Control of Problematic *Halanaerobiales* that Limit the Reuse of Hydraulic Fracturing Fluids

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Oil and gas in Oklahoma (OK) are regularly extracted from unconventional plays using a combination of horizontal drilling and hydraulic fracturing techniques. Such practices use remarkable volumes of water (up to 1 x 10^6 barrels/d) (1) to fracture formations and increase permeability. Roughly 10-60% of the injected water returns as produced water (PW) with total dissolved solids content up to 225,000 mg/liter. Wells generate PW throughout their operational lifetimes. In 2014, nearly 1.5 billion barrels of PW were disposed into Class II disposal wells in OK[1]. In 2016, concerns over the correlation of this practice with increased seismic activity, prompted the OK Corporation Commission to reduce the volume of PW disposal. Around the same time, OK's Governor launched a study (1) to explore options for alternatives to injecting PW in disposal wells. The key findings of this group include, i) PW re-use is the near-term and most cost-effective alternative to deep well disposal, ii) evaporation of PW is a more expensive medium-term option and iii) PW treatment and desalination scenarios, while technically feasible, are a long-term option since they are currently too expensive.

Regrettably, microbial activity in general, and sulfide producing microbes in particular, have the potential to undermine the success of all three reuse scenarios via the production of acids and sulfides, leading to human health risks, corrosion, gas souring, as well as clogging or fouling events (2). Biocides are routinely used to control such activities, but the effectiveness of this approach is limited (3). Yet a suitable method to control undesired microbes in hypersaline PW should be possible since upwards of 99% of the microbial community in PW is dominated by a single bacterial Order, the *Halanaerobiales* (2, 4-7). Specifically, bacteria affiliated with the genus *Halanaerobium* dominate PW in the Barnett formation (6). There are other bacteria that can also be problematic, but the *Haloanaerobiales* tend to do best in the high salt conditions typical of fractured shales. The dominance of the *Halanaerobiales* is the consequence of its metabolic versatility. These organisms can tolerate broad salt ranges and ferment carbohydrates ranging from simple sugars to complex polymers like guar gum (6). They also can oxidize the same substrates with thiosulfate as an electron acceptor resulting in production of undesirable sulfides. As carbohydrate and thiosulfate are utilized by the resident microflora, acetic acid and sulfide are the resulting corrosive metabolic end products, respectively. The activity of these organisms makes recycling of PW more problematic and thus, suitable methods to control these types of bacteria are of paramount importance. This project was designed to explore the environmental tolerance limits of a *Halanaerobium* isolate obtained from Barnett formation PW (6). **The goal of this study was to find environmental conditions that will restrict the growth and activity of this model organism. We also looked into the feasibility of bacteriophage**

lysis of *Halanaerobiales* as suggested by the recent study of Daly (4). In addition, we examined the genome of the Barnett shale isolate *Halanerobium,* Lake-01 for possible targets to use inhibitors to interrupt critical metabolic pathways.

Our search for the ecological boundaries that restrict the proliferation of *Halanaerobiales* spp. focused on salinity as it is relatively easy, inexpensive and technologically feasible approach to large-scale control of the resident microflora. The lower limit of salinity for the proliferation of *Halanaerobium* Lake-01 is 2%. However, the upper salinity limit of this organism was unknown prior to the implementation of this project. Our previous study with the isolate revealed that the optimal salinity for bacterial growth was 10% NaCl. This level of salinity was chosen as a positive control for the current set of experiments. The culture was grown in a basal mineral medium with 0.2% guar gum as an electron donor and 20 mM sodium thiosulfate as an electron acceptor (6). The salinity level was then adjusted upwards from 14% - 30% (w/v).

Protein was measured colorimetrically with the Coomassie Plus assay as previously described (8). Sulfide was also measured colorimetrically as described in Trüper and Schlegel (9).

As shown in **Figure 1, t**he growth of the isolate was slightly inhibited at 20% NaCl, significantly inhibited at 25% and effectively stopped at 30% NaCl.

