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Aerobic Bacteria Induce Corrosion

K. Y. Mataqi[1], Research Scientist
Biotechnology department
Kuwait Institute for Scientific Research
Shuwaik, Kuwait
E-mail:kmataqi@kisr.edu.kw

B. Mathew[2], Research Assistant
Biotechnology department
Kuwait Institute for Scientific Research
Shuwaik, Kuwait
E-mail: bmathew@kisr.edu.kw

***Abstract*—The aim of this study was to isolate, identify and study the effect of the aerobic bacteria (AB) that causes pitting corrosion on the mild steel. During this study, principal groups of AB were isolated from four locations in Kuwait. In total, corrosion principal groups were found, as well as other microorganisms were isolated and cultured. The noncorrosive or non-principal groups of microorganisms were also of high importance, since they could be pathogenic and may affect the health of the people working in this industry. A total of 17 samples were screened and cultured using the standard microbiological culture methods. As well as a highly specific diagnostic method, such as sequencing and the molecular identification was done during this study. The DNA was extracted from the pure bacterial cultures and the 16S rDNA was amplified which was then sequenced in order to give the optimum clarity of results. Moreover, although being the same genera, some of the principal groups were of different subtypes. These subtypes play an important role in corrosion; therefore, identifying the microorganism using conventional methods would have missed the presence of such subtypes and could have given false negative results which were possible through the DNA sequencing method. The bacterial principal groups showed to cause biofilm formation, spotting, and pitting to the mild steel used. Knowing the principal groups of corrosion would make the selection of biocides and decontamination methods much obvious and more useful to the petroleum industry.**

Keywords—aerobic bacteria; pitting corrosion; mild steel coupon; 16s rDNA amplification; petroleum; oil installations

I. INTRODUCTION

The petroleum industry has suffered great financial loss due to damage, maintenance and operational costs caused by microbial contamination in fuel storage tanks. In addition, funds have also been depleted on new capital equipment, modernization, and maintenance of refinery equipment that have been damaged by microbial corrosion. In order to reduce, prevent, or eliminate microbial proliferation, careful evaluation on the influence of microbial contamination in fuel systems is needed. [17].

The main types of AB associated with corrosion of cast iron, mild and stainless steel structures are sulfur oxidizing bacteria (5], iron oxidizing/ reducing bacteria [14], manganese oxidizing bacteria [6], and bacteria secreting organic acids and exopolymers or slime [21]. They can coexist in naturally occurring biofilms and often form synergistic communities that are able to affect electrochemical processes through cooperative metabolism not seen in the individual species [7].

The use of molecular markers to study the ecological roles of the AB diversity in oil sector tanks is necessary. The 16S ribosomal ribonucleic acid (RNA) gene is found in all the organisms and has both conserved and variable regions that make it suitable for designing specific primers and probes. In addition to its use as a unique molecular marker, it can be used to conduct phylogenic and evolutionary analysis of bacterial populations [13]. Molecular identification methods involve the use of polymerase chain reaction (PCR) techniques to amplify the specific regions of deoxyribonucleic acid (DNA) sequences by the use of specific primers [10].

Another advanced technique that can be used to study biofilms and biocorrosion phenomena is scanning electron microscopy (SEM) [3]. Presently, the SEM is considered to be one of the most reliable methods for detecting biofilm formation, bacterial adhesion, and corrosion at early stages. The microscopic techniques provide information about the morphology of microbial cells and colonies, their distribution on the surface, the presence of extracellular polymeric substances, and the nature of corrosion products. The type of attack (e.g., pitting or uniform corrosion) can also be studied by visualizing the changes in microstructure and surface features after the removal of the biofilm and corrosion products.

II. MATERIALS AND METHODS

A. Site locations and oil sample sources

Oil samples were obtained from four sites in Kuwait. A total number of 17 oil samples were collected from various sites in 500-ml sterile glass containers. Designated code, date, and location were assigned to each sample based on the type of the oil layer.

