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# SULPHATE REDUCING BACTERIA IN BIOFILMS ON THERMOSETTING POLYMERS/Zn COMPOSITE LAYERS

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## ABSTRACT

*Bacterial adhesion to surfaces is the first step in the formation of a biofilm and has been studied extensively over the past decades in many diverse applications. Sulphate Reducing Bacteria (SRB) is a group of phylogenetically diverse anaerobic microorganisms that were first discovered by Beijerinck in 1895. This work investigates the attachment of Sulphate Reducing Bacteria and the modification of roughness before and after the attachments on the surfaces of zinc and thermosetting polymers/zinc composite layers obtained by electro co-deposition. There were used two types of thermosetting polymers: phenol – formaldehyde resin (type NOVOLAC) and epoxi resin. For investigations of the surfaces were used atomic force and epifluorescence microscopy methods (AFM and EFM, respectively). Sessile bacteria on coupons were stained with 4’, 6-diamidino-2-phenylindol (DAPI) and visualized by EFM as well as AFM. The best imaging conditions for AFM were assessed.*

KEYWORDS: biofilm, composite layers, Sulphate Reducing Bacteria, Atomic Force Microscopy, Epifluorescence Microscopy, roughness

## 1. Introduction

Bacterial adhesion mechanism is complex and many factors affect cell adhesion [1]. Some bacteria have been reported to accelerate the corrosion of metals, while others influence the corrosion behavior in a beneficial way. Desulfovibrio desulfuricans, Pseudomonas sp. and Bacillus sp. can accelerate corrosion. On the other hand, Bacillus subtilis has been shown to inhibit the corrosion of aluminum 2024 by secreting polyglutamate and polyaspartate [2, 3], while Pseudomonas flava inhibits corrosion by forming a phosphate film [4].

Central to the phenomenon of microbially induced corrosion is the formation of biofilm on the metal surface. The biofims are formed by microbial aggregates and extracellular polymeric substances (EPS). The EPS creates a microenvironment for sessile bacteria and allow for the development of synergistic relationship. Their main components are not only polysaccharides, but also proteins, lipids and nucleic acids in minor proportion [5].

Sulphate Reducing Bacteria (SRB) is a group of phylogenetically diverse anaerobic microorganisms that were first discovered by Beijerinck, in 1895. At present, 14 genera have been identified, the two most established genera of SRB being Desulfovibrio and Desulfotomaculum [6, 7]. The biofilms are involved in both beneficial and detrimental effects: one beneficial aspect is their potential use as biosurfactancts in tertiary oil production and their capacity to trap heavy metals; as detrimental effect, biofouling increases friction resistance and produces changes in metallic surface properties (hydrophobicity, roughness, color, etc.); finally, biofilms participate in biocorrosion by bind with metal ions [8].

The atomic force microscope is a mechanical imaging device that requires minimal sample preparation and creates three-dimensional images with high spatial resolution. By combining AFM and EFM, two techniques with complementary strengths and weaknesses are joined to yield a powerful tool for the investigation of biological samples [9 - 12]. Using this methods, it was reported the attachment of SRB on different type of materials: 316 stainless steel [13 - 14], D36 carbon steel [15], C1018 carbon steel [16], alloy 625 and austenitic stainless steel [17], Q235 steel [18], heat resistant steel 1Cr18Ni9Ti [19], ASTM grade 2 titanium [20], cerium-doped $TiO_2$ film on 304 stainless steel [21], polyurethane foam Ringlace® and lava rock [22], polyurethane foam (PU), vegetal carbon (VC), low-density polyethylene (PE) and alumina-based ceramics (CE) [23].

The literature is penurious referring to attachment of SRB on metal and especially on composite layers. For this reason, in the present study the work was focus on performing AFM coupled with EFM studies in order to observe the influence of materials structure (pure zinc, PF resin/Zn composite layers and epoxi resin/Zn composite layers prepared by electro co-deposition) on SRB attachment.

