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Variation in sessile microflora during biofilm formation on AISI-304 stainless steel coupons

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Coupons of stainless steel type AISI-304 were exposed to the industrial cooling system of a petrochemical plant fed by seawater from the Guanabara Bay, Rio de Janeiro, Brazil, in order to study the *in situ* formation of biofilms. Bacteria, microalgae and fungi were detected on the coupons as soon as 48 h after exposure. Their respective numbers were determined at times 48, 96 and 192 h and over the following 8 weeks. Aerobic, anaerobic and sulfate-reducing bacteria were quantified according to the technique of the most probable number, and fungi by the pour plate technique. The number of microorganisms present in the forming biofilm varied over the experimental period, reaching maximal levels of 14×10^{11} cells cm^{-2}, 30×10^{13} cells cm^{-2}, 38×10^{11} cells cm^{-2} and 63×10^{5} cells cm^{-2}, respectively, for aerobic bacteria, anaerobic bacteria, sulfate-reducing bacteria and fungi, and the dynamics of this variation depended on the group of microorganisms. *Bacillus* sp, *Escherichia coli, Serratia* sp and *Pseudomonas putrefaciens* were identified among the aerobic bacteria isolated. Additionally, microalgae and bacteria of the genus *Gallionella* were also detected. Nonetheless, no evidence of corrosion was found on the stainless steel type AISI-304 coupons over the experimental period.

Keywords: biofilm; cooling water; microbiologically influenced corrosion; microbial fouling; stainless steel

Introduction

Microorganisms rarely occur or act singly in nature. They typically form communities, with their members possessing an array of biochemical and physiological functions [14].

The formation of a biofilm takes place when a solid surface comes into contact with a liquid medium. Organic substances and minerals are transported to the surface and create a conditioning film, where nutrients are concentrated and allow the replication of microorganisms present in the aqueous environment [4].

Due to their ability to form biofilms on solid surfaces, microorganisms and their varied metabolic processes have been studied in relation to metal corrosion, and the results demonstrate that microbial activities accelerate fouling processes. According to Videla [17], the non-correlation between electrochemical and microbiological approaches has long prevented an adequate interpretation of microfouling, a problem that so often affects industrial activities.

Biofilm formation may cause important damages to industrial appliances, such as reduction in the efficiency of heat exchangers, obstruction of filters and metal corrosion [8]. In addition, in the food industry, adhesion of microorganisms to processing equipment may bring about serious consequences, like the organoleptic alterations of products [12].

Petrochemical plants tend to be built at coastal locations due to the ease of loading or product transportation at these sites, and also because of the availability of water for use as coolant fluid. Sea water contains relatively high concentrations of salts and can therefore bring about electrochemical corrosion. Furthermore, its high organic content facilitates the proliferation of sessile microorganisms, which are known to accelerate the corrosion process.

The most studied metals in relation to microfouling are those preferentially used for the manufacture of industrial equipment, such as carbon steel, copper and nickel alloys, titanium and stainless steel. Of these, the most resistant to seawater corrosion is titanium, yet its high cost represents a great disadvantage when decisions on metal choice are to be made [3]. Stainless steel type AISI-304, on the other hand, despite being considered susceptible to the action of corrosive salt water, is employed in refrigeration systems of petrochemical plants.

The aim of this work was to evaluate the sequence of establishment and variation of microorganisms during biofilm formation on stainless steel type AISI-304 coupons exposed to salt water. Seawater was from the intake basin used by the Petroflex petrochemical plant to feed its cooling system.

Materials and methods

Field (in situ) testing

Field experiments were carried out in a petrochemical plant (Petroflex) located by the side of the Guanabara Bay, Rio de Janeiro, Brazil.

The 2.0×1.7 cm^2 coupons used for testing were of stainless steel type AISI-304 because part of the equipment at the plant was made of this metal. The coupons were degreased in acetone and kept in a dessicator before being fixed to PVC ducts at the seawater catchment area of the

