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Biofilm formation on brass coupons exposed to a cooling system of an oil refinery

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Brass coupons (70% Cu 30% Zn) were exposed to a cooling freshwater system of an oil refinery, in order to investigate susceptibility of the metal to biofilm formation. The coupons were fixed on bypasses at points which allowed the circulation of makeup, cooling and return water. The number of aerobic, anaerobic and sulfate-reducing bacteria was determined in both the planktonic and the sessile phases. Maximum bacterial concentrations were detected in the cooling water, corresponding to $2.1 \pm 0.1 \times 10^6$ CFU ml^{-1} (planktonic phase) and $1.3 \pm 0.2 \times 10^5$ CFU cm^{-2} (sessile phase) for aerobic bacteria and to $3.2 \pm 0.3 \times 10^5$ cells ml^{-1} (planktonic phase) and $6.2 \pm 0.7 \times 10^5$ cells cm^{-2} (sessile phase) for anaerobic bacteria. Sulfate-reducing bacteria (SRB) were observed only in the planktonic phase, being found in greater numbers in the return water. Scanning electron microscopy (SEM) analysis indicated that biofilm formation occurred at the three monitored sites and showed a diversity in cell morphology. Nonetheless, no evidence of corrosion was observed on the brass coupons during the experimental period.

Keywords: biofilm; biocorrosion; brass; cooling water

Introduction

Biodeterioration of metal surfaces is usually accompanied by the formation of a biofilm. According to Sifdlarek *et al* [16], biofilms are composed of a mixed consortium of bacteria, fungi and a small number of algae, associated with extracellular anionic polymers, in general related to microbially induced corrosion (MIC). The thickness of the biofilms and the complexity of the bacterial populations in a biofilm create an aeration gradient throughout the various layers of its structure; in this way, hypothetically, adequate conditions are established for the development of anaerobic microorganisms, including sulfate-reducing bacteria (SRB) [4,17]. Different types of biofilm can be formed depending on the flow rate and water chemistry in a system, which in turn affect the rate of microbial corrosion and probably the mechanism(s) involved in this process [10].

Copper alloys, despite showing a good resistance to corrosion, are susceptible to MIC, which may take place through various mechanisms, such as differential aeration, cathodic depolarization, selective leaching and corrosion under deposits [20]. According to Gentil [8], brass (copper-zinc alloys) may undergo a process of selective corrosion (dezincification) whereby zinc is preferentially oxidized, leaving a residue of copper and corrosion products. Donlan [7], studying the relationship between colonization by SRB and microbial metabolism on selected metals exposed to recirculating coolants, observed that the processes of colonization on brass and steel were similar, despite the toxic effect of Cu(II).

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In the present study, brass coupons were placed at various sites within the cooling system of an oil refinery, in order to study biofilm formation and obtain microbiological data relevant to an evaluation of treated recirculating water.

Materials and methods

Operational characteristics of the cooling water system

The cooling system of the oil refinery operates with recirculating water. Fresh water was used for feeding (makeup), which was first clarified by flocculation with aluminum sulfate and submitted to chlorination. Chemical analysis of water samples was performed according to APHA [2]. Feeding and recirculating water analysis revealed the following compositions (mg L^{-1}): makeup water – alkalinity as $CaCO_3$ 18, sulfate 1.7, silica 12, chloride 14, total solids 31, total iron 0.5; recirculating water – alkalinity as $CaCO_3$ 15.3, sulfate 3.5, silica 10, chloride 10, total solids 600, total iron 20, phosphate 7.9, zinc 2.7. Inhibitors were used to control the corrosion process. Chlorine was added to maintain a residual concentration of 0.4–0.6 ppm (free Cl_2) and biocide (izothiazolone, Kurita) was used only when a fall in chlorine concentration occurred.

A diagram of the cooling water system with monitored sites is shown in Figure 1.