B. Detection and quantification of microbial activities

A total of 17 samples were screened for microbial activities by using the standard microbiological culture

method. Three different media were used for samples screening such as nutrient agar (NA), which is a broad spectrum bacterial medium; MacConkey Agar, and selectively enhanced gram-negative bacteria. All media were prepared using deionized water and were sterilized by autoclaving at 121^{o}C for 20 min. A 0.1 ml of each sample was spread onto plates with different media in duplicates. The samples were incubated at 37°C for AB. The plates were examined after 1 to 7 d of incubation. The colonies were aseptically picked and streaked onto plates from the same media for pure isolates. Pure isolates were cultured, stained, and examined under light microscope.

III. Aerobic bacterial isolates molecular identification

A. *DNA extraction from bacterial pure cultures*

Genomic DNA was purified from each pure aerobic bacterial culture using the Wizard Genomic DNA purification kit as recommended by the manufacturer (Promega). DNA was quantified by fluorometry with a model TK 100 fluorometer (Hoefer Scientific Instruments) by using the extended assay protocol of the manufacturer. DNA extracts were then stored at −20°C prior to amplification.

B. *Amplification of 16S rDNA gene*

For the purpose of identifying isolated bacteria, 16S rDNA gene was amplified from extracted bacterial DNA by PCR using 27F (5`-AGAGTTTGATCACTGGCTCAG-3`) and 1492R (5`-GAATTCTACGGYTACCTTGTT ACGACTT-3`) primers. All reactions were carried out in 25-µl volumes, containing 12.5 pmol of each primer, 200 µM of each DNA triphosphate, 2.5 µl of 10x PCR buffer (100-mM Tris-HCl, 15-mM $MgCl_2$, 500-mM KCl; pH 8.3), and 0.5 U of Taq DNA polymerase (ABI), increased to 25 µl with sterile water. PCR was performed in a thermocycler (ABI) with the following thermocycling program as follows: 5-min denaturation at 95°C, followed by 30 cycles of 1-min denaturation at 95°C, 1-min annealing at 55°C, 1-min extension at 72°C, and a final extension step of 5 min at 72°C. PCR products were visualized by electrophoresis in 2% (wt/vol) agarose gels and with ethidium bromide (0.5 µg ml^{-1}) staining.

C. *Sequencing of amplified rDNA*

The amplified 16S rDNA sequences from extracted genomic DNA from bacterial pure cultures were purified by Nucleospin Extract II kit (Macherey Nagel, USA) followed by sequencing PCR using a big dye terminator cycle sequencing kit according to manufacturer's instructions (ABI). The mixtures were incubated in a thermocycler at 96°C for 1 min followed by 25 cycles of denaturation at 96°C for 1 min, annealing at 50°C for 5 s, and extension at 60°C for 4 min. The final products were further purified, using 3 µl of 3-M sodium acetate pH4.6, 62.5 µl of non-denatured 95% ethanol and 14.5 µl of deionized water. Samples were incubated in the dark at room temperature for 20 min before being centrifuged at 15,000 xg in room temperature for 20 min. The pellet formed was washed with 250 µl of 70% ethanol and recentrifuged at 15,000 xg at room temperature for 5 min. The pellets were air-dried for 15 min in the hood, resuspended in 20 µl of Hi-Di-Formamide, and loaded directly in the 3130xl Genetic Analyzer (Applied Biosystems, USA).

D. *Identification of isolated aerobic bacteria*

The 16S rDNA sequences obtained were run against the databases using the basic alignment search tool (BLAST). Sequences were assigned to recognized representatives of the main eubacteria based on scores of 97% or higher.

IV. Detection of microbe-corrosion on the mild steel

A. *Coupon preparation*

Coupons of steel (10x5x5 mm) were cut on screw shape, polished with abrasive paper, defatted with alcohol, weighed, and placed in test tubes. The coupons were then sterilized either at 120^{o}C for 30 min or washed with 100% ethanol and flamed.

