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Interactions Between Anaerobic Oil Bacteria – Monitoring by Classic and Molecular Microbiology

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Abstract

The biogenic production of sulphide is one of the main problems in oil and gas industry, causing corrosion in storage tanks and pipes. This is possible by the injection of seawater during the secondary oil recovery. In the present work high levels of sulphide and acid producers were detected in water/oil samples from several sites from the petroleum industry. In a further stage, a broader range of microbial cells were detected, and finally, metagenomic analysis confirmed the presence of a diversity of microbes, indicating the complexity of the consortium in the production of sulphide, and based on the activity of acid producing cells and associated species.

Keywords: Acid producing bacteria; Sulphate reducing bacteria; Sulphide; Metanogenics; Petroleum industry.

1. Introduction

The oil industry faces serious problems related to the corrosion of metallic surfaces, such as platform structures, storage tanks, and production lines. The corrosion of metals is an extremely common and costly process with 20% of metal corrosions reported as microbiologically induced corrosion (MIC) [1-3]. It is assumed that 50% of corrosive failures in pipelines are related to MIC [4-7]. Biocorrosion is the consequence of the deterioration of metals caused by the metabolic activity of some microorganisms, the main ones sulphate-reducing bacteria (SRB) [8].

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Many types of industries, such as mining companies and the oil industry face serious damage, often irreversible, due to the biocorrosion of their metallic structures, which normally affects pipes [9, 10]. The most common groups found, such as SRB, iron reducing bacteria (IRB) and acid producing bacteria (APB) have been frequently reported in the microbial communities in the reservoirs and pipes that suffer from this type of corrosion [11]. However, new genera and bacterial species are being discovered in these reservoirs, indicating, this way, a great potential for corrosion, not yet discovered [12-15]. Many oil reservoirs around the world have been extensively flooded with water for secondary oil recovery [16-18]. The injection water produced from the oil-water separation from production waters is recycled to be injected through a semi-open system, which provides the perfect environment for the growth of various microbial groups such as those mentioned above. According to Almeida [19], APB constitutes a very important group, since they are able to use long chains of hydrocarbons in the oil as substrate to produce smaller organic acids, which will serve as a substrate for SRB to proliferate and produce H_2S, a toxic and corrosive agent. In view of this scenario, it is very important to identify the different groups existing in the microbial consortia of these reservoirs, especially the existing species of APB, since they are at the beginning of the chain of the corrosive agents that damage and degrade not only metal structures, but also the oil itself. Thus, the present work has the purpose of monitoring these microorganisms through classical microbiology techniques, such as the most probable number technique (MPN). As a complementary analysis, we performed the identification of some metabolites produced by APB through HPLC technique, with UV detection with electrospray ionization technique coupled to mass spectrometry (HPLC-UV-IES-MS) and also metagenomic sequencing, a very powerful technology to identify genetic information contained in an environmental sample for non-culturable microbes.

2. Materials and methods

2.1. Samples

Table 1: Samples from phases 1 and 2 and microbial groups investigated.

Number of samples	Sample description	Microbial groups investigated
Phase 1		
12	Water from oil storage tanks and drains	Aerobic/Anaerobic acid-producing bacteria (APB) and Sulphate-reducing bacteria (SRB)
8	Internal oil transportation lines	
2	Water from tanks with a high content of oil	
4	Effluent treatment station	
3	PIG residues	
6	Water from water storage tanks	
Phase 2		
5	Water from water storage tanks	Culturable heterotrophic aerobic bacteria (CHAB), Culturable heterotrophic anaerobic bacteria (CHAnB), Iron-precipitating bacteria (IPB), Aerobic acid-producing bacteria (APB), Anaerobic acid-producing bacteria (AnPB) and Sulphate-reducing bacteria (SRB)
7	Water from oil storage tanks	

The samples used in this work came from different locations, such as production water storage tanks, oil storage tanks, oil drain lines, and fuel flow lines of an oil company. In a first phase 35 different samples were studied,

and in a second phase 12 different samples were studied, all of them at 3.5 % (v/v) salinity (Table 1).

2.2. Culture media

The anaerobic culture media used in this work were solubilized in distilled water under agitation and mild heating, to decrease the concentration of oxygen in the medium, since the basic condition for the growth of the anaerobic microorganisms is the absence of oxygen. For the same purpose, nitrogen gas was purged during the entire preparation and distribution of the medium, in the case of anaerobic cells quantification. The following media were used: Phenol Red Broth, for APB; Postgate E, for the SRB; Iron broth, for IRB; CHAB medium, for cultivable heterotrophic anaerobic bacteria; and Nutrient Broth, for aerobic cultivable bacteria.

