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ORIGINAL RESEARCH

Aerobic and Facultative Microorganisms Isolated From Corroded Metallic Structures in a Hydroeletric Power Unit in the Amazon Region of Brazil

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Abstract: Aerobic and facultative bacteria belonging to the *Enterobacteriaceae*, *Pseudomonadaceae*, *Bacillaceae*, *Corynebacteriaceae* and *Streptococcaceae* families have been isolated from corroded metallic structures of a hydroelectric power unit in the Amazon region of Brazil. In addition to anamorphic dematiaceous and moniliaceous fungi, members of the archeobacteria kingdom were also detected in the same samples. Scanning electron micrographs of metal bars cultivated with consortia of the isolated microorganisms depicted suggestive images of biofilm formation and corroded metallic structures questioning the possible role of these microorganisms in the corrosion activity. We also found Amazonian medicinal plants exhibiting inhibitory activity against some of the isolated microorganisms. Our new findings need additional studies to confirm the participation of some isolated microorganisms in the process of metallic degradation despite our main question if are there particular microorganisms involved in the corrosion process? or if physicochemical conditions would favor the development of a particular microbiota and consequently the corrosion process would result from its metabolism? Therefore we hypothesize that any microorganism could be potentially involved in the genesis of corrosion process. This is the first report in the literature dealing with microbiologically induced corrosion in the Amazon region which is especially characterized by its high humidity and elevated temperature all year round.

Keywords: biocorrosion, microorganisms, metallic surfaces, hydroeletric power unit, amazon region of Brazil, Amapá state

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Introduction

Microbiologically induced corrosion is a matter of great concern all over the world. It costs so much to prevent the corrosion process and to replace periodically deteriorated metal structures.[1] The isolation and identification of microorganisms involved in the corrosion process is a necessary and mandatory step in order to design methods to prevent and control it.[2,3] The biofilm formation which is mainly determined by the interaction of a consortia of microorganisms represents the main factor involved in the etiological mechanism of biocorrosion. Composed basically of 95% of water and 5% percent of extracellular polymeric substances including organic molecules produced by the microbial metabolic activity and inorganic molecules, the biofilm generates a highly dynamic environment, promoting the exchange of ions between the metallic surface and itself. Such ionic interactions weaken the arrangement of metallic bonds' structures, causing fissures in its surface that in an amplified scale and at a practical view result in great economic loss.[1,2,4] The algae, fungi and bacteria have been reported to be involved in corrosion processes.[5,6] The members of the Enterobacter and Pseudomonas genera were found to be potential candidates in the etiology of biocorrosion related processes as these microorganisms are capable to interchange electrons with the adhered surface representing the fundamental mechanism of ionic interplay over the metallic surfaces leading to the corrosion process.[7,8] Also, Sulfate-Reducing Bacteria (SRB), a group of phylogenetically diverse anaerobes that perform the dissimilatory reduction of sulfur compounds including sulfate, sulfite, thiosulfate and even sulfur to form sulfide, is involved in the corrosion process, in mechanisms producing cathodic depolarization by the removal of hydrogen ions (protons) from the cathodic area on the iron surface catalyzed by bacterial hydrogenases, coupled to sulfate reduction to sulfide that could account for the severe corrosion of iron in an anoxic environment under neutral pH.[9,10]

Here we report for the first time, the isolation and biochemical characterization of microorganisms cultivated from fragments of corroded metallic structures collected at the facilities of Coaracy Nunes hydroelectric power unit, in Amapa state, in the Amazon region of Brazil and found also the suggestive role of the isolated consortiated microorganisms in the corrosion process, as also preliminary results of antimicrobial activity of Amazonian medicinal plants against the isolated microorganisms. Taking into account our results we are strongly inspired to hypothesize that any microorganism is capable to trigger mechanisms of metallic corrosion dependly mainly on the microenvironment physicochemical conditions and the molecular composition and architecture of the material surface exposed to the local microbiota.