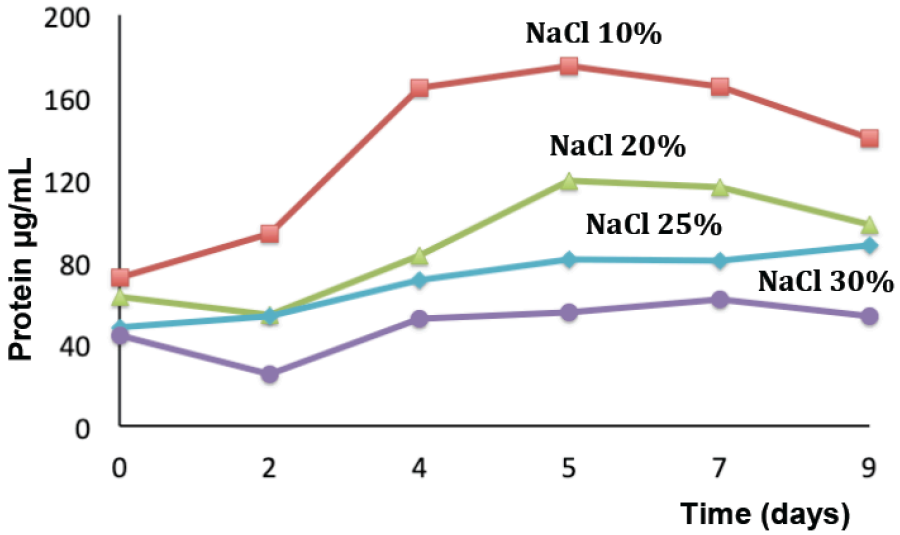


Figure 1. Growth Of *Halanaerobium*, str. Lake-01 on Guar Gum and Thiosulfate with Increasing Salinity

Salinities below 20% did not substantially influence the growth of the organism relative to the positive control.

The same trend was observed when sulfide formation was considered instead of microbial growth. That is, significant inhibition of sulfide formation was achieved at 25% NaCl and almost completely stopped at 30% NaCl (**Figure 2)**. Thus, it seems clear that manipulating the salinity levels of PW has the potential to both inhibit microbial growth and limit the metabolic formation of sulfide.