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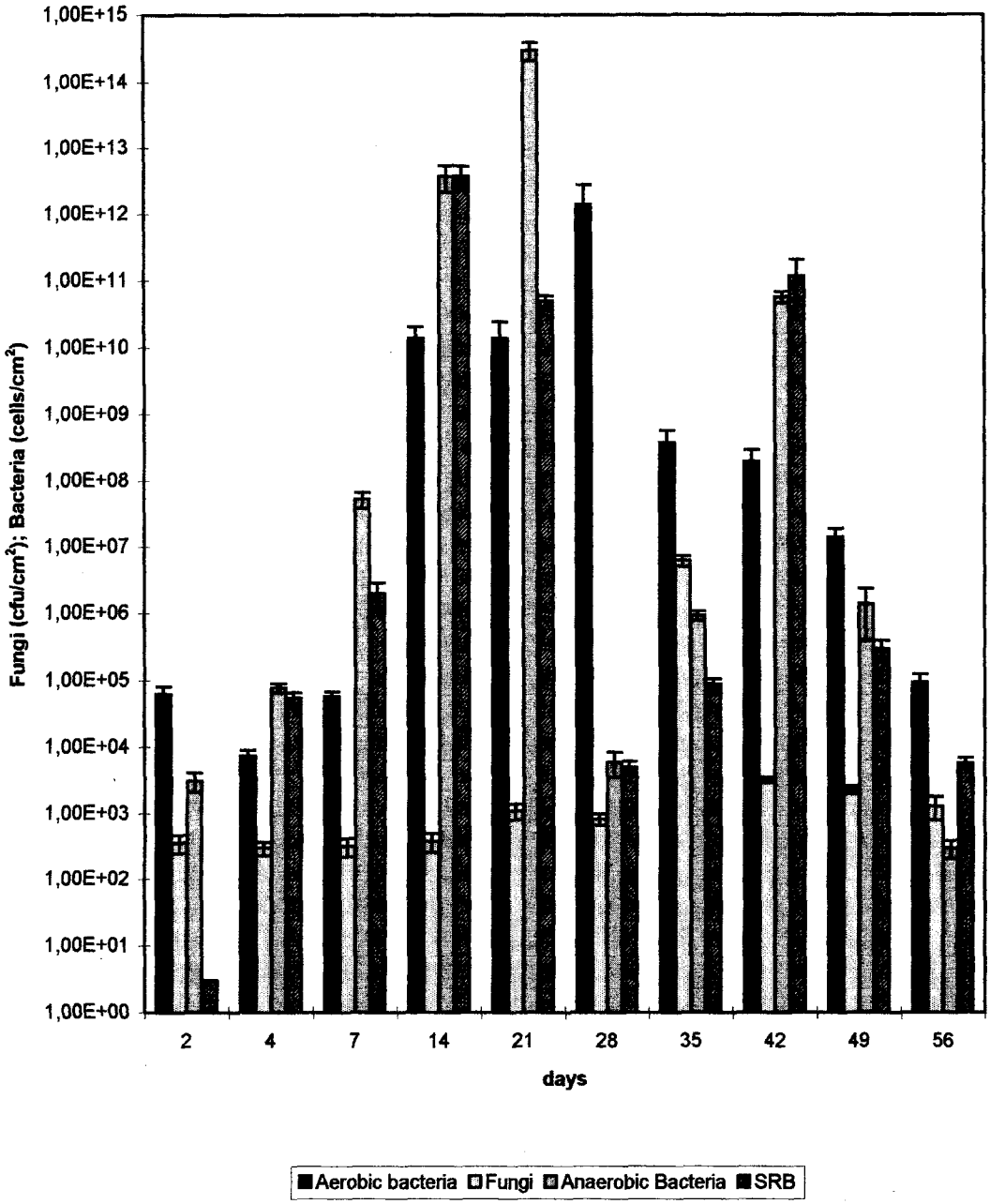

Figure 1 Number of microorganisms detected on biofilms on the surface of stainless steel type AISI-304 coupons exposed to seawater. Mean value of four experiments.

plant. Abrasive treatment of the coupons was avoided in order to minimize interferences with the capacity of the metal surface to promote microbial adherence [18].

The seawater used by the Petroflex plant is frequently not submitted to previous treatment, as is the case with the cooling water fed to its open heat exchanger system. Due to the location, in an industrial setting, this seawater is susceptible to contamination by waste discharge from neighboring plants. Chemical analysis revealed the following composition: Mg 740 mg L^{-1}; Al < 1.0 mg L^{-1}; Ca 323 mg L^{-1}; Fe 0.35 mg L^{-1}; Mn 0.1 mg L^{-1}; K 253 mg L^{-1}; Na 6.4 g L^{-1}; and Cl^- 12.85 g L^{-1}.

During the experimental period, the water temperature varied from 30°C to 32°C and the pH from 7.7 to 7.9. The mean industrial water flow was 1.9 m^3 h^{-1}.

Test coupons were withdrawn for analysis at various time intervals over a period of 56 days of exposure to seawater.

Laboratory testing

The direct effect of seawater on stainless steel AISI-304 was tested in the laboratory. To this end, metal coupons were placed inside flasks containing 100 ml of seawater collected at the plant's water intake basin. The system was sterilized at 121°C for 20 min.

