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Electrochemical Analysis of the Microbiologically Influenced Corrosion of AISI 304 Stainless Steel by Sulphate Reducing Bacteria Associated with *Bacillus Cereus*

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The associative effect towards the corrosion of AISI 304 stainless steel caused by the main two bacterial species present in marine environments around the Canary Islands (Spain) has been investigated using electrochemical techniques. The test environments were sterile conditions, with SRB (sulphate-reducing bacteria) *Desulfovibrio desulfuricans* (strain DSM 642) isolated from its natural marine environment, with *Bacillus Cereus var. mycoides* (CECT 193), and with a mixture of both. EIS data demonstrate the development of a synergic effect of both bacteria, where *Bacillus cereus mycoides* favours the development of SRB and, consequently there is a greater tendency to the progress of corrosion.

Keywords: stainless steel, Electrochemical Impedance Spectroscopy, microbiological corrosion, Sulphate Reducing Bacteria, Bacillus Cereus

1. INTRODUCTION

Bacterial colonisation on any surface is considered a bacterial survival strategy for its development in nature. Numerous examples have been described in marine environments, and in all cases the bacterial colonisation follows a sequence in stages: a) cell adhesion to the substrate, b) irreversible adhesion of extracellular polymers (EPS) which act as a bridge between the two surfaces, and c) growth as a result of the formation of microcolonies which can join by coalescence to the formation of the biofilm [1]. The microorganism induced corrosion (MIC) is related to modifications

in the electrolyte, such as changes in the pH, oxygen, or production of aggressive metabolites like sulphides, acids or oxidizing substances [2-12].

Biocorrosion of stainless steels can be catalysed by a series of metabolic activities of a wide range of microorganisms. Generally, the major bacteria involved in microbiologically influenced corrosion are anaerobic *sulphate reducing bacteria* (SRB) [13-16], whose role in corrosion processes was evidenced several decades ago [17]. These are obligate anaerobic microorganisms which carry out the reduction of sulphate with the formation of either sulphide, or mixtures of disulphide and hydrogen sulphide, and which are well known aggressive species for their corrosive action [18-23]. On the other hand, aerobic bacteria and fungi may also participate in the corrosion process [24-26]. In general, bacteria can oxidize a wide variety of chemicals and use them as nutrient source and enhance the proliferation of bacteria [27,28]. The microorganisms induce corrosion by altering the chemistry at the interface between the metal and the bulk fluid [29,30].

Bacteria with aerobic metabolism generally have a faster rate of development than anaerobic organisms, with a much higher metabolic activity. Consequently, they are organisms with greater potential in terms of the influence on the corrosion rate [31]. Therefore, most of the research on MIC has focused on SRB. However, recent studies suggest that SRB need not be present in abundance in every microbial communities responsible for MIC [32,33].

The bacteria *Bacillus cereus ACE4*, very similar to *Bacillus cereus* (99%), is the predominant species in the corrosive processes in diesel pipelines, in the degradation of the diesel itself, and in the corrosion of API 5LX steel [14]. Subsequent studies have demonstrated that the capacity for Cr(VI) reduction is widespread and found in such organisms as *Bacillus cereus* [34]. Electron transport systems to Mn(IV) have been investigated in several *Bacillus* species that reduce Mn(IV) [35-37]. However, the available evidence suggests that this Mn(IV) reduction is a minor side reaction that does not conserve energy to support growth for these organisms, and thus this metabolism may have little relevance to the bulk of dissimilatory Mn(IV) reduction in sedimentary environments [38]. The development of this type of bacteria is enhanced in the presence of oxygen. However, when the oxygen tension on the metallic surface decrease or disappear, the metabolism is now predominantly anaerobic and, therefore, the combination with SRB has a synergic effect which favours corrosion through alteration of the metallic surface [11,12].

This paper investigates a possible synergic effect for MIC by *Desulfovibrio desulfuricans* and *Bacillus cereus var. mycoides* (*strain CECT 193*), both simultaneously present in marine environments around the Canary Islands (Spain), which is performed using electrochemical techniques.

2. EXPERIMENTAL

AISI 304 stainless steel samples were processed from 8 mm diameter cylindrical rods. Cut sections of the steel rods were hot embedded in acrylic resin to form the test sample with an exposed area of 0.50 cm^2. The samples were ground with a sequence of emery papers ranging from 150 to 1000, and then polished with 3 μm alumina slurry.

The SRB (*strain DSM 642*) were isolated from seawater taken from the Atlantic Ocean on the south coast of the island of Gran Canaria (Canary Islands, Spain) [39]. An analysis of the seawater gave the following composition in g l^{-1}: chlorides 35.70, sulphate 2.55, sodium 10.6, sulphides 0.75, and other components in low concentrations. The measured pH was 8.14, and the temperature was between 20 and 22ºC. The SRB were grown using Postgate medium [40], made with sterile seawater, whose composition per litre was as follows: K_2HPO_4 0.5 g; NH_4Cl 1 g; $CaCl_2.2H_2O$ 1 g; Na_2SO_4 1 g; $MgSO_4.7H_2O$ 2 g, 70% sodium lactate 2g; yeast extract 2 g. This medium is partially selective for the SRB, with the sodium lactate as electron donor and source of carbon for growth. The enrichment medium was sterilised in autoclave for 15 minutes at 121 ºC. The SRB were inoculated in the enrichment medium and developed at 37ºC in anaerobic conditions, this being achieved by the application of a surface layer of sterile glycerine. In the case of the bacteria *Bacillus cereus var. mycoides* (*strain CECT 193*), the same techniques were employed, though in this case the enrichment medium was made from seawater which contains (per litre): meat extract 10 g, peptone 10 g, NaCl 5 g, bacteriological agar 15 g. This enrichment medium was sterilised in autoclave for 15 minutes at 121ºC. Four different conditions (test environments) were chosen for the study, namely:

a) Sterile seawater with sodium lactate.
b) Sterile seawater with sodium lactate + *Bacillus cereus var. mycoides*.
c) Sterile seawater with sodium lactate + *SRB.*
d) Sterile seawater with sodium lactate + *SRB* + *Bacillus cereus var. mycoides*.