## 2. Materials and methods

### *2.1 Substratum and biofilm formation*

Three types of surfaces were preparated by electro co - deposition: pure zinc, PF resin/Zn composite layers and epoxi resin/Zn composite layers. They were electrochemically deposited from a bath with the following composition: 310g/L $ZnSO_4 \cdot 7H_2O$; 75g/L $Na_2SO_4 \cdot 10H_2O$; 30g/L $Al_2(SO_4)_3 \cdot 18H_2O$. The pH of the solution was 3.8.

These layers were electrodeposited on DC04 steel as substrate. Suspension for electro co - deposition of composite layers was prepared by adding phenol formaldehyde resin particles, respectively epoxi resin particles (mean diameter 6 – 10μm) to the solution to give a concentration of 10g/L in the zinc electrolyte plating bath. Electro co - deposition took place in the bath at a temperature of $25^{0}$C, current density of 4A/dm$^2$, time for electrodeposition 30min. The suspension bath was stirred by a mechanical stirrer at a constant rotational speed of 800rpm.

In this investigation, SRB from the University of Duisburg Essen Biofilm Centre, Aquatic Biotechnology were used for bacterial adhesion tests. The pH of solution with cells suspension was 6.2. The attachment of cells was made in the following steps: putting a drop from the preparated solution cells on the tested surfaces; waiting to dry (15-20min); next, coupons were incubated in bacterial suspension of SRB (1 ppb organic matter $10^{9}$cells/mL) for 24h to allow for the attachment and biofilm formation with 2, 5% glutaraldehyde.

### *2.2. Instrumentation*

Biofilm and attached cells on pure zinc, PF resin/Zn composite layers and epoxi resin/Zn composite layers were investigated with combined AFM and EFM methods. Subsequently, they were stained with 0.01% (wt/vol) DAPI for 10min and visualized at the epifluorescence microscope.

A NanoWizardII atomic force microscope (JPK Instruments, Germany) and an upright epifluorescence microscope (AxioImager A1m; Zeiss, Germany) were combined using the BioMaterialWorkstation (JPK Instruments). Throughout the present study the prototype of this new system was used. The key feature of the BioMaterialWorkstation was a shuttle stage that carried the actual sample precisely fixed on a glass slide. This shuttle stage could be transferred between the atomic force microscope and the epifluorescence microscope, giving a precise positioning of the stage on both microscopes.

For AFM images, silicon cantilever CSC37 A (Mikromasch, Estonia) with the following features was used: typical length, 250 μm; width, 35 μm; thickness, 2 μm; resonance frequency, 41 kHz; and nominal force/spring constant, 0.65 N/m. Each AFM image consists of 512 by 512 pixels. AFM images were performed by contact mode in air.

## 3. Results and discussion

The surface structure of pure zinc layer and thermosetting polymers/Zn composite layers under atomic force microscope are presented in Figs. 1 – 3. It can be observed that the surface of zinc is made up of regular crystals.

