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Microbiologically induced corrosion (MIC) causes the degradation of coatings and it can be attributed to either direct or indirect microbial metabolic activity. In this study, we report the ability of sulphate reducing microorganisms (SRM) and marine strain bacteria to attach onto UNS1008 carbon steel, and zinc rich epoxy coatings with a content of carbon nanotubes (CNTs) respectively. In aerobic conditions the outer layer presents a micro-crack appearance and many semi-sphere products, attributed to spore formation. In anaerobic conditions, evidence of iron sulphide surrounded by a mixture of sulphur-containing extracellular polymer substance was observed by SEM images and EDS analysis. The presence of hybrid coatings (Zn rich epoxy with CNT content) affected the level of microbial adherence and the concentration of corrosion products (Fe_2O_3, $Fe(OH)_2$, FeS); the cell attachment was lower when the steel surface was coated with Zn/CNTs.

Keywords: sulphate-reducing microorganisms, marine microorganisms, zinc rich epoxy coatings, carbon nanotubes, EDS.

Introduction

Biocorrosion or microbially influenced corrosion (MIC) is the damage caused on metal surfaces by microorganisms due to their metabolic activities. Some organisms associated with metals in terrestrial and aquatic habitats are sulphate-, iron- and CO_2-reducing bacteria, sulphur-, iron- and manganese-oxidizing bacteria [1]. Among them, sulphate-reducing bacteria (SRB) are recognized as a major group of microorganisms associated to anaerobic corrosion. These microorganisms can coexist in naturally occurring biofilms with a wide bacterial community including fermentative bacteria, often forming synergistic communities (consortia) that are able to affect electrochemical processes through co-operative metabolism [2].

The biocorrosion process may be recognized by a combination of events: corrosion, presence of microbial slime masses, presence of hydrogen sulphide and ferrous or ferric hydroxide, mainly in anaerobic systems [2]. A wide variety of bacteria have been isolated

or detected in the petroleum industry, but because of their detrimental effects, sulphate-reducing bacteria (SRB) have been the most commonly studied group. Concerning the mechanisms of action, most of the basic theories on electrochemical corrosion are relevant to biocorrosion and could be employed to interpret the acceleration of the corrosion in different media both in anaerobic or aerobic conditions. Most coatings in industry used for microbiologically influenced corrosion (MIC) control are designed to provide an effective barrier against corrosion processes and biocidal effect to inhibit either or both conditions. They have been synthetized in organic, inorganic or hybrid schemes; however, some of them have been degraded when exposed to MIC due to the microbial attack, either as a consortium or specific bacteria strain, specially under anoxic conditions, like marine water or in oil and gas pipelines [3]. Recently, many researchers have reported investigations of the action of Sulfate Reducing Bacteria (SRB) or Sulfate Reducing Microorganisms (SRM), characteristic of anaerobic substrate conditions when exposed to epoxic coatings [4,5]. SRM-induced biocorrosion, associated to anoxic sulfate-rich environments is recognized the severe corrosion damage they can cause. Nowadays, environmentally-friendly coatings have improved their physicochemical and geometric properties, especially for resistance to microbiological corrosion attack. A new generation of coatings denominated “hybrid nano-architected sacrificial coatings” has emerged in a context of double control protection mechanism: the galvanic or cathodic protection, by the incorporation of active particles within the coating; and a barrier effect due to the presence of the polymeric matrix itself. Inclusion of -nanoadditives can improve both properties [6,7]. Such an additive, like carbon nanotubes (CNTs) influences the interconnectivity of active particles, and synergistically, can act filling spaces formed during the fabrication process of the coating within the polymeric matrix, as shown by Cubides and Castaneda when using zinc rich epoxy coatings with contents of CTNs [7].

In the present study, impedance analysis, scanning electronic microscopy (SEM) and energy-dispersive X-ray spectroscopy (EDS) were used to evaluate the adherence, biofilm formation and corrosion effect of SRM and marine bacteria onto UNS1008 carbon steel anaerobic and aired conditions, respectively. And the anticorrosive performance of hybrid nanostructured coatings on UNS1008 coupons against marine bacteria.

Materials and Methods

Cultivation of microorganisms

The SRM were isolated from samples collected with instrumentation from the interior of pipelines. The consortium was inoculated in an electrolyte known as ATCC Medium 1249 for Desulfovibrio desulfuricans [8]. The pH value was adjusted to 7.2 after de-aeration using nitrogen. The medium was autoclaved at 120 °C for 15 min. The SRM consortium was incubated at 37 °C.