The electrochemical tests were performed during the immersion of stainless steel specimens in the different environments during 3 weeks. A conventional three-electrodes electrochemical cell configuration was employed, using a saturated calomel electrode (SCE) as the reference electrode, a platinum mesh as the counter electrode, and the working electrode was the stainless steel specimen. The measurements were performed using an EG&G PAR potentiostat-galvanostat model 263A, and an EG&G PAR lock-in amplifier model 5210. The electrochemical impedance spectra (EIS) were recorded over the frequency range comprised between 10 kHz and 1 mHz, and the sinusoidal voltage signal being 10 mV around of the corresponding OCP, E_{corr}. The impedance spectra were analysed in terms of an equivalent circuit using the software developed by Boukamp [41].

3. RESULTS AND DISCUSSION

The bacterial count of SRB in the test environments was carried out using the Most Probable Number (MPN) method, while the plate technique was used for the *Bacillus cereus var. mycoides* (*CECT 198*) count. Table 1 lists the bacterial counts obtained from the four test environments determined every week.

Table 1. Bacterial count obtained during 3 weeks of immersion in Postgate medium and bacteriological agar, for sulphate reducing bacteria (SRB) and *bacillus cereus var. mycoides* (*CECT 198*), respectively.

Time (days)	SRB isolated from seawater (MPN/100 ml)	*Bacillus cereus var. mycoides* (cfu/100 ml)	SRB + *bacillus cereus*	
			(MPN/100 ml)	(cfu/100 ml)
0	$2 \cdot 10^8$	$12 \cdot 10^5$	$21 \cdot 10^3$	$4 \cdot 10^7$
7	$1 \cdot 10^6$	$5 \cdot 10^4$	$15 \cdot 10^3$	$6 \cdot 10^6$
14	$4 \cdot 10^4$	$4 \cdot 10^3$	$93 \cdot 10^2$	$4 \cdot 10^4$
21	$21 \cdot 10^3$	$1 \cdot 10^3$	$12 \cdot 10^2$	$3 \cdot 10^4$

The open circuit potentials, E_{corr}, of stainless steel samples immersed in the four test environments were measured at different times of exposure, and the data are listed in Table 2. The linear polarisation curves were also measured for the AISI 304 stainless steel after one, two and three weeks of immersion, thereby allowing the pitting potential, E_p, and polarisation resistance, R_p, values to be determined over three weeks of immersion (cf. Table 3).

Table 2. Corrosion potential values (E_{corr}) of 304 steel during 3 weeks of immersion in Postgate medium and bacteriological agar, for the study of SRB and *Bacillus cereus var. mycoides*, respectively.

Time (days)	Corrosion Potential, E_{corr} (mV *vs* SCE)			
	Without bacteria	*Bacillus Cereus*	SRB	SRB + *Bacillus Cereus*
7	-112	-123	-157	-136
14	-105	-122	+81	-192
21	-97	-107	-15	-190

Table 3. Results obtained through linear polarisation curves of AISI 304 stainless steel after 3 weeks of immersion in the four test environments.

	Without bacteria	*Bacillus Cereus*	SRB	SRB + *Bacillus Cereus*
E_p (mV *vs* SCE)	476	346	682	570
R_p (Ω cm^2)	177.9	290.3	276.1	835.1

The time evolution of the polarisation resistance values from AISI 304 steel specimens exposed to the four test environments under consideration can be observed from the inspection of Figure 1. A major increase of the polarization resistance of the metal occurs at the beginning of the experiments for the samples immersed in the test environments containing SRB, though it subsequently decreases with the elapse of time, eventually merging with the values occurring in SRB-free environments. It is also interesting to notice that *Bacillus Cereus* also originates a variation of the R_p values relative to those

determined in the blank condition, though its magnitude is significantly smaller than for SRB-containing conditions, and its maximum occurs at longer exposures (i.e. 2 weeks).