Quantitative determinations

Preparation of suspensions for analysis: In order to remove planktonic microorganisms from the coupons, immediately after withdrawal the metal surface was rapidly flushed with sterile distilled water containing 2% NaCl.

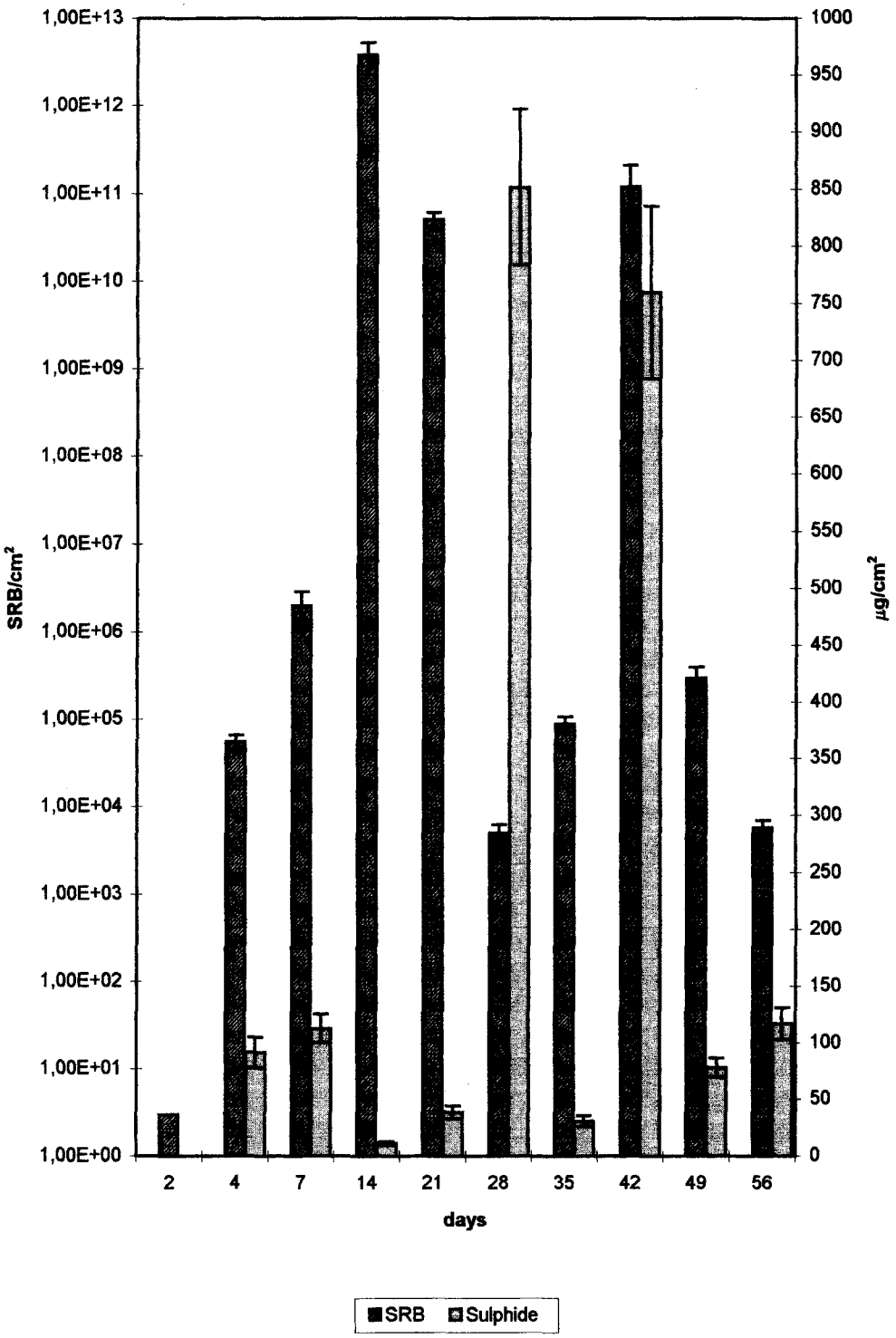

Figure 2 Number of SRB and total sulfide content detected on biofilms on the surface of stainless steel type AISI-304 coupons exposed to seawater. Mean value of four experiments.

Microbial suspensions for analysis of aerobic microorganisms were obtained by scraping off one of the surfaces of the metal coupon with a sterile spatula under aseptic conditions. Biofilm samples were suspended in 30 ml of 2% NaCl solution and sonicated at an amplitude of 4 μm for 1 min (Soniprep, MES Instruments, Thornton), as described by Cook and Gaylarde [6].