The marine strain was isolated from samples collected from the Mexican Gulf at a depth of 1500 m. Sediment sample was inoculated in nutrient broth with 0.8 % NaCl (NB-NaCl) for 24 h and the strain was isolated in nutrient broth agar plates for isolation of single colony. The strain was maintained in glycerol 15 % at -80°C. The cells were cultivated on NB-NaCl for 24 h at 21 ºC for all the tests performed.

Hybrid coatings

Zinc rich epoxy base coatings with CNTs as additives were used. Substrate UNS1008 steel is the common base alloy for all the aerobic and anaerobic samples. All the

experiments were carried out in triplicate and the controls (without cells and the abiotic medium) were included for the analysis.

Impedance Analysis

Experiments were performed by using a 50 mL three-electrode glass cell. The coating/substrate samples and bare steel samples are considered as the working electrode with dimensions of 10 x 11 mm (0.39 x 0.43 in) and a 3 mm width rectangular case was mounted as working electrodes with an epoxy resin. The reference electrode is a saturated calomel electrode (SCE) and a platinum screen was used as a counter electrode. External and internal surfaces of the EIS glass cells were autoclaved. Working electrodes, reference and auxiliary electrode are sterilized with 70 % ethanol and acetone and set under UV light and laminar flow).

The electrochemical testing procedure consisted in a measurement of open circuit potential and electrochemical impedance spectroscopy for a period of 28 days. Open circuit potential was measured during 10 min prior electrochemical impedance spectroscopy measurements. EIS was performed at the OCP in a frequency range from 100 KHz to 10 mHz with 10 mV amplitude. All electrochemical experiments were performed by duplicate to ensure reproducibility. All tests were performed at 23 °C (room temperature). The electrochemical set up was performed on a potentionstat/galvanostat Biologic VSP 300.

Scanning electron microscopy sample preparation

Carbon steel coupons (UNS1008) and hybrid coatings with adhered cells were rinsed with PBS 1X (8.0 g of NaCl, 0.2 g of KCl, 1.4 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.2 g of KH_2PO_4 per liter, pH 7.2). Steel coupons (UNS1008) with adhered cells were rinsed with PBS 1X (8.0 g of NaCl, 0.2g of KCl, 1.4 g of $Na_2HPO_4 \cdot 2H_2O$, and 0.2 g of KH_2PO_4 per liter at pH 7.2) and fixed with four different methods (after washing): glutaraldehyde 2.5% w/v in PBS for 2 h (washing every 30 min), paraformaldehyde 4% w/v in PBS (washing every 30 min) and ethanol/acetic acid (3:1) for 10 min. All fixations were conducted at room temperature. After fixation, the cells were washed twice in PBS and then re-suspended, in sterilized ultrapure water to avoid salts crystallizing during the drying process and subsequent influence on SEM measurement. The samples were covered with gold and observed on JEOL JSM-700F field emission SEM for analysis.

Results and Discussion

Effect of fixation methods on sample preparation for SEM analysis

Figure 1 shows that different cellular structures from the SRM consortium were detected on SEM images after 38 incubation days, including extracellular polymeric substance (EPS) under different fixation treatments, and the corresponding EDS results show the corrosion products. The results suggest that the fixation methods could significantly affect the morphology of bacterial cell as well as the surface ultrastructure. The fixation methods containing alcohols such as ethanol/acetic acid (Figure 1A) are less suitable than those containing aldehydes (2.5% glutaraldehyde, and 4% paraformaldehyde; Figures 1B and 1C) for evaluating the cell morphology during corrosion processes. The cells fixed by 2.5% glutaraldehyde and 4% paraformaldehyde were preserved better than those treated with ethanol/acetic acid. This could be caused by the alcohols in these fixation solutions which could dissolve the membrane lipids, form large pores in the cell, causing

detachment [9]. The fixation methods applying aldehydes showed medium preservation ability for cell morphology judging from the images showed in Figure 2. It is postulated that aldehydes fixed cells by forming covalent chemical bonds between proteins and therefore could maintain the integrity of membrane lipids as well as the surface macromolecules [10]. In the present study, 2.5% glutaraldehyde showed the best performance for fixation with 4% paraformaldehyde. Paraformaldehyde, the polymerized form of formaldehyde, would be depolymerized to formaldehyde when dissolved; therefore, the 4% paraformaldehyde solution contained pure formaldehyde.

