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INFLUENCE OF SiC/Ni NANOCOMPOSITE COATINGS ON SRB ATTACHMENT AND BIOFILM FORMATION

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ABSTRACT

Bacteria adhesion is a very complicated process affected by many factors: bacterial/material properties and environment. Materials characteristics and chemistry of surfaces are the most important factors in bacterial adhesion and biofilm growth. Cells initially attach by physico–chemical interactions or extracellular matrix protein secretion to form a cell monolayer, in which cells express pili and have twitching motility and/or the ability to undergo chemotaxis. Cells proliferate in the monolayer and other microbes attach to form an active biofilm, the development and distortion of which is influenced by environmental factors such as hydrodynamic and mechanical stress. Cells in the mature biofilm are motile and undergo chemotaxis, which leads to spreading of biomass and an increased rate of horizontal gene transfer. As cells die, active bioconversion and/or biodegradation leads to solute transfer to or from the bulk liquid which results in eventual biofilm detachment.

The work was focused on performing surface modifications studies by co-deposition of dispersed nano particles with metals in order to observe the influence of materials structure (nano-structured coatings prepared) on bacteria cells (Sulphate Reducing Bacteria) attachment. Sessile bacteria on coupons were stained with 4, 6-diamidino-2- phenylindol (DAPI) and visualized by EFM as well as AFM. These types of bacteria are well known as very corrosive for metals in natural seawater.

KEYWORDS: biofilm, bacteria attachment, surface modification, electrodeposition, nanocomposite coatings, sulphate reducing bacteria.

1. Introduction

Many microorganisms in the natural environment exist in multicellular aggregates generally described as biofilms, associated with solid surfaces and in intimate contact with other microbial cells [1–3]. Cells adhere to surfaces and each other through a complex matrix comprising a variety of extracellular polymeric substances (EPS) including exopolysaccharides, proteins and DNA. Biofilm configurations range in complexity from flat, relatively featureless films, to tightly clustered aggregates, to complex heterogeneous cellular arrangements such as towers and streamers. Cells within biofilms are physiologically distinct from the same cells grown in dispersed culture [4-6]. Biofilm cells respond to nutrient and waste product diffusion gradients, modulate their metabolism as a function of their position within the biofilm, contact adjacent cells, and engage in cell–cell communication. Adherent populations exhibit elevated antimicrobial tolerance as a consequence of biofilm structure and physiological adaptation [3]. Biofilms have tremendous practical importance in industrial, medical and agricultural settings, exhibiting both beneficial and detrimental activities. The biofims are formed by microbial aggregates and extracellular polymeric substances (EPS). The EPS creates a microenvironment for sessile bacteria and allow the development of synergistic relationship. Their main components are not only polysaccharides, but also

proteins, lipids and nucleic acids in minor proportion [3]. The biofilms are involved in both beneficial and detrimental effect. One beneficial aspect is their potential use as biosurfactancts in tertiary oil production and their capacity to trap heavy metals; as detrimental effect, biofouling, increase friction resistance, and produce changes in metallic surface properties (hydro phobisity, roughness, color etc.); finely biofilms participate in biocorrosion by bind with metal ions [4]. Microbiologically Influenced Corrosion, MIC refers to corrosion that is influenced by the presence and activities of microorganisms and/or their metabolites (the products produced in their metabolism). Bacteria, fungi and other microorganisms can play a major part in bio corrosion [3-8]. Spectacularly rapid corrosion failures have been observed due to microbial action and it is becoming increasingly apparent that most metallic alloys are susceptible to some form of MIC. The mechanisms potentially involved in MIC are summarized as:

-Cathodic depolarisation, whereby the cathodic rate limiting step is accelerated by micro-biological action

-Formation of occluded surface cells, whereby microorganisms form "patchy" surface colonies. Sticky polymers attract and aggregate biological and non-biological species to produce crevices and concentration cells, the basis for accelerated attack.

-Fixing of anodic reaction sites, whereby microbiological surface colonies lead to the formation of corrosion pits, driven by microbial activity and associated with the location of these colonies.

-Underdeposit acid attack, whereby corrosive attack is accelerated by acidic final products of the MIC "community metabolism", principally short-chain fatty acids.

Certain microorganisms thrive under aerobic conditions, whereas others thrive in anaerobic conditions. Anaerobic conditions may be created in the micro-environmental regime, even if the bulk conditions are aerobic. The pH conditions and availability of nutrients also play a role in determining what type of microorganisms can thrive in a soil environment. Microorganisms associated with corrosion damage are classified as follow:

-Anaerobic bacteria that produce highly corrosive species as part of their metabolism.