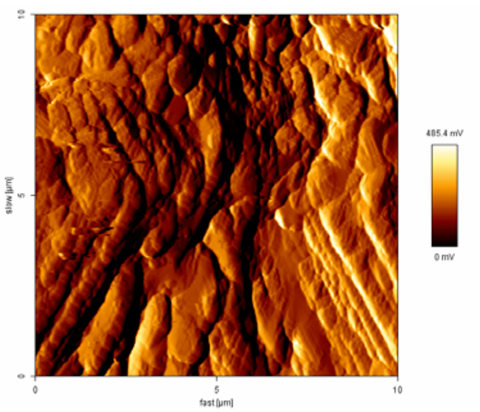

***Fig. 1****. 2D - AFM image of untreated pure zinc surface*

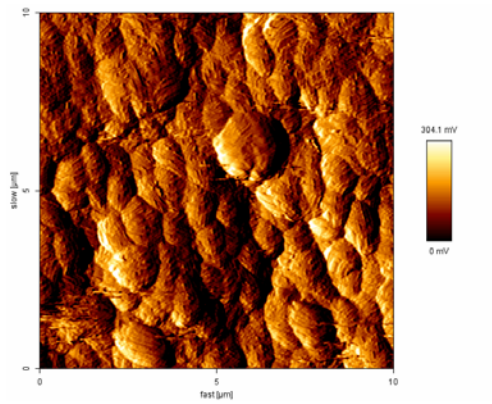

***Fig. 2****. 2D - AFM image of untreated surface PF resin/Zn composite layer*

The thermosetting polymers particles co - deposited with zinc disorder the regular crystal structure and the structure of the zinc matrix becomes finely crystalline.

The pure zinc layers have a rather regular surface, whereas the composite layer surfaces have finer grains structure with particles of resin uniform by distributed on the surfaces. The thermosetting polymer could have an inhibition effect of zinc crystals growth and a catalytic effect in increasing nucleation sites.

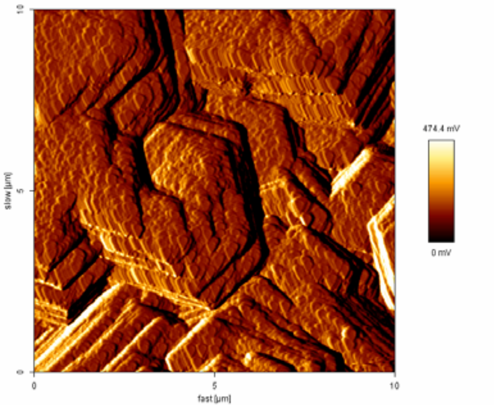


***Fig. 3****. 2D - AFM image of untreated surface epoxi resin/Zn composite layer*

Epifluorescence microscopy (EFM) images of a DAPI – stained biofilm sample of SRB on the surface of zinc and thermosetting polymers/Zn composite layers are presented in Figs. 4 – 6.

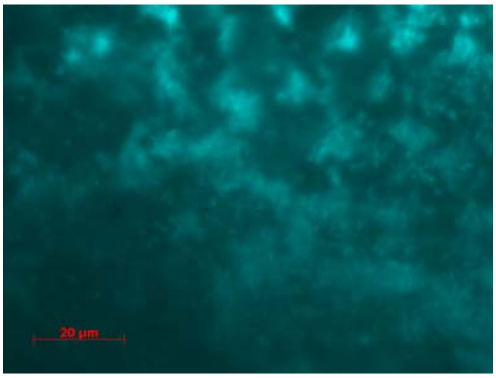


***Fig. 4.*** *EFM image of the SRB attachment and EPS formed on pure zinc surface*

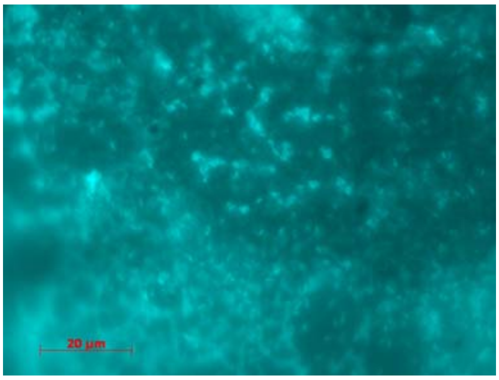


***Fig.5.*** *EFM image of the SRB attachment and EPS formed on PF resin/Zn composite layers surface*

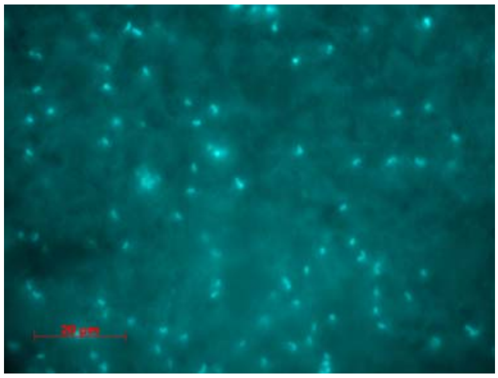

***Fig.6.*** *EFM image of the SRB attachment and EPS formed on epoxi resin/Zn composite layers surface*

From the EFM images, it was observed that the attachment of SRB on thermosetting polymers/Zn composite layers surface is lesser than on pure zinc surface. Those facts indicated that the thermosetting polymers/Zn composite layers are more resistant to the attack of microorganisms like SRB.