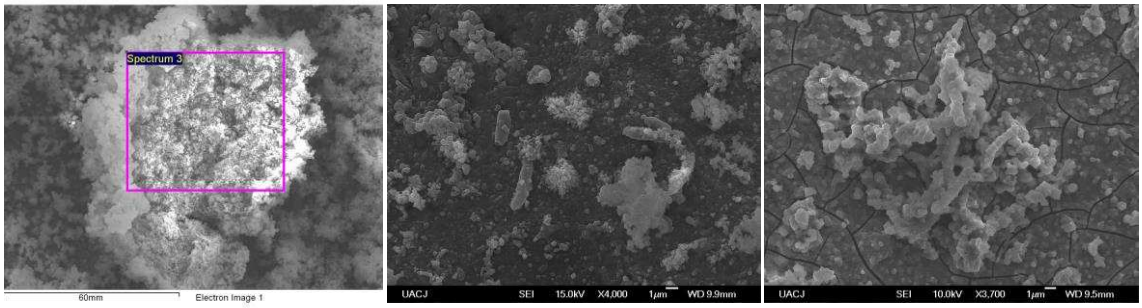

Fig. 1. SEM images of microorganisms over USN1008 steel surfaces. Coupons of SRM consortium cells fixation with ethanol/acetic acid (A), paraformaldehyde 4% w/v (B) or glutaraldehyde 2.5% w/v (C).

Compared with formaldehyde, glutaraldehyde could fix samples more tightly since it has a longer molecule and two aldehyde groups which has potential to link more distant protein molecules [11]. This might explain the superior performance of glutaraldehyde in fixing the bacterial filaments among the applied fixation methods.

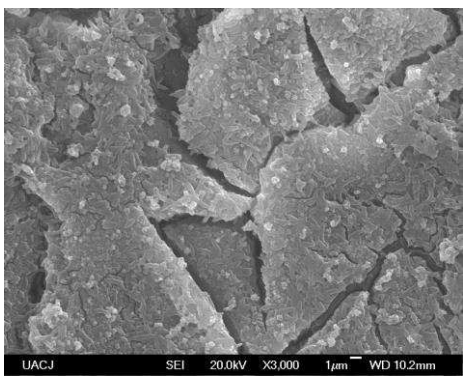

Fig. 2. SEM image of marine-strain MGG12 biofilm formed over UNS1008 carbon steel. Cells were fixed with glutaraldehyde 2.5% w/v.

SRM consortium adherence on UNS1008 steel coupons

Figure 3 shows SEM and EDS results for SRM consortia. Two kinds of the corrosion product layers were observed on the surface of the steel in the presence of SRM. The outer layer presented a micro-crack appearance. The inner layer under the detached film was a compact layer including many semi-sphere products which might be sulphur-free carbonates surrounded by a mixture of sulphur-containing extracellular polymer substances [12]. SRM were observed on the surface of the steel. As can be seen in Figure 3B, the corrosion products were mainly iron oxides and the element O should be ascribed to the iron oxides produced by the oxidation of sulphide when small quantities of oxygen enter the anaerobic incubator. These results are similar of those found by Sheng et al. [13]; they have concluded that the morphology of cells adhered to steel coupons has a significant influence on the corrosion, and when they are settled in a compact way they could act as a protective film on the metal surface.

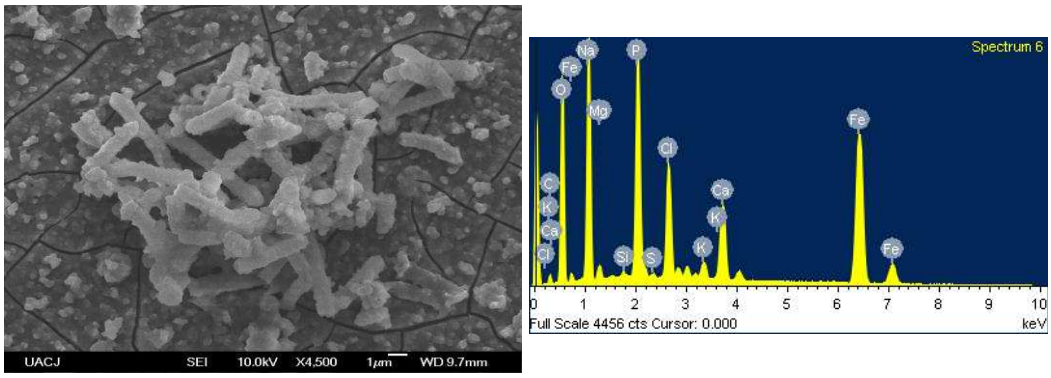

Fig. 3. SEM and EDS analysis for SRM consortia on UNS1008 steel surfaces, (A) SEM images of SRM treated with glutaraldehyde 2.5% w/v, (B) EDS results corresponding to the corresponding treatment.