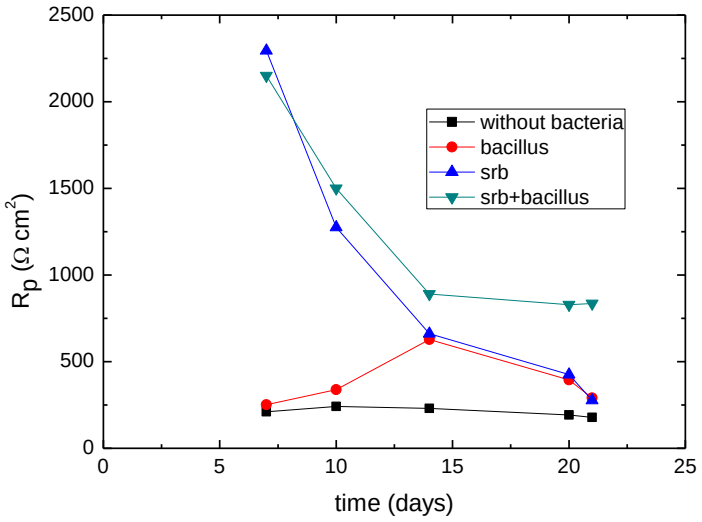
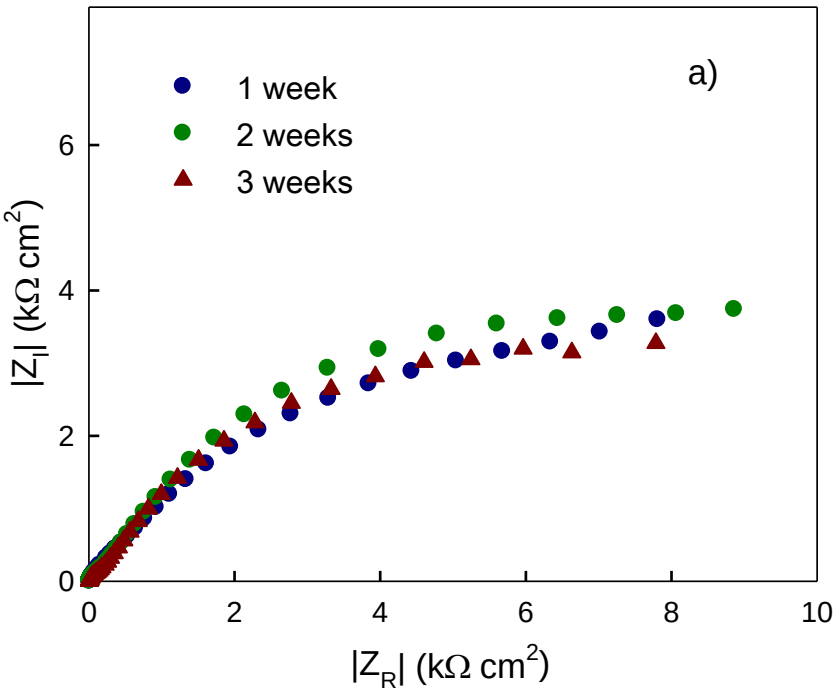


Figure 1. Evolution of the polarisation resistance (R_p) over 21 days of immersion of 304 stainless steel samples in the four test environments.

These observations concerning the bacterial effect on the electrochemical behaviour of the 304 SS samples were further investigated using Electrochemical Impedance Spectroscopy (EIS). Figures 2-5 show the measured impedance spectra plotted as Nyquist and Bode phase diagrams, for various exposure times, in the four test environments considered in this work.



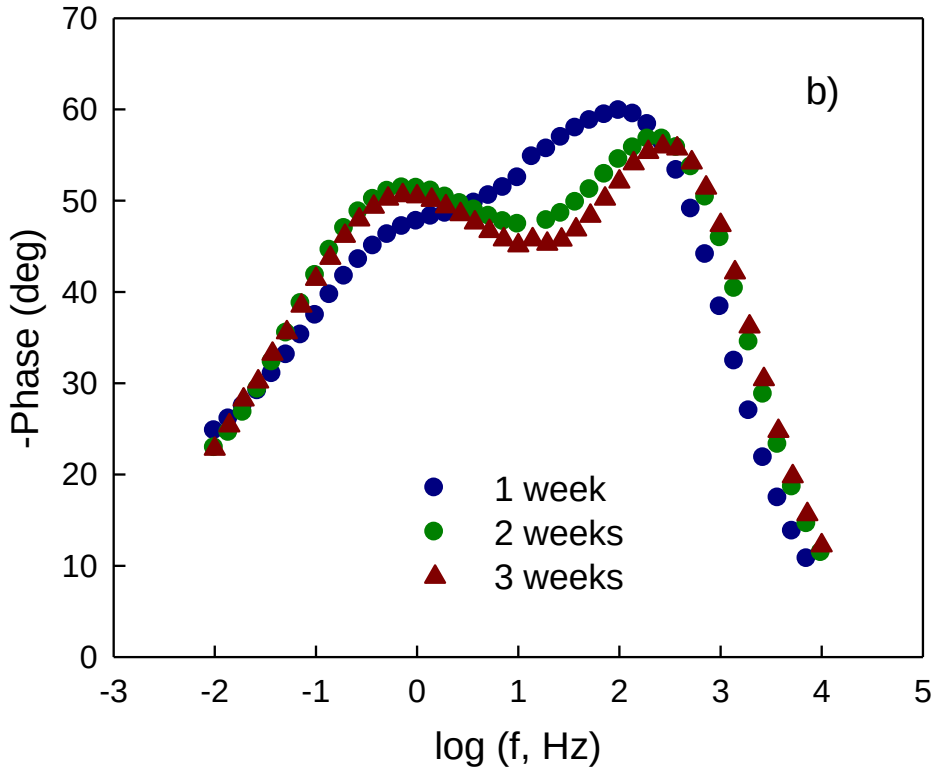
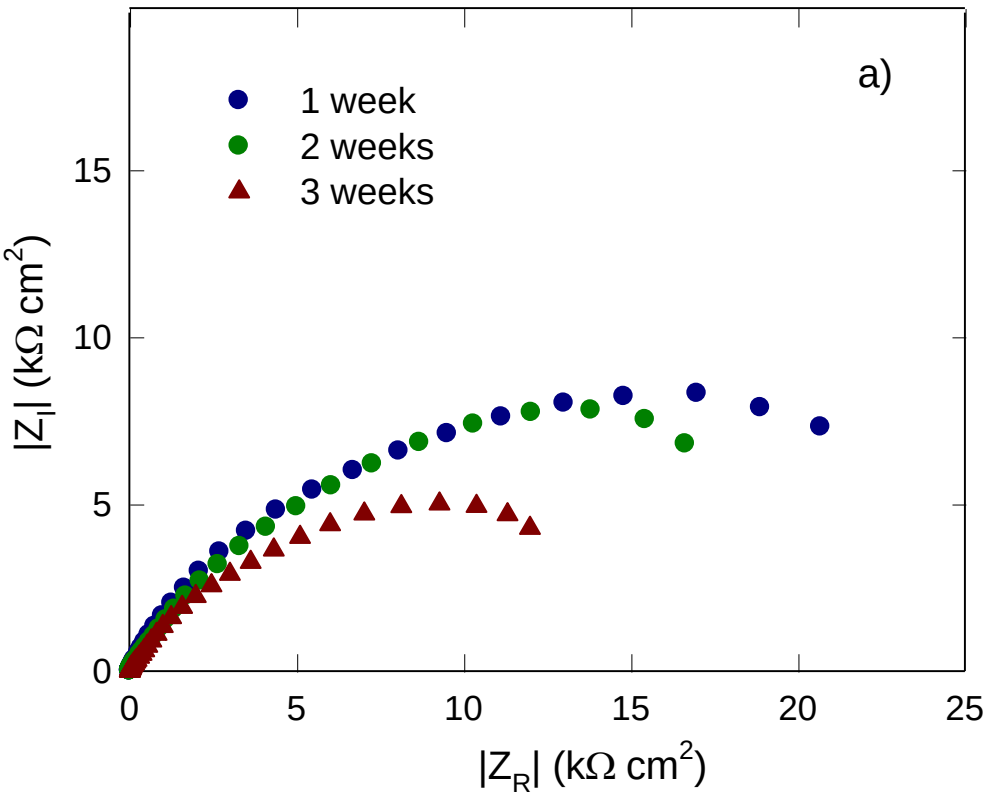