2.3. Microbial quantification

For the quantification of SRB and APB cells present in each sample, the Most Probable Number (MPN) method was used [20]. Dilutions ranging from 10^0 to 10^8 were prepared for all microbial groups. For the inoculation of each triplicate of each dilution, sterile syringes were used, with all procedures performed inside a vertical laminar flow cabin. All flasks inoculated with the different samples were incubated for 28 days at 30° C and their growth was monitored every 7 days, for 28 days.

2.4. High performance liquid chromatography

In order to identify some metabolites produced by APB, some samples were initially inoculated in a selective medium (Phenol Red Broth) for the growth of this microbial group. After growth, cultures were filtered using a 0.2 μm filter syringe to sterilize the samples. Some solutions were filtered accordingly, to serve as control solutions: Glucose Solution (10g/L), Pure Phenol Red Broth (15g/L) and APB growth medium. The chromatographic technique used in this work was the high-performance liquid chromatography with UV detection and electrospray ionization technique coupled to mass spectrometry (HPLC-UV-IES-MS). Samples were diluted in spectroscopic grade methanol (Tedia, Brazil) at a concentration of 200 μL/mL. Afterwards, they were filtered (0.45 μm Millipore membrane, Merck Germany) and subjected to chromatographic analysis. The equipment used was produced by Ultra Shimadzu® with degasser (DEU20AS), two pumps (LC20AD), automatic injector (SIL20AC), fixed wavelength UV detector (SPD20A), oven (CTO20A) and interface (CSM20A). Samples were separated by a Thermo-Scientific® RP18 column (250 mm x 4.6 mm with 5Å particle). Elution of the mobile phase occurred by a ramp-type gradient, starting at 95% ultrapure water (MilliQ®) acidified with trichloroacetic acetic acid PA, pH 2.5 (VETEC) and 5% acetonitrile (Tedia) (0-2 min), ultrapure acidified water and 100% acetonitrile (2-45 min), 100% acetonitrile (45-55 min) and returning to the initial condition in the final 5 minutes (55-60 min) . Samples were separated on Thermo-Scientific® RP18 column (250 mm x 4.6 mm with 5Å particle). After UV detection, with a fixed lamp at 270 nm, the mobile phase was carbonated by electrospray ionization (200 ºC - 8 μL/min - 4 psi) and followed by the mass spectrometry analysis (Bruker MicrOTOF - QII). Mass spectra were obtained in negative mode, with the voltage of a 4000V capillary, in the mass/charge (m/z) range from 50 to 1000. The gas used during desolvation was nitrogen, with a flow of 400 L/h and submitted to the temperature of 250 ºC. The ionization energy was 10 eV,

with mass scan analysis. The flow rate was 1 mL/min and the injection volume was 10 µL.

2.5. Metagenomic analysis

In the metagenomic study, DNA from only one sample highly contaminated with APB cells was extracted after growth on Red Phenol medium. The DNA was quantified and standardized at 3ng/µL by fluorescence from the Qubit® 3.0 Fluorometer and Qubi dsDNA HS assay kit (Thermo Fisher Scientific). The PCR reaction for amplification was performed with samples with a final volume of 20µl, containing 10µl of GoTaq® Master Mix Incolor 2x (Promega, USL), 0.3 µM foward primer and 0.3 µM reverse primer, 1µg/µL of genomic DNA and sterile ultrapure water. The amplification program used followed the protocol: 94°C for 3 min, followed by 29 cycles of denaturation at 94°C for 45 sec, annealing at 50°C for 1 min, extension at 72ºC for 1.5 min and a final extension at 72°C for 10 min. Amplification reactions were conducted in thermocycler Veriti™ Thermocycler (Applied Biosystems). As initiator, the Foward Universal Primer 5'-AATGATACGGCG ACCACCGAGATCTACACTATGGTAATTGTGTGCCAGC-3' was used [21]. After amplification of each piece, amplification by electrophoresis in 2% agarose gel stained with 0.03% (v/v) UniSafe Dye, with approximately 300bp (amplicon size) was confirmed. PCR triplicates were combined into a single aliquot and subjected to purification using the Agencourt AMPure XP magnetic (Beckman Coulter), for the removal of small fragments from the total population of primers, molecules and residues. Subsequently, quantification was performed using the Real Time PCR methodology using QuantStudio 3 Real Time (Applied Biosystems) and KAPA-KK4824 (Library Quantification Kit - Illumina / Universal) thermocycler, all according to manufacturer's protocol. An equimolar pool of DNA was generated by normalizing all samples at 2nM for sequencing, which was conducted using the Illumina MiSeq next-generation sequencing system (Illumina® Sequencing).

3. Results and discussion

3.1. Samples from the first phase

These determinations were necessary in order to map the most critical environments suitable for the growth of APB and SRB microbial cells. All samples were submitted to microbial quantification of 3 distinct groups, aerobic APB, anaerobic APB and SRB, using the MPN methodology based on the statistical determinations from Harrigan's table.

