EFFECT OF SAMPLE STORAGE CONDITIONS ON THE MOLECULAR ASSESSMENT OF MIC

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SUMMARY: Microbiological surveys play a fundamental role in diagnosing and monitoring microbiologically influenced corrosion (MIC) in oil and gas production systems. Currently, microbiological characterization is being carried out by the implementation of molecular microbiological methods (MMMs) such as the 16S rRNA gene diversity profiling. Molecular characterization of microorganisms provides information to assess the risk of MIC in the production facilities. Even though MMMs have been included in NACE standards, standardized protocols for collection, storage and preservation of oilfield samples have not been written. In this study, the effect of sample storage conditions on the microbial composition, community structure, alpha diversity and functional capability of oilfield samples was investigated. The effect of storage samples at room temperature or refrigeration on the molecular MIC assessment was statistically evaluated by comparison with samples pre-processed and preserved on-site straight upon collection. Sample storage resulted in changes in the relative abundance of the microbial populations, which had a significant impact on the alpha diversity and structure of the community. Likewise, the functional capability of the microbial community in oilfield samples was affected by storage conditions. Abundances of genes associated with corrosive pathways such as sulphate reduction, iron utilization and methanogenesis decreased under conditions evaluated. Results of this research provide evidence of the importance of sample handling for an accurate microbial characterization and subsequent assessment of the MIC risk in industrial systems. Thereby, on-site pre-processing of the samples and addition of nucleic acids preservation solutions is recommended for an optimal microbiological survey, and in cases where this is not feasible, refrigeration is preferred over room temperature storage conditions.

Keywords: Microbiologically Influenced Corrosion (MIC), Molecular Microbiological Methods (MMM), 16S rRNA Microbial Diversity Profiling, oilfield samples, storage conditions.

1. INTRODUCTION

Microbiologically Influenced Corrosion (MIC) is defined as the deterioration of metals that results from the presence and activity of microorganisms on their surfaces. The participation of microorganisms in the corrosion process can significantly increase the corrosion rates, representing a big concern for the integrity of industrial infrastructure particularly oil and gas production facilities (1, 2). Annually, this phenomenon generates millions of dollar losses to the world's industry (3, 4). The most recent NACE report estimated the global cost of corrosion as US$2.5 trillion, without including safety or environmental consequences (5). In fact, several studies have estimated that MIC contributes between 20% and 50% of total corrosion failures (6-9).

A way to prevent corrosion failures is the opportune detection of the threats. This process is usually acknowledged as corrosion management and has been described by Skovhus *et al.*, (10) as a cycle of three fundamental steps: 1) identify the corrosion threats, 2) Mitigate the threats and, 3) Monitor of mitigation effectiveness. The first step of this cycle is essential for the appropriate corrosion control. For the MIC risk, this step refers to an early detection of the presence of corrosive microbes in the system. Periodic microbiological surveys are executed in the oil and gas systems following guidelines of the NACE standard TM-0194 (11, 12).

In the last decades, microbiological characterization of oilfield samples has been mostly carried out by the implementation of traditional culture-based techniques (13-16). However, knowing the limitations of these methods such as the inability to recover all the microorganisms present (17, 18), molecular microbiological methods (MMMs) have been developed and implemented for studying, monitoring and management of MIC (19-25). Microbial diversity profile analysis based in next-generation sequencing (NGS) of the 16S rRNA gene is one of the methodologies being used by the industry to help identify and characterize the total microbial community living in oil and gas production systems (26-30). Apart from the taxonomic classification, 16S sequencing data can be used for predicting the functional capabilities of the community as a proxy to more complex metagenomic analysis (31). Identifying the metabolic potential and the dominant genes in the microbial population living in oilfields could suggest what the microorganisms are doing in the system (32). Nevertheless, it is important to have in mind that analysis based on nucleic acids (DNA or RNA) are susceptible to many biases in the complete procedure of data acquisition. De la Cuesta-Zuluaga & Escobar (33) presented the most common challenges in the 16S rRNA workflow which include steps from sampling to the operational taxonomic unit (OTU) table generation and emphasized on the need of standardized protocols to reduce variations. Although the latest updates of the NACE standards (TM0106, TM0194 and TM0212-2018) have included the MMMs as complementary tests for the monitoring and diagnosing of MIC (34), standardized protocols for collection, storage, preservation and processing of oilfield samples have not been written.

Proper preservation and storage of samples is essential in maintaining the microbial composition and structure of field samples and in the accurate assessment of MIC risk. Transportation of samples from the production facilities can take days, or even weeks, to arrive at specialized laboratories for molecular analysis, time period where the DNA in the samples can change if it is not well preserved (20, 35). Several investigations have focused on the effect of storage conditions on the microbiome analysis of environmental and human samples (36-38). However, there is limited research on the influence of those conditions in the assessment of complex microbial communities in the oilfield systems. A previous study based on culture-dependent methods showed that storage conditions affect significantly the concentration of corrosive microbial groups in the samples (39), nonetheless, the effect on culture-independent methods was not evaluated. To address these knowledge gaps, we assessed the effect of sample storage conditions on the molecular characterization of the microbial composition and structure of water and sludge samples collected from an oilfield facility in Australia. This is the first study that used bioinformatics of the 16S rRNA sequencing data to predict the functional capability of an oilfield microbial community.