-Aerobic bacteria that produce corrosive mineral acids.

-Fungi that may produce corrosive by products in their metabolism, such as organic acids. Apart from metals and alloys they can degrade organic coatings and wood.

-Slime formers that may produce concentration corrosion cells on surfaces.

Microorganisms pervade our environment and readily "invade" industrial systems wherever conditions permit. These agents flourish in a wide range of habitats and show a surprising ability to colonize water rich surfaces wherever nutrients and physical conditions allow. Microbial growth occurs over the whole range of temperatures commonly found in water systems, pressure is rarely a deterrent and limited access to nitrogen and phosphorus is offset by a surprising ability to sequester, concentrate and retain even trace levels of these essential nutrients.

Many engineers continue to be surprised that such small organisms can lead to spectacular failures of large engineering systems. The microorganisms of interest in Microbiologically Influenced Corrosion (MIC) are mostly bacteria, fungi, algae and protozoan.

Bacteria are generally small, with lengths of typically under 10 μm, collectively, bacteria tend to live and grow under wide ranges of temperature, pH and oxygen concentration. Carbon molecules represent an important nutrient source for bacteria.

Fungi can be separated into yeasts and molds. Corrosion damage to aircraft fuel tanks is one of the well-known problems associated with fungi. Fungi tend to produce corrosive products as part of their metabolisms; it is these by-products that are responsible for corrosive attack. Furthermore, fungi can trap other materials leading to fouling and associated corrosion problems.

Protozoan are predators of bacteria and algae and therefore potentially mitigate microbial corrosion problems.

MIC is responsible for the degradation of a wide range of materials. Bacteria can exist in several different metabolic states. Those that are actively respiring, consuming nutrients, and proliferating are said to be in a "growth" stage. Those that simply exist, not growing because of unfavourable conditions, are said to be in a "resting" state.

Some strains, when faced with unacceptable surroundings, form spores that can survive extremes of temperature and long periods without moisture or nutrients, yet produce actively growing cells quickly when conditions again become acceptable.

The latter two states may appear, to the casual observer, to be like death, but the organisms are far from dead. Cells that actually die are usually consumed rapidly by other organisms or enzymes.
When looking at an environmental sample under a microscope, therefore, it should be assumed that most or all of the cell forms observed were alive or capable of life at the time the sample was taken.

Microorganisms can be categorized according to oxygen tolerance.

Sulphate reducing bacteria (SRB) are anaerobes that are sustained by organic nutrients. Generally they require a complete absence of oxygen

and a highly reduced environment to function efficiently. Nonetheless, they circulate (probably in a resting state) in aerated waters, including those treated with chlorine and other oxidizers, until they find an "ideal" environment supporting their metabolism and multiplication. SRB are usually lumped into two nutrient categories, those that can use lactate and those that cannot. The latter generally use acetate and are difficult to grow in the laboratory on any medium. Lactate, acetate, and other short chain fatty acids usable by SRB do not occur naturally in the environment. Therefore, these organisms depend on other organisms to produce such compounds. SRB reduce sulphate to sulphide, which usually shows up as hydrogen sulphide or, if iron is available, as black ferrous sulphide. In the absence of sulphate, some strains can function as fermenters and use organic compounds such as pyruvate to produce acetate, hydrogen, and carbon dioxide. Many SRB strains also contain hydrogenase enzymes, which allow them to consume hydrogen. Most common strains of SRB grow best at temperatures from 25° to 35°C. A few thermophilic strains capable of functioning efficiently at more than 60°C have been reported. SRB have been implicated in the corrosion of cast iron and steel, ferritic stainless steels, 300 series stainless steels (also very highly alloyed stainless steels), copper nickel alloys, and high nickel molybdenum alloys. They are almost always present at corrosion sites because they are in soils, surface water streams and waterside deposits in general. Their mere presence, however, does not mean they are causing corrosion.

The key symptom that usually indicates their involvement in the corrosion process of ferrous alloys is localized in corrosion filled with black sulphide corrosion products. Sulphate Reducing Bacteria are responsible for extreme damage to piping and support equipment in many industries. Sulphate Reducing bacteria are a group of anaerobic bacteria (Do not need air or oxygen) that generate hydrogen sulphide (H_2S). H_2S can cause a number of significant problems in water.