Materials and Methods

The hydroeletric power unit "Coaracy Nunes"

The Coaracy Nunes hydroelectric power unit (UHE) is located in northern Brazil, at Ferreira Gomes county, in the Amapa state, delimited at southeast by the Amazon river and northeast by the Oiapoque river, in the boundaries of French Guyana (00°55'00' North and 51°15'00' West) as depicted in Figure 1 (Map of Amapá, Brazil).

Corroded metallic structures

At the UHE, 10 samples of corroded fragments and 2 water samples from pipelines designated AB1 to AB12 were collected from the metallic surfaces of the pipelines/valves/heat exchanger and from the refrigeration system. The crusts were removed

Figure 1. Geographic localization of Coaracy Nunes Hydroeletric Power Unit in the Amapa state, in Ferreira Gomes county.

utilizing a sterile bistouri and blade and collected on sterile falcon tubes containing transport medium (vermiculite) as also water from the pipelines refrigeration system.

Microbiological culture of corroded metallic fragments and water

In order to isolate and biochemically characterize the microorganisms, the collected samples were inoculated into sterile brain heart infusion medium (BHI) and incubated at 35 ± 1 °C for 24 hours and transferred to blood agar, MacConkey agar and azide agar. The gram staining was performed at this stage. Additional 24 hours incubation period was carried out at the same conditions. The agar grown colonies were recorded considering morphological and staining characteristics. The pure cultures were obtained by reinoculation of isolated colonies in the same media. The complementing biochemical characterization was performed in a semiautomatic Autoscan Walkaway (W/A) system (MicroScan, Sacramento, Calif.) and by the analysis based on the metabolic characteristics of isolated microorganisms.[11]

Also the previously suspended samples in BHI media were transferred to the Sabouraud agar dextrose and Mycosel agar, and kept under incubation for 10 days at 25 °C. The fungi grown colonies were transferred to the Potato dextrose agar and kept at room temperature. The phenotypic identification was based on the macroscopic and staining characteristics likewise the texture, topography and colour of the colonies as also the microscopic features displayed by the reproductive structures, the hyphae and conidia.[12,13]

Detection of unculturable microorganisms by molecular methods

The samples collected from the metallic corroded structures in BHI medium were submitted to DNA extraction and amplified by the polymerase chain reaction utilizing the Archaebacteria universal primers.[14] As briefly described, 500 uL of each collected sample, enriched in BHI medium was incubated under agitation for 24 hours at 35 °C. After growth, DNA was extracted utilizing the PureLink Genomic DNA kit (Invitrogen), following the steps of proteinase K digestion of bacterial lisates and RNA degradation by RNase treatment. DNA was extracted on columns (PureLink Spin Column), washed out and eluted. The quality of obtained DNA was assessed by visualization on 1% agarose gel and quantified in a spectrophotometer.

DNA samples were amplified by the polymerase chain reaction utilizing 0.5 uM of archael primer pair A571F and UA1204R (16S rRNA), 0.2 mM of dNTPs, 1.0 U of Taq polymerase in 2.5 uL of buffer (Roche) and 1.5 mM of Mg^{++} in a final volume of 25 uL. The thermocycler was programmed to 94 °C/2 min., followed by 30 cycles of 94 °C/1 min., 55 °C/1 min., 72 °C/1 min. and finishing at 75 °C/10 minutes. After amplification, 12.5 uL of reaction volume was analyzed by electrophoresis on 1% agarose gel and ethidium bromide staining.

Analysis by scanning electron microscopy

The corroded fragments and water (AB1 to AB12) collected in individual sterile falcon tubes were transferred to BHI sterile medium in tubes containing a metallic bar measuring 1 cm long × 1 cm large and 1 mm deep. Each 24 hours after inoculation, the turbidity of the medium was measured in an ELISA reader at 600 nm wavelength. After 14 days, the metal bars were washed out in distilled water, dried at room temperature and observed under the scanning electron microscope, in a JEOL-JSM, T-330 A model, coupled to an analyzer of dispersive energy, a photographic camera and a LEO microscope Leica-Zeiss, 440 model coupled to EDX and WDX detector in a 50× magnifier.[15]