B. *Corrosion of mild steel by aerobic bacteria principle groups in carbon source media*

Nutrient media were distributed in 50ml sterile screw capped vials; in each vial a sterile, previously weighed coupon was placed. The vials were then enriched with bacteria at a concentration of 1:25 and 1:50. For each location, the isolated bacteria from that particular site were incubated individually, and the changes in coupon weight were measured every 5d upto 30d.

C. *The role of aerobic bacteria principal groups on mild steel corrosion using diesel as a carbon source*

Four genera of bacteria were identified and used in this experiment as principal bacterial groups which included *Bacillus* sp., *Branchybacterium* sp., *Staphylococcus warneris* and *Kocuria rizophila*. In this experiment, sterile diesel (200 ml) with 2% water was used as control system, while diesel (200 ml) with 2% water and 10^6 colony forming unit (CFU)/ml of the inoculate (2 ml) were used as sample. Coupons were placed in all flasks, and the weight loss study was performed in duplicates after 7, 14 and 21d, the coupons were then removed, washed and soaked with 1M HCl. The final weights of air-dried coupons were taken and the corrosion rates were calculated using the weight loss formula.

D. Measurement of coupon weight loss

The corrosion rate was determined using coupon weight measurement after incubation with the principle AB. The coupons were washed with water and incubated with 1N HCl for 1−2 min. This treatment could quickly remove the corrosion products without reducing metal weight. The coupons were weighed on BP210S scales (Sartorius, Germany)

E. Determination of coupon weight loss

The coupons were incubated with bacterial groups and the percentage of weight loss of the coupons were measured using the following formula

$$\% \text{ of weight lost} = \frac{\text{Weight of the coupon after incubation}}{\text{Weight of the coupon before incubation}} \times 100$$

For example, 0.3% means that for every 100g of metal, there would be 0.3g loss. Therefore, for each kilogram (1000g), there would be a 3g loss.

F. Detection of corrosion on coupons using electron probe micro analyzer

The images of mild steel coupons that were exposed to the AB were taken using electron probe micro analyzer (EPMA) that allows a much higher resolution images, JXA 8230-Japan, with magnification range from 40 to 30000 times.

V. RESULTS

A. Bacterial growth under aerobic conditions

All 17 samples were cultured on the aforementioned three different types of media, and their growth periods were analyzed. Since each media has selective growth properties, this gave insight into the potential identity of the unknown microbial species within the samples, based on the biochemical and physiological conditions favoring their growth.

Since nutrient agar (NA) had been used for a broad range of bacterial growth, most of the samples showed a growth in such medium. In addition, under aerobic condition with 37°C incubation temperature, it may be the optimal temperature for bacterial growth, as less time was taken for colonies to appear on the NA plate (Table 1). Samples obtained from Sabhan two diesel tanks and two ultra-super tanks showed no microbial growth. This indicated that Sabhan refinery has been maintaining proper microbial control over these three tanks.

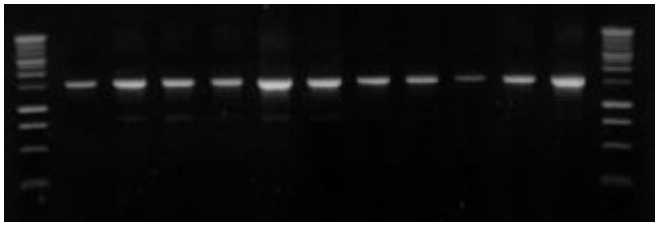
B. Microbial counts

The screening of the 17 fuel samples resulted in the detection and isolation of 33 aerobic bacteria. This demonstrated that in each sample, multiple types of bacteria could coexist in the same tank environment and reside symbiotically. Such symbiotic relationship may in turn lead to potential problems such as fuel tank degradation.