Metal samples and monitored sites

Brass coupons ($14 \times 12 \times 1.0$ mm in size) were fixed to PVC ducts installed at three points, which were selected to allow the recirculation of fresh water prior to treatment, cooling water and return water, (Figure 1, sites 1, 2, 3 respectively). Metal coupons were placed in the ducts with the help of a screwed plastic support, in the same direction of the water flow (Figure 2). The coupons were degreased with acetone and kept in a desiccator before being fixed

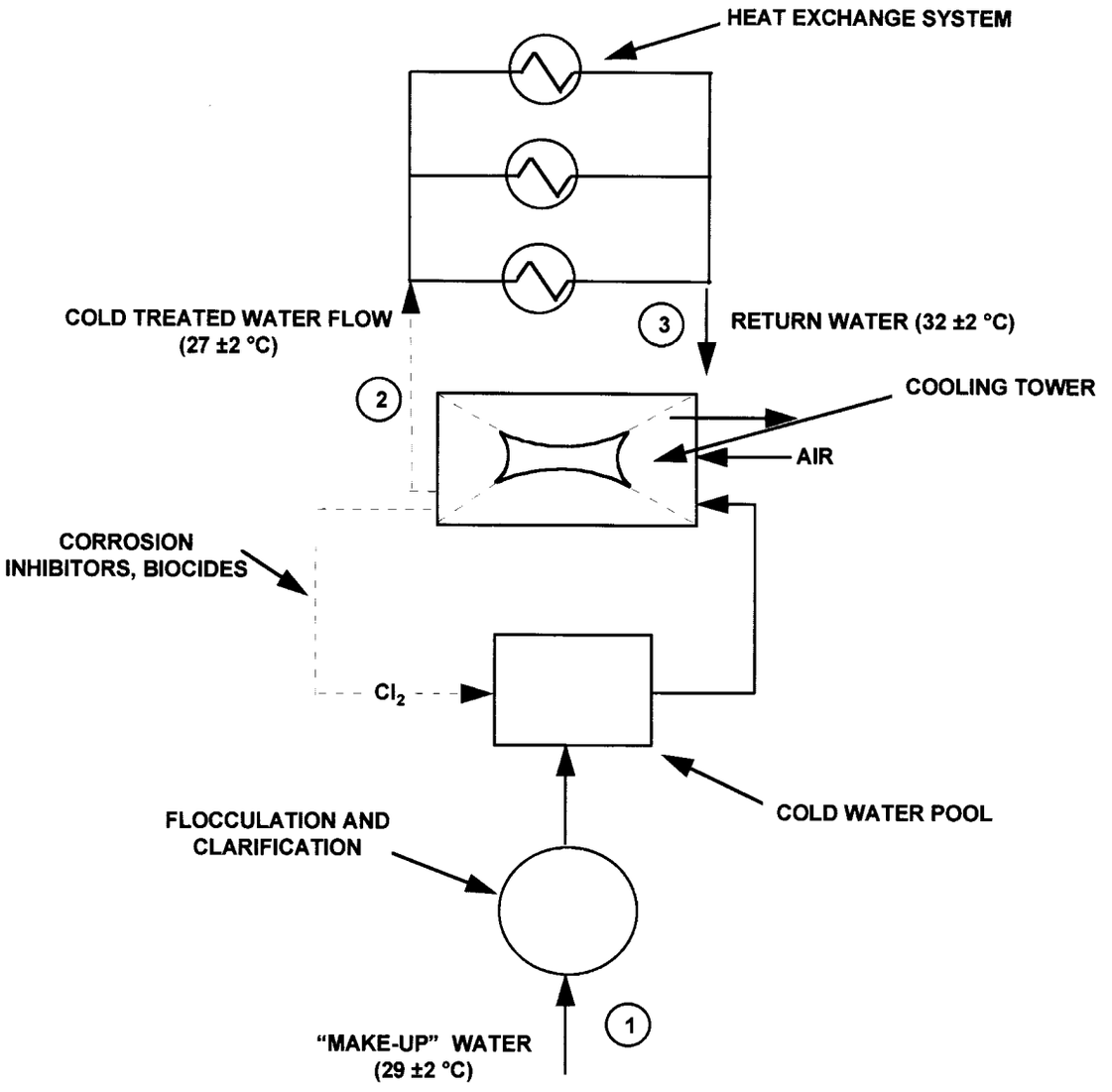

Figure 1 Diagram of the cooling water system. Monitored points: (1) makeup water, (2) cooling water, (3) return water.

to the PVC ducts. To avoid possible interference with the attachment of microorganisms onto the metal surfaces, coupons were not submitted to abrasive pretreatment [19,21].