Figure 2. Experimental impedance spectra of an AISI 304 stainless steel specimen after immersion in sterile seawater with sodium lactate for the exposures indicated in the plots: a) Nyquist, and b) Bode-phase diagrams.



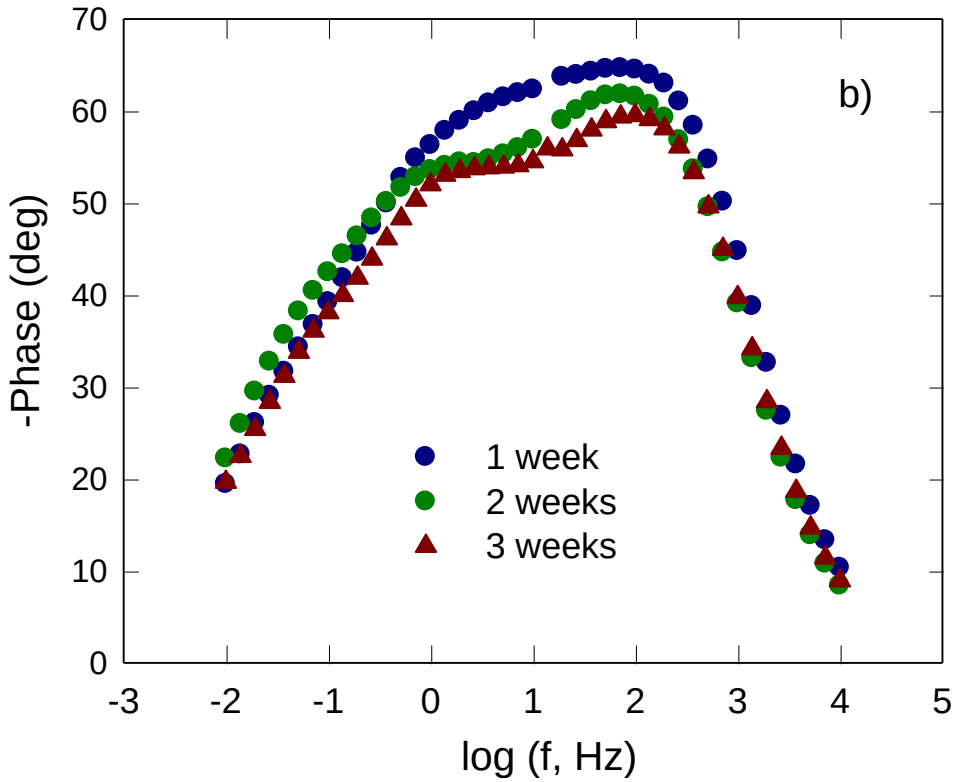
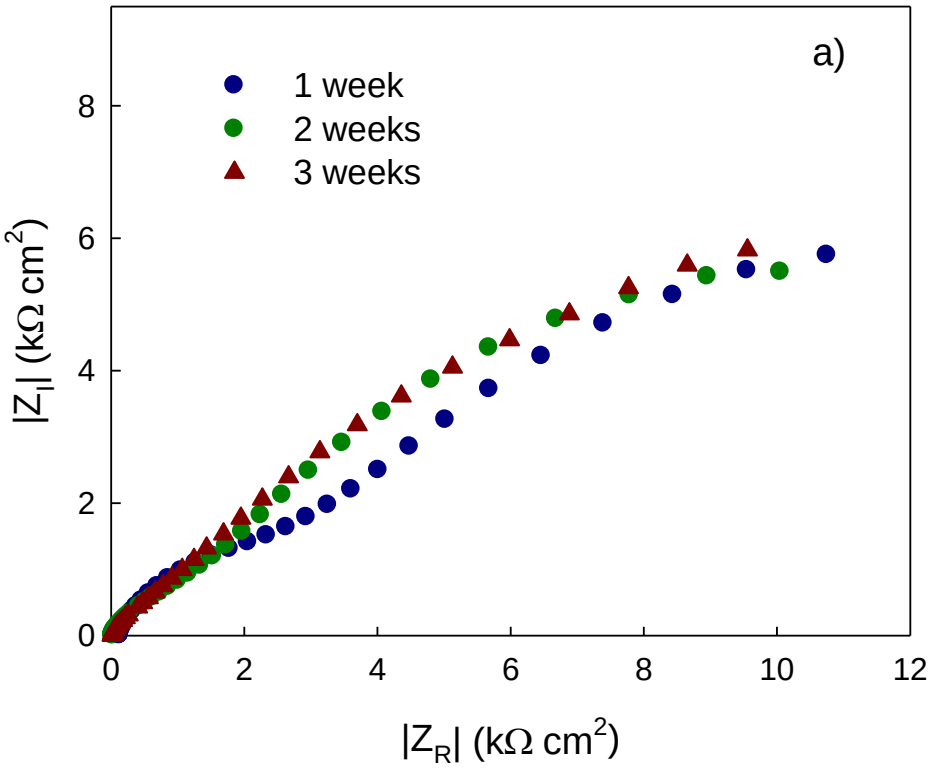