3.1.1. Water samples

Samples of water collected in different drains and tanks from a petroleum industry showed significant growth for total APB (aerobic and anaerobic) and SRB groups. In most samples, it can be observed the growth of both groups, particularly SRB cells (Figure 1), in accordance to the work of Okoro and his colleagues [22], which mentions the great potential of microbiological growth in water samples due to the great supply of nutrients needed for the growth of these microbial groups. From Figure 1, it can be seen the microbial growth of all groups investigated in the 12 samples, presented average values around 10^4 MPN/mL. Half of the samples presented no growth of APB cells, while SRB cells presented growth in most of the samples. It is important to

emphasize that SRB cells were mostly higher than APB cells. This may be an indication that the presence of APB cells may produce short-chain compounds, amenable to be used by SRB cells, thus, stimulating their growth. This indicates that water samples constitute strong candidates for a deeper investigation of other microbial groups that may be involved in corrosion, in order to understand the correlation between microbial groups. It is also interesting to note that some samples (11 and 12) presented no APB and SRB growth, probably due to the presence of high levels of biocide, widely used by petroleum industry. This is in agreement with the work performed by Sugai and his colleagues [23] who confirmed the rapid kinetics of SRB cells associated to high levels of biogenic sulphide production.

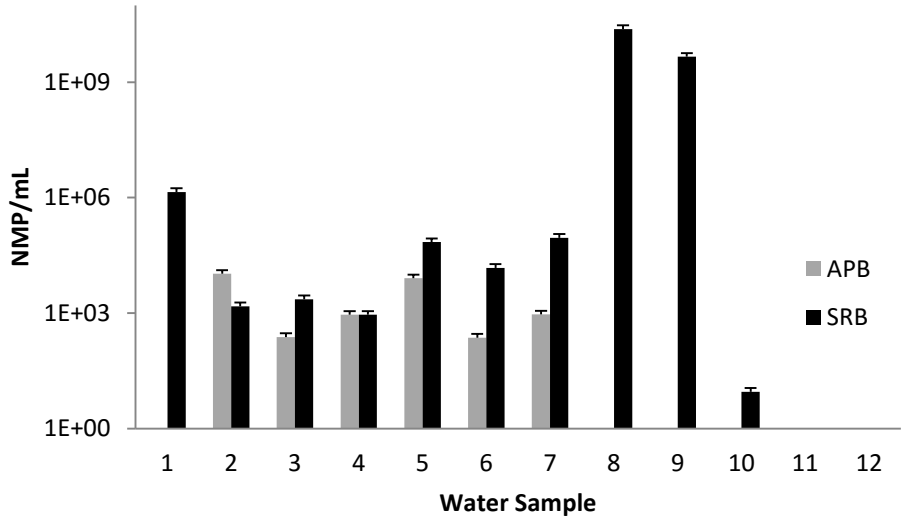

Figure 1: Quantification of APB and SRB cells in water samples

3.1.2. Water storage tanks samples

According to Ren and his colleagues [24], several microbial groups can be found in storage tanks, demonstrating a significant growth under favorable conditions. This is in accordance to the results obtained in the present work, where SRB cells were found in all samples tested. Here, again, APB cells were not present in all samples tested, and, not all samples presented APB cells. Two samples indicated the presence of SRB cells, even though APB cells were not detected. From Figure 2, it can be observed that all samples presented high levels of SRB growth, usually above 10^5 MPN/mL, a high average value if compared to the other samples from the present research. On the other hand, APB growth presented concentration levels from 102 to 1010 MPN/mL. Here, again, SRB cells were present in higher concentrations in comparison to APB cells, when both groups were present in the same sample (except sample 3), corroborating the previous observation that APB cells may be stimulating the growth of SRB cells, due to the production of metabolites that serve as energy source for SRB cells.