2. EXPERIMENTAL DETAILS

2.1 Sample Collection

One sludge sample and six produced oil-water samples were collected from an Australian oil production facility. Sludge sample was collected in a sterile 50 mL Falcon tube. Oil-water samples were decanted in sterile separatory funnels for oil phase removal. The water phase was then divided into two equal volumes. One volume was used for immediate filtration and used as a reference sample. Reference samples were immersed in RNAprotect® Bacteria Reagent, which prevents nucleic acids degradation and gene induction. The remaining volume of three of the water samples was stored under refrigerated conditions (4°C) for one week, whereas the remaining volume of the other three water samples was stored at room temperature for 24 hours (transport period) before refrigeration and processing. Samples were treated as follow.

Room temperature

Water samples: 500 mL of samples PW-1, PW-2 and PW-3 were stored at room temperature ≅ 25°C for 24 h and then stored at 4°C for one week. After refrigeration period, sample was filtered through sterile 0.2 µm pore size membranes and membranes filters were stored at -20°C.

Sludge sample: 5 g of sludge were stored at room temperature ≅ 25°C for 24 h and then stored at 4°C for one week.

Refrigeration

Water samples: 500 mL of samples PW-4, PW-5 and PW-6 were stored at 4°C in sterile glass bottles for one week. After the refrigeration period, samples were filtered through sterile 0.2 µm pore size membranes and membranes filters were stored at -20°C.

Sludge sample: 5 g of sludge were placed in a Falcon tube and stored at 4°C for one week.

Immediate pre-processing (reference samples)

Water samples: 500 mL of samples PW-1, PW-2, PW-3, PW-4, PW-5 and PW-6 were filtered through sterile 0.2 µm pore size membranes, then, membrane filters were immersed in RNAprotect® Bacteria Reagent

(QIAGEN) and stored at 4°C for 2 days until arrival to the laboratory. In the laboratory, RNAprotect was washed from the samples and membrane filters were stored at -20°C.

Sludge sample: 5 g of sludge were placed in a Falcon tube with 10 mL of RNAprotect® Bacteria Reagent and stored at 4°C. Sample was processed twice, DNA was extracted after 2 days and after one week of sample storage to determine the effect of time of processing in samples preserved in RNAprotect® solution.

2.2 DNA extraction and quantification.

Water samples

DNA was isolated using the DNeasy PowerWater Kit (QIAGEN) according to the manufacturer's instructions with the following modifications: filters were placed into the PowerWater DNA Bead Tube containing solution PW1 and heated at 65°C for 10 min before the Vortex step. Total DNA was eluted in 100 µL of free DNA water. DNA concentration was quantified fluorometrically with the Qubit dsDNA HS Assay kit (Life Technologies).

Sludge samples

DNA was isolated using the DNeasy PowerSoil Kit (QIAGEN) according to the manufacturer's instructions with the following modifications: 0.25 g of sludge were placed into the PowerBead Tube containing solution C1 and heated at 65°C for 10 min before the Vortex step. Total DNA was eluted in 100 µL of free DNA water. DNA concentration was quantified as mentioned for water samples.

2.3 16S rRNA gene sequencing

The hypervariable region V3–V4 of the 16S rRNA gene was amplified using the universal primers 341F (5' CCTAYGGGRBGCASCAG 3') and 806R (5' GGACTACNNGGGTATCTAAT 3') (40), and adapters for Illumina MiSeq sequencing were included. Amplicons were pooled in equimolar concentrations and quantified with Quant-iT dsDNA HS assay kit (Life Technologies). PCR products indexing was performed with Nextera XT Index kit (Illumina), and sequencing was completed using the dual index paired-end approach. Both, polymerase chain reaction and sequencing were performed by the Australian Genome Research Facility (AGRF).

2.4 Bioinformatics

The Quantitative Insights Into Microbial Ecology Software (QIIME, v1.9.1) (41) was used for the analyses of the 16S rRNA gene sequences generated with the Illumina MiSeq. Paired-ends reads were merged using PEAR (v0.9.10 - 64 bit) (42) with default parameters. Then, sequences with an average quality score lower than 20 or containing unresolved nucleotides were removed from the dataset. Unclipped primer sequences were also removed by employing Cutadapt (v1.10) (43) using default settings. Afterwards, USEARCH (v10.2) (44) was used for OTU clustering with UNOISE algorithm to dereplicate, remove singletons, and sort all quality-filtered sequences by length (400 bp). Subsequently, OTUs were clustered at 100% sequence identity. Chimeric sequences were removed using UCHIME (45) with SILVA as reference database (SILVA v132) (46). Quality-filtered sequences were mapped to chimera-free OTUs and an OTU table was created using VSEARCH (v1.1.3) (47). Taxonomic classification of the reference sequences (zOTUs) was performed by similarity searches using BLAST against the same database. Sample comparisons were performed at the same surveying effort, utilizing 22,000 by random selection. Species richness, alpha and beta diversity estimates, and rarefaction curves were determined using the QIIME algorithms.