TABLE 1 INCUBATION PERIOD AND MICROBIAL GROWTH

Incubation period (days)	
Sample Code	**NA Aerobic**
MMA1	4
MMA2	4
Tank 251 Diesel (1)	-ve
Tank 251 Diesel (2)	-ve
Tank 223 Super (1)	5
Tank 223 Super (2)	8
Tank 224 Premium (1)	4
Tank 224 Premium (2)	8
Tank 231 Ultra Super (1)	-ve
Tank 231 Ultra Super (2)	-ve
CB-34-413	3
CB-34-429	5
CB-34-412	7
CB-34-416	7
CB-34-428	14
CB-34-427	3
CB-34-414	7

-ve: Indicates no growth, Nutrient Agar (NA) aerobic conditions at 37°C.



C. Molecular analysis of isolated aerobic bacteria

Figure 1 The 1.5 % agarose gel represents the 16s rDNA gene amplification with the 1500bp molecular size after PCR on bacterial DNA

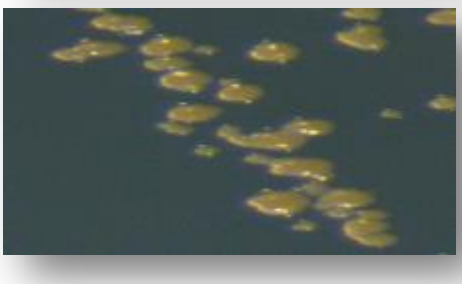
samples Lane 1: 1kbp DNA ladder; lane 2: sample 1; lane 3: sample 2; lane 4: sample 3; lane 5: sample 4; lane 6: sample 5; lane 7: sample 6; lane 8: sample 7; lane 9: sample 8; lane 10: sample 9; lane 11: sample 10; lane 12: sample 11; lane 13: 1kbp DNA ladder. The gel was electrophoresed for 120 min at 80 volts

Individual colonies were selected for DNA analysis based on their source of origin for comparative reasons. Moreover, colonies from different growth media were also selected for characterization to increase the possibility of identifying diverse microbial species. Selected aerobic bacteria isolates were characterized based on 16S rDNA sequences of 1500-bp amplified fragment as shown in Fig. 1. A total of four different bacterial genera were identified that included *Bacillus* in abundance than the others. The most predominant bacterial genera found in all samples were the gram positive bacteria *Bacillus* and *Staphylococcus.*

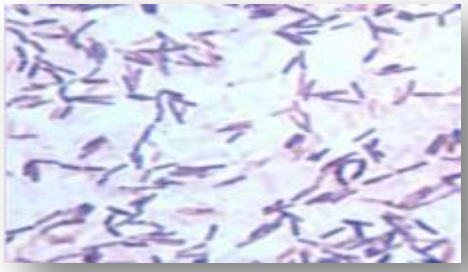
The 16s rDNA sequencing showed a number of bacterial strains collected from Kuwait oil refineries (Table 2). Among them, 57% of bacteria isolated from Al-Shuaiba storage tank were *Staphylococcus sp;* 40% were *Bacillus sp;* and 3% were *Kocuria*. Among the *Staphylococcus* isolates identified, were *S. pasteuri, S. warneri*, and S. *haemolyticus.* Furthermore, members of *Bacillus sp* genus identified included *B.subtilis, B.pumilus*, and *B. licheniformis*. On the other hand, bacteria isolated from Al-Ahmadi refinery storage tank showed higher percentage for *Bacillus* 50% and the other 50% was divided equally between *Staphylococcus warneri* and *Brachybacterium* sp. Also in Al-Ahmadi local market, the *Bacillus* sp. viz *B.subtilis* and *B.licheniformis*, as well as *staphylococcus* sp. and *S.pasteuri* accounts were distributed equally (50% each). In Sabhan refinery storage tanks, *Staphylococcus* sp., *S.warneri*, and *S.epidermidis*, accounted for 53% of identified bacteria. *Bacillus* sp. and *B.licheniformis* accounted for 41%, and the remaining 6% accounted for *Kouria rhizophila* sp. Images of each bacterium are shown in Fig. 2.