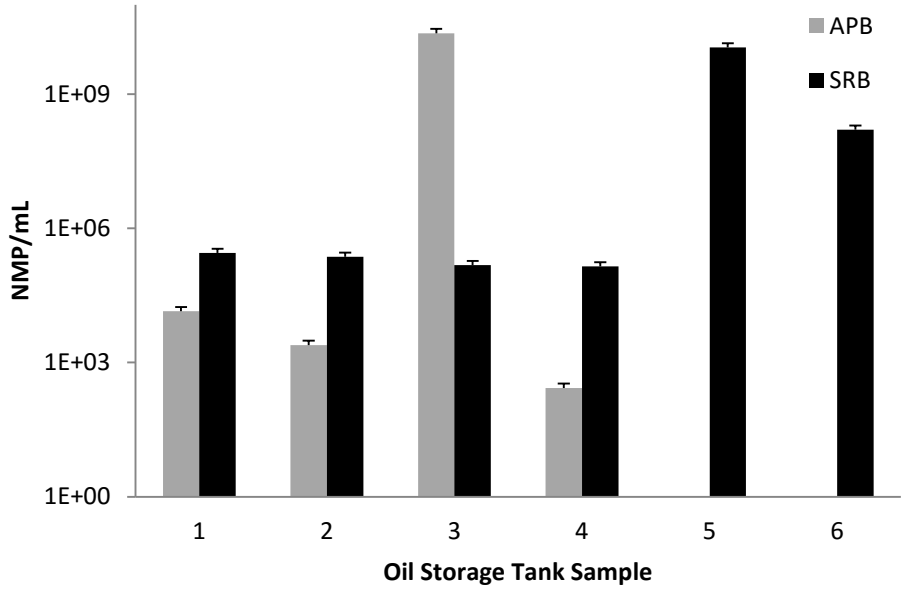

Figure 2: Quantification of APB and SRB cells in storage tanks

3.1.3. Oily water samples

These samples were here segregated from the remaining water samples from Figure 1 due to the fact that they had much higher oil content. Only two among many water samples studied presented this characteristic. The results from Figure 3 indicate the presence of SRB cells in both samples, one of them associated to a high level of APB cells (Sample 2). Here, again, the presence of SRB cells was superior to the number of APB cells, once again indicating a possible relation between these two microbial groups.

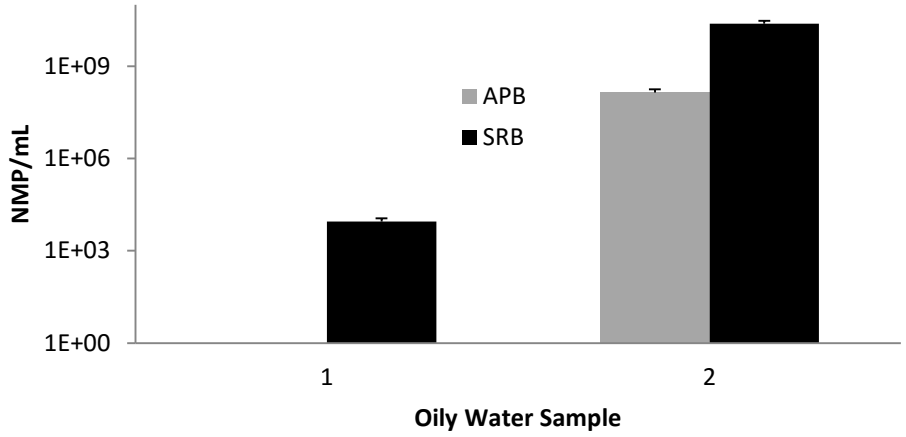

Figure 3: Quantification of APB and SRB cells in oily waters

3.1.4. Water transport lines, production water treatment station and PIGs samples

The results observed from samples from water transport lines, production water treatment station and PIGs presented similar qualitative results (Figures 4, 5 and 6). For all samples, SRB cells were present at higher concentrations than APB cells, or SRB cells were present in absence of APB cells. These results suggest that APB may stimulate the growth of SRB cells by providing suitable substrates, or, in the absence of APB cells the medium may provide suitable conditions for sulphate reducers growth.

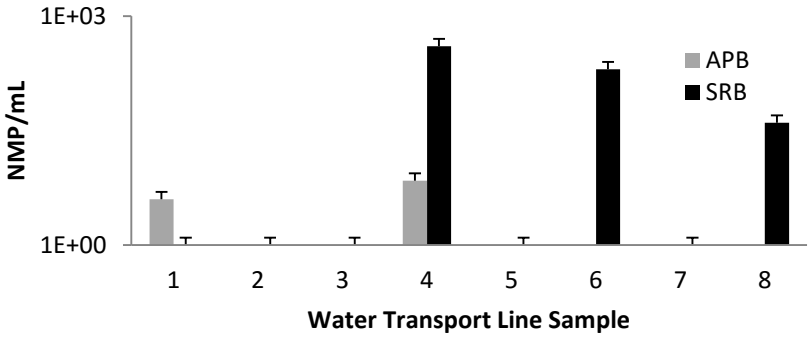

Figure 4: Quantification of APB and SRB cells in water transport lines

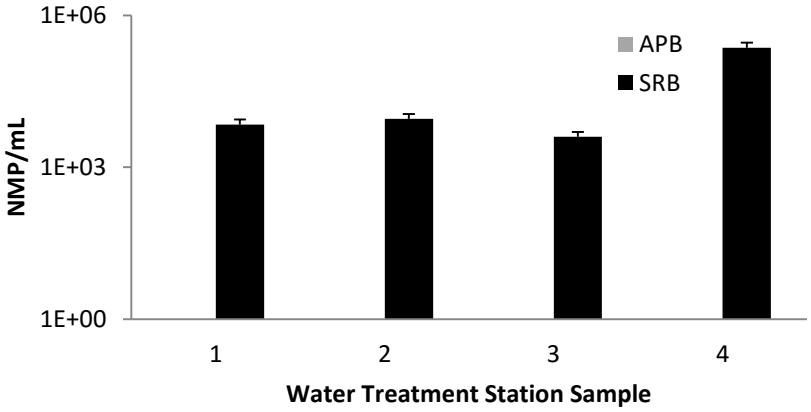

Figure 5: Quantification of APB and SRB cells in production water treatment station