Results

Aerobic and facultative bacteria belonging to the Enterobacteriaceae, Pseudomonadaceae, Bacillaceae, Corynebacteriaceae and Enterococcaceae families were isolated from the corroded metallic structures and biochemically characterized. The isolates of the Enterobacteriaceae family included the *Enterobacter cloacae*, *Escherichia coli*, *Hafnia alvei* and *Serratia marcescens*, and pertaining to the Bacillaceae family we isolated the *Bacillus polymyxa*, *Bacillus thuringiensis*, *Bacillus brevis* and *Bacillus alvei*. The *Corynebacteriumpseudodiphthericum*,*Pseudomonas aeruginosa* and *Enterococcus faecalis* were the sole representatives of the Corynebacteriaceae, Pseudomonadaceae and Enterococcaceae families

respectively. All biochemical parameters analyzed, manually and automatically, were displayed in Tables 1, 2 and 3. Phenotypical and metabolic profiles of isolated microorganisms were assorted and based on these data, identified in the W/A system's computer software at the genera and species level.[11] All identified members of the Enterobacteriaceae family, *Enterobacter cloacae*, *Escherichia coli*, *Hafnia alvei* and *Serratia marcescens* presented bacilar morphology, gram negative staining, oxidase and cetrimide negative test, ornithine and ortho-nitrophenyl-β-D-galactosidase positive tests, being all of them capable to utilize glucose, allowing us to differentiate them from the Pseudomonadaceae family member, *Pseudomonas aeruginosa* (Table 1). Members of the Bacillaceae and Corynebacteriaceae family reacted positively by the gram staining, being identified mainly by their property to ferment glucose, sucrose and lactose in the TSI test. The *Bacillus polymyxa* and *Bacillus thuringiensis* utilized all carbohydrates and the *Bacillus brevis* and *Bacillus alvei* just fermented glucose and, the solely identified species in the Corynebacteriaceae family, the *Corynebacterium pseudodifhtherium* did not ferment any sugar (Table 2). Just one species, *Enterococcus faecalis*, in the Enterococcaceae family was identified, yielding catalase negative test, growth on 6.5% NaCl broth and esculine positive test and did not hemolyze blood in agar (Table 3).

The *Myceteae* kingdom was represented by the isolates of anamorphic dematiaceous and moniliaceous fungi. The morphological and staining characteristics of colonies of fungi grown in Sabouraud agar are shown in Figure 2A and 2B. The dematiaceous fungi exhibited dark coloured, grayish or grayish-brown powdery and felt-like colonies, and their dark reverse showed curves to straight conidia, containing three to four septs and septated and dematiaeous hyphae, characteristic of *Curvularia* spp (Fig. 2A). The moniliaceous fungi presented white cotton-like colonies and a brown reverse colour with double verticillated conidiophores exhibiting globolous conidia and septated and hyaline hyphae, characteristic of *Penicillium* spp (Fig. 2B).

Preliminary metagenomic analysis of DNA extracted from collected mixed water and corroded metallic samples detected a band in the gel after

Table 1. Biochemical characterization of Enterobacteriaceae and Pseudomonadaceae species isolated from corroded metallic structures in the Coaracy Nunes Hydroelectric Power Unit, Amapa state.

Metabolic markers	Fermentation																OF													
Microorganisms Family/species	Glucose	Sucrose	Sorbitol	Raphnose	Ramnose	Arabinose	Inositol	Adonitol	Melibiose	Urea	Hydrogen sulfite	Indole	Lysine	Arginine	Ornithine	Tryptophan deaminase	Esculin hydrolisis	Voges-Proskauer	Galactosidadse (ONPG)	Citrate	Malonate	Tartarate	Acetamide	Cetrimide	Nitrate	Base	Glicose	Oxidase
Enterobacteriaceae																														
Enterobacter cloacae	+	+	+	+	+	+	–	–	+	+	–	–	–	+	+	–	–	+	+	+	+	–	+	–	+	–	+	–
Escherichia coli	+	+	+	+	+	+	–	–	–	–	–	+	+	–	+	–	–	–	+	–	–	+	+	–	+	–	+	–
Hafnia alvei	+	–	–	–	+	+	–	–	–	–	–	–	+	–	+	–	–	+	+	–	+	+	–	–	+	–	+	–
Serratia marcescens	+	+	+	–	–	–	+	–	–	–	–	–	+	–	+	–	+	+	+	+	–	+	+	–	+	–	+	–
Pseudomonadaceae																														
Pseudomonas aeruginosa	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	+	+	+	+	+	+	–	–	+

Abbreviations: +, positive; –, negative; ONPG, ortho-nitrophenyl-β-D-galactosidase; OF, oxidation and fermentation.