Figure 3. **E**xperimental impedance spectra of an AISI 304 stainless steel specimen after immersion in sterile seawater with sodium lactate + *Bacillus cereus var. mycoides* for the exposures indicated in the plots: a) Nyquist, and b) Bode-phase diagrams.



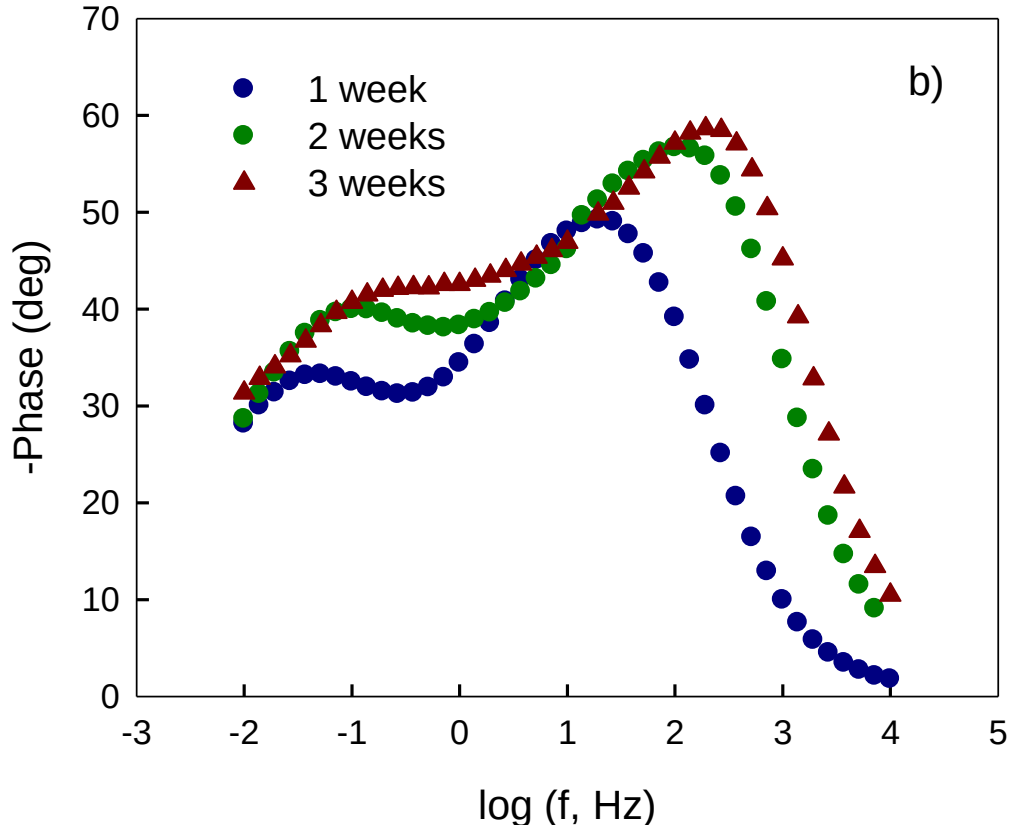
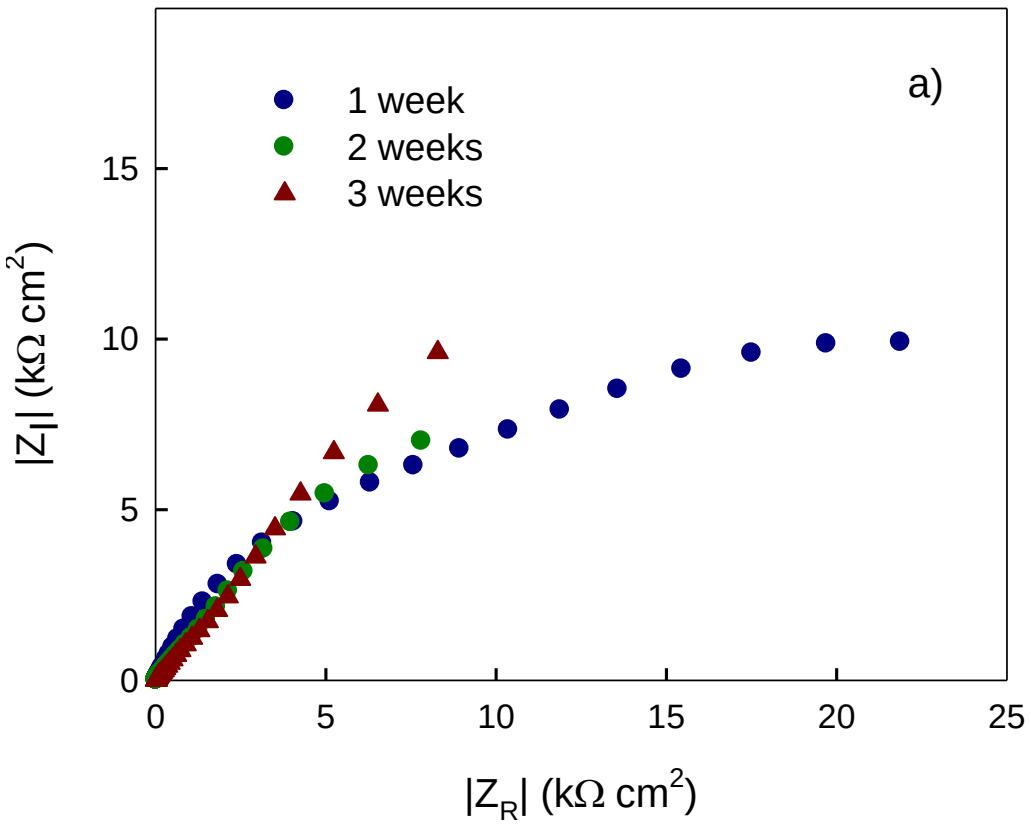