2.5 Statistical analyses

Statistical analyses and graphs were conducted employing R (v3.4.3) (48), and PAST (v3) (49) software. Results of statistical tests were considered significant with $p \leq 0.05$. A t-test was used for comparison of alpha diversity between storage conditions. To visualize the multivariate dispersion of the community composition a non-metric multidimensional scaling (NMDS) was performed based in the Weighted UniFrac distance (50), lines for joining samples collected in the same sampling point were projected onto the ordination, utilizing the function ordiellipse in the Vegan package. Permutational analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) were used to test for significant differences in beta diversity, Bray-Curtis distance (51) was used in these tests.

2.6 Prediction of bacterial functional profiles.

The functional profile was predicted by using the R package Tax4Fun (31). Tax4Fun is a software that links the 16S rRNA gene sequences with the functional annotation of sequenced prokaryotic genomes by the identification of the nearest neighbour. Results of Tax4Fun predictions are highly correlated with the functional profiles obtained from whole metagenome sequencing (31).

For this analysis, the genes encoding key enzymes involved in metabolic pathways associated with corrosion were identified in the resulting profiles using their KEGG orthologs. The sum of the abundances of genes selected for each metabolism was used for comparison among storage condition.

3. RESULTS

3.1 Sample codification and storage conditions

Six water samples were collected from distinct locations of an oil production facility. Produced water samples PW-1, PW-2 and PW-3 were stored at room temperature (RT) for 24 hours, whereas PW-4, PW-5 and PW-6 were stored refrigerated (4°C) to determine the impact of the sample storage conditions in the diversity profile of oilfields microbial communities. One portion of sample from each location was processed on-site, immediately after collection, and was preserved in RNAprotect® Bacteria Reagent and used as reference (Ref) in the analysis.

To determine if storage conditions have the same impact on water and solids samples, one sludge sample was collected from a corroded pipe. Similarly to the water samples, a portion of the sample was stored at RT for 24 hours, another portion stored at 4°C for one week, and another placed in RNAprotect solution. Sample in RNAprotect solution was processed twice, after 2 days (Ref) and after one week of being stored at 4°C. This analysis was made to determine if there is an impact of the time of processing in samples preserved in RNAprotect® solution.

3.2 Effect of sample storage on the diversity profile of the microbial community

Water and sludge samples were processed separately. DNA was extracted and V3-V4 region of the 16S rRNA gene was sequenced for determining the microbial composition of the oilfield community. A total of 983,520 (water) and 449,548 (sludge) high-quality sequences were obtained after removal of singletons, chimeras and low-quality sequences. The number of sequences per sample fluctuated from 39,301 to 180,907. Data normalization was executed by subsampling at 22,000 sequences per sample to correct the unequal sequencing depth. After normalizing the number of OTUs ranged from 56 ± 9 to 258 ± 20.

The relative abundance of microbial populations at domain level (Bacteria; Archaea) as a function of storage conditions is shown in Figure 1 for water samples and in Figure 2 for sludge sample. The analysis of the microbial structure at domain level revealed that the relative abundance of the archaea community diminished after storage conditions (both temperatures) with respect to the reference samples (processed on-site). This pattern was seen in all sampling points with higher reduction observed in the water samples stored at room temperature for 24 hours (Figure 1). Sludge sample showed a similar decrease in the archaea community under RT and 4°C storage conditions (Figure 2). The sample preserved for a longer time (1 week) in RNAprotect® Bacteria Reagent did not exhibit significant differences in the community compared to samples preserved in the same solution for shorter time (2 days). This indicates that there is not an impact of storage time on the abundances of bacteria and archaea species in samples preserved in RNAprotect®.

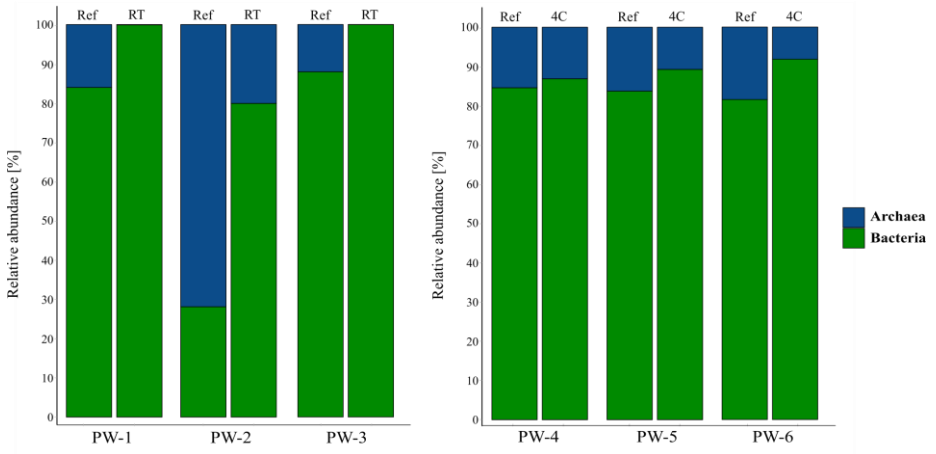

Figure 1 Relative abundance of Bacteria and Archaea in water samples (PW-1 to PW-6) assessed by 16S rRNA sequencing. Sample processed on-site (Ref), sample stored at room temperature 24 hours (RT), sample stored under refrigeration (4C).