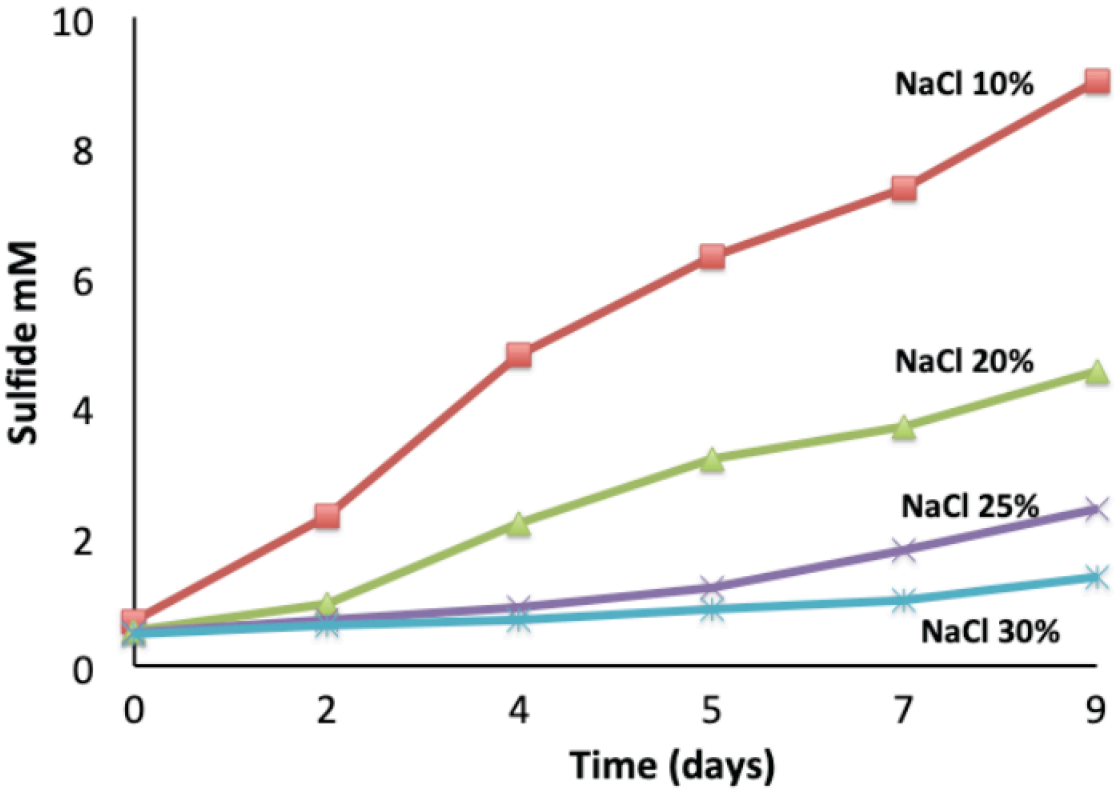


Figure 2. **Sulfide Formation By *Halanerobium,* Strain Lake-01 Grown On Guar Gum And Thiosulfate With Increasing Salinity**

Previous geochemical studies of flowback and produced waters from shale formations reveal that these resources are also enriched in cations other than Na^{+}. That is, the high TDS value of PWs are also a function of Ca^{2+}, Mg^{2+}, Sr^{2+} salts (10). We wondered if the salt combinations in actual PWs might elicit a synergistic impact on *Halanaerobiales* activity at a lower TDS concentration relative to NaCl. Therefore, we investigated the influence of Mg^{2+}/Na^{+} combinations on *Halanaerobium*, Lake-01 activity by examining the rates of sulfide production at optimal NaCl concentration as a function of varying amounts of Mg^{+2}.

As depicted at **Figure 3,** sulfide production by the model organism was only significantly (but not completely) inhibited at 1M Mg^{2+} (24 g/L). This concentration was much higher than the typical magnesium content in the microbiologial medium or in flowback water taken as an example: maximum content detected in the Marcellus shale was 2,550 mg/L or 106 mM (10). The chloride in the cultures that received both NaCl and $MgCl_2$ was calculated as 16.8%. As indicated above (Fig. 1), the equivalent amount of pure NaCl would not have inhibited either growth or sulfide production by *Halanaerobium*, Lake - 01. However, in combination with the magnesium salt, the rate of sulfide formation decreased about 3 fold. This finding suggests that for practical applications, the use of PW evaporates containing multiple cations would have

an equivalent impact on the growth and activity of microorganisms at lesser concentrations relative to pure NaCl.