Impedance Analysis

Figure 4 shows the Nyquist diagram and the phase angle representation for the interfacial mechanisms in electrolyte with SRM exposed to UNS1008. Figure 4(b) shows

the phase angle representation, where there are one maxima points, which is associated with the coating resistance during the electrolyte uptake process at medium frequencies. The time constant at medium frequencies at day 25, indicates the contribution of an extra layer formed on the coating surface, which can be attributed to the formation of a biofilm by the SRM consortium and corrosion products. The results suggest that the biofilm formed during the first 5 days and continued to grow until 15 days. After 15 days only one time constant keeps increasing in the phase angle representation, as shown in Figure 4(a).

As the SEM images in Figure 3 show the presence of a porous polysaccharide layer after 28 days of exposure. The low-frequency maximum point increased from approximately 20 degrees to 60 degrees and shifted towards low-medium frequencies. This can be attributed to a decrease of the active area due to the electrical contact between particles and their distribution, while the charge transfer promotes interface activity.

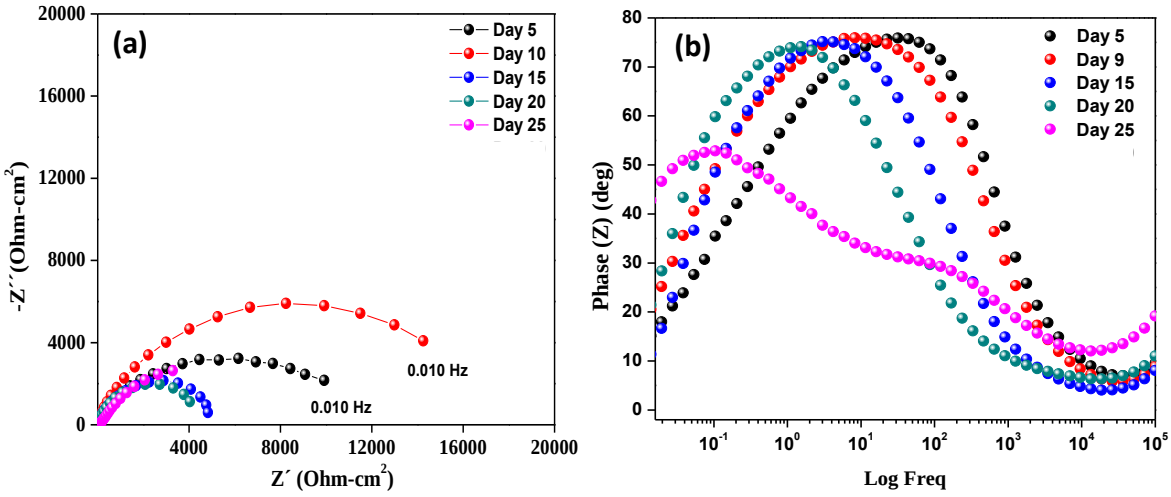

Fig. 4. Nyquist and Bode diagram of SRM onto G1008 carbon steel

Marine bacteria adherence on Zn hybrid coatings with CNTs

Zinc base coatings on steel surface are widely used to enhance their life time [6]. Zinc composite coatings are a field that have been increase interest among the oil industry. In these case the zinc metal is coated mainly with polymers and metal oxides, and their properties depend on electrochemical parameters that will confer functional properties like corrosion resistance [14]. To make these composites more attractive, they have been combined with nanoparticles due to their increasing availability and particularly the carbon nanotubes (CNTs) have attracted interest in different areas including applied perspectives [15]. Nevertheless, in corrosion studies there are few studies using CNTs in combination with metals, specifically zinc. In the present study, the effect of microbial adherence on zinc and zinc-CNTs surfaces, and their corrosion products were evaluated. Figure 4 shows marine stain adherences on the steel USN1008 coated with zinc (A) and Zn-CNTs (B) after 7 days of incubation under aerobic