Figure 4. Experimental impedance spectra of an AISI 304 stainless steel specimen after immersion in sterile seawater with sodium lactate + SRB isolated from natural seawater for the exposures indicated in the plots: a) Nyquist, and b) Bode-phase diagrams.



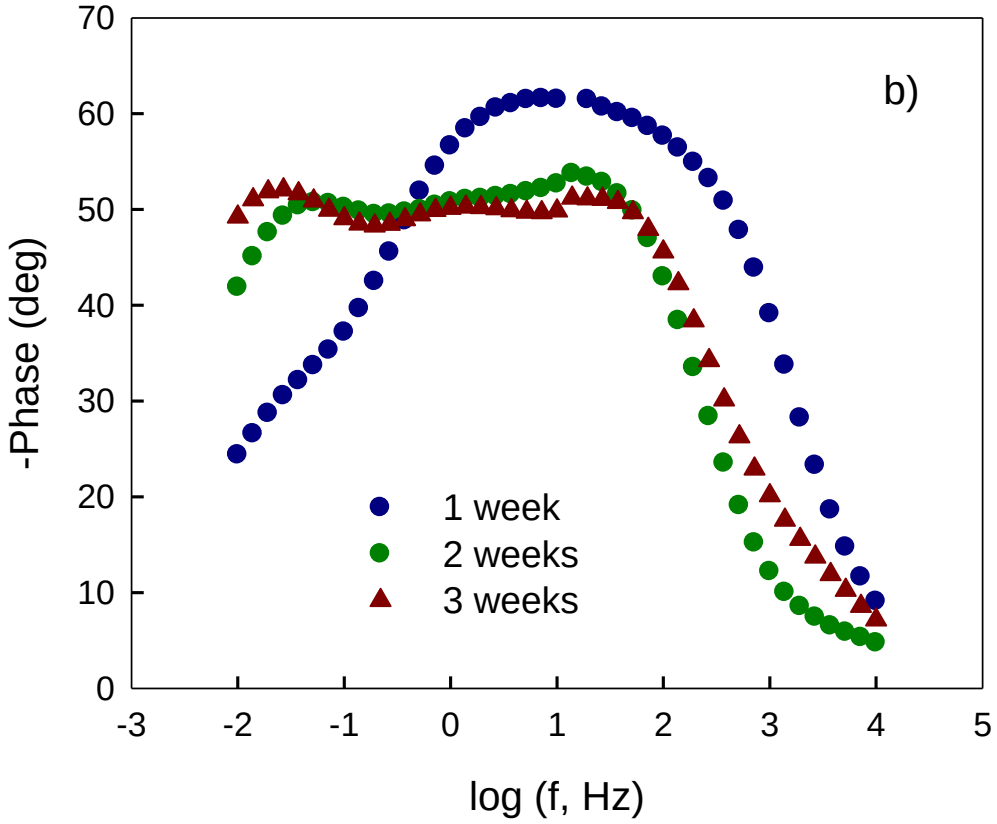


Figure 5. Experimental impedance spectra of an AISI 304 stainless steel specimen after immersion in sterile seawater with sodium lactate + SRB isolated from natural seawater + *bacillus cereus var. mycoides* for the exposures indicated in the plots: a) Nyquist, and b) Bode-phase diagrams.