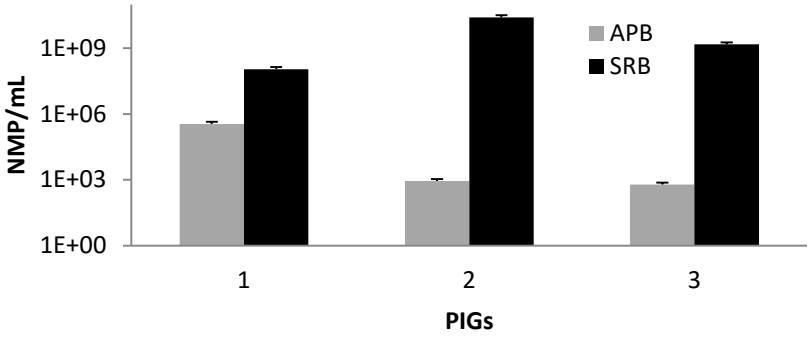

Figure 6: Quantification of APB and SRB cells in PIGs

An overall discussion of the results obtained in the first phase of the work, clearly indicates the need to search for additional microbial groups. Even though it seems to have a close relationship between APB and SRB cells, the presence of other microbial groups can help to understand the complex nature of microbial growth on petroleum substrates, that cannot be easily explained based on quantification of just two microbial groups.

3.2. Samples from the second phase

Due to the growth behavior observed in the first phase of the work, it was decided to investigate additional microbial groups, as suggested by Ren and his colleagues [24]: iron precipitating bacteria (IPB), and other anaerobic and aerobic heterotrophs that may also be found. At this stage of the present study, 6 new samples were selected, 3 samples from water storage tanks and 3 samples from oily water storage tanks. In order to investigate the effect of a high content of oil in combination with water, the results obtained will be discussed in two main groups: initially results from 3 samples from tanks containing only water will be presented, and then, the remaining 3 samples, including water and oil will be shown. In order to facilitate comparison, results will be presented in Figures 7 and 8. Results from Figure 7 indicate the presence of high levels of sulphate reducing bacteria, followed by a smaller number of culturable heterothrophic anaerobic bacteria, and then anaerobic acid producing cells, and finally a smaller number of aerobic cells. This is in agreement with the previous results, where SRB cells constitute the major microbial group in produced water, followed by a smaller number of anaerobic acid producing cells.

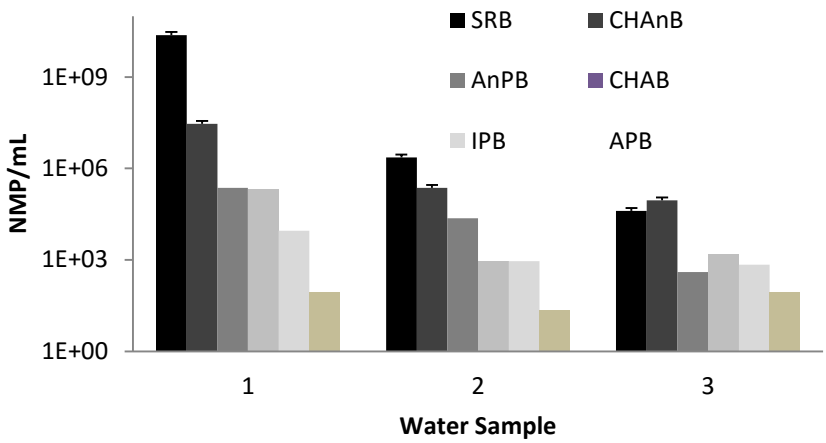

Figure 7: Microbial groups from water tanks

However, it can be seen, that the interaction between sulphate reducing cells with other microbial groups, is not solely restricted to acid producing cells with other culturable cells (both aerobic and anaerobic) probably contributing in the chain of sulphide production. This microbial consortium of microbial cells probably interacts inside the tanks, providing suitable conditions for biogenic sulphide production. This is what is being investigated, that in several environments of the petroleum industry, sulphide production occurs as a function of a much broader action of microbial cells, not restricted to sulphate reducers. One can see that in the present investigation, initially we concluded that in several environments sulphate reducers and acid producers seem to

be in close association. Here, it can be seen that not only these two groups seem to be responsible for the production of undesirable metabolites, but, other cells, not deeply investigated, may take part in the phenomenon. Water storage tanks, due to the lack of a homogeneous composition, seem to be one of the most contaminating environments, due to the presence of seawater, residual additives and some other compounds that may support the growth of several groups. According to Figure 8, the combination of production water and oil inside the tanks, may have changed the conclusions previously designed. Here, the presence of aerobes was considerably higher in comparison to the tanks lacking the presence of oil. Once the oil content is not the same in both tanks, this may be the reason for a higher concentration of aerobic cells in comparison to the results obtained from tanks containing water only. Different oil/water layer heights leads to distinct oxygen gas diffusion, thus contributing to the levels of aerobic cells. Some aerobes may be responsible for the initial degradation of the oil fraction, providing suitable substrates for anaerobes, a conclusion that needs to be confirmed due to the low number of tanks here evaluated.