Problems range from "rotten egg" odors to the blackening of equipment, slime formations, and extensive corrosion. SRB microorganisms are difficult to detect because they are anaerobic and tend to grow deep down within biofilms (slimes) as a part of a microbial community.

Our knowledge of the mechanisms behind these interactions is limited because they are complex, and because we lack suitable model systems to study the interactions.

Our paper is a part of a big project concerning the nanocomposite coatings obtained by electrodeosition of nano SiC with nickel on different surface materials in order to improve their corrosive and wear properties [7-8].

This part will present few results about SRB attachment and biofilm formation on nanostructured SiC/Ni surfaces compared topure Ni coatings, by Atomic Force Microscopy (AFM) and Epifluorescence Microscopy (EFM).

In an attempt to determine the relative importance of nano SiC included in the nickel during electrodeposition, experiments were designed to evaluate the electrochemical corrosion [9] and the relationship between SRB bacteria and two types of protecting coating on steel:

i) Nano-structured composite coating with SiC (20nm) embedded during nickel electrodeposition from a disperse nickel plating bath

ii) Pure nickel coating electrodeposited at the same thickness from a sulphate -chloride plating bath.

Biofouling has been recognized as a widespread problem in design and operation of processing equipment such as heat exchangers, cooling water systems and food processing equipment. The objective of this work is to study the influence of SiC/Ni nanocomposite coatings on biofilm formation compared to pure nickel coatings.

2. Experimental set-up

For the SRB biofilm investigations on SiC/nickel nanostructured coatings cold rolled steel panels (20mm x 100mm) were coated with the following combinations:

i) SiC (20 nm) + nickel with two coating thicknesses:

1) 54 μm Ni+SiC
2) 28 μm Ni+SiC

The content of SiC nano particles was determined by EDX analysis on electronic microscope and was found to be between 12 and 13 as volume percent of SiC particles inside the nickel matrix.

ii) pure nickel with two coating thickness:

1) 54 μm Ni
2) 28 μm Ni

In the bacteria attachment and biofilm formation, the Sulphate Reducing Bacteria was used. SRB cells were prepared in University of Duisburg Essen Biofilm Centre. The pH of all solutions with cells suspension was 6.2.

Attachment of cells was made in the following steps: putting a drop from the prepared solution with cells on the surface of coatings; waiting to dry (15-20min); incubation in bacterial suspension of SRB (about 10^9 cells / mL) for 24h to allow attachment and biofilm formation with 2,5% glutaraldehyde. Subsequently, they were stained with 0.01% (wt/vol) DAPI for 10min and visualized at the EFM. AFM imaging was performed by contact mode in air.

Biofilms and attached cells on pure nickel and SiC/Ni nanostructured coatings samples were investigated with combined AFM and EFM. 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. Furthermore, a precision sample clamp guaranteed a tight and accurate fixation of the sample to the shuttle stage, thereby allowing the retrieval of the same sample location with AFM and EFM with an error of no more than 3 µm to 5µm. For sequential investigations, this shuttling could be repeated as often as required without losing position. For a successful combination of both microscopes, meaning the visualization of the same sample location, the variable position of the AFM cantilever had to be aligned to the static optical axis of the epifluorescence microscope in order to match the AFM scan region with the epifluorescence microscope's field of view.

3. Results and discussions

3.1. Structural aspects of SiC/Ni nano composite coatings

Micrographies presented allow comparison between a nanostructured composite surface (Fig. 1) and pure nickel surface (Fig. 2). The pure nickel deposit has a rather regular surface, whereas the composite coating develops in a nodular disturbed surface structure.