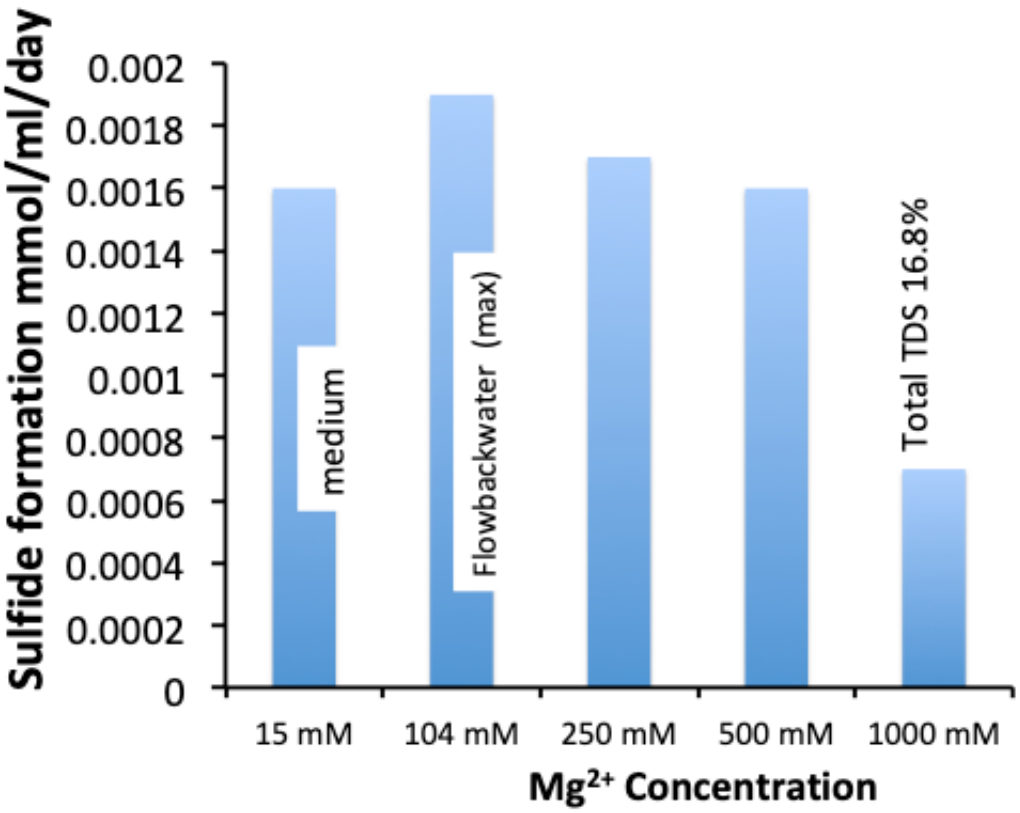


Figure 3. Rate Of Sulfide Formation By *Halanaerobium*, strain Lake-01 At Optimum NaCl (10%) With Various Amounts Of Mg^{2+}

Another potential approach to the control of *Halanaerobiales* populations was the induction of lytic prophages. Superficially, such an approach would seemingly be justified by low diversity of bacterial communities inhabiting shale formations in the north eastern and central plains of the United States. In many of these formations, ≥ 80% of the total microbial population were affiliated with the *Halanerobiales*. About the time this project was initiated, a study (11) appeared and reported detecting prophages in 16 *Halanaerobium* isolates and 21 metagenome-assembled genomes that originated from a number of shale formations. Theoretically, the induction of the prophages would cause the bacterial cells to lyse and thereby limit the pernicious activity of the host organisms. However, viruses tend to be species and even strain specific. Thus, even if a virus impacted one segment of the Halanaebiales community, the other organisms would remain intact and proliferate. On this basis alone, a decision was made to minimize our effort to investigate the role of viruses for the control of problematic *Halanaerobiales*. Rather we considered it more appropriate to evaluate the recently reported findings (11) in terms of their potential applicability to control the growth and sulfide formation in our model organism. To that end,