Changes in the impedance characteristics as a result of the exposure of the specimens to the test environments could be observed from the comparison of the spectra. From a cursory observation of these plots it can be observed that two time constants are operating in these systems at all times. Thus, the spectra could be analysed in terms of the equivalent circuit depicted in figure 6, which corresponds to the case of a two-layer film developed over the metallic substrate [42]. The components of this equivalent circuit are namely: the ohmic resistance of the test electrolyte and electrical connectors, R_e; the constant phase element of the passive layer, Q_p; the resistance of the passive layer, R_p; the resistance related to the biofilm, R_b; and the capacitance of the biofilm, Q_b. In the case of the spectra measured in bacteria-free electrolytes, R_b is the polarization resistance of the metal substrate, and Q_b is the double-layer capacitance at the substrate/electrolyte interface. Analysis of the impedance spectra in terms of this equivalent circuit allowed for the various impedance parameters to be determined. The fitting procedure (see Table 4) revealed that better agreement between theoretical and experimental data was obtained if frequency dependent constant-phase elements (CPE) were used instead of pure capacitances. Generally, the appearance of a CPE is due to the presence of inhomogeneities in the electrode-material system. In all the cases the constant phase elements (Y_p, and Y_b) are included, as well as the number n, an empirical exponent which can vary between one for a perfect capacitor and zero for a perfect resistor [43].

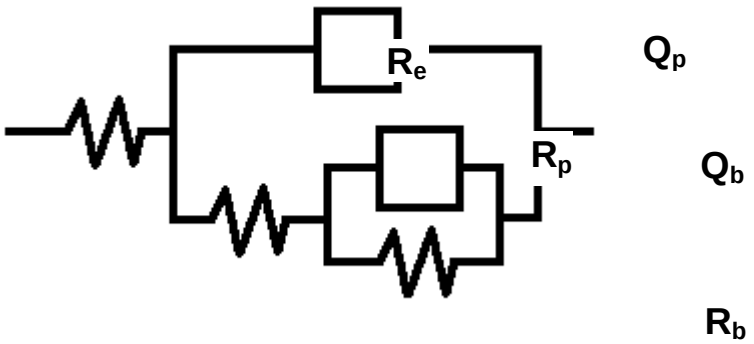


Figure 6. Equivalent circuit with two time constants used to model the electrochemical impedance spectra. The components are: R_e, ohmic resistance of the electrolyte; Q_p, capacitance of the passive layer; R_p, resistance of the passive layer; R_b, resistance related to the biofilm; Q_b, capacitance of the biofilm.

Table 4. Time evolution of the impedance parameters of AISI 304 steel samples immersed in the four test environments under consideration.

	Time (days)	R_e (Ω cm^2)	R_p (Ω cm^2)	Q_p		R_b (Ω cm^2)	Q_b	
				Y_p (Ω^{-1} cm^{-2})	n		Y_b (Ω^{-1} cm^{-2})	n
Without bacteria	7	$2.053\cdot10^{-2}$	0.4227	$1.858\cdot10^{-2}$	0.862	30.03	0.1169	0.510
	14	$2.107\cdot10^{-2}$	0.466	$1.673\cdot10^{-2}$	0.859	26.48	0.1136	0.627
	21	$1.985\cdot10^{-2}$	0.3554	0.1438	0.874	23.62	0.1397	0.612
Bacillus Cereus	7	$2.455\cdot10^{-2}$	0.5223	$1.485\cdot10^{-2}$	0.866	62.83	$3.72\cdot10^{-2}$	0.541
	14	$3.131\cdot10^{-2}$	0.1308	$1.89\cdot10^{-2}$	0.840	57.33	$5.348\cdot10^{-2}$	0.566
	21	$2.728\cdot10^{-2}$	0.7872	$2.111\cdot10^{-2}$	0.825	38.00	$6.203\cdot10^{-2}$	0.538
SRB	7	0.2398	4.599	$2.066\cdot10^{-2}$	0.783	67.41	0.144	0.516
	14	$3.911\cdot10^{-2}$	1.365	$1.615\cdot10^{-2}$	0.819	66.31	0.128	0.504
	21	$3.283\cdot10^{-2}$	0.58	$9.599\cdot10^{-3}$	0.882	76.03	0.1173	0.481
SRB + *Bacillus Cereus*	7	$3.743\cdot10^{-2}$	26.86	$3.446\cdot10^{-2}$	0.731	70.83	$8.144\cdot10^{-2}$	0.342
	14	$4.775\cdot10^{-2}$	1.646	$9.5\cdot10^{-2}$	0.762	76.12	0.1635	0.600
	21	$4.302\cdot10^{-2}$	3.19	0.1104	0.679	538.00	0.1146	0.559

The exposure of AISI 304 stainless steel in sterile seawater with sodium lactate for 21 days of immersion led to an increase of the charge transfer resistance during two weeks after immersion, and thereafter decreased (cf. Figure 2). The corrosion potentials (E_{corr}) shifted to slightly more positive values along the duration of the tests, indicating that the formation of a passive film characteristic of stainless steels [7] is taking place on the metallic surface. It can be seen in the Bode-phase diagrams that there is no change in the process mechanism between the second and third week of immersion, the pattern only being modified between the first and second week. We can see that the polarisation

resistance (R_p) remains practically constant, with a slight decrease only after three weeks of immersion.