Figs. 7 – 9 show the 3D images of the AFM scan acquired by contact mode in air on pure zinc layers and thermosetting polymers/Zn composite surfaces untreated and after SRB attachment with biofilm and EPS formation.

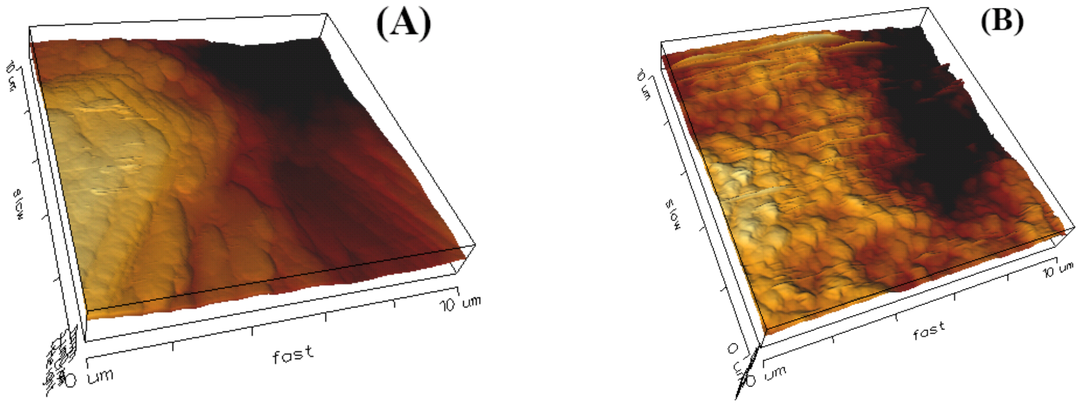


***Fig. 7.*** *3D - AFM images of pure zinc surface: (A) - untreated; (B) - with SRB bacteria*

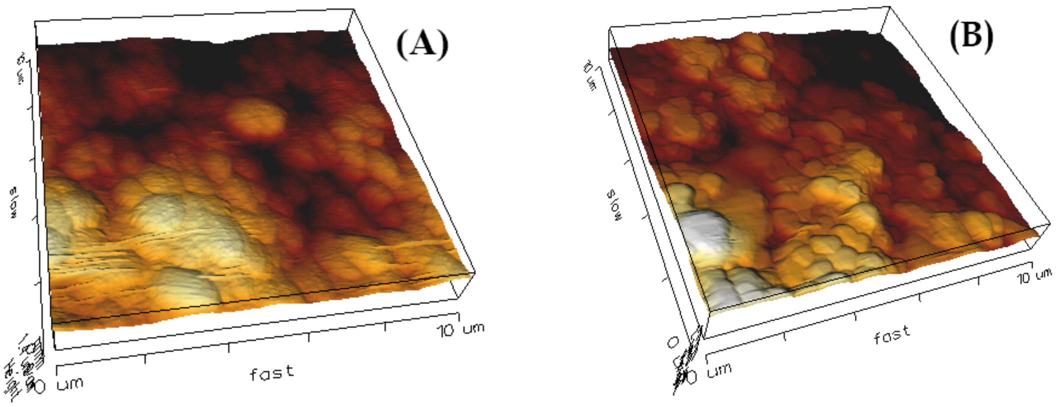


***Fig. 8.*** *3D - AFM images of PF resin/Zn composite layer surface*
*(A) - untreated; (B) - with SRB bacteria*

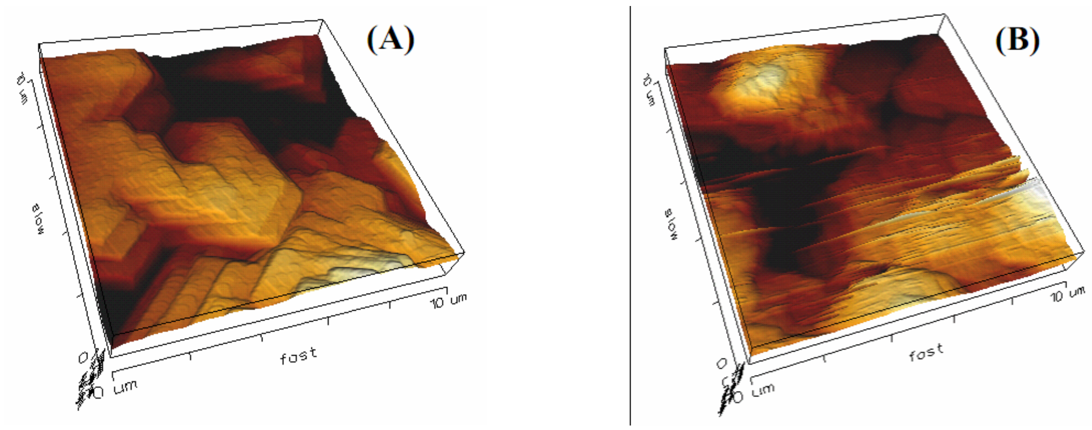


***Fig. 9.*** *3D - AFM images of epoxi resin/Zn composite layer surface*
*(A) - untreated; (B) - with SRB bacteria*

The differences between untreated surfaces and treated with SRB are visible, representing the attached cells of Sulfate Reducing Bacteria on the surfaces, biofilm and EPS formation. EFM – AFM images indicate an adherence process of the microorganisms on the tested surfaces. The use of microscopy to count adhered cells on surfaces is a viable technique, since, on a microscopic scale, surfaces can be found to have cracks and crevices, quite unlike the macroscopic appearance. These surface imperfections protect the microorganisms against removal by swab or rinse. The histograms of the scanned surfaces before and after the attachment of SRB are presented in Fig.10 - 12.