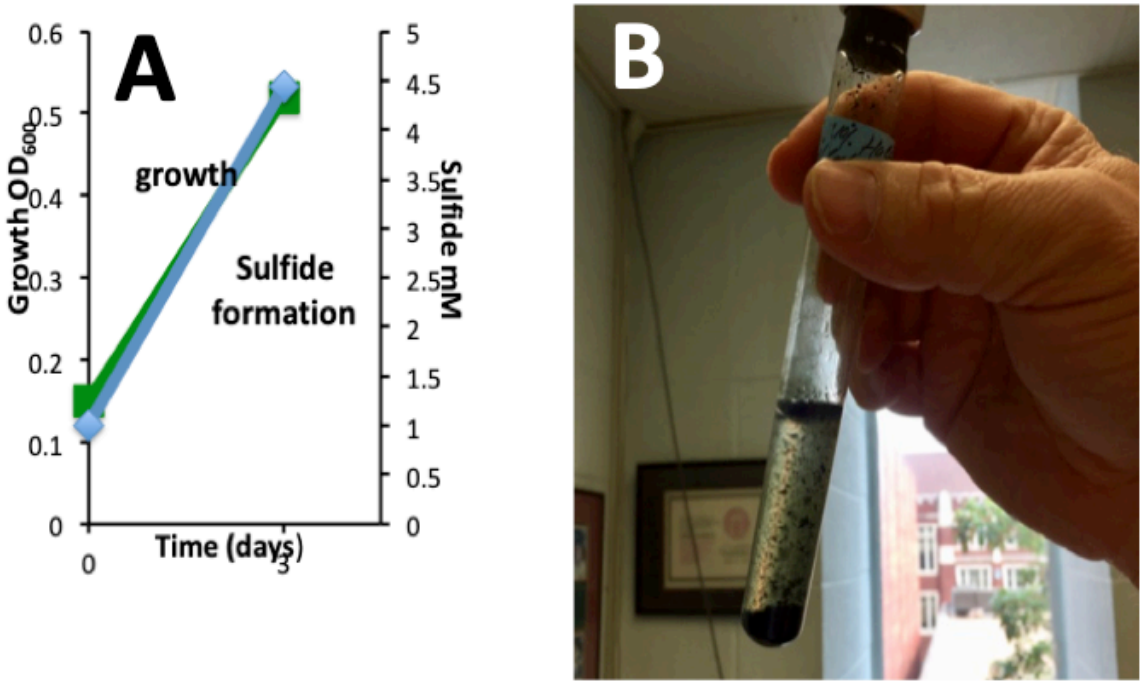
we considered three approaches for the induction of lytic viral cycles. These included an evaluation of mitomycin C (0.5 µg/ml), succinic acid (100 mM) and copper (II) chloride (10mM).

Detecting prophages with mitomycin C-mediated induction is a common practice in microbiology. As indicated above, the *Halanaerobiales* strains recovered from shale formations (11) are known to harbor one or more prophage genomes within their chromosome. However, the true extent of the inducibility and functionality of such prophages cannot be readily deduced by sequence analysis. The authors used mitomycin C (0.5 µg/ml), but were unable to demonstrate prophage induction. Thus, the effectiveness of this drug would appear to be limited at best. Moreover, mitomycin C is a well-known chemotherapeutic agent and used for many purposes other than phage induction. The literature suggests that higher concentrations of micromycin C (1-2 mg/ml) are poorly soluble in normal saline solutions and would almost certainly exhibit similar problems at the typical salinities of PWs. It is also a thermolabile compound that decomposes at temperatures ≥ 50^{o}C. Typically, refrigeration is required for the transportation and storage of this drug. Lastly is prohibitively expensive even at the low concentration of (0.5 µg/ml). That is, 40 mg of mitomycin C ranged in price from $350-800 over the last three years and it would cost over $60 to treat a liter. Thus, even if is was effective at prophage induction in *Halanaerobiales*, the use of this drug is simply not practical for the treatment of large volumes of highly saline PW.

A similar argument can be made for succinic acid. Phage induction was observed at 100 mM concentration of succinic acid (11). Such a high concentration of succinic acid also seems impractical as it is an easily biodegradable substrate and could potentially promote the growth of undesired microorganisms. Like mitomycin C, it is also expensive even though commodity prices of succinic acid are relatively cheap. More specifically, 10 kg ($366) of succinic acid is enough to provide the desired concentration for only 600 liters of PW.

Our consideration of copper (II) chloride (10 mM) revealed that it was far more affordable as well as effective. Thus, we experimentally evaluated the ability of this compound to induce prophage and to lyse our model organism. The *Halanaerobium*, Lake-01 culture was grown for three days and reached an optical density of 0.5 units and formed 4.5 mM of sulfide. (**Figure 4A**). At this point, the culture was amended with10mM $CuCl_2$. However, an immediate black precipitate was observed, presumably due to a chemical reaction between the copper and the pre-formed sulfide (**Figure 4B**). The reaction effectively rules out the use copper (II) chloride treatment in the field as the black precipitate would easily clog the formation and reduce injectivity. Presumably the authors used fermentatively growing cells in their experiments (11) to test various prophage inducing agents and not when the cells were grown as thiosulfate-reducing bacteria. Based on this evaluation, we suggest that induction of viral lysis for the control of problematic *Halanaerobiales* is impractical for now and cannot be recommended.