a. *Brachybacterium* sp. at magnification 100X

b. *Brachybacterium* sp. at magnification 6.3X

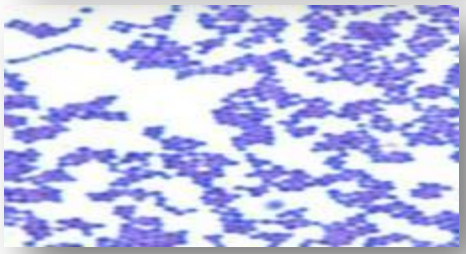


c. *Bacillus* sp. at magnification 100X



d. *Bacillus* sp. at magnification 6.3X

e. *Staphylococcus* sp. at magnification 100X



f. *Bacillus* sp. at magnification 6.3X

Figure 2 Images of isolated bacterial samples and their colonies

TABLE 2 THE PRINCIPAL GROUPS OF BACTERIA

Sample Code	Bacteria Type
MAA1-b	*Staphylococcus warneri*
Tank 223 Super (1)	*Staphylococcus sp.*
Tank 224 Premium (2)	*Bacillus sp.*
CB-34-412	*Staphylococcus haemolyticus*
Tank 224 Premium (2)	*Bacillus subtilis*
Tank 223 Super (2)	*Kocuria rhizophila*
Tank 224 Premium (1)	*Staphylococcus pasteuri*

Tank 223 Super (4)	*Staphylococcus epidermidis*
CB-34-416	*Bacillus pumilus*
MAA2	*Brachybacterium sp.*

D. Detection of aerobic bacteria induced corrosion on mild steel using nutrient media as carbon source

The effects of corrosion produced by the isolated AB on the mild steel were studied. Table 2 lists all the AB used. The metal coupons that were placed in 50ml sterile screw capped vials containing rich nutrient media and glucose as a carbon source showed considerable weight loss of 0.15% to 0.3% as per the formula as well as corrosion to an extent when analyzed using the electron probe micro analyzer.

E. The influence of aerobic bacteria principal groups on mild steel corrosion using diesel as a carbon source

The four bacterial principal groups; namely, *Bacillus* sp., *Branchybacterium* sp., *Staphylococcus warneris,* and *Kocuria rizophila* all showed certain types of corrosion which included small pitting, large pitting, color changes which indicated the initiation of corrosion and biofilm formation (Fig. 3). In addition to this changes in coupon weight were also observed.

F. Assessing corrosion in diesel as a carbon source using EPMA

The contribution of AB to corrosion was assessed using electron probe micro analyzer (EPMA) microscope. EPMA examination of corrosion on mild steel coupon surfaces is useful when detecting corrosion.

Coupons were incubated for 14 d in diesel medium. The images of coupon after incubation with *Bacillus* sp. showed changes in metal color (spotting) which indicates the initiation of corrosion. As well as evidence of small corrosion pits was clearly observed where spotting and small pitting became larger as corrosion progressed. The images in Fig. 3 show localized attack on the coupon's surface. Secretions of extracellular polymeric substances by *Brachybacterium* could lead to a development of biofilm layer and bacterial attachments on the stainless steel coupon.

The image also showed localized attack on the coupons, in which *Staphylococcus warneris* corrosion induction on mild steel is clearly observed. Also, fiber-like formations, indicating biofilm initiation which can cause potential corrosion were observed. The change in color indicates the beginning of pitting. The control coupons showed a uniform texture without any noticeable changes or any corrosion forms (Fig. 3).