Microbial quantitation

Sampled coupons were removed from the ducts, after 15, 30, 60, and 90 days of exposure, placed inside sterile flasks filled with water from the system and immediately taken to the laboratory for processing. After being withdrawn from the flasks, the coupons were washed gently in sterile distilled water to remove planktonic microorganisms [3,6]. For determination of aerobic bacteria, biofilms were recovered by scraping with a sterile spatula under aseptic conditions in 30 ml of sterile distilled water as described by Lutterbach and de França [11]. Then the suspensions were vortexed to disperse the microorganisms, as recommended by Walker *et al* [22]. For determination of anaerobic microorganisms the same procedure was used, by substituting the sterile distilled water with a reducing solution [15] previously degassed with N_2 [6].

Quantitation of aerobic bacteria

Nutrient agar (Merck No 5443, Germany) was used as the culture medium and the aerobic bacteria were quantified by plate counts. Serial dilutions of bacterial suspensions were made using sterile water and triplicate replicates of each dilution were used for plating. The number of colonies was counted in a Phoenix CP600 (Brazil) colony counter after 72 h incubation at 30 ± 1.0°C.

Quantitation of anaerobic bacteria and sulfate-reducing bacteria (SRB)

The most probable number technique (MPN) [14] was used to quantify the anaerobic bacteria. Dilutions were made on a reducing solution [15] and fluid thioglycollate (Merck No 8191) degassed with N_2 was used as medium. The assay was carried out inside tubes sealed airtight with a rubber stopper and a metal ring belt. To ensure complete anaerobiosis, strict precautions were taken in the preparation and handling of the tubes. Anaerobic conditions were ensured by handling the tubes in a glove box, in an atmosphere

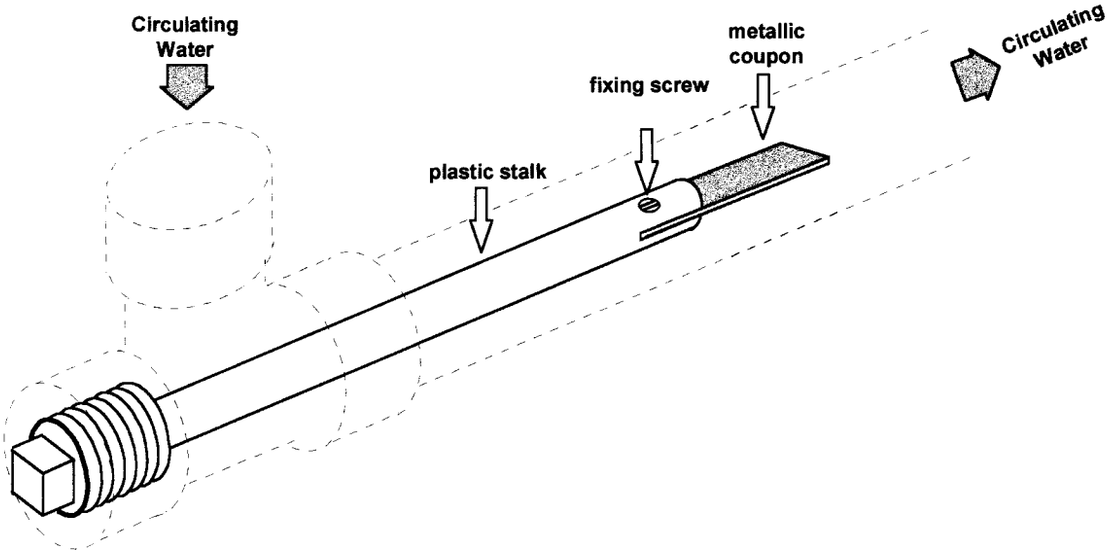

Figure 2 Schematic representation of the coupons' plastic support.

containing 80% N_2, 10% CO_2, and 10% H_2. A syringe and needle were used to inoculate through the rubber seal. Incubation was carried out at 30 ± 1.0°C for 28 days.

The same technique described for anaerobic bacteria was applied to quantify the number of SRB present in the biofilms, using Postgate E as the culture medium [15].

The number of cells cm^{-2} were obtained by dividing the total number of cells by the top surface area of the metal coupons.

Quantitation of total sulfides

Total sulfide content was determined colorimetrically using N-N-dimethyl-*p*-phenylenodiamine and ferric chloride (Merck), as described by APHA [1], after treatment of the biofilm suspensions with concentrated HCl to dissolve insoluble sulfides. Readings were taken at 670 nm in a UV-visible spectrophotometer (CAMSPEC, model 302, UK). The standard curve used as reference was obtained with Na_2S-monohydrate PA (Merck).