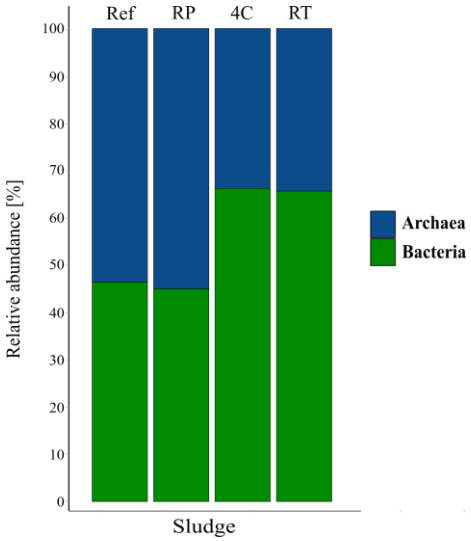

Figure 2 Relative abundance of Bacteria and Archaea in sludge sample assessed by 16S rRNA sequencing. Sample stored in RNAprotect® solution and processed after 2 days (Ref), sample stored in RNAprotect® solution and processed after one week (RP), sample stored sample stored under refrigeration (4C) and sample stored at room temperature 24 hours (RT).

Taxonomic classification of the OTUs was assigned to genus level, species representing <1% of the population were grouped as rare taxa. Bar charts of the diversity profile are presented in Figure 3 for water sample and in Figure 4 for sludge sample. A total of 84% of the species in water samples and 58% of species in sludge sample were classified to the genus level, the remaining 16% and 42%, respectively, belonged to uncultured bacteria living in the oilfield.

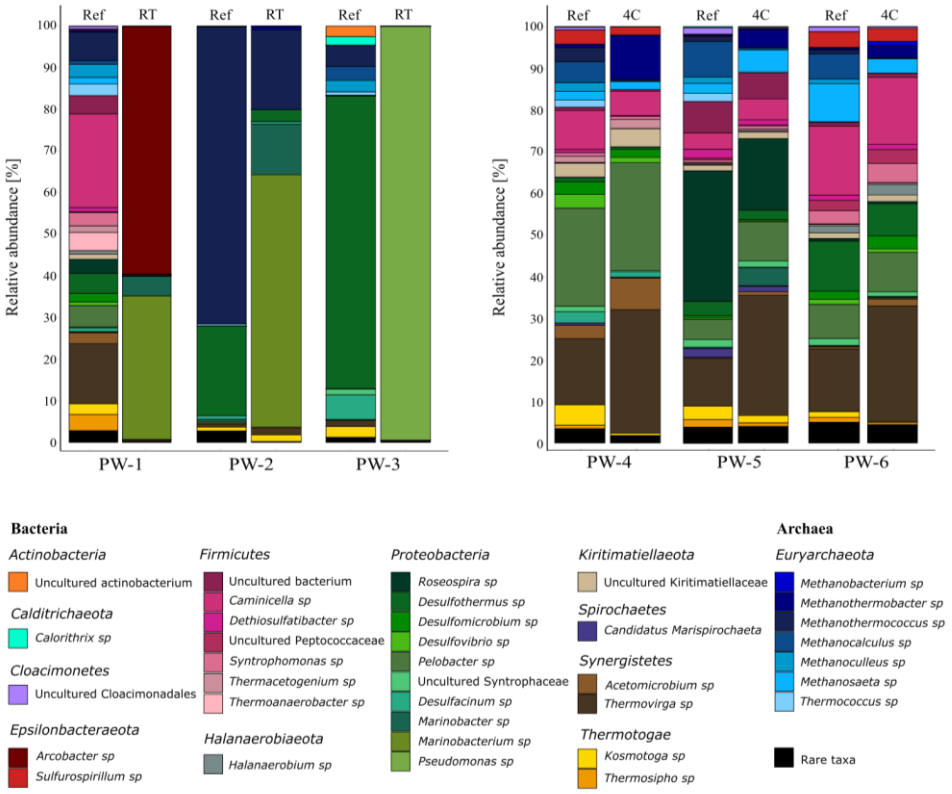

Figure 3 Microbial community composition of water samples at genus level. Sample processed on-site (Ref), sample stored at room temperature 24 hours (RT), sample stored under refrigeration (4C). Bacterial and archaeal genera with relative abundances > 1% are visualized; genus contributing ≤ 1% were summarized as rare taxa.

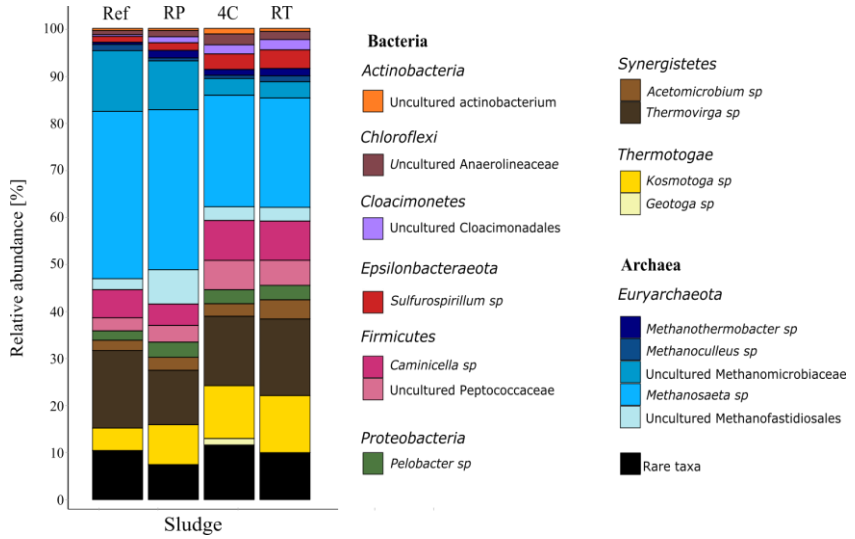

Figure 4 Microbial community composition of sludge sample at genus level. Sample stored in RNAprotect® Bacteria Reagent and processed after 2 days (Ref), sample stored in RNAprotect® Bacteria Reagent and processed after one week (RP), sample stored sample stored under refrigeration (4C) and sample stored at room temperature 24 hours (RT),. Bacterial and archaeal genera with relative abundances > 1% are visualized; genus contributing ≤ 1% were summarized as rare taxa.