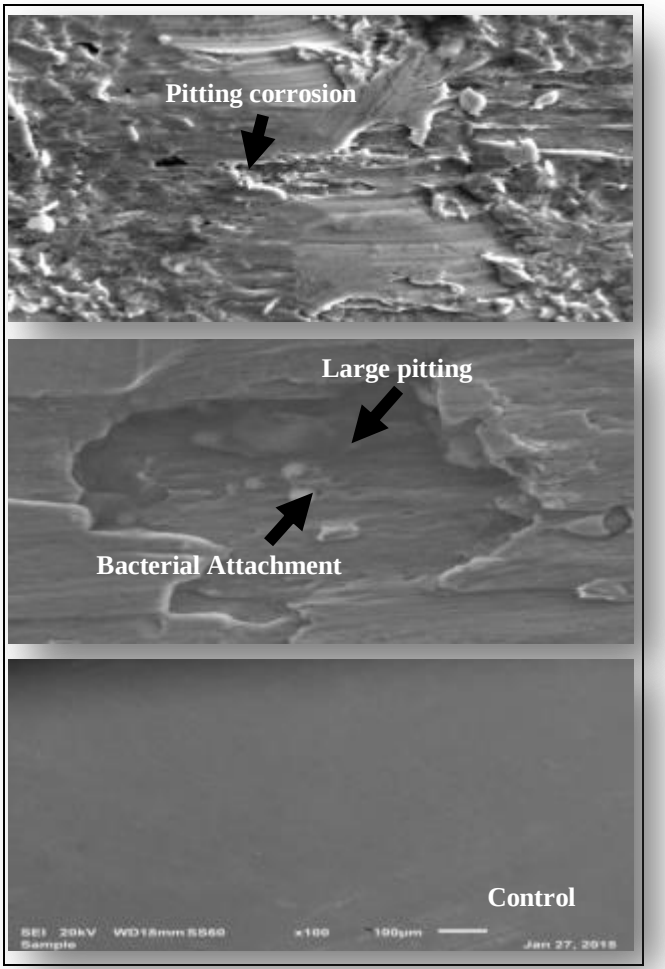


Figure 3 Images of mild steel coupons after exposure with aerobic microbes captured by electron probe micro analyzer (EPMA) micrograph at3000X magnification power and the control.

G. Assessing corrosion in diesel as a carbon source using weight loss

The contribution of microorganisms to corrosion was assessed using weight loss measurements in which the coupons weight was observed with various bacteria; isolates at different incubation periods (Fig. 4).

The coupons incubated with *Bacillus* sp showed a 0.15% decrease in stainless steel coupon weight. This indicated that *Bacillus* sp can cause a loss of 1.5 g/kg in the alloy within 21 d. These results are supported by EPMA images which showed evidence of large corrosion pits (Fig. 3). Coupon incubation up to 28 d resulted in the formation of deeper corrosion pits, as well as extracellular polymeric substances as a result of biofilm formation that were sharply visible.

For the coupons incubated with *Brachybacterium* sp., no significant decrease in weight was observed. However, the images obtained from EPMA for this coupon showed bacterial attachment and biofilm formation. Upon increasing the magnification power, large pitting corrosion was noted on the coupon surface. Coupon incubation up to 28 d resulted in deeper corrosion pits and extracellular polymeric substances, as well as bacterial attachment were observed more clearly (Fig. 3).

For the coupons incubated with *Staphylococcus warneris* sp, a significant decrease in weight was observed. There was a 0.31% decrease in the coupons weight. This means that there was a weight loss of 3.1 g/kg in the alloy within 21 d. This is supported by EPMA images which showed pitting corrosion (Fig. 3). As the coupon incubation time increased to 28 d, deeper pitting corrosions were observed as well as the formation of extracellular substances.

In Fig. 4, diesel was used as a carbon source, in which the effect of the four bacterial principal groups on stainless steel coupon weight was monitored over incubation periods. For the control sample, there was no change in the coupon weight throughout all incubation periods. In addition, examination of EPMA images confirmed that there were no occurrences of corrosion on the control coupon surface.