Table 2. Biochemical identification of species belonging to the bacillaceae and Corynebacteriaceae families isolated from corroded metallic structures in Coaracy Nunes Hydroeletric Power Unit, Amapa state.

Microorganisms	Blood agar hemolysis	Catalase	Urea	TSI up/bottom	H_2S
Bacillaceae					
Bacillus polymyxa	β	+	–	A/A	–
Bacillus brevis	β	+	+	Alc/A	–
Bacillus alvei	β	+	+	Alc/A	–
Bacillus thuringiensis	β	+	+	A/A	–
Corynebacteriaceae					
Corynebacterium pseudodifhtherium	β	+	+	Alc/Alc	–

Abbreviations: +, positive; –, negative; β, β-hemolysis; TSI, triple sugar iron; Alc/A, alkaline and acid reaction in TSI.

submission to electrophoresis of amplified DNA fragments by the PCR assay, corresponding to 1,300 bp fragment stained by ethidium bromide as depicted in Figure 3. The band was not resolved in sequential gel analysis in order to check out if more than one band was present.

Previously mentioned isolated microorganisms were found to be associated in the corroded fragments and water removed from the metallic surfaces and pipelines respectively. The scanning electron micrographs exhibiting colonies of the isolated bacteria and biofilm formation on the surface of the metallic structures are depicted in Figures 4 and 5 as also the metallic dissolution represented by the spectra of energy dispersion of molecular oxygen and iron ions (Fig. 5). The bacterial growth over the surface of the metallic bars in the liquid medium in consortiated groups as designated in material and methods from AB1 to AB12 did not show any significative difference in turbidity parameters, all starting the lag phase in the second day of cultivation, determined by the measurement of the optical density values in an ELISA reader at 600 nm wavelength (Fig. 6). Also we detected fissures in the surface of the metallic bars colonized in vitro by the consortiated microorganisms that were previously biochemically characterized. They were not assayed individually as in the natural environment those microorganisms are found associated as we corroborated in our experiments.

Of 30 distinct medicinal plants collected in the Amapa state, we detected preliminarily 5 plant extracts exhibiting antimicrobial activity. All plants assayed in this research work are traditionally utilized by native people in the Amazon region to cure their diseases. The ethanolic extracts of *Copaifera reticulata, Tabebuia serratifolia, Brosimum rubescens* and *Carapa guianensis* presented activity against gram positive bacilli of the Bacillus genera, and the ethanolic extract of *Aspidospermum carapanauba* showed activity against the gram positive bacilli and *Pseudomonas aeruginosa*. Despite these data be preliminary, most of the microorganisms susceptible to the plants extracts are multiresistant to commonly used antibiotics (data not shown).

Discussion

The Amazon region is characterized by high humidity and elevated temperatures all over the year. In Amapa state, the Ami and Amw climates predominate. According to Köppen classification, the former

Table 3. Biochemical identification of the Streptococcaceae family species isolated from corroded metallic structures of the Coaracy Nunes Hydroeletric Power Unit, Amapa state.

Microorganism	Hemolisys in blood agar	Catalase test	NaCl at 6.5%	Bile + Esculin test
Streptococcaceae				
Enterococcus faecalis	Without hemolisys	–	+	+

Abbreviations: +, positive; –, negative.

