

Impact of Preservation Method, Storage Temperature, and Processing Time on Microbiological Community Shift in Solid Samples Collected from Crude Oil Transmission Pipelines

Mohita Sharma
University of Calgary
2500 University Drive NW
Calgary, AB, T2N 1N4
Canada

Nicole Taylor
University of Calgary
2500 University Drive NW
Calgary, AB, T2N 1N4
Canada

Lisa Gieg
University of Calgary
2500 University Drive NW
Calgary, AB, T2N 1N4
Canada

Trevor Place
Enbridge Inc.
10108 8th Street NW
Edmonton, AB, T6P 1V8
Canada

Yin Shen
University of Calgary
2500 University Drive NW
Calgary, AB, T2N 1N4
Canada

Danika Nicoletti
University of Calgary
2500 University Drive NW
Calgary, AB, T2N 1N4
Canada

Jennifer Sargent
Suez Water Technologies & Solutions
15125 137 Avenue NW
Edmonton, AB, T5V 1R9
Canada

ABSTRACT

Microbiological monitoring of samples collected from oilfield operations is crucial for understanding microbial impacts on metal infrastructure. As many oilfields and related infrastructure are in remote locations, the time between sample collection, shipping to laboratory and sample processing for microbiological analysis can vary from days to weeks. Hence, robust sample handling and preservation methodology to arrest microbiological activity at the time of sample collection is needed.

To identify the best preservation methodologies that can be adopted for solids, oily sludge samples were collected twice from the nose of the same pig trap during a routine pipeline pigging operation of a crude oil transmission pipeline over a span of two years. Samples were chemically preserved at the time of sample collection (with different alcohols or commercially available nucleic acid

preservation reagents) or remained unpreserved, and all samples were stored either at 4°C or at room temperature following collection. Samples were assessed using 16S rRNA gene sequencing to determine microbial community composition at day 0 (time of sample collection) and at different intervals for up to 50 days of storage, and comparisons in microbial community composition were made to the day 0 results.

A substantial shift in microbial community composition was observed in samples that were not chemically preserved or not kept cold at 4°C in as little as 7 days following sludge collection. In addition, greater microbial diversity and community shift was observed in samples kept at room temperature compared to cold storage. Chemical preservation reagents showed varying efficacies at preserving microbial community composition with increased lengths of storage. These results emphasize the need for careful sample preservation and handling of sludge samples for accurate monitoring of microbial communities, an analysis that can play a pivotal role in the early identification of potentially corrosive microorganisms during the routine inspection of pipelines.

Key words: Chemical preservatives, microbial communities, crude oil pipeline pigging sludge, microbiologically influenced corrosion (MIC), next generation sequencing (NGS)

INTRODUCTION

Robust integrity management plans are critical for ensuring the lifespan and preventing failures of man-made infrastructure, including the metal (carbon steel) infrastructure that dominates the oil and gas industry. In this sector and others, many types of corrosion can occur on metal infrastructure, including corrosion that involves the participation of microorganisms, commonly referred to as microbiologically influenced corrosion, or MIC. MIC can be difficult to diagnose as the cause of a given infrastructure failure because it is not a stand-alone mechanism – the physical and chemical properties of a system can influence the types of microorganisms that are present and active, while the metabolisms of these microorganisms can influence the surrounding chemistry and physical properties of a system. Thus, identifying the types of microorganisms associated with different oil and gas operations is becoming increasingly important to determine whether detrimental microorganisms may be present so that integrity management plans can be made accordingly.¹ Notably, culture-independent methods, commonly referred to as molecular microbiological methods (MMM), are becoming increasingly used in the oil and gas sector.²⁻¹¹ MMM are known to capture much more microbial diversity than culture-based methods such as most-probable-number determinations, which are limited to only those organisms that can grow in defined laboratory media (approximately 1% of microorganisms present in a sample).^{2, 12}