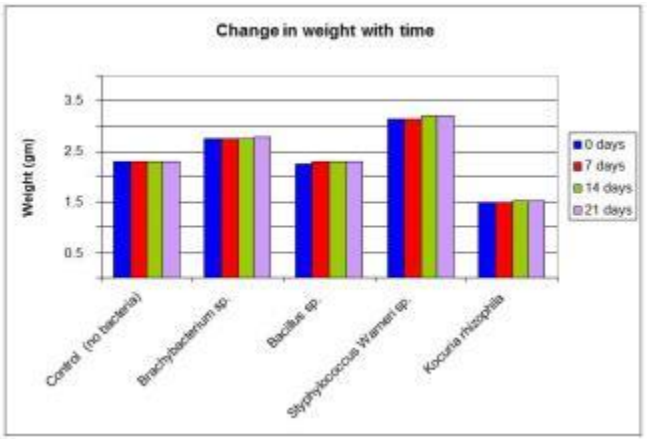


Figure 4 The effect of bacterial principal groups on coupons weight over time. The four principle bacterial groups were incubated in diesel medium with stainless steel coupons for 0, 7, 14, and 21d. The control does not contain any microorganism in the medium.

A decrease of 0.15 % in coupon's weight was observed with *Kocuria rhizophila* incubation. Therefore, there was a weight loss in the alloy by 1.5 g /kg after 21 d. This is further supported by EPMA images which showed bacterial attachment, small pitting as well as large ones. Coupon incubation up to 28 d resulted in larger pitting, and the observation of bacterial attachment was noticed.

VI. Discussion

A major issue in the petroleum industry is the growth of microbes in fuel tanks which could lead to refinery operational challenges. In the last few years, the rate of MIC occurrence and severity appeared to have been significantly increasing [9; 12]. In fuel storage tank, microbial contamination had been well-studied [11]. Studies on bacterial enumeration and identification in fuel tanks showed the occurrence of several bacterial species as follows: *Staphylococcus* sp*. Serratia marcescens*, *Bacillus subtilis, Bacillus cereus, Pseudomonas aeruginosa, Klebsiella oxytoca, Pseudomonas stutzeri, Bacillus litoralis, Bacillus pumilus, Bacillus carboniphilus, Bacillus megaterium, Pseudomonas* sp*., Bacillus* sp*., Gallionella* sp*., Legionella* sp*., and Vibrio* sp*., Thiobacillus* sp*., Thiospira*, and *Sulfolobus* sp.

In this study, many principal groups of AB were isolated and can result in blockage of pipes, valves, and filters, and can also lead to the formation of sludge and surfactant, corrosion of tanks and pipe lines. [11; 17; 20].

All isolated AB were gram-positive, and the generic distribution was found to be *Staphylococcus* sp. (51%); *Bacillus* sp. (43%); *Kocuria rhizophila* (4%); and less than 2% for *Brachybacterium* sp. Rajasekar, Ponmariappan, Maruthamuthu and Palaniswamy (2007) [16] suggested that gram-positive bacteria are more aggressively involved in the corrosion of steel which was in line with the study isolates. In addition, *Staphylococcus* sp. and *Bacillus* sp*.* contribute to biofilm formation by producing polysaccharide intercellular adhesion facilitating the attachment of microorganisms [8].The present study indicated that *Staphylococcus* sp. was the dominant species in the storage tank followed by *Bacillus* sp. Members of the genus staphylococcus are nonmotile, gram-positive cocci that usually form irregular clusters [15]. Both bacteria utilize hydrocarbon as a sole carbon source [16] and in their presence in fuel tank, pipeline would lead to the reduction in the quality of fuel, and in turn, financial loss. Therefore, these four isolated bacterial types were used in these experiments as the bacterial principal groups. The selection of biocides is important to the petroleum industry. Bacterial reaction to biocides is basically determined by the nature of the chemical in the biocide and the type of organism involved. Bacterial spores of the genera *Bacillus* sp. are the most resistant among the different types of bacteria to biocides [4; 19]. As a result, identification of the AB communities within fuel tanks is essential for the potential control of MIC.