In the seawater system with sodium lactate containing *Bacillus cereus* the formation of a biofilm can be observed on the steel surface, which becomes particularly evident after 14 days of immersion as indicated by the progressive increase in the polarisation resistance (R_p) values up to 628.2 Ω cm^2, and later falls to 290.3 Ω cm^2 at 21 days of immersion, this being a higher value than that after 7 days of immersion. Though the bacterial count fell towards the end of the test, the production of silt in the first few days caused the formation of a biofilm which was particularly indicated by the increase in R_p after 14 days of immersion. The impedance spectra obtained (cf. Figure 3) show that the charge transfer resistance decreased sharply with the time of immersion, with no major changes in the pattern of the Bode-phase graphs, with a maximum phase angle of 65 degrees. This can be explained by the fact that the development of *Bacillus cereus* brings about the formation of a large quantity of slime as extracellular polymeric substances (EPS) which helps in the formation of a biofilm on the metallic surface. This feature is confirmed by the corrosion potential value (E_{corr}) after 21 days of immersion (see Table 3), where it can be seen that the potential moved towards more positive values due to the presence of slime in the medium [44].

When AISI 304 stainless steel was subjected to seawater with sodium lactate in the presence of SRB isolated from their natural environment, the impedance spectra (see Figure 4) and data obtained show that the charge transfer resistance increases with the time of immersion. Major changes can be seen in the Bode-phase diagrams in the pattern of the curves in the range of low frequencies, due to the action of the microorganism under study (generation of hydrogen sulphide, formation of iron sulphides, exopolymers, etc.) [4-6]. In this system the tendency of the corrosion potential to shift to more positive values than for the sterile system after 14 days of immersion (namely +81 mV vs SCE) is observed, while thereafter turning again to negative values as a consequence of the anodic depolarisation created by the formation of iron sulphides.

In the seawater system with sodium lactate containing both SRB isolated from their natural medium and *Bacillus cereus* there was a clear decrease in the polarisation resistance (R_p) values throughout the test. The corrosion potential value (E_{corr}) tended to negative values in a greater extent than the other systems, which could indicate a synergic effect of both bacteria, given that *Bacillus cereus var*. *mycoides* is a facultative anaerobic bacteria and, therefore, favours the development of SRB and consequently there is a greater tendency to the phenomenon of corrosion. The impedance spectra depicted in Figure 5 show that with the time of immersion the charge transfer resistance increases after three weeks, while the corrosion potentials (E_{corr}) move throughout the test to more negative values. A change can be observed in the pattern Bode-phase diagram between the first and second week of immersion, and later there is no change in the process mechanism between the second and third week of immersion. The formation of slime through the development of *Bacillus cereus* in the first days of immersion favours the development of SRB because of the anaerobic conditions which are generated in the biofilm, influencing the metabolism of *Bacillus cereus* through the anaerobic process. Though it can be seen that the synergic effect of both microorganisms causes an overall decrease in the polarisation resistance (R_p) over the total immersion time, these values are higher than for the other systems tested, which indicates that the formation of a biofilm on the metallic surface

gives an initial protective character, but the later development of SRB favoured by the anaerobic conditions causes an increase in the corrosion phenomenon.

It can be seen that the highest capacitance of the passive film (Q_p) occurs for the system with the mixture of organisms (191.5 Ω^{-1} cm^{-2}) after 21 days of immersion, while additionally it exhibits the lowest value of n (0.727), which indicates a more resistive character than the other systems. Analogously, the highest capacity of the substrate-electrolyte interface (Q_b) also belongs to the microorganism mixture system (383.1 Ω^{-1} cm^{-2}), which indicates the formation of a biofilm with a comparatively greater protective character than the other systems.

4. CONCLUSIONS

In the seawater system with sodium lactate without bacteria, there can be seen, as would be expected for stainless steels, the formation of a passive film on the surface giving a protective character to the AISI 304 steel. On measuring the corrosion potentials (E_{corr}), a tendency to shift towards more positive values with the elapse of the time of immersion is found, whereas a slight increase in the charge transfer resistance in the impedance graphs is observed .

In the seawater system with sodium lactate containing *Bacillus cereus*, the formation of a biofilm can be observed on the steel surface, with the production of slime during the first days of immersion, as is clear from the impedance diagrams, causing an increase in R_p for the initial fourteen days.

The seawater system with sodium lactate with SRB isolated from natural seawater, shows that after 14 days of immersion the corrosion potential (E_{corr}) shifts to a positive value (+81 mV vs. SCE), as a consequence of the formation of a biofilm that does not have a high protective quality, as can be seen from the Bode phase diagrams with a phase angle at low frequencies of 40º. In addition, there is a progressive decrease in R_p over the time of immersion. This effect is due to the formation of a biofilm on the metallic surface together with the formation of iron sulphide as a consequence of the metabolism of the SRB which generates hydrogen sulphide and, therefore, creates anodic depolarisation.

In the seawater system with sodium lactate containing both SRB isolated from their natural medium and *Bacillus cereus var. mycoides* (*CECT 193*), there is a clear decrease in the polarisation resistance (R_p) throughout the test duration. The value of the corrosion potential (E_{corr}) tends to negative values in a greater extent than the other systems, which indicates the synergic effect of both bacteria, given that *Bacillus cereus var. mycoides* is a facultative anaerobic bacteria and, therefore, favours the development of SRB and consequently there is a greater tendency to the phenomenon of corrosion after an initial phase of enhanced protection.

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