Figure 4. *Halanaerobium* Lake-01 growth and sulfide formation before Cu(II)chloride amendment (A) and the precipitate formed after Cu^{2+} addition (B).



Other potential targets for the control of sulfide formed during thiosulfate-reduction could possibly be evaluated through the interrogation of the *Halanaerobiales* genomes. Such an approach could reveal critical metabolic metabolic pathways and allow investigators to differentially target functional enzymes associated with essential reactions. With specific regard to the metabolic options for thiosulfate reduction described in bacteria, such an approach is somewhat promising.

First, a thiosulfate sulfurtransferase or rhodanese pathway exists in most *Halanaerobiales* where rhodanese or thiosulfate: cyanide sulfurtransferase (EC 2.8.1.1) catalyzes transfer of a sulfur moiety to cyanide yielding sulfite and thiocyanate (12). Sulfite, in turn, is reduced by dissimilatory sulfite reductase or anaerobic sulfite reductase. Thiosulfate reduction involving rhodanese was detected in some *Halanaerobium* spp. and other thiosulfate-reducing bacteria (13, 14). Secondly, thiosulfate reduction can be catalyzed by a thiosulfate: thiol sulfatransferase (EC 2.8.1.3). This enzyme transfers the sulfane sulfur to a thiol, such as glutathione and results in the formation of sulfite and sulfide (15). Other types of thiosulfate reductases are also known including a heme-iron protein cytochromes C_3 (EC 1.8.2.5) that catalyzes the stoichiometric production of sulfide and sulfite from thiosulfate (16). Finally, a third option for the conversion of thiosulfate to sulfide in a living organisms involves a thiosulfate disproportionation reaction, when one sulfur atom is oxidized and the other one reduced (17,18)

$$S_2O_3^{2-} + H_2O \longrightarrow SO_4^{2-} + HS^- + H^+$$

A genome interrogation of strain Lake-01 performed by in our laboratory revealed that it is most likely this organism reduces thiosulfate via the rhodanese pathway. Biochemical studies of crystallized rhodanese from other organisms detected sufhydryl groups at the catalytic center (four cysteines) of this enzyme. The oxidation of sulfhydryl groups (SH) to sulfenyl groups (-S-OH) effectively inactivated the enzyme (12). Such a finding suggests that common oxidizing chemicals, such as peroxide (H_2O_2), perchlorate (ClO_4^-) or nitrite ($NO2^-$) could potentially react with the sulfhydryl groups of this critical enzyme and limit sulfide formation in this organism. The efficacy, working concentration and cost of such oxidants would have to be experimentally evaluated. While this is a realistic prospect, it was beyond the limited scope of this project and additional funding would be necessary to pursue these objectives.

Important conclusions

- *Halanaerobiales* are the most abundant and the most salt tolerant organisms in shale formations. Controlling growth and sulfide formation by adding NaCl to reach salinity concentrations of ≥ 25% NaCl can control the proliferation of such organisms as well as their ability to reduce thiosulfate to sulfide. We suspect that this approach is also one that is likely to be economically feasible.
- Sulfide production was also suppressed by raising the Mg^{2+} concentration ≥ 24 g/L.
- The above finding suggests that PW evaporate may be useful to reduce the total TDS value of the treated PW and still maintain effectiveness. In effect the recovery of salts from volume-diminishing operations could be more efficient than use of NaCl alone.
- The induction of prophages used to lyse *Halanaerbiales* biomass seems impractical or overly expensive as of this writing and cannot be recommended.
- Other approaches for the control sulfide formation from thiosulfate reduction can possibly be evaluated in the future. An examination of the genome and an understanding of the requisite physiology of these organisms has been revealing in that it suggests various hypothesis than can be experimentally evaluated.

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