For the determination of anaerobic bacteria, removal of biofilms was carried out as described above, with the substitution of a reducing solution [15] previously degassed with N_2 for the NaCl solution.

Quantification of aerobic bacteria: Nutrient broth (Merck, Darmstadt, Germany) was used as culture medium, and aerobic bacteria were quantified by the most probable

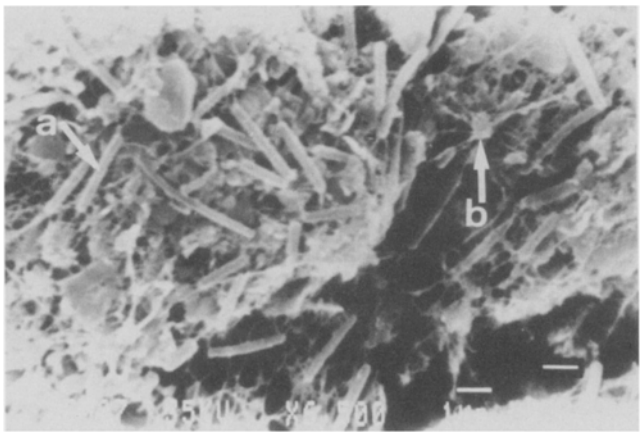

Figure 3 SEM photomicrograph showing biofilm on stainless steel type AISI-304 coupons after 21 days of *in situ* exposure to seawater. a, bacteria; b, extracellular matter (bar = 1 μm).

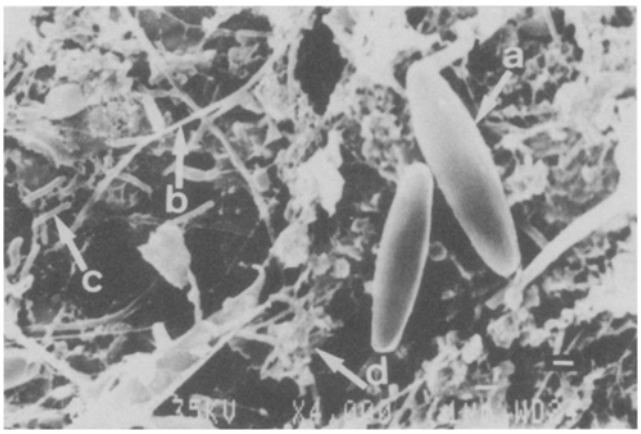

Figure 4 SEM photomicrograph showing biofilm with colonization by algae on stainless steel type AISI-304 coupons after 21 days of *in situ* exposure to seawater, a, microalgae; b, filaments; c, rod-shaped cells; d, deposits (bar = 1 μm).

number technique (MPN) [10]. Incubation was at 32 ± 1°C for 48 h.

Quantification of anaerobic bacteria: The MPN technique was applied using as culture medium fluid thioglycollate (Difco No. 0256, Difco Laboratories, Detroit, MI, USA) supplemented with ferric ammonium sulfate and degassed with N_2. The assay was carried out in tubes sealed with a rubber stopper and a metal ring belt. After addition to the tubes, the medium was degassed once more with N_2, to ensure total removal of O_2 prior to use. Incubation was at 32 ± 1°C for 28 days. Essentially the same technique was used to estimate the number of sulfate-reducing bacteria (SRB), substituting Postgate B medium [15] as the culture medium.

Quantification of fungi: Fungi were quantified by enumeration of colony forming units on Sabouraud agar (Merck). Incubation was at 25 ± 1°C for 7 days.

Quantification of total sulfides: Total sulfide content was quantified by a colorimetric method using N,N-dimethyl-*p*-phenylenodiamine and ferric chloride [1] after treating the biofilm suspensions with concentrated HCl. The standard curve used for reference was obtained with Na_2S-monohydrate PA (Merck). Readings were taken at 670 nm.

Qualitative analyses

To verify the presence of bacteria of the genus *Gallionella*, samples of the suspensions prepared for analysis of aerobic microorganisms were inoculated into the culture medium recommended by APHA [2].

The same microbial suspensions were also inoculated into modified Chú medium [16] for evaluation of recovered microalgae.

Identification of bacteria

The predominant aerobic bacteria were identified according to Bergey's Manual of Systematic Bacteriology [9].