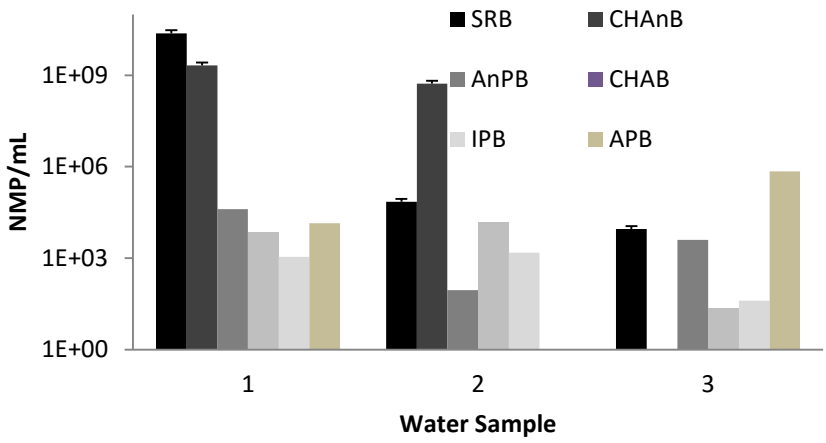

Figure 8: Microbial groups from oily water tanks

High number of cells from tank samples is due to the fact that these microorganisms are dependent on oxygen for their growth and the diffusion of this gas into water occurs more easily than in oil. This is particularly true in the quantification of APB and CHAB, both requiring oxygen for growth. Sharma and his colleagues [25] indicates that APB around 10^3 MPN/mL constitutes an extremely significant number of cells considering microbial attack on metal structures. This approximation of values can be due to the existence of facultative APB, growing both under aerobic and anaerobic environments. Observing the values found for the group of culturable anaerobic bacteria, it can be observed that these bacteria reached 10^9 MPN/mL while those from the water samples reached values of maximum 10^7 MPN/mL, corroborating the results that show the need for anaerobic environments for suitable growth of these microorganisms. Under aerobic environments, bacteria that have anaerobic growth, require biofilms formed by aerobic bacteria, which promote the anaerobic environment, thus maintaining the necessary conditions for their growth. Okoro and his colleagues [22] quantified sulphate reducing cells using the most probable number technique reaching values from 10^7 to 10^8 MPN/mL, relating such values to the effective power of microbiological corrosion promoted by these microorganisms. These numbers agree with the values found in the present work, where we obtained even greater values for some

samples, which demonstrates a significant value of sulphate reducing bacteria, being those potential sources of future corrosion for metal components of structures in the oil industry. Although this work focused on APB, it is necessary to emphasize that SRB are as important as APB, since APB produces the favorable environment for SRB to grow, with the consumption of organic acids produced by breaking the long hydrocarbon chains of the oil, thus causing SRB action to promote corrosion [11]. According to Kim and his colleagues [26] SRB together with APB are the main bacteria involved in MIC processes, SRB being responsible for the production of enzymes that remove cathodic hydrogen from the metal causing pitting corrosion on metal surfaces. On the other hand, APB produces organic acids that will not only serve as a substrate for SRB but also lower pH, thus promoting the perfect environment for corrosion of surfaces [27].

3.3. Chromatographic analysis

For the identification of the metabolites through the HPLC technique, 200µL from 7 samples were analyzed, including: 1) Blank under Storage Conditions; 2) Blank under Growth Conditions; 3) Sample from one water storage tank; 4) Sample from a second water storage tank; 5) Sample from an oily water tank; 6) Glucose - Solution 10g/L (blank from culture medium); and, 7) Phenol Red - Solution 15g/L (blank from culture medium). The chromatograms obtained at 270 nm (better condition for HPLC), it can observed the typical peaks of phenol red (that appears in all samples because it is an indicator), as well as the typical peaks from glucose. The distinct peaks will not be here discussed, but the most interesting result can be seen in the time of approximately 3-4 minutes, a peak referring to the formic acid, the acid most widely used by SRB cells (Figure 9).