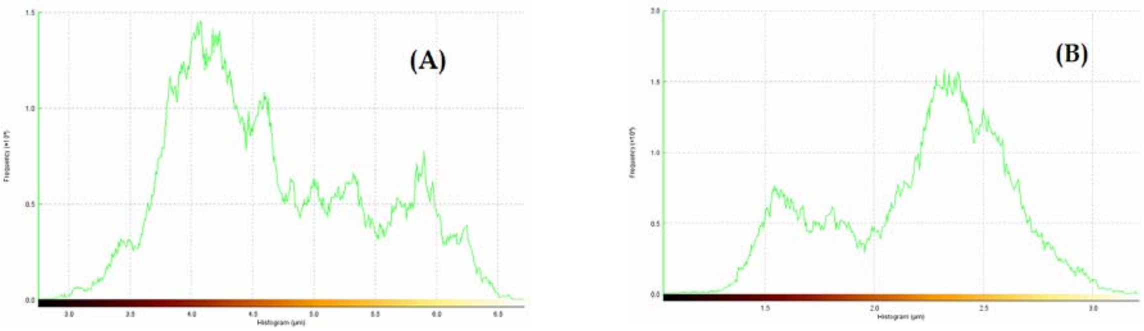


***Fig. 10.*** *Histograms of the scanned surfaces for pure zinc before (A) and after the SRB attachment (B)*

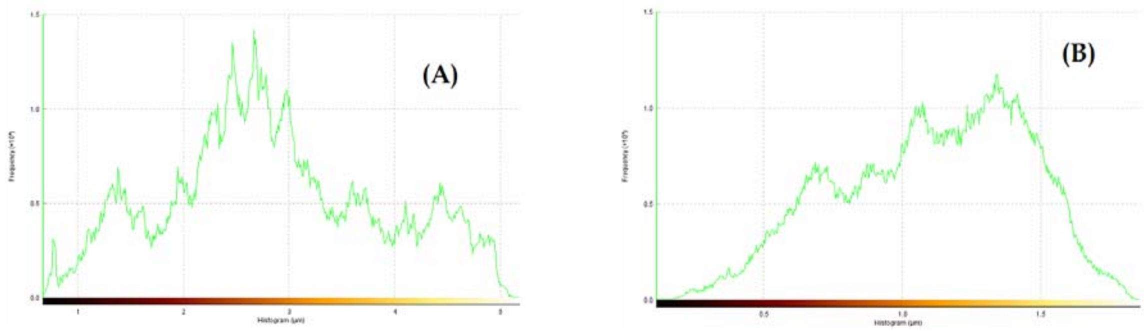


***Fig. 11.*** *Histograms of the scanned surfaces for PF resin/Zn composite layers before (A) and after the SRB attachment (B)*

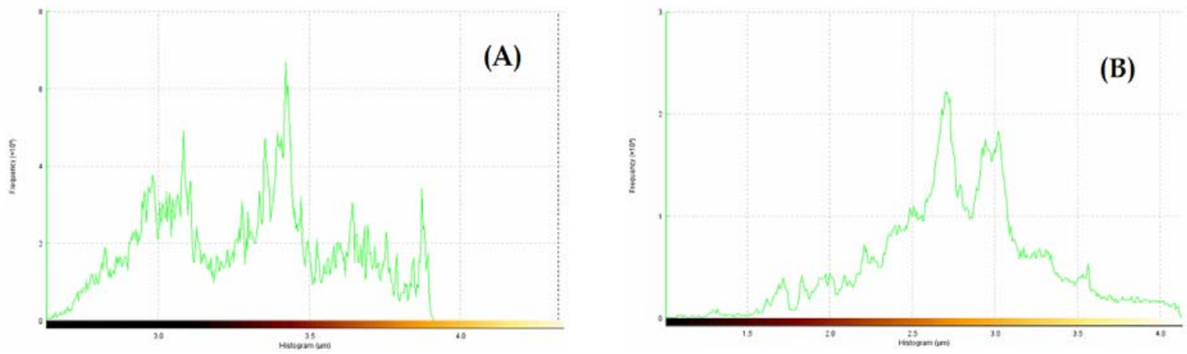


***Fig. 12.*** *Histograms of the scanned surfaces for epoxi resin/Zn composite layers before (A) and after the SRB attachment (B)*

These histograms were used to calculate the roughness of the tested surfaces before and after the attachment of SRB, biofilm and EPS formation.

Roughness is a measure of the texture of a surface and plays an important role in determing how a real system will interact with the environment.

It is quantified by the vertical deviations of a real surface from its ideal form. If these deviations are large, the surface is rough; if they are small the surface is smooth.

The variation of the surfaces roughness for pure zinc and thermosetting polymers/Zn composite layers before and after attachment of SRB is shown in Fig. 13.

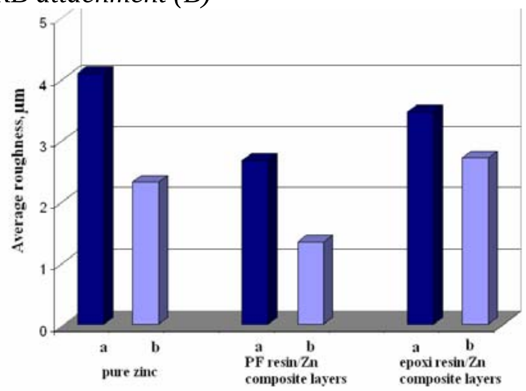


***Fig.13.*** *Variation of roughness surfaces of pure zinc and thermosetting polymers/Zn composite layers before (a) and after the attachment of SRB (b)*

It could be observed that the values of roughness for composite layers without bacteria are smaller than the roughness of pure zinc.