conditions. When the cells are in contact with the Zn base coatings, the resistance to the adherence is low, the cells are deposited on the steel on and under the coating layer and the corrosion products are in higher concentrations. However, when the cells are in contact with the Zn-CNT coating the resistance to the adherence is higher and in consequence, the attachment is low as it is showed in the Figure 4B. In this case, the corrosion products are at low concentration. Show et al [15] has shown the effectiveness of CNTs coatings on corrosion studies where weight loss measurements, salt spray test and electrochemical methods were matching with each other when the coatings were in contact with acidic solutions. Finally, when the microbial adherence is evaluated, the results are similar, the attachment is lower when the surface is coated with Zn-CNTs, likely due to the ability of CNTs of acting as physical barrier to the corrosion process by filling in crevices, gaps, and micro holes on the surface of deposit. During corrosion

processes, the Zinc is washed away, leaving the CNTs on the metal surface, reducing the corrosion rates and as result, the metal loss. Further studies in electrochemistry (impedance measurements) are necessary to confirm that the resistance of Zn-CNTs are higher that the Zinc on its own in corrosion processes.

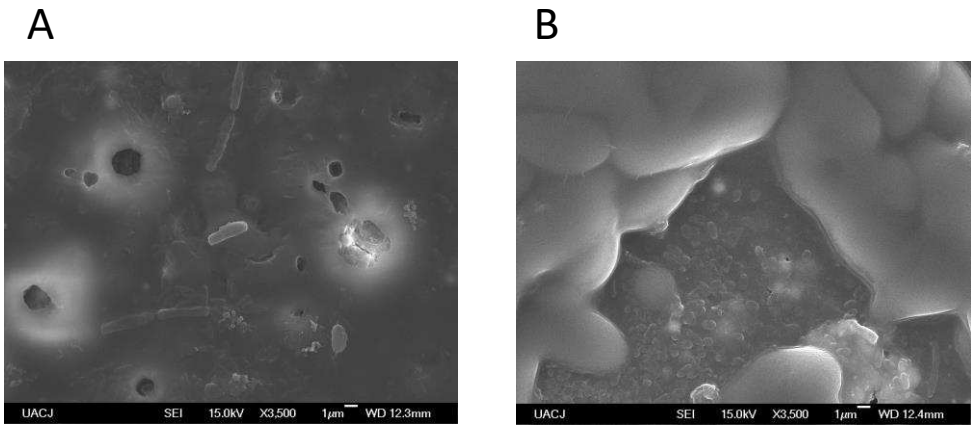

Fig.6. SEM images for marine strain MGG12 biofilm on USN1008 steel surfaces: (A) SEM images of marine bacteria on Zn coating and (B) SEM images of marine bacteria on Zn-CNTs coating.

Impedance Analysis

The complex diagram showed in Figure 5a for Zn rich epoxy shows a loop with a finite semicircle, an equivalent circuit represented in Figure 7b can describe the characteristics of this system by using electrical elements. The R_{ic} magnitude represents the resistance of the Zn particle reacting with the sterile electrolyte. The magnitude or charge transfer decreases meaning the reactive surface is increasing with time; more active particles are reacting while the bioelectrolyte is reaching more sites within the coating. When the CNT is added to the coating the electrical connection between particles produce a larger activation area, the charge transfer resistance prevails in this sample with a decreasing magnitude with time by comparing with the no CNT sample as it is shown in Figure 5b.