Microbial composition analysis in water samples demonstrated marked differences between the samples processed immediately and the samples stored at room temperature for 24 hours. Under this storage condition, the percentage of abundance of the genera *Marinobacter*, *Marinobacterium*, *Arcobacter*, and *Pseudomonas* increased significantly and became the dominant populations in the microbial community. On the other hand, microbial communities of samples stored at 4°C presented variations in the abundances with respect to the microbial composition of references samples, however, those deviations were less significant compared to samples stored at RT. Storage at cooling conditions had a positive effect in genera such as *Methanothermobacter, Pelobacter, Acetomicrobium* and *Thermovirga* which showed an increase in their percentage of abundance in samples but had a negative effect on the genera *Sulfurospirillum, Kosmotoga, Thermosipho, Thermococcus, Methanocalculus, Methanothermococcus* and *Methanoculleus* which exhibited lower abundances compared to the reference sample.

On the other hand, microbial composition analysis in sludge samples showed a different pattern. Both, room temperature and refrigeration conditions had a very similar microbial composition among them but different respect to the reference sample (Figure 4). The main impact was seen in the reduction of relative abundances of methanogenic species such as *Methanosaeta* and the uncultured *Methanomicrobiaceae*. Other genera like *Methanothermobacter, Sulfurospirillum, Kosmotoga* and *Caminicella* showed an increase in their percentage of abundance under storage conditions. Contrary to what was seen in the community structure at domain level, the analysis at genus level revealed that time of sample processing has a slight impact on the microbial composition of samples stored in RNAprotect® Bacteria Reagent. However, variation in storage time of samples stored in RNAprotect resulted in less significant changes compared to storage conditions without preservation solution.

3.3 Effect of sample storage on microbial diversity and richness

The effect of storage conditions on the alpha diversity of oilfield microbial communities was evaluated based on richness (Chao1) and diversity (Shannon) indexes. Richness measures the number of species found in a community whereas diversity combines richness and evenness into a single indicator. This means diversity measures the probability that two randomly selected microorganisms in a community belong to different species.

Analysis of alpha diversity indexes differed significantly between storage conditions in most of the cases ($p \leq 0.05$, T-test) (Table 1). Water samples stored at room temperature presented lower richness values while refrigerated samples had higher richness values compared to the reference samples. In the case of sludge sample, all storage conditions showed higher richness than the reference sample. In terms of Shannon diversity index, the differences between storage conditions and pre-processed samples did not follow a unique pattern of increase or decrease according to the storage conditions. Some stored samples presented higher diversity values than reference samples and some others lower diversity values.

Table 1. Alpha diversity analysis of oilfield samples

Sample	Treatment	Number of sequences	Number of OTUs	Chao1	Shannon
PW-1	Ref	86050	113	105 ± 6	4.6 ± 0.012
PW-1	RT	39301	65	73 ± 7*	1.5 ± 0.005*
PW-2	Ref	55034	45	56 ± 9	1.4 ± 0.137
PW-2	RT	61788	54	57 ± 11	2.0 ± 0.005*
PW-3	Ref	124841	62	57 ± 17	2.0 ± 0.015
PW-3	RT	53015	21	26 ± 12*	0.5 ± 0.003*
PW-4	Ref	141813	142	133 ± 18	4.8 ± 0.010
PW-4	4C	60548	152	153 ± 10*	4.3 ± 0.012*
PW-5	Ref	88721	157	149 ± 12	4.3 ± 0.020
PW-5	4C	65896	214	213 ± 16*	4.6 ± 0.013*
PW-6	Ref	87220	275	258 ± 20	4.9 ± 0.013
PW-6	4C	119293	278	253 ± 19	4.9 ± 0.011
Sludge	Ref	49119	137	146 ± 14	3.0 ± 0.013
Sludge	RP	118463	148	154 ± 8	2.9 ± 0.017
Sludge	4C	180907	152	170 ± 18*	3.4 ± 0.008*
Sludge	RT	101062	148	154 ± 10	3.3 ± 0.009*

(*p<0.05 compared to reference sample).

3.4 Effect of sample storage on microbial structure

The effect of sample storage on the microbial structure was only assessed for water samples due to the number of sludge samples collected not being enough for statistical analysis. NMDS was the method selected for the ordination of samples based on the Weighted Unifrac distance matrix. According to this analysis, the community structure of samples that are ordinated closer to each other are likely to be more similar than those that are ordinated apart.