The thermosetting polymer type PF resin and epoxy resin act as reducing the crystals size of electrodeposited zinc during co-deposition. For all systems tested the surfaces roughness decreases after the attachments of bacteria. That could indicate an increase of the uniformity for all tested surfaces after the attachments of SRB, biofilm and EPS formation.

From the EFM images and the values of the roughness it was observed that the attachment of SRB on thermosetting polymers/Zn composite layers is more reduced than on pure zinc surface. For pure zinc surfaces, the difference between the value of roughness for untreated and surface with SRB is bigger than other tested surfaces. That indicates a lot of bacteria attached on this surface, creating biofilm, EPS and corrosion product conducting to a smooth surface. Those facts indicated that the thermosetting polymers/Zn composite layers are more resistant to the attack of microorganisms like SRB.

## 4. Conclusions

The Sulfate Reducing Bacteria were attached on the pure zinc and thermosetting polymers/Zn composite layers.

Bacterial attachment on the surfaces is a complicated process that is affected by material surface (pure metal or composite layers).

From the epifluorescence microscopy and atomic force microscopy images it could be observed that the thermosetting polymers/Zn composite layers are more resistant to the attack of the Sulphate Reducing Bacteria than pure zinc layers.

The surface roughness decreases after the attachments of bacteria, biofilm and EPS formation.

The new system for combining imaging of AFM and EFM on pure zinc and thermosetting polymers/Zn composite layers is feasible for the application to study the biofilm formation by Sulfate Reducing Bacteria on these surfaces.

## References

**[1]. Q. Zhao, C. Wang, Y. Liu, S. Wang**, *Bacterial Adhesion on the Metal- Polymer Composite Coatings*, International Journal of Adhesion and Adhesives, vol. 27, issue 2, p.85- 91, 2007

**[2]. D. Ornek, T.K. Wood, C.H. Hsu, Z. Sun, F. Mansfeld**, *Pitting Corrosion Control of Aluminum 2024 Using Protective Biofilms That Secrete Corrosion Inhibitors*, Corrosion, vol. 58, issue 9, p. 761 – 768, 2002

**[3]. F. Mansfeld, H. Hsu, D. Ornek, T.K. Wood, B.C. Syrett**, *Corrosion Control Using Regenerative Biofilms on Aluminum 2024 and Brass in Different Media*, Journal of the Electrochemical Society, vol. 149, issue 4, p. B130 –B138, 2002

**[4]. G. Gunasekaran, S. Chongdar, S.N. Gaonkar, P. Kumar**, *Influence of bacteria on film formation inhibiting corrosion*, Corrosion Science, vol. 46, issue 8, p. 1953-1967, 2004

**[5]. I. B. Beech, J. A. Sunner, K. Hiraoka**, *Microbe-surface interactions in biofouling and biocorrosion processes,* International Microbiology, vol. 8, issue 3, p. 157 - 168, 2005

**[6]. L. L. Barton,** *Sulphate-reducing bacteria*, Plenum Press, New York, 1995

**[7]. R. Javaherdashti,** *A review of some characteristics of MIC caused by sulfate-reducing bacteria: past, present and future,* Anti – Corrosion Method and Materials, vol. 46, issue 3, p. 173 – 180, 1999

**[8]. H. C. Flemming and J. Wingender**, *Relevance of microbial extracellular polymeric substances (EPSs) - Part II: Technical aspects,* Water Science Technology, vol. 43, issue 6, p. 9 – 16, 2001

**[9]. W. Zhang, A. G. Stack, Y. Chen**, *Interaction force measurement between E. coli cells and nanoparticles immobilized surfaces by using AFM*, Colloids and Surfaces B: Biointerfaces, vol. 82, issue 2, p.316-324, 2011

