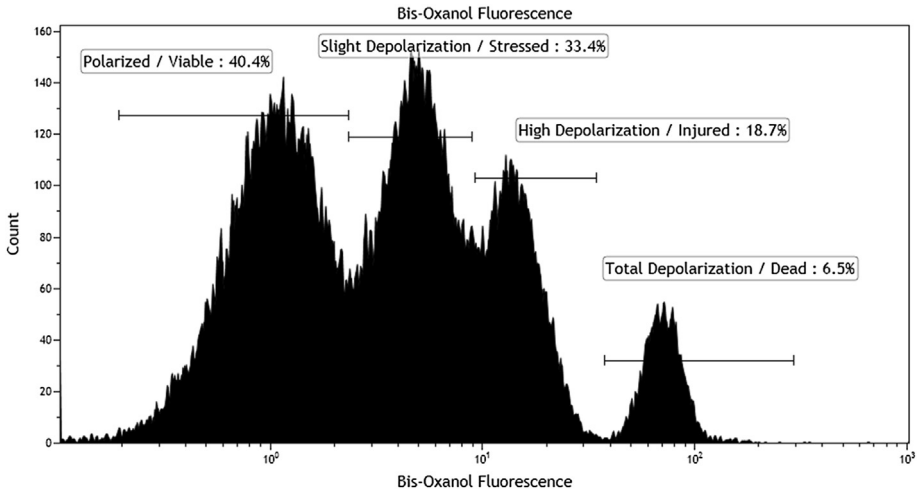

Fig. 1. Polarization. Multiple and reversible polarization states in microbes: *E. coli* were treated with 20% methanol for 4 h at 37 °C under aerobic conditions and stained with bis-oxanol. A total of 50,000 cells are shown in four distinct populations of cells. These are gated in increasingly depolarized states as shown by the brackets.