NMDS of water samples displayed a clear separation of the structure community at room temperature and the reference samples (Figure 5). Consistent with these findings, the PERMANOVA and ANOSIM tests on the Bray-Curtis dissimilarity indicated that there is a significant difference ($p = 0.03$ and $p = 0.01$ respectively) between the beta diversity of room temperature samples and their references. On the contrary, no significant difference was detected in the microbial community structure of refrigerated samples with respect to their references ($_{PERMANOVA}$ $p = 0.30$, $_{ANOSIM}$ $p=0.37$). NMDS displayed a close ordination of the refrigerated and reference samples (Figure 3).

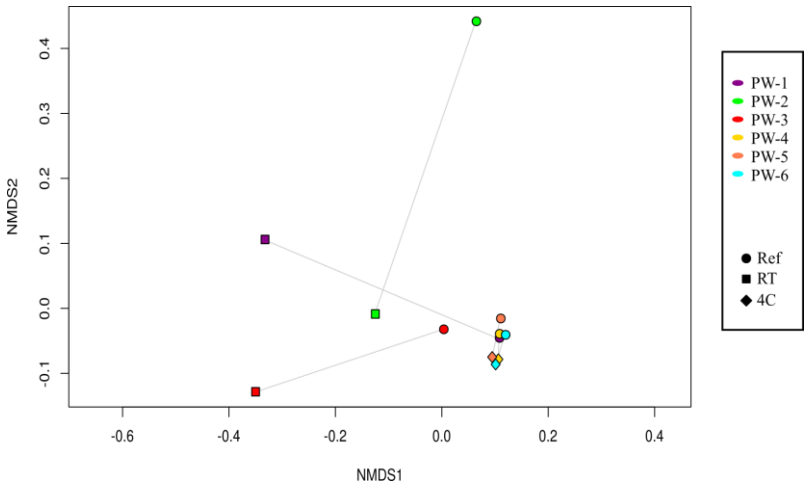

Figure 5 Non-metric multidimensional scaling (NMDS) of microbial community composition in water samples based on weighted Unifrac distance matrix. Storage conditions are represented by shape and sampling points by colours.

3.5 Effect of sample preservation on the functional capability

The functional profile analysis was focused on the microbial metabolic pathways that have been associated with the initiation or acceleration of corrosion processes. Relative abundances of key enzymes encoding genes participating in sulphate reduction, thiosulphate reduction, methanogenesis, acid production, iron oxidation, iron reduction and nitrate reduction were calculated with Tax4Fun R package. The impact of the sample storage on the functional profile of the community followed the same pattern in water (Figure 6) and sludge (Figure 7) samples. Sulphate reduction, methanogenesis and iron oxidation/reduction capabilities were shown to be reduced under both storage conditions compared to reference samples. On the other hand, fermenting and thiosulfate reducing capabilities increased after storage while nitrate reducing capabilities remained the same after storage compared to reference samples. Sludge sample preserved in RNAprotect® solution for a longer time (RP) did not show significant differences in the functional capabilities of the community with respect to the reference.