Identification of bacteria

The most frequently found aerobic bacteria were isolated and identified according to Bergey's Manual of Determinative Bacteriology [9].

Scanning electron microscopy (SEM)

Biofilm-containing coupons were analyzed by SEM at the end of the experimental period of exposure (90 days). Specimens were fixed with 5.0% glutaraldehyde in 0.1 M sodium cacodylate buffer 1:1 (vol/vol) for 24 h at 4°C. Next, samples were dehydrated through an acetone series to 100% (30%, 40%, 50%, 60%, 70%, 80%, 90%). Then samples were dried by injection of CO_2 in a critical point drying apparatus (CPD-030 Balzers, Germany) and coated with a gold layer with a Balzers Union SCD-040 (Germany) [5]. They were observed using the JEOL JXA-840A Electron Probe Microanalyser (Japan).

Results

Planktonic phase

Table 1 shows that the number of bacteria in cooling and return water samples was greater than that found in makeup water samples. This is probably due to water evaporation and to the drawing of sessile microorganisms from the system itself. Anaerobic bacteria, including SRB, were found in lower numbers than aerobic bacteria at the three monitored points, possibly because of the significant oxygen concentration in the water as a consequence of aeration in the cooling tower.

Sessile phase

The attachment of both aerobic and anaerobic bacteria to the surface of the brass coupons from the makeup water point was detected after 15 days of exposure, and a decrease in bacterial number was observed at 90 days (Table 2). Biofilm formation was similarly evidenced in coupons immersed for 15 days in cooling water (Table 3), and was verified by a subsequent reduction of aerobic bacteria with time. Maximal numbers of anaerobic bacteria were observed after 60 days of exposure, these values were significantly reduced on day 90. In relation to return water (Table 4), the data show that the number of aerobic bacteria was constant although the number of anaerobic bacteria increased over the monitored time period. No attachment of SRB was found at any of the monitored points. The low numbers of bacteria recovered from the biofilms, in comparison to the results obtained by Lutterbach and de França [11], were possibly due to the fact that they were formed upon exposure to freshwater, which tends to carry smaller numbers of microorganisms. Also, since this water was used as recirculating coolant, it was submitted to treatment that included the use of biocides.

SEM analysis of the coupons immersed in makeup, cooling and return water for 90 days confirmed the formation of biofilms at the three monitored points (Figures 3–5).

Table 1 Number of microorganisms and sulfide content of water samples from the three monitored points of the cooling system

Water	Aerobic bacteria (CFU ml^{-1})	Anaerobic bacteria (cells ml^{-1})	SRB (cells ml^{-1})	Sulfides (μg ml^{-1})
Make-up	$1.5^{a} \pm 0.1^{b} \times 10^{2}$	20 ± 1	12 ± 1	27 ± 3
Cooling	$2.1 \pm 0.1 \times 10^{6}$	$3.2 \pm 0.3 \times 10^{5}$	$1.3 \pm 0.2 \times 10^{2}$	51 ± 6
Return	$2.9 \pm 0.3 \times 10^{5}$	$1.2 \pm 0.1 \times 10^{3}$	$2.1 \pm 0.3 \times 10^{2}$	48 ± 5

[a]Mean value of experiments; [b]mean standard deviation; CFU, colony-forming units.

Table 2 Number of microorganisms and sulfide content of biofilms recovered from coupons exposed to makeup water

Days	Aerobic bacteria (CFU cm^{-2})	Anaerobic bacteria (cells cm^{-2})	Sulfides (μg cm^{-2})
15	$6.5^{a} \pm 0.7^{b} \times 10^{3}$	7.8 ± 0.5	48 ± 5
30	$3.1 \pm 0.3 \times 10^{3}$	8.0 ± 0.6	45 ± 4
60	$2.5 \pm 0.3 \times 10^{3}$	6.2 ± 0.7	76 ± 8
90	$3.8 \pm 0.4 \times 10^{2}$	2.2 ± 0.3	24 ± 3

[a]Mean value of experiments; [b]mean standard deviation; CFU, colony-forming units.