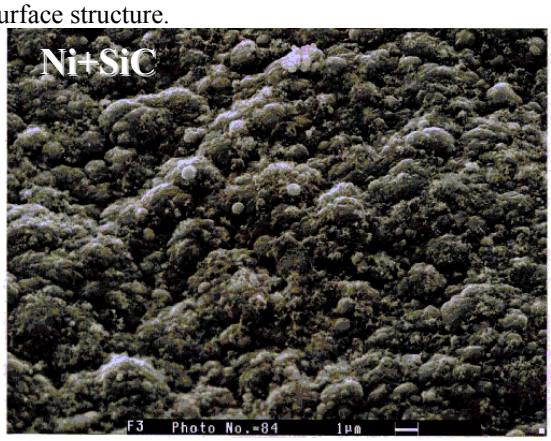

Fig. 1. *SEM surface morphology of SiC/Ni nano-structured composite coating.*

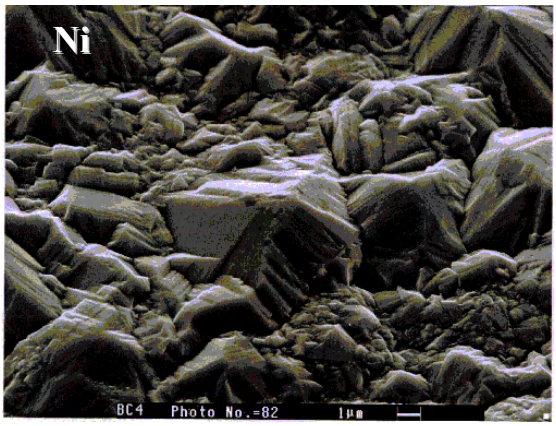

Fig. 2. *SEM surface morphology of pure Ni coatings.*

The surface analysis was performed on composite surface with X-ray disperse energy system (EDS) on the same surface area of the samples. From the general EDS analysis, the total amount of nano SiC particles inside the deposit was calculated at 8.99% as weight percent or 25% as volume percent. The volume percent of nano –SiC is higher because of lower density of particles (3.2 gdm^{-3}) compared to nickel matrix (8.9 gdm^{-3}).

3.2. AFM-EFM study of biofilm formation

Epifluorescence microscopy (EFM) images of a DAPI – stained biofilm sample of SRB on the surface of nickel and SiC/Ni nano composite coatings obtained at different parameters for electrodeposition are presented in Figs. 3-4.

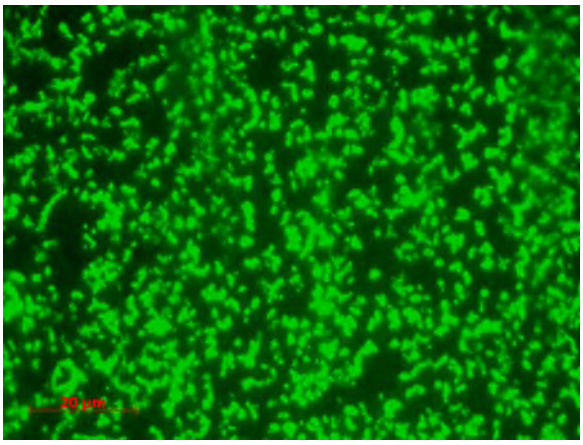

Fig. 3. *Fluorescence microscopy image of SRB on pure nickel surface obtained at current density 4A/dm^2, 60min.*

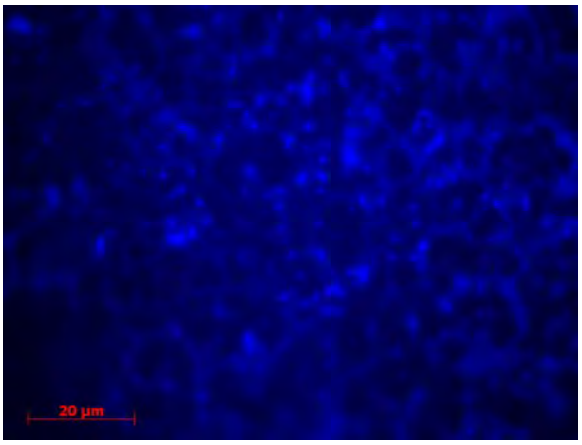

Fig. 4. *Fluorescence microscopy image of SRB on SiC/Ni nano composite coatings surface obtained at current density 4A/dm^2, 60 min.*