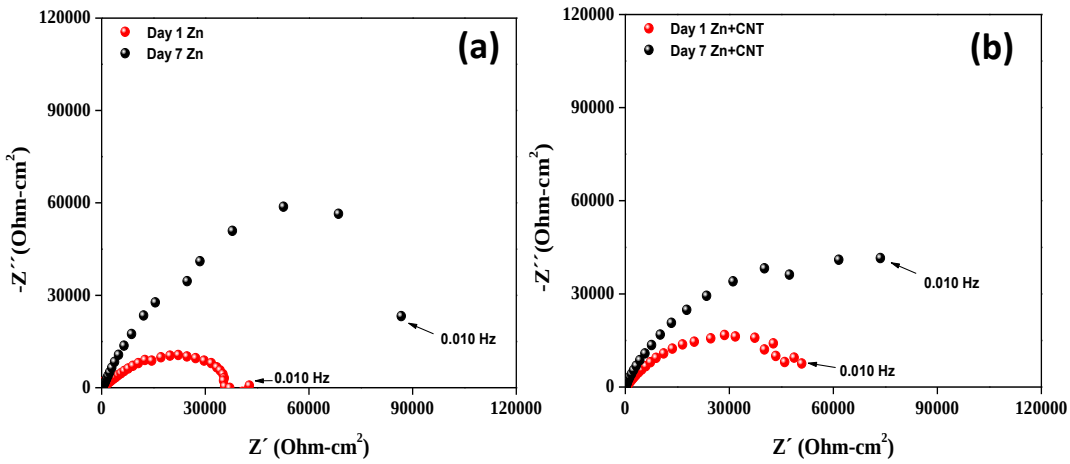

Fig. 5. Electrochemical Impedance spectra in Nyquist diagram obtained during 7 days of immersion with Zn hybrid coating and Zn+CNTs hybrid coating onto UNS1008 carbon steel in presence of MGG12 bacteria strain.

Table 1

Fitting parameters from equivalent circuit simulation for Zn and Zn+CNTs hybrid coating in presence of marine bacteria

MGG12	Time (day)	Rs (ohm cm^2)	R_{ic} ($R_{biofilm}$) (ohm cm^2)	Q_{ic} ($Q_{biofilm}$) (10^{-10}F cm^{-2})	n_b [1]	R_{ct} (ohm cm^2)	Q_{dl} (10^{-09}F cm^{-2})
Zn	7	$8.6x10^{-4}$	350	48.3	0.7	49855.4	55214
ZnCNT	7	23.5	207	31.7	0.6	36432.2	13256

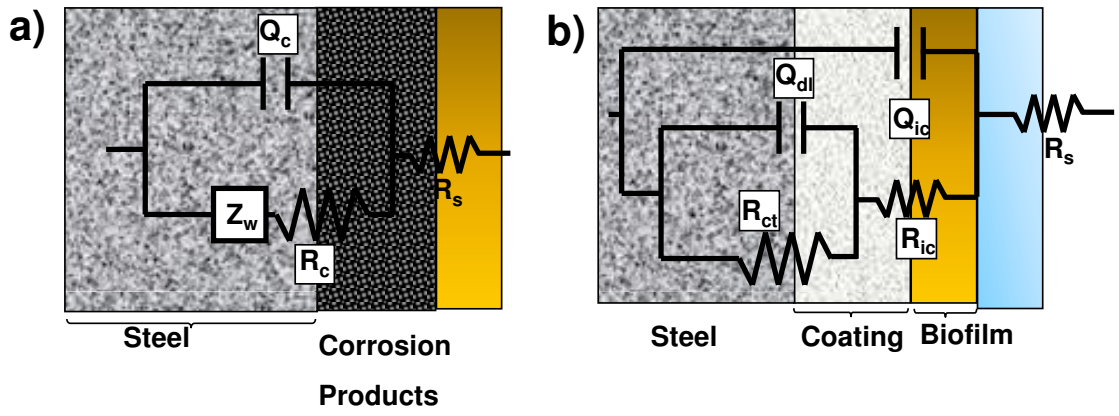

Fig. 6. Electrochemical analogs proposed during a) 25 days of immersion with UNS1008 carbon steel in presence of SRM. b) 7 days of immersion with Zn hybrid coating and Zn+CNTs hybrid coating onto UNS1008 carbon steel in presence of MGG12 bacteria strain.

Conclusions

The strategy applied in the present study was to evaluate different fixation methods to observe corrosion mediated by different microorganisms on aerobic and anaerobic conditions using scanning electronic microscopy. The glutaraldehyde (GA) 2.5% w/v was the more reliable method, either using marine consortiums or SRM. The artefacts were reduced and the different layers corresponding to corrosion were clearly visible (cracking, microspheres, EPS and cell morphology). For further evaluation, it would be ideal to include the critical dry point along with the GA as a fixation method, on steel as well as on surface with different coatings to observe the effect of microorganisms on corrosion mechanisms from a wider point of view. The microbial adherence on USN1008 was dependant on its nature. The nature of the composite affected the microbial adhesion, and as consequence the corrosion mechanism. The CNTs provided a barrier to the corrosion medium and the microbiological environment. This study opens a window for further evaluations of CNTs associated with metals as active materials to assess the corrosion on extreme corrosive environments (like oil and gas pipelines) were the microorganisms play an important role either, to increase or reduce the corrosion processes.

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