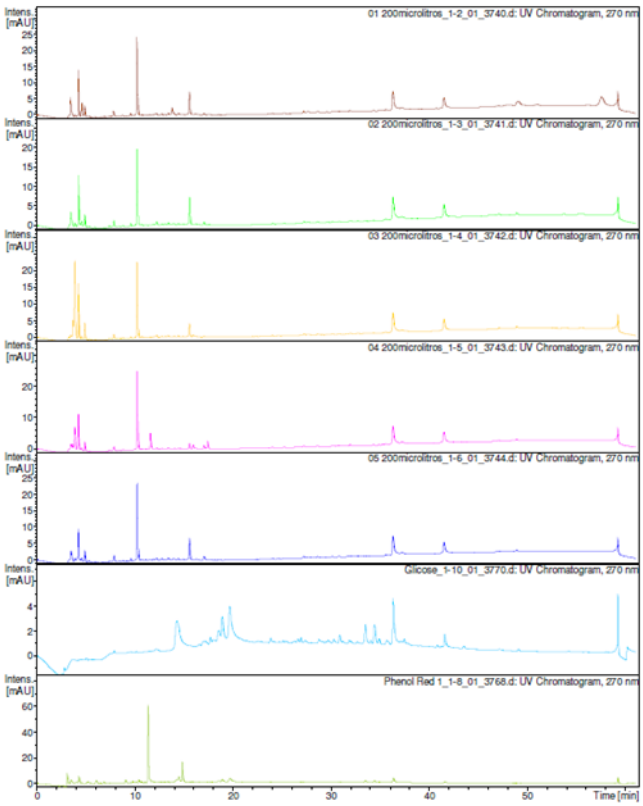

Figure 9: HPLC spectrum for glucose degradation, phenol red marker and formic acid detection

This may be a confirmation of the previously discussed results: APB cells are probably degrading the oil from water/oil mixtures, thus providing suitable conditions for SRB proliferation. When evaluating the mass spectrum of the samples, the formic acid profile could be detected as one of the products formed through comparison of the masses with reference materials used on the calibration of the equipment using a standard solution of sodium formate (Figure 10). Formic acid production decreases de pH of the medium, modifying the staining pattern of phenol red indicator, a result that corroborates with studies from Adams (2010). It can also be seen from Figure 9 that the production of this acid was more intense in sample 3, which is in agreement with the studies of Okoro and Armund [28], confirming that water samples have a higher susceptibility to the growth of microorganisms. Jacobs and Severin [29] also investigated the metabolites produced under anaerobic conditions. The main focus of the authors was on the accuracy of the several analytical methods and their application limits.

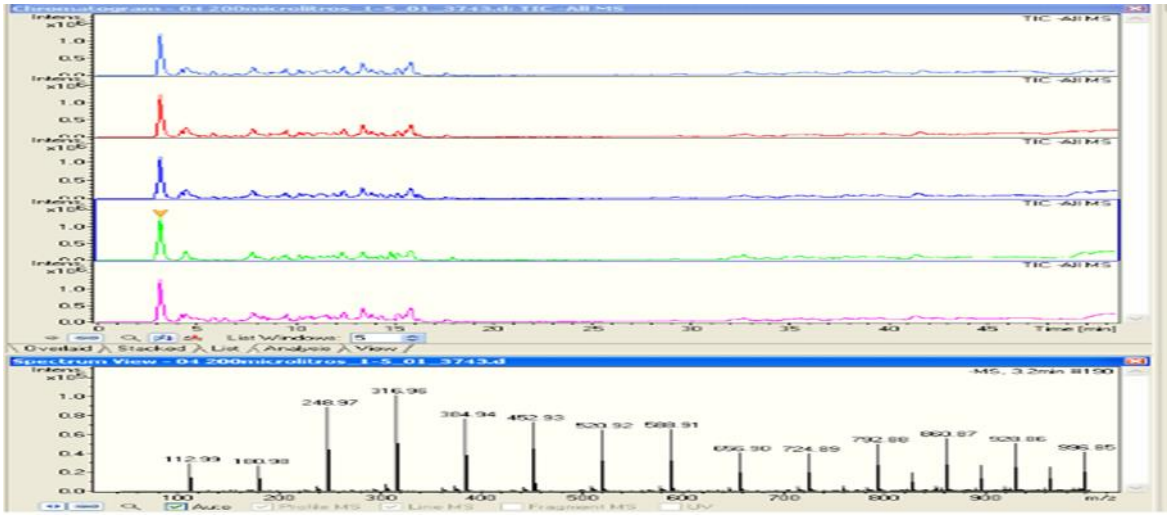

Figure 10: HPLC spectrum for glucose degradation, phenol red marker and formic acid

3.4. Metagenomic analysis

As previously observed, during the first phase of the present work it was demonstrated the predominance of SRB and APB cells; in the second phase of the study, other aerobic and anaerobic microbial groups were quantified, indicating the complexity of the consortium involved in the degradation of oil organic compounds in composition with saline water. However, in those two initial steps of the work, just culturable cells were quantified. In this final step of the work, metagenomic analysis was performed in order to detect the presence of non-culturable cells in the most contaminated sample. The importance of this genomic detection lies on the possible involvement of other microbial groups in biocorrosion and biogenic production of sulphides in the petroleum industry [30]. From data generated by sequencing, it is possible to detect taxonomic groups, to characterize intra-sample diversity (alpha diversity) and to assess the classification of taxonomic groups. The QIME 2018.4 program group was used. The alpha-diversity corresponds to the variability of species observed within the same sample (Figure 11).