Figure 2A. Fungus *Curvullaria* spp. cultivated in Agar Sabouraud. Agar surface (left). Agar reverse side (midlle). Optical microscopy (40×), stained with lactophenol, showing conidia and septated dematiae hyphae (right).

is characterized by tropical rainy weather, presenting a well defined dry season, lacking pluviometric precipitation from August to December, and the latter is also characterized by tropical rainy weather but without a defined dry season, thus raining regularly during all year round.[16] The Coaracy Nunes hydroeletric power unit is localized in the transition limits of both climate pattern above described. The proper maintanance of metallic strucutures in a hydroeletric power unit, particularly in the amazon region requires periodic painting and substitution of corroded structures, demanding excessive financial costs making the final product, the eletricity, so expensive to the population.

The rich microbiota in the Amazonian tropical forest greatly contributes to the widespread colonization of microorganisms in all surfaces of any material such as wood, metals, plastics whatsoever. The metallic structures composing important industrial facilities, including the electricity power unit, suffer enormous losses due to the corrosion processes, mainly induced by microorganisms. In our work, we could confirm the findings of many laboratories and also, observe *in vitro* the suggestive role of the

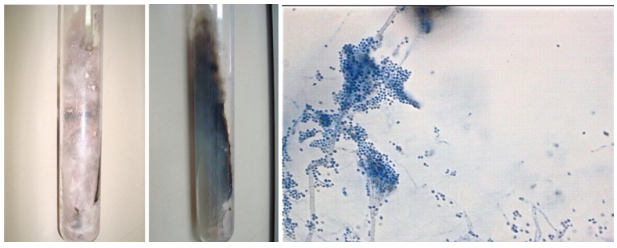

Figure 2B. Fungus *Penicillium* spp. cultivated in Agar Sabouraud. Agar surface (left). Agar reverse side (midlle). Optical microscopy (40×), stained with lactophenol cotton blue, showing double verticillated conidiophores exhibiting globolous conidia and septated and hyaline hyphae (right).

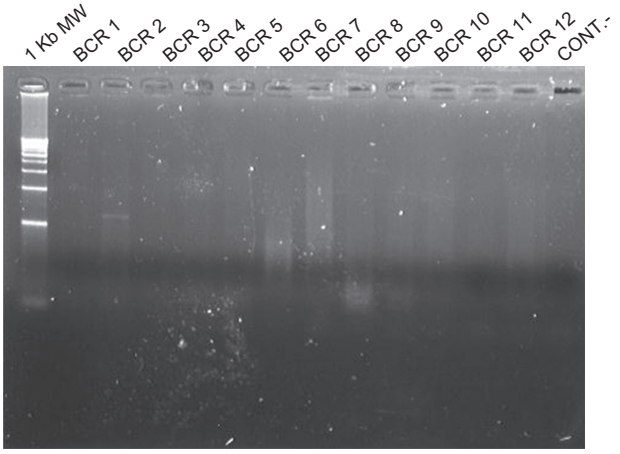

Figure 3. PCR assay of uncultured microorganisms DNA amplified utilizing Archae universal primers (16S ribossomal RNA gene). First Lane/1 Kb MW (left) showing bands of DNA molecular markers and lane/BCR 2 showing a 1,300 bp fragment and last Lane/CONTR as a negative control.

isolated microorganisms consortiated in the corrosion process. The detection of biofilm production as also the precipitated salts on the metallic bars allied to the detection of spectral patterns of metallic ions (Figs. 3 and 4) likewise, decrease in iron ions concentration, carbon atoms and molecular oxygen increase besides the alteration in the concentration of other elements (Fig. 5) suggests the occurrence of corrosion processes of microbial etiology. Interestingly, the microorganisms usually reported in pathological processes in humans and animals as *Serratia marcescens, Escherichia coli, Enterobacter cloacae, Pseudomonas aeruginosa, Enterococcus faecalis, Hafnia alvei*