Table 3 Number of microorganisms and sulfide content of biofilms recovered from coupons exposed to cooling water

Days	Aerobic bacteria (CFU cm^{-2})	Anaerobic bacteria (cells cm^{-2})	Sulfides (μg cm^{-2})
15	$1.3^{a} \pm 0.2^{b} \times 10^{5}$	$1.5 \pm 0.2 \times 10^{3}$	$2.0 \pm 0.3 \times 10^{2}$
30	$2.8 \pm 0.3 \times 10^{4}$	$8.3 \pm 0.9 \times 10^{3}$	$1.1 \pm 0.2 \times 10^{2}$
60	$1.4 \pm 0.2 \times 10^{4}$	$6.2 \pm 0.7 \times 10^{5}$	$1.7 \pm 0.1 \times 10^{2}$
90	$1.3 \pm 0.2 \times 10^{3}$	$4.3 \pm 0.5 \times 10^{2}$	$3.4 \pm 0.4 \times 10^{2}$

[a]Mean value of experiments; [b]mean standard deviation; CFU, colony-forming units.

Table 4 Number of microorganisms and sulfide content of biofilms recovered from coupons exposed to return water

Days	Aerobic bacteria (CFU cm^{-2})	Anaerobic bacteria (cells cm^{-2})	Sulfides (μg cm^{-2})
15	$1.0^{a} \pm 0.2^{b} \times 10^{4}$	$1.5 \pm 0.2 \times 10^{2}$	$7.0 \pm 0.5 \times 10^{2}$
30	$2.9 \pm 0.3 \times 10^{3}$	$5.2 \pm 0.6 \times 10^{3}$	$1.2 \pm 0.1 \times 10^{2}$
60	$8.7 \pm 0.9 \times 10^{4}$	$9.0 \pm 0.8 \times 10^{3}$	$1.7 \pm 0.2 \times 10^{2}$
90	$2.3 \pm 0.3 \times 10^{4}$	$1.2 \pm 0.2 \times 10^{4}$	$4.0 \pm 0.5 \times 10^{2}$

[a]Mean value of experiments; [b]mean standard deviation; CFU, colony-forming units.

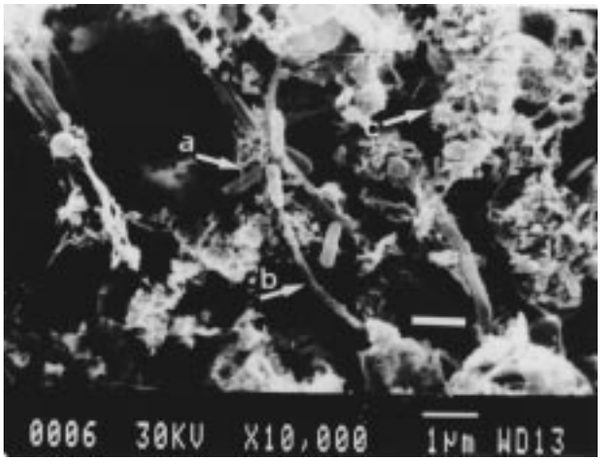

Figure 3 SEM photomicrographs of biofilms formed on brass coupons after 90 days of exposure to makeup water: (a) rod-shaped cell; (b) filaments; (c) deposits. Bar = 1 μm.

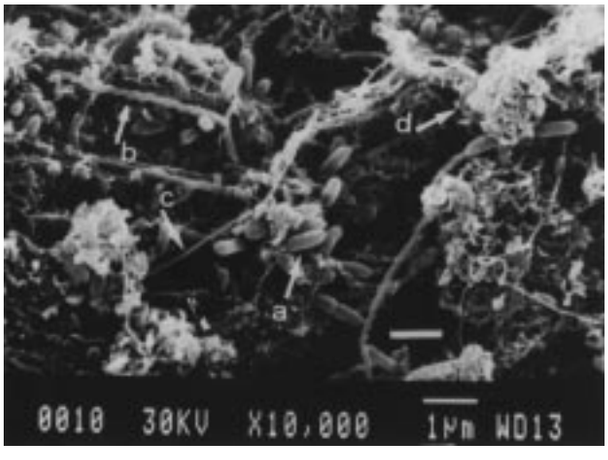

Figure 4 SEM photomicrographs of biofilms formed on brass coupons after 90 days of exposure to cooling water: (a) rod-shaped cell; (b) spiral-shaped cell; (c) filaments; (d) extracellular material. Bar = 1 μm.