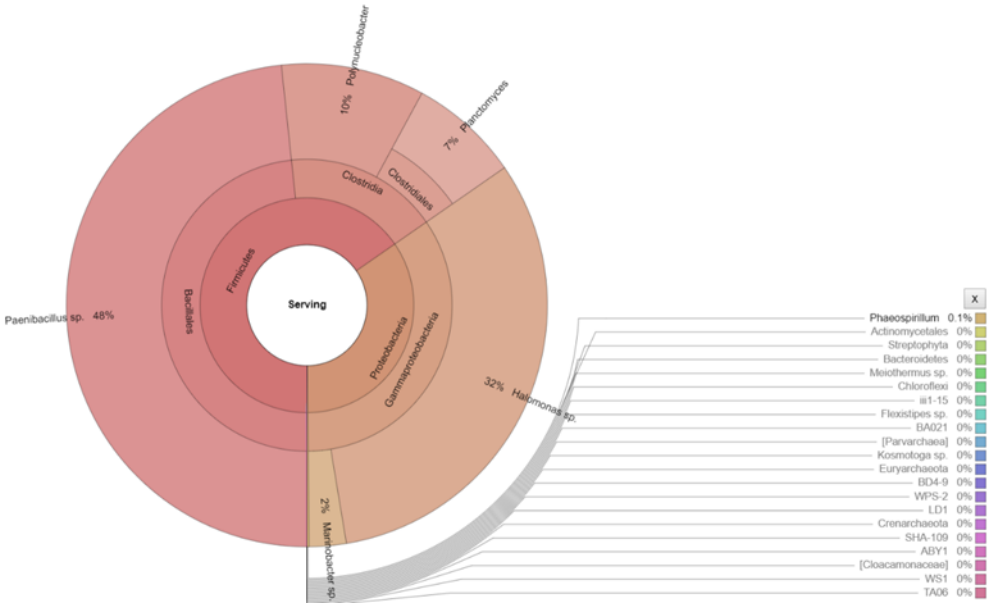

Figure 11: Average community composition at the phylum/class level

After analysis by bioinformatics of the sequences obtained in the sequencing of the genomic material of the sample, it was observed that the presence of Bacillus sp., which may have occurred due to the selectivity of the medium [31, 32]. Several other groups, more representative of the clusters include: Paenibacillus sp. (48.3%), Halomonas sp. (32%), Polynucleobacter sp. (10%), Planctomyces (7%) and Marinobacter sp. (2%) as can be seem in Figure 11. Okoro and Amund [28] also investigated the microbial community structure of a low sulfate oil producing facility using 16S rRNA gene sequencing technique, in production water and oil samples. The authors identified the massive presence of Marinobacter sp., as observed in the present work, associated to several other potentially corrosive Archaea species. Purwasena and his colleagues [33] also searched for new strategies based on phylogenetic analysis discovering a new strain of Petrotoga, confirming that a broad understanding of the action of a microbial community in several sites of the petroleum industry, still requires further investigations.

4. Conclusions

- It could be observed a high level of sulphate reducers and acid producers in several operations of the petroleum industry, including water tanks, water/oil tanks, PIGs and water treatment stations. Usually, the number of sulphate reducers was higher than the number of acid producers. This was a first indication that probably these two bacterial groups interact, with one group producing short-chain organic acids to supply sulphate reducers with energy source.

- The presence of iron bacteria and other anaerobic and aerobic bacterial groups in similar samples, proved that the final production of biogenic sulphide is not mediated solely by acid producers and sulphate reducers.

- Metanogenic analysis proved the involvement of other bacterial groups, not easily culturable under

laboratory conditions that may take part in the complex consortium of cells that may occur in several petroleum industries.

- The proper understanding of the role of each microbial group in a petroleum facility, may bring a considerable reduction in biocide consumption, to prevent the uncontrolled generation of biogenic sulphides.

5. Recommendations

Some recommendations are included in the present work. Initially, it is suggested to enlarge the number of sites for sample samplings, beyond the ones chosen in the present work. This will give a broader spectrum of microbial species present in the petroleum industry, other than the ones here selected. Beyond this, authors also recommend the evaluation of microbial species in a higher number of samples for each site, due to the associated uncertainty in the evaluation of some microbial groups.

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