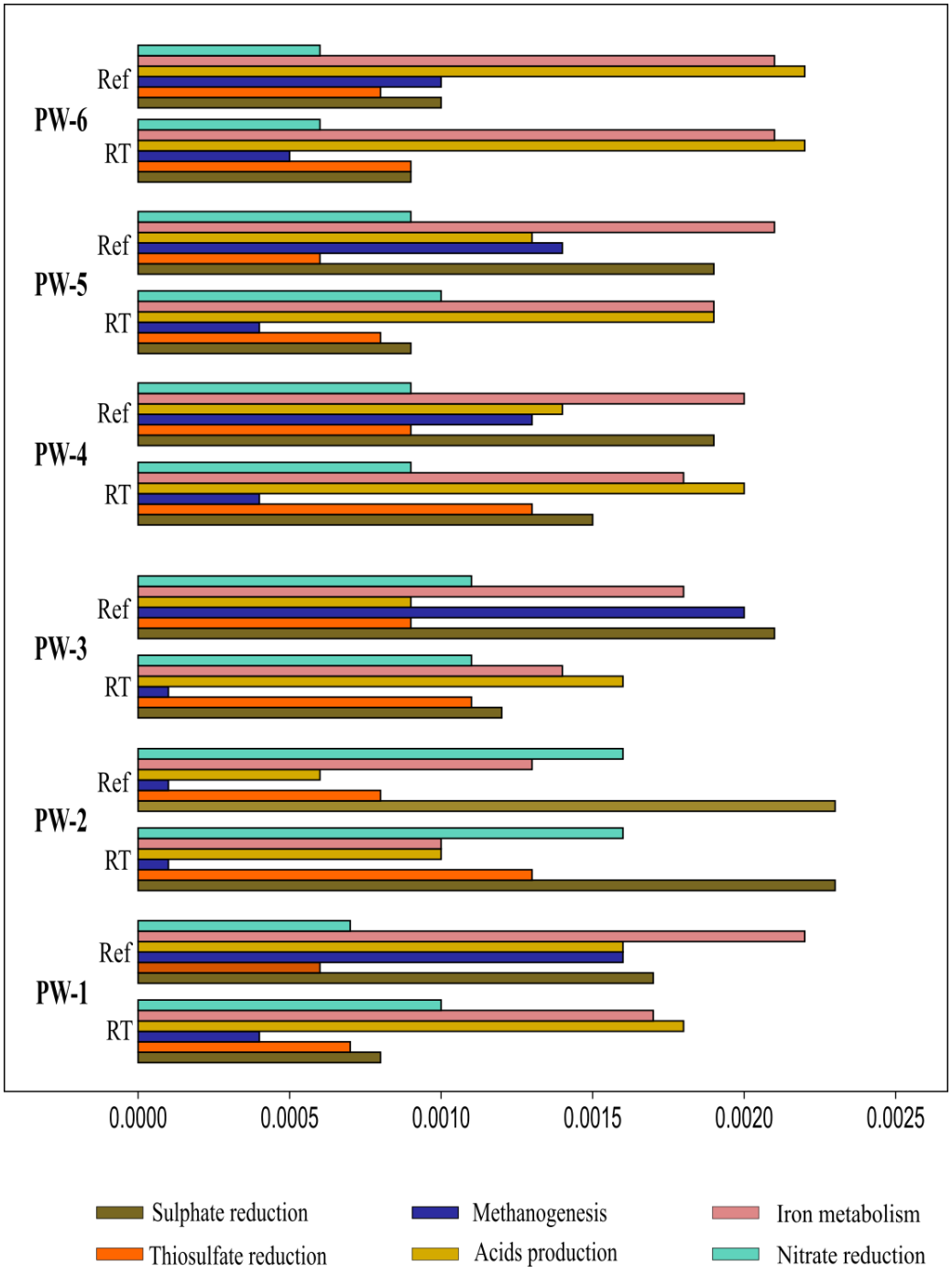

Figure 6 Functional profile of water samples. Relative abundances of key enzymes involved in microbiologically influenced corrosion processes.

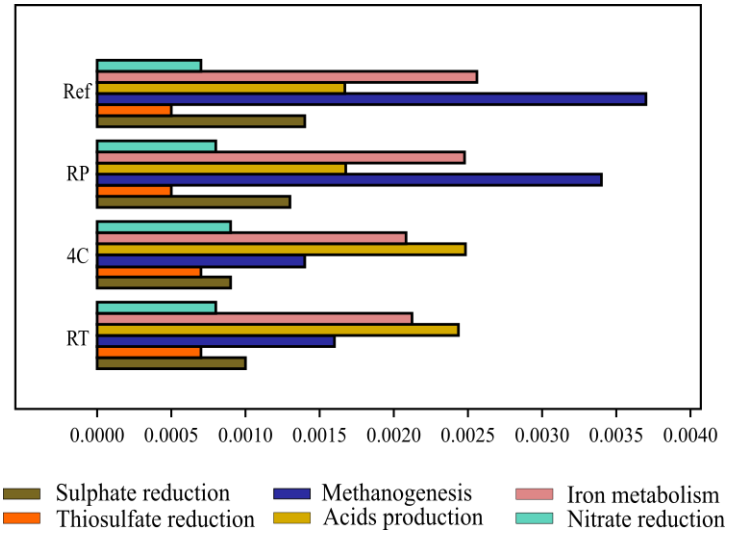

Figure 7 Functional profile of sludge sample. Relative abundances of key enzymes involved in microbiologically influenced corrosion processes.

4. DISCUSSION

Microbiological characterization of samples from remote oil production facilities is a challenging task since samples must be transported long distances after collection and before arriving at the laboratory for analysis. In particular, this is the case when microbiological assessment is done using molecular microbiological methods where sample processing requires more delicate protocols and trained analysists. During transportation, samples are rarely delivered under refrigeration and in most instances, samples are delivered at room temperature. By assessing the impact of storage temperature on the assessment of the microbial community in oilfield samples we provide a base understanding of the implications of this crucial procedure in the MIC assessment.

In this study, the microbial structure of the community was significantly influenced by storage conditions, particularly by room temperature conditions. Predominant populations in the water samples PW-1, PW-2 and PW-3 were replaced by aerobic or aerotolerant mesophilic microorganisms. This can be explained by the fact that during sample collection, samples can be exposed to air developing more favourable conditions for these microorganisms to thrive compared to the oxygen-free environment of the oilfield facilities. At room temperature conditions, microorganisms such as *Marinobacter, Marinobacterium, Arcobacter*, and *Pseudomonas* thrived in the samples and became the major genera in the community. Distinct to the room temperature, refrigeration did not affect considerably the community structure of the water samples. Other genera not related to aerobic respiration, e.g., *Methanothermobacter, Acetomicrobium* and *Thermovirga* exhibited higher relative abundances after storage, however, it is uncertain if this increase was related to the growth of microorganisms or to a higher resistance to the cooling conditions compared to other thermophile species that may be more sensitive to cold temperature and did not survive. According to Canganella & Wiegel, (52) thermophilic anaerobes can survive at mesobiotic and even psychrobiotic environments but cells at these conditions are unlikely to be able to multiply. A previous study developed by Kilbane (39), who investigated the effect of storage conditions on the recovery of microbial groups by serial dilution method reported relatively similar results, showing that higher microbial concentrations were detected on samples stored at room temperature and lower microbial concentrations on samples stored at 4°C. The author attributed the decrease of microorganisms to temperature or metabolic shock and to the depletion of energy reserves during storage.