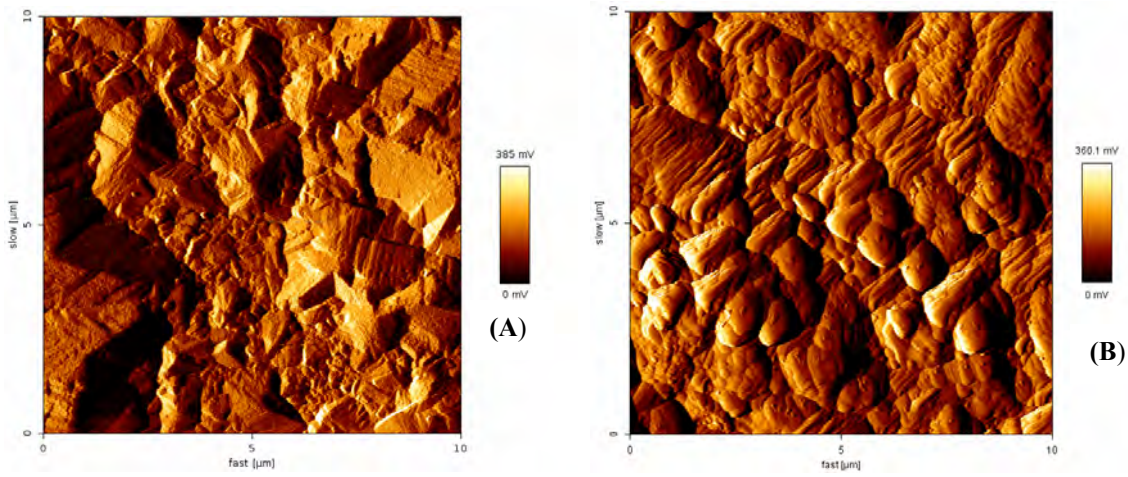

Fig. 5. *AFM of nickel surface (i = 4 A/dm^2 for 1h): A- untreated surface; (B) with SRB, 2D – Vertical deflection.*

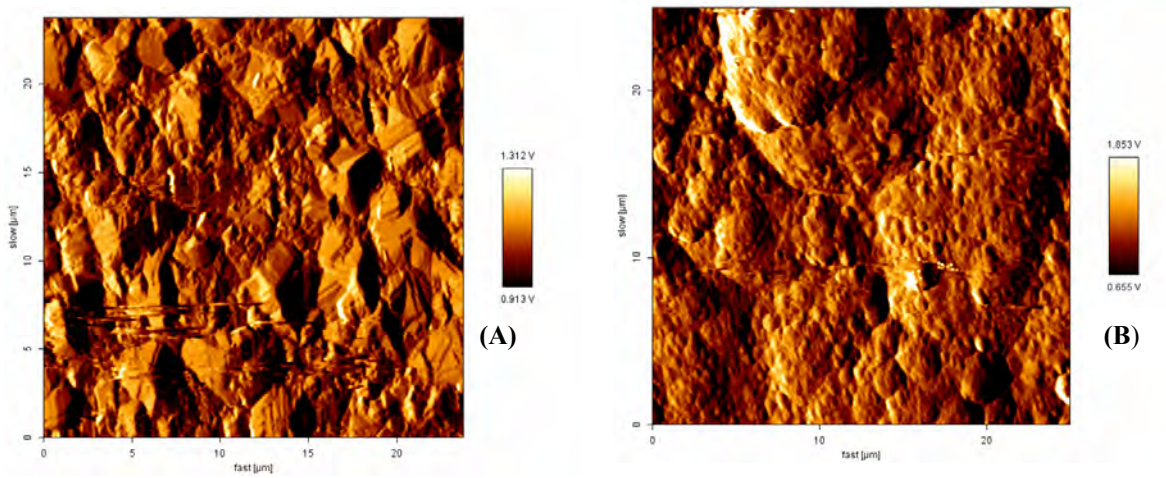

Fig. 6. *AFM of SiC/Ni nano composite coating surface 2D – Vertical deflection (i = 4 A/dm2 for 1h): (A) – untreated; (B) – with SRB bacteria.*

Figs 5-6 show the vertical deflection images of the AFM scan acquired by contact mode in air on pure nickel coating and SiC/Ni nano composite surfaces untreated and after SRB attachment and biofilm formation. The differences between untreated surfaces and treated with SRB are visible, representing the attached cells of Sulfate Reducing Bacteria on the surfaces.

The differences of SRB biofilm formation on two types of surfaces could be observed also by AFM images presented in 3D high mode, see Figs. 7-8.