Some examples of MMM that can be used to identify and/or quantify microorganisms include 16S rRNA gene sequencing - also known as next generation sequencing (NGS), which is used to identify all the microorganisms (also termed microbial taxa) in a given sample, and qPCR, which can be used to quantify different types of microorganisms or functional genes of interest.^{1-11, 13} These approaches are now included in and/or recommended for use in industry standards involving microbial characterization of oil and gas samples.¹⁴

To accurately determine the types of microorganisms found in a given environment using MMM, it is critical that samples are carefully collected and stored prior to analysis. In the past few years, several studies have shown that microbial community compositions will change over time if improperly stored for any length of time. For example, Kilbane showed using serial dilution tests that the numbers of different types of microorganisms found in oilfield fluids increased by 2 orders of magnitude when stored for only 2 days at warm temperatures (25°C and 35°C).¹⁵ Rachel and Gieg also showed dramatic changes in microbial community composition as determined using NGS when oilfield produced waters remained chemically unpreserved at room temperature for a week compared to the Time 0 community.¹³ They also found some preservatives or protocols to be more effective than others when added to unfiltered produced water samples, wherein alcohols, a lab-made buffer solution, and freezing were more effective at preserving the microbial community for up to 28 days after sampling than using commercially available nucleic acid preservation reagents.¹³ Salgar-Chapporo and Machuca tested the effects of produced water filtration and chemical preservation of the filter with unfiltered samples stored for up to one week.⁶

Using these different storage protocols for produced water samples, their results showed dramatic changes in the microbial community taxa identified using NGS.⁶ This study also examined the effect of different storage conditions on a sludge sample collected from the same facility as the produced water samples. With the sludge sample, the effects of differential storage conditions (cold, warm, chemical preservation) revealed changes in the relative abundance of microbial taxa rather than different microbial communities as was observed with the produced water samples, showing that different sample types may respond differently with prolonged storage.⁶ De Paula et al. carried out a comparative study when produced water samples were handled differently in the field (unfiltered water sample treated with a commercial nucleic acid preservative and kept on ice versus filtration of the water sample, treating with a commercial nucleic acid preservative, and freezing storage) prior to NGS analysis by two different laboratories.⁵ The microbial community results were significantly different between samples preserved and stored differently and processed differently using MMM, unsurprising considering the variables deliberately tested in the study.⁵ The authors stressed the need for standardized protocol for sample handling and processing for accurate microbial determinations.⁵ A recent review of the literature that examined sampling and preservation protocols for many different biological and environmental sample types revealed that few studies exist that are relevant to oilfield systems.¹⁶ Thus, there is a clear need to develop robust sampling and handling procedures for different types of oilfield samples that may be more challenging to work with including oily sludges.

Given the paucity of published sample handling and preservation protocols for sludge samples associated with oilfield infrastructure, a chemical preservation study was conducted with crude oil transmission pipeline pigging solids to determine how long these types of samples could be stored prior to observing substantial changes in microbial community composition. The chemicals selected for the study included alcohols and selected commercially available nucleic acid preservation reagents.^{4-6, 8, 13, 16} The alcohols chosen were those readily available to the public ('off-the-shelf' alcohols), while the commercially available nucleic acid preservation reagents were generally only available from chemical or laboratory suppliers. Results from studies such as these can help to inform standards and best practices for sampling and handling different types of samples for MMM analysis and that can be used to provide microbiological data as part of integrity management plans.

EXPERIMENTAL PROCEDURE

Sludge samples were collected on two occasions (in September 2019 and September 2020) from the nose of a cleaning pig run through the same segment of a crude oil transmitting pipeline.^{9, 10} The pipeline transports primarily heavy crude oil, and undergoes routine corrosion prevention using cleaning pigs and batch applications of various treatment chemicals (corrosion inhibitor, biocide, and biodispersant).^{9, 10} On both occasions, samples were collected immediately following the opening of the pig trap using sterilized implements to conduct the preservation experiments.