Both microbiological and molecular-based identification of AB isolates were performed in this study which confirmed the presence of various bacteria in Kuwait fuel storage tanks. Identification of AB based on ribosomal/ ITS (internal transcribed spaces) DNA sequences is gaining importance due to the improvement in sequencing technologies and availability of sequence information for a large number of DNA sequence [1]. The current study clearly demonstrated the effectiveness of sequencing-based methods for the rapid identification of AB in oil refineries' storage tank. The occurrence of these microorganisms in storage tank has suggested that the microbial control methodology used in these refinery needs to be adjusted.

Furthermore, the generic principal groups of bacteria (*Staphylococcus* sp*, Bacillus* sp*, Kocuria rhizophila* and *Brachybacterium* sp) were grown in diesel as a carbon source in addition to nutrient rich

media in order to mimic what is happening in fuel tank in which these bacteria actually live.

Earlier studies showed that most cases of microbially induced corrosion are associated with localized attacks. The interactions of microorganisms with metals lead to pitting, crevice attack, and stress corrosion cracking. EPMA images of mild steel showing localized attack, following exposure to *Pseudomonas* sp and *sulfate-reducing* bacteria have been obtained in studies done by Beech & Gaylarde [2]. In this study, the working team showed that *Staphylococcus* sp*, Bacillus* sp, *Kocuria rhizophila,* and *Brachybacterium* sp principal groups isolated from different Kuwait storage fuel tanks were able to cause pitting, local spotting (start of corrosion), and biofilm formation.

Earlier studies showed that most cases of microbially induced corrosion are associated with localized attacks. The interactions of microorganisms with metals lead to pitting, crevice attack, and stress corrosion cracking. EPMA images of mild steel showing localized attack, following exposure to *Pseudomonas* sp and *sulfate-reducing* bacteria have been obtained in studies done by Beech & Gaylarde [2]. In this study, using EPMA on the same pattern of corrosion indicated by Beech & Gaylarde [2] was found. Moreover, in this study, the project team showed that *Staphylococcus* sp, *Bacillus* sp, *Kocuria rhizophila,* and *Brachybacterium* sp principal groups isolated from different Kuwait storage fuel tanks were able to cause pitting, local spotting (start of corrosion), and biofilm formation (Fig. 4).

Rossum [18] discussed the increase and decrease in pH values and its influence on corrosion process, showing its importance in corrosion occurrence and progression. In addition to pH changes, the effect of high temperature on accelerating corrosion rates were discussed which may play an important role in understanding what is happening in reality. Thus in Kuwait, in addition to pH changes caused by the microorganisms, the high temperature may facilitate the corrosion process. Hence, it is important to monitor and control any pH changes all the time.

During this study, principal groups of AB were isolated from four locations in Kuwait (Al-Ahmadi Refinery, Sabhan Refinery, Al-Shuaiba, and Al-Ahmadi local market). In total, corrosion principal groups were found, as well as other microorganisms. The noncorrosive or nonprincipal groups of microorganisms were also of high importance, since they could be pathogenic and may affect the health of the people working in this industry.

Moreover, although being of the same genera, some of the principal groups were of different subtypes. These subtypes play an important role in corrosion; therefore, identifying the microorganism using conventional methods would miss the presence of such subtypes and could give false negative results. Furthermore, the need for a highly specific diagnostic method, such as sequencing, which was done during this study, is highly important.

The bacterial principal groups showed to cause biofilm formation, spotting, and pitting to the mild steel used. Knowing the principal groups of corrosion would make the selection of biocides and decontamination methods much obvious and more useful to the petroleum industry.

VII. Acknowledgements

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