None of the coupons processed showed an even distribution of the biofilm and, in fact, presented areas of higher cell density associated with deposits. A great variety of cell morphologies was also observed, including filamentous, rod-shaped and spiral-shaped organisms.

Gram-positive and Gram-negative bacteria were present in the biofilms, with a predominance of the latter. The genera *Pseudomonas*, *Bacillus* and *Flavobacterium* were isolated and identified as the most frequently found aerobic bacteria in the biofilms formed on the metal surface.

Total sulfide content

Sulfide determinations were made in both aqueous phases and in the biofilms. Low levels of sulfide were observed in water samples (Table 1), in comparison to the sulfide measured in the biofilm, from the three monitored points in spite of the SRB presence. The sulfide content detected in the biofilms formed on coupon surfaces at the three monitored points (Tables 2–4) did not change significantly during the experimental period.

In spite of the biofilm formation, microscopic stereo-

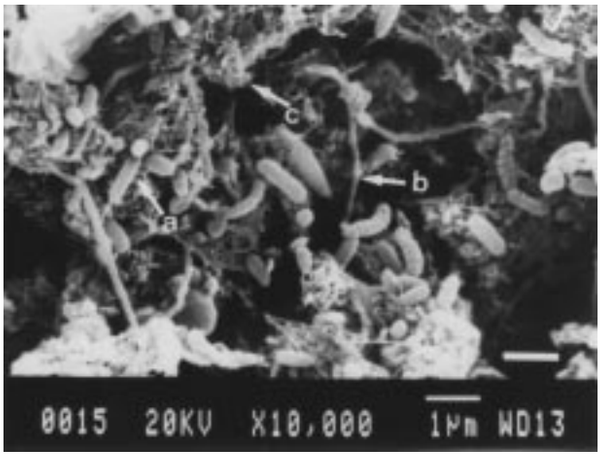

Figure 5 SEM photomicrographs of biofilms formed on brass coupons after 90 days of exposure to return water: (a) rod-shaped cell; (b) filaments; (c) extracellular material. Bar = 1 μm.

scopic analysis of the brass coupons after biofilm removal detected the presence of reddish spots only, with no evidence of corrosion, showing that the metal was resistant to corrosion under the conditions tested.

Discussion

The data suggest that biofilm formation occurred at all three monitored points of the cooling system. The planktonic bacteria attached to the metallic surfaces, even though copper is toxic to a variety of microorganisms.

A greater number of adhered microorganisms was observed when the number of planktonic bacteria was higher. This showed that there was a correlation between the attachment of microorganisms and the planktonic cells. It is important to remark that the heat exchange within the cooling towers causes temperature variation which interferes in the amount of microorganisms transported in the aqueous phase. Biofilm sloughing, caused by the water velocity, also influences the number of planktonic cells.

The colonization of copper alloys, including brass, probably occurs due to the presence of mechanisms of protection of some microorganisms. The production of exopolymers (EMP) appears to contribute to the settling of microbial cells on brass surfaces [7,12,18]. The acidic groups in these exopolymers may interact with the copper, protecting the cells from the metallic ions.

Pseudomonas spp frequently found in cooling systems, are often associated with biofouling and are known to be active producers of exopolysaccharides (EPS) that play a role in protecting the cells from metallic ions and help to trap other species of microorganisms [13,18]. *Flavobacterium* was also found in the cooling water and bacteria of the genus *Bacillus* are often associated with microfouling.

The presence of anaerobic bacteria in the biofilms at the three monitored points shows that the growth of aerobic bacteria created suitable microenvironments for the development of these bacteria, through consumption of oxygen, and secretion of exopolymers which limit the diffusion of oxygen to the base of the biofilm. The lack of attachment of SRB to the coupons may be a consequence of the low number of planktonic cells.

The variation in the number of bacteria, both aerobic and anaerobic, as a function of time of exposure may be due to environmental characteristics. This work is based on field experiments and factors such as water turbulence within the bypass ducts, water chemistry, nutrient availability and the toxic nature of the metal must be accounted for.