For the September 2019 experiment, samples were weighed on-site into sterile conical tubes in 5 g aliquots. Some of the replicates were treated with chemical preservatives, while others were not chemically preserved. Duplicate tests were established for each of the following storage conditions: **(1)** no chemical preservative, stored at 4°C; **(2)** no chemical preservative, stored at room temperature (20-22°C); **(3)** amended with 1 mL of 70% isopropanol (designated Alcohol 1); **(4)** amended with 1 mL of Reagent A (a commercially available guanidine-based nucleic acid preservative, ThermoFisher); or **(5)** amended with 1 mL of Reagent B (a commercially available ammonium sulfate-based nucleic acid preservative, Invitrogen). Samples were transported the same day to the laboratory for further storage and processing.

All samples were stored at 4°C for up to 50 days, except for the duplicates stored at room temperature, to assess the efficacy of the various preservation protocols. Samples were removed and processed for microbial community analysis using 16S rRNA gene sequencing (overviewed below) on the day of sample collection (Time 0), and after 10, 30, and 50 days of storage. In this experiment, only one sample

from each treatment was sequenced following each storage time. Figure 1 overviews the sample treatments for this initial preservation experiment.

Samples were collected in an identical manner for the September 2020 experiment (5 g of sample were aliquoted into sterile conical tubes on-site following the opening of a cleaning pig trap). Like the September 2019 experiment, some aliquots were chemically preserved, while others received no chemical preservative. For this experiment, each condition was tested in triplicate (rather than in duplicate), and all preservatives were stored at both 4°C and at room temperature (rather than at 4°C only) to examine the effects of two temperatures on preservative efficacy. Triplicate tests were established for each of the following storage conditions: **(1)** no chemical preservative; **(2)** amended with 1 mL Alcohol 1; **(3)** amended with 1 mL Alcohol 2 (95% alcohol, Luxco Inc.); **(4)** amended with 1 mL Reagent A; **(5)** amended with 1 mL Reagent B; and **(6)** amended with 1 mL of Reagent C (a commercially available nucleic acid preservative, Biomatrix). Samples were removed and processed for microbial analysis using 16S rRNA gene sequencing (overviewed below) on the day of sample collection (Time 0), and after 7, 14, 28, and 42 days of storage. Figure 1 also overviews the sample treatments for this second preservation experiment.