In terms of alpha diversity and richness, both storage conditions significantly affected the OTUs recovered in the analysis of water and sludge samples. Shannon index (diversity) did not follow a unique pattern of increase or decrease according to the storage condition in both water and sludge samples, the reason for this is unclear but it is likely related to particular characteristics of each sampling point. Richness measured in sludge samples increased under both storage conditions while the pattern was different in water samples. The number of species decreased under room temperature and increased during refrigeration conditions. As mentioned before, room temperature favoured the growth of aerotolerant species generating an uneven distribution of species in the community. On the other hand, cooling conditions resulted in a reduced abundance of mesophilic and thermophilic taxa, all of them classified as strict anaerobes. This phenomenon could be related to temperature sensitivity of species or to the fact that oxygen solubility increases with the decrease of temperature thus harming strict anaerobes by exposing cells to toxic levels of oxygen (53). Lee *et al.,* (20) suggested that increases of DNA concentration during storage conditions could be the result of growing microbes while decreases can be the consequence of the presence of nucleases that degrade the DNA molecules. To avoid the activity of these enzymes and preserve the DNA and RNA integrity in the samples, immediate freezing of samples in liquid nitrogen or dry ice is the most suitable method. However, when sampling is carried out in remote areas, stable freezing of samples in liquid nitrogen tank or dry ice during transportation is rather impractical and in most cases logistically impossible (54). An alternative to the freezing protocol is the use of nucleic acids preservation solutions such as RNAprotect® Bacteria Reagent that protects the sample from enzymatic degradation and stops bacterial activity.

The functional capability of the community was assessed using Tax4Fun, a novel bioinformatics software that provides data on the potential metabolic capabilities of the microbial community in a sample. Results showed that the functional capability was also affected by storage conditions which is associated with the variations in the relative abundances of microbial populations. Determining the presence and abundance of corrosive microorganisms is the key step in microbiological surveys in terms of MIC prediction. Defining if a microorganism can cause corrosion it is closely related to their metabolic function within a community. Generally, MIC prediction tools are centred in the corrosive activity of sulphate reducing bacteria (55-57) and some have included the effect of methanogenic species (58). Implementing these methodologies for the MIC assessment in samples stored at room temperature or cooling conditions will underestimate or overestimate the risk of MIC because of the effect of these storage conditions have on the abundance of corrosive microorganisms. According to the functional profile analysis realized in this study, sulphate reduction and methanogenesis pathways reduced their abundances under both storage conditions. Other metabolic pathways such as acid production and thiosulphate reduction presented a different pattern, suggesting that these populations have the ability to tolerate aerobic environments. Considering the results of this research and those obtained by Kilbane (39), sample storage conditions have an effect on microbiological surveys executed using both culture-independent and culture-dependent methods.

Overall, results of this research were in agreement with previous studies. For instance, Choo et al., (59) studied the microbiome in faecal samples. The authors detected significant changes in the community structure of samples stored at room temperature and not significant changes in samples stored at 4°C. Similar to our findings, aerotolerant species increased abundances under room temperature conditions, which was also correlated with the exposure of their faecal samples to aerobic conditions. Conversely, some studies have shown no significant effect of storage conditions on the microbial composition (37, 38, 60, 61). However, the difference between this investigation and other published work is the predominance of thermophile microbes in the samples evaluated here. Oilfield microbial communities are mainly constituted by anaerobic thermophile microorganisms whereas most of the studies developed in this area relate to the microbiome of mesophilic communities in soil and human body environments. Considering that temperature is one of the key factor in the microbial growth, the preservation of samples at a different temperature of the sampling point, with exception of freezing, can lead to a complete shift in the microbial community recovered during 16S rRNA gene sequencing. For this reason, results from this work demonstrate that on-site pre-processing along with addition of DNA/RNA preservation solutions is the preferred approach for the molecular assessment of microbial communities in oilfield samples where the field conditions make unlikely the immediate freezing of samples. This research did not evaluate the effect of different preservation solutions on the recovery of the oilfield microbial community and further analysis is required for a complete sampling protocol optimization.

5. CONCLUSIONS

We assessed the effect of sample storage on the assessment of oilfield microbial communities by 16S rRNA sequencing. Results demonstrated that diversity profiling, microbial structure and functional capability of oilfield microbiomes are affected by sample storage conditions, named room and cold temperature conditions, as compared to reference samples pre-processed and preserved immediately on-site. Alpha diversity measured by richness and diversity indexes was significantly affected in most of the samples under both conditions whereas microbial structure was only significantly affected by storage at room temperature. The functional capability of the community was also affected by both storage conditions evaluated.

Abundances of genes associated with sulphate reduction, iron utilization and methanogenesis pathways decreased whereas genes related to acid production and thiosulphate reduction increased. Therefore, changes in the microbial composition of oilfield samples due to inadequate sample storage can lead to inaccurate MIC prediction due to the facility and quick response of the microorganisms to environmental changes such as those experienced during sample storage. Due to the above, if on-site freezing of samples is not feasible, on-site pre-processing is the preferred choice for an accurate assessment of the microbiological content in the samples. The on-site pre-processing refers to the sample immersion on a nucleic acids preservation solution, in the case of water samples a previous filtration through membrane filters of 0,2 um pore size is required. When on-site processing is not feasible or practical, samples should be at least stored at 4°C to prevent the growth of mesophilic populations.

6. ACKNOWLEDGMENTS

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7. REFERENCES

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