Evaluation by scanning electron microscopy (SEM)

SEM analysis was carried out on coupons containing biofilms after exposure to seawater. Specimens were fixed immediately after *in situ* collection with 2.5% glutaraldehyde solution and 0.1 M cacodylate buffer, 1 : 1 (vol : vol) in sea water, for 24 h at 4°C. Next, samples were rinsed with 0.1 M cacodylate buffer and subsequently with decreasing concentrations of sea water (30%, 20%, 5% in triple-distilled water) for desalination. Samples were then dehydrated through an acetone series to 100% (30%, 50%, 70%, 90%, 100%). The samples were then dried by injection of CO_2 in a critical point drying apparatus (CPD-030 Balzers) [7]. The dried samples then received a layer of gold with a Balzers Union SCD-040 (Aktiengesellschaft, Fürstentum, Liechtenstein) and were observed through the Jeol JXA 840 Electron Probe Microanalyzer (Tokyo, Japan).

Results and discussion

After 2 days exposure to seawater from the industrial setting, a biofilm was observed on stainless steel type AISI-304 test coupons. Not only bacteria but also fungi and microalgae were detected, although the latter microorganisms are normally cited in the literature as the last colonizers to settle and contribute to biofilm formation [17].

The numbers of adherent microorganisms varied during the process of biofilm generation. After 21 days exposure, the biofilm formed contained a greater number of microbial cells, attaining levels of $1.4 \pm 0.16 \times 10^{10}$ cells cm^{-2} and $3.0 \pm 0.14 \times 10^{14}$ cells cm^{-2} for aerobic and anaerobic bacteria, respectively. It was also observed that the fungi adhered to the metal surface at the start of biofilm formation, despite being considered among the last colonizers. However, contrary to what was observed for attached bacteria, the number of fungal cells was constant throughout the time of exposure, varying only after 35 days when their number reached 63×10^5 CFU cm^{-2} (Figure 1). As reported in the literature, settled microbial cells undergo metabolic changes and start to secrete large amounts of exopolysaccharides (EPS). These extracellular polymers improve the adherence capacity to a metal surface and promote further trapping of microorganisms in the substratum. The

microenvironment that results thus limits diffusion of oxygen through the layers of the biofilm. This effect, added to the consumption of oxygen by already settled aerobic microorganisms and microaerophiles, creates ideal conditions for the growth of anaerobic species [4]. The establishment of conditions for anaerobiosis was confirmed by the finding of a great number of adhered anaerobic bacteria.

After 28 days of exposure, a gradual reduction in the number of microorganisms was observed. This variation may be due to the action of external factors such as water turbulence, which can cause a sloughing effect and lead to detachment of the outermost portions of the biofilm [5]. The nature of the biofilm is thus not static and varied with time.

Qualitative analysis of microbial components of the biofilm by specific assays revealed the presence of microalgae and bacteria of the genus *Gallionella* throughout the experimental period. *Gallionella* spp are important because they contribute to the generation of conditions favorable to colonization by SRB, and also because they function as Fe biooxidants, with consequent formation of iron precipitates that become deposited on pipelines and cause unwanted changes in water turbidity, color, odor and taste [11].

With respect to aerobic bacteria, *Pseudomonas putrefaciens, Escherichia coli, Bacillus* sp and *Serratia* sp were identified. *Pseudomonas* species are normally found in aquatic environments and are capable of producing EPS, which facilitates the attachment of other species to the forming biofilm [13]. Bacteria of the genus *Bacillus* have been isolated frequently from tubercles formed on metals and associated with microfouling. The presence of *Escherichia coli*, an enteric bacterium, can be explained by the inflow of water contaminated with organic matter, drained from areas contaminated with organic waste near the experimental site.

Bacteria of the genus *Serratia* have not been reported in biofilms formed on metal surfaces. However, in our study, their presence was observed frequently. As these microorganisms produce outer capsules, they probably tend to adhere easily to a forming substratum and may facilitate the attachment of other microorganisms.

A lack of correlation between cell counts for SRB and total sulfide content was observed, despite the fact that biogenic sulfide derives from the activity of SRB species (Figure 2). This could be explained by the sloughing of tubercles from the metal surface due to water turbulence.

Laboratory analysis of a possible direct action of sterile seawater on metal coupons did not demonstrate a detectable effect on stainless steel type AISI-304 over the experimental period.

SEM evaluation of biofilm formation in field experiments revealed the presence of microorganisms on the stainless steel surface 21 days after exposure of coupons to seawater. A large number of bacteria was detected, as well as adhered extracellular matter (Figure 3), microalgae, various filaments, rod-shaped cells and deposits (Figure 4).

Microbial species normally associated with microfouling of metals were recovered from biofilms formed *in situ* in this study; nonetheless, no evidence of corrosion was detected on the test surface of stainless steel type AISI-304 coupons, observed through a stereoscopic microscope, showing that this metal was resistant to seawater corrosion under the conditions tested.

Acknowledgements

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