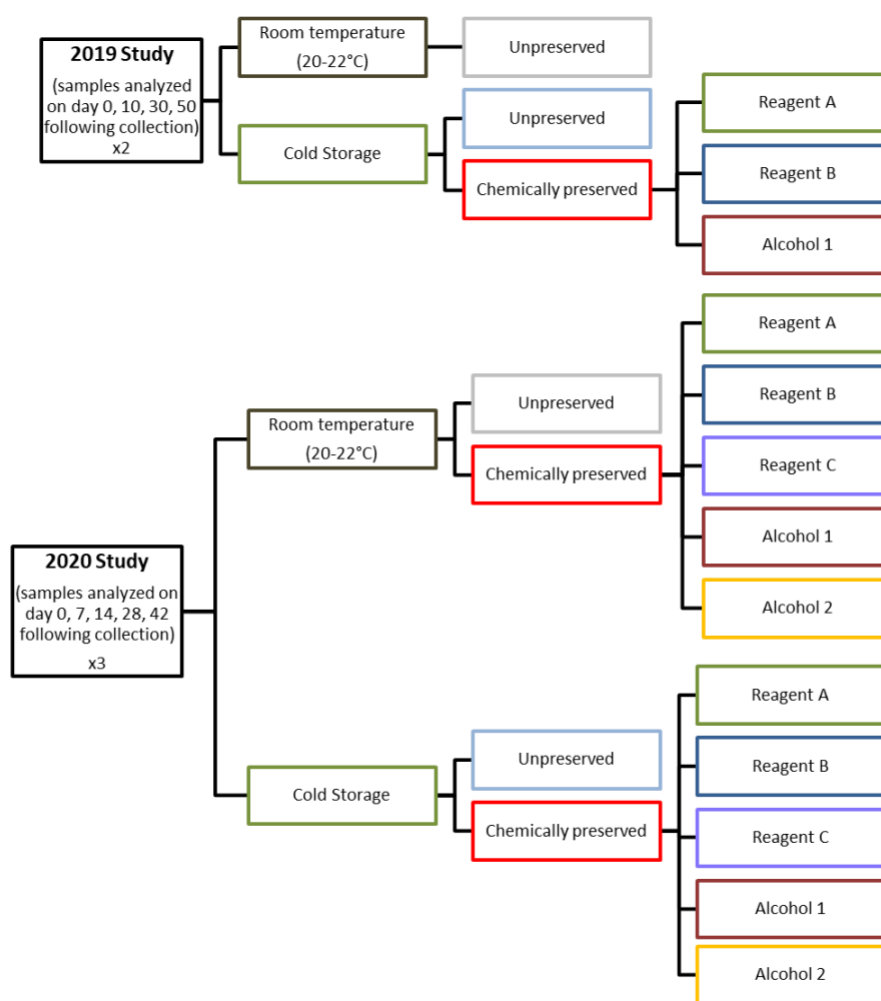


Figure 1: Flowchart summarizing the treatments and incubation times used for each of the preservation experiments using crude oil pipeline pigging sludge as described in this study.

At each time point, 0.5 g sample were aseptically removed from each conical tube, and DNA was extracted using a commercially available DNA extraction kit. The V4-V5 region of the 16S rRNA gene was amplified by PCR and amplicons were sequenced using Illumina sequencing at a University of Calgary sequencing facility. Raw sequences underwent a variety of quality control steps, and sequences were assigned as amplicon sequence variants (ASV) to the lowest taxonomic level. Samples were subject to diversity analysis and non-metric multidimensional scaling (NMDS) analysis, an ordination analysis that measures dissimilarity amongst samples, was conducted in R with the Vegan package (Version 2.5-6). Heat maps showing % relative abundances of ASVs in all samples were prepared using commercially available graphing software (GraphPad Software™). Details for DNA extraction, PCR steps, and gene sequence analysis used in this study are available in a previous open-source publication.¹³

RESULTS

Preservation experiment, Fall 2019

The initial sludge preservation experiment carried out with cleaning pig sludge samples collected in September 2019 showed that different preservation treatments and storage times affected the microbial community composition of the samples as determined using 16S rRNA gene sequencing and diversity analysis. Samples not receiving chemical treatment were stored either at 4°C or at room temperature, while chemically treated samples were stored at 4°C; all samples were stored for up to 50 days. The box plots shown in Figure 2 compare the distributions of total observed taxa (ASVs) sequenced from the samples stored under different conditions in the preservation experiments. For the box plots shown in Figure 2 the vertical size of the box indicates the boundaries of the 25th and 75th percentiles, while the line within each box indicates the median (50th percentile) of the data set. The larger the box, the more distribution within a given data set, meaning that there is more 'spread' or diversity in the data set. Box plots that have outlier points not associated with a box indicate a sample that is very different from the rest of the samples within that data set. With these principles in mind, some initial trends emerged from the experiment conducted in September 2019. Increased diversity (data spread) was observed when sludge samples were stored at room temperature compared to those stored cold, as seen in Figure 2A by the larger size of the box plot prepared from the microbial community sequence data following room temperature-storage samples compared to the size of the box plot prepared using the cold stored samples. This initial observation suggested increased microbial growth when samples were stored at a warmer temperature (Figure 2A). Of the chemical reagents tested in the September 2019 study, the tightest species distribution was found with Reagent A, followed by Alcohol 1 and then Reagent B (Figure 2A, not taking outliers into account). However, as only single samples could be analyzed in this experiment after 10, 30, and 50 days of storage, and the efficacy of chemical preservatives was only tested at 4°C, a second experiment using pig solids from the same pipeline and location was carried out in September 2020 to incorporate additional replication and to include room temperature storage as well for all preservatives.

TM = Trade Name