**[10]. L. Kailas, E.C. Ratcliffe, E.J. Hayhurst, M.G. Walker, S.J. Foster, J.K. Hobbs**, *Immobilizing live bacteria for AFM imaging of cellular processes*, Ultramicroscopy, vol. 109, issue 7, p. 775-780, 2009

**[11]. J. Li, J. Li, W. Yuan, Y. Du**, *Biocorrosion characteristics of the copper alloys BFe30-1-1 and HSn70-1AB by SRB using Atomic Force Microscopy and Scanning Electron Microscopy*, International Biodeterioration & Biodegradation, vol. 64, issue 5, p. 363-370, 2010

**[12]. X. Sheng, Y. P. Ting, S. O. Pehkonen**, *The influence of sulphate-reducing bacteria biofilm on the corrosion of stainless steel AISI 316*, Corrosion Science, vol. 49, issue 5, p. 2159-2176, 2007

**[13]. C. Xua, Y. Zhanga, G. Chenga, W. Zhu**, *Pitting corrosion behavior of 316L stainless steel in the media of sulphate-reducing and iron-oxidizing bacteria*, Materials Characterization, vol. 59, issue 3, p. 245 – 255, 2008

**[14]. F. Kuang, J. Wang, L. Yana, D. Zhang**, *Effects of sulfate-reducing bacteria on the corrosion behavior of carbon steel,* Electrochimica Acta, vol. 52, issue 10, p. 6084 – 6088, 2007

**[15]. Jie Wen, Kaili Zhao, Tingyue Gu, Issam I. Raad**, *A green biocide enhancer for the treatment of sulfate-reducing bacteria (SRB) biofilms on carbon steel surfaces using glutaraldehyde*, International Biodeterioration & Biodegradation, vol. 63, issue 8, p. 1102 – 1106, 2009

**[16]. D. G. Enos and S. R. Taylor**, *Influence of Sulfate-Reducing Bacteria on Alloy 625 and Austenitic Stainless Steel Weldments*, Corrosion, vol.*52*, issue 11, p. 831 – 843, 1996

**[17]. Y. Wan, D. Zhang, H. Liu, Y. Li and B. Hou**, *Influence of sulphate-reducing bacteria on environmental parameters and marine corrosion behavior of Q235 steel in aerobic conditions*, Electrochimica Acta, vol. 55, issue 5, p. 1528 – 1534, 2010

**[18]. J. Liu, X. Liang and S. Li**, *Effect of sulphate-reducing bacteria on the electrochemical impedance spectroscopy characteristics of 1Cr18Ni9Ti*, Journal of University of Science and Technology Beijing, vol. 14, issue 5, p. 425 – 430, 2007

**[19]. T.S. Rao, Aruna Jyothi Kora, B. Anupkumar, S.V. Narasimhan, R. Feser**, *Pitting corrosion of titanium by a freshwater strain of sulphate reducing bacteria (Desulfovibrio vulgaris)*, Corrosion Science, vol. 47, issue 5, p.1071–1084, 2005

**[20]. H. Wanga, Z. Wang, H. Honga, Y. Yina,** *Preparation of cerium-doped $TiO_2$ film on 304 stainless steel and its bactericidal effect in the presence of sulfate-reducing bacteria (SRB)*, Materials Chemistry and Physics, vol. 124, issue 1, p. 791–794, 2010

**[21]. O. Basu; S. A. Baldwin**, *Attachment and Growth of Sulphate-Reducing Bacteria on Different Support Materials,* Environmental Technology, vol. 21, issue 11, p. 1293 – 1300, 2000

**[22]. A.J. Silva, J.S. Hirasawa, M.B. Varesche, E. Foresti, M. Zaiat,** *Evaluation of support materials for the immobilization of sulfate-reducing bacteria and methanogenic archaea*, Anaerobe, vol. 12, issue 2, p. 93–98, 2006.