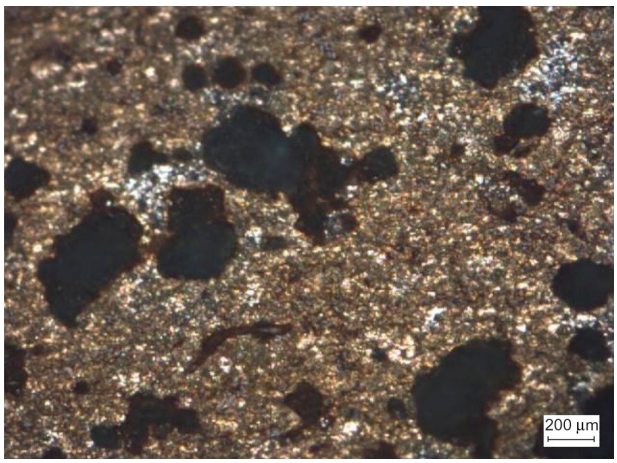

Figure 4. Optical micrograph magnified 50× showing deposits of metallic salts and corroded areas (black spots) in metal bars incubated with consortiated microorganisms isolated from corroded metallic structures of Coaracy Nunes Hydroeletric Power unit in Amapa state.

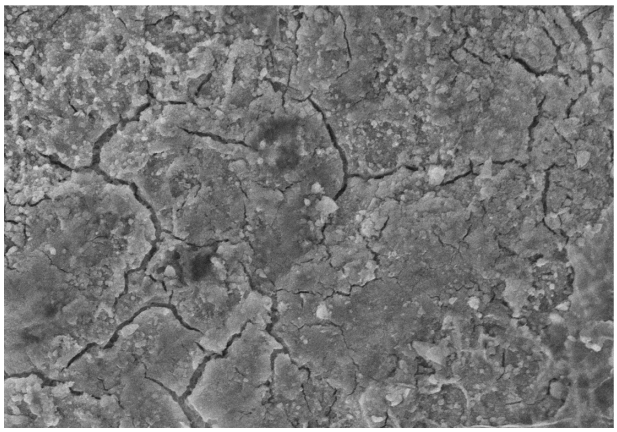

Figure 5. Scanning electron micrograph magnified 200.000× showing surface fractures over the metal bar after microbial growth of samples obtained from corroded metallic structures of the Coaracy Nunes Power unit in Amapa state.

and *Corynebacterium pseudodiphtheriticum* were detected in metallic corrosion processes too.[17–19] Anyway, other microorganisms uncommonly causing diseases, known as opportunistics organisms are usually widely spread in the environment, victimizing immunosupressed humans, animals and plants.[20,21] Therefore, under this viewpoint, bioorganisms defend dynamically themselves against invading and colonizing microorganisms[22] contrary to inert material like metals that need technological devices[23] to protect their surfaces against environmental microorganisms' colonization and consequential ions exchange between the microorganisms metabolic products and metal ionic surface, which generally early or later results in corrosion processes, as generally proposed to certain microorganisms.[24]

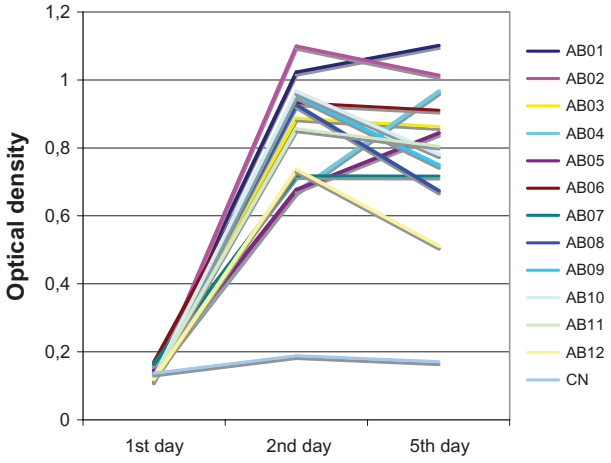

Figure 6. Growth curve (lag phase) of consortiated groups of bacteria (AB01 to AB12) in the experiment to reproduce biocorrosion *in vitro*.