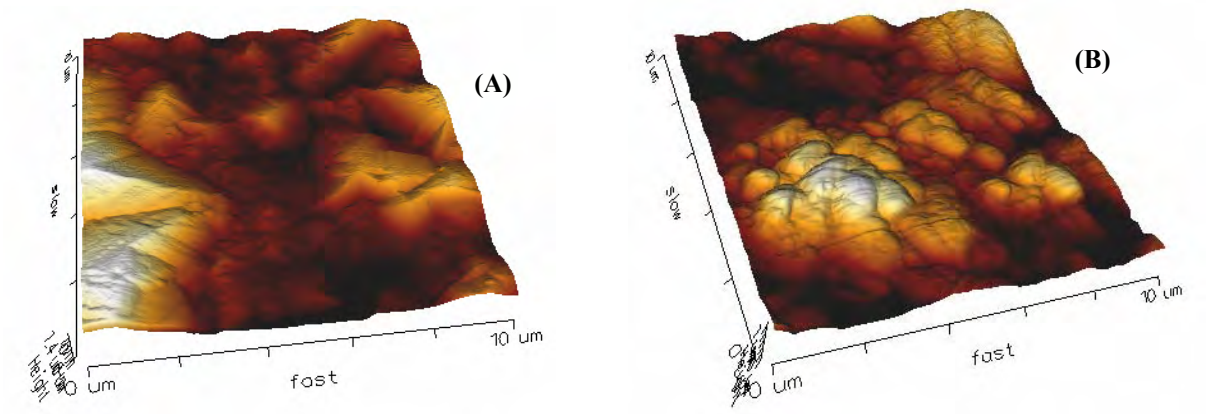

Fig. 7. *AFM of pure nickel surface 3D – Heigh (i = 4 A/dm^2 for 1h): (A) - untreated; (B)- with SRB bacteria*

From the EFM and AFM microscopy images we could observe that the attachment of SRB bacteria on nano composite coatings is less than that on pure nickel coatings. Those facts indicated that the SiC/Ni nano composite coatings are more resistant to the attack of microorganisms like SRB. These AFM-EFM images indicate an adherence process of the microorganisms on the studied 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.

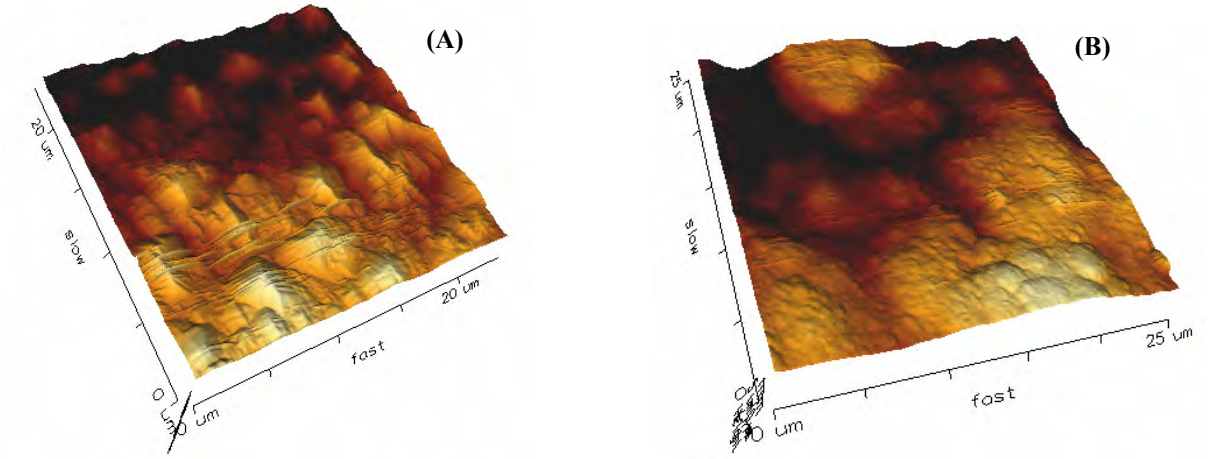

Fig. 8. *AFM of SiC/Ni nano composite coating surface 3D - Heigh (i = 4 A/dm^2 for 1h): (A) – untreated; (B) - with SRB.*

These surface imperfections protect the microorganisms against removal by swab or rinse, for example. Microscopes coupled with image analysis systems can help the count process of adhered cells on surfaces by EP.

For all systems tested we could observe that the surface roughness decreases after the attachments of bacteria, that indicate the uniformity of biofilm and extra-cellular polymer formation [10].

Decreases of the surface roughness was also reported for two types of stainless steel austenitic AISI type 304 and the superduplex UMS S32750 after immersed in seawater containing Desulfovibrio vulgaris ssp vulgaris DP4 [11].

SiC/Ni nanocomposite coatings seem to have better resistance to SRB attachment and biocorrosion followed by biofilm formation.

4. Conclusions

The Sulfate Reducing Bacteria (concentration about 10^9cells/mL) are attached on the pure nickel and less on the SiC/Ni nano composite coatings after an incubation of 24h.

From the epifluorescence microscopy and atomic force microscopy images we could observe that the SiC/Ni nano composite coatings are more resistant to

the attack of the Sulfate Reducing Bacteria compared to pure nickel coatings.

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

The new system for combining imaging of AFM and EFM on nickel and SiC/Ni nano composite coatings is feasible for the application to study the biofilm formation by Sulfate Reducing Bacteria on these surfaces.

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