Just a minimum percentage of microorganisms in distinct environments are cultivated by conventional microbiological methods.[25] Uncultured microorganisms have been detected by preliminary metagenomic analysis. In our study, the solely band found by the PCR assay of the isolated DNA samples of consortiated microorganisms could represent the amplification of multiple microorganisms DNA fragments.[26] Utilizing molecular tools to detect ribosomal DNA segments from fragments of industrial pipelines carrying crude oil, about 31 distinct archaebacteria composing the *Methanobacteriales, Methanomicrobiales* and *Methanosarcinales* order were found, despite it is not known the role of these microorganisms in biocorrosion processes.[27] As expected, common soil microorganisms were found among the analyzed samples, mainly represented by members of the Bacillaceae family. Ongoing studies are aiming for the isolation and characterization of anaerobes which were partially initiated by metagenomic studies and considering their important roles in biocorrosion processes.[9,10]

Despite the current knowledge to find culprits for corrosion processes, our results allied to other reports in the literature suggest that there are not particular microorganisms involved in the mechanisms of corrosion but, the physicochemical conditions of the environment determines that the particular microbiota of any surface and therefore the metabolic activity of any microorganism would interact with metallic surfaces or in other words, the redox reactions would occur initiating the corrosion process of metallic surfaces.

Presently we are looking for local solutions for the problem, besides to what actually has been done, likewise the removal of damaged metallic structures and their periodic painting. As an alternative way to chemical pollutants utilized in paintings composition, we are testing Amazonian medicinal plant extracts looking at growth inhibition or destruction of microorganisms found colonizing the metallic structures. We have shown growth inhibition of multiresistant bacteria involved in human nosocomial infections by extracts of Amazonian medicinal plants.[28] As many of our microorganisms isolates from corrosion processes are also involved in human and animal diseases, we proposed in this study to assay Amazonian plants as antimicrobial agents to eliminate corrosion. If from the plants assayed, crude antimicrobial agents against

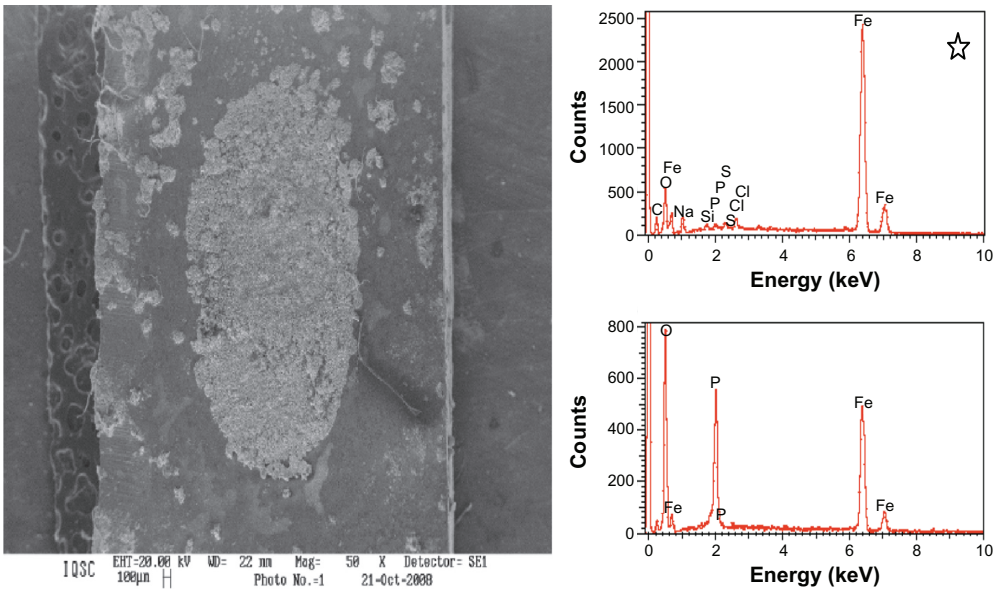

Figure 7. Scanning electron micrograph magnified 50× showing metallic salts deposit (white area) and smooth zone displaying metallic dissolution as confirmed by energy spectral dispersion showed in the graphics (right side) of mainly iron and oxygen íons picks dislodgment.

consortiated microorganisms could be developed, the benefits would prevail as the plants are not toxic to the environment, they could be cultivated and locally processed and would also contribute to the improvement of quality of life of local communities.

Disclosures

This manuscript has been read and approved by all authors. This paper is unique and not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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