The content of sulfide in the biofilms (Tables 2–4), unlike that found in the water samples where SRB were present (Table 1), may be a consequence of chemical reactions of compounds containing sulfur or derivatives, or by compounds from the oil refinery itself, since no SRB were found in the sessile phase. It is known that H_2S can be formed from the decomposition of organic matter, residual gases in petroleum refineries, and other organic wastes [8].

One possible explanation for the differences in sulfides in biofilms formed in the return water and cooling water sampling points may be associated with oil leakage that occurred in the industrial unit during the experiments.

SEM analysis (Figures 3–5) revealed that biofilms developed at the three monitored points contained a variety of microorganisms in close contact with extracellular material. Several authors [7,12,18] have suggested that colonization of toxic metals may be related to the ability of adhered microbial species to secrete extracellular mucous material. Similar biofilms were found [11] on brass coupons exposed to a seawater cooling system.

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References

1 American Public Health Association (APHA). 1972. Tentative methods of analysis for hydrogen sulphide content of the atmosphere. In: Methods of Air Sampling and Analysis. pp 426–432, APHA, Washington.
2 American Public Health Association (APHA). 1992. Standard Methods for the Examination of Water and Wastwater, 18th edn. Washington, DC.
3 Benbouzid-Rollet ND, M Conte, J Guezennec and D Prieur. 1991. Monitoring of a *Vibrio natriegens* and *Desulfovibrio vulgaris* marine aerobic biofilm on a stainless steel surface in a laboratory tubular flow sistem. J Appl Bacteriol 71: 6–10.
4 Characklis WG, KC Marshal and GA McFeters. 1990. The microbial cell. In: Biofilms (Characklis WG and KG Marshal eds), pp 131–159, John Wiley & Sons, New York.
5 Coutinho CMLM, FCM Magalhaes and TCA Jorge. 1983. Scanning electron microscope study of biofilm formation at different flow rates over metal surfaces using sulphate-reducing bacteria. Biofouling 7: 19–27.
6 de França FP and MTS Lutterbach. 1996. Variation in sessile microflora during biofilm formation on AISI-304 stainless coupons. J Ind Microbiol 17: 6–10.
7 Donlan RM. 1992. Correlation between sulfate reducing bacterial colonization and metabolic activity on selected metals in a recirculating cooling water system. Corrosion, Paper No 183: 1–16.
8 Gentil V. 1996. Corrosion, 3rd edn. Livros Tecnicos e Cientificos Editora SA, Rio de Janerio, Brazil.

9 Holt JG, NR Krieg, PHA Sneath, JT Stanley and ST Williams (eds). 1994. Bergey's Manual of Determinative Bacteriology, 9th edn. Williams & Wilkins, Baltimore.
10 Lee W, Z Lewandowski, PH Nielsen and WA Hamilton. 1995. Role of sulfate reducing bacteria in corrosion of mild steel. A review. Biofouling 8: 165–194.
11 Lutterbach MTS and FP de França. 1997. Biofilm formation on brass coupons exposed to cooling water. Braz J Chem Eng 14: 81–87.
12 Marszalek DS, SM Gerchakov and LR Udey. 1979. Influence of substrate composition on marine microfouling. Appl Environ Microbiol 38: 987–995.
13 Page S and CC Gaylard. 1990. Biocide activity against *Legionella* and *Pseudomonas*. Intern Biodet 26: 139–148.
14 Oblinger JL and JA Koburger. 1975. Understanding and teaching the most probable number technique. J Milk Food Technol 38: 540–545.
15 Postgate JR. 1984. The Sulphate Reducing Bacteria. Cambridge University Press, Cambridge.
16 Sifdlarek H, D Wagner, WR Fischer and HH Paradies. 1994. Microbiologically influenced corrosion of copper. The ionic transport properties of biopolymers. Corr Sci 36: 1751–1763.
17 Stupak ME, MC Peres and AR di Sarli. 1990. Relacion entre la fijacion de microy macrofouling y los procesos de corrosion de estructuras metalicas. Rev Iberoam Corr y Prot 21: 219–225.
18 Tevors JT and CM Cotter. 1990. Copper toxicity and uptake in microorganisms. J Ind Microbiol 6: 77–84.
19 Videla HA and MFL de Mele. 1988. Assessment of corrosion and microfouling of several metals in polluted seawater. Corr Sci 44: 423–426.
20 Wagner P and B Little. 1993. Impact of alloying on microbiologically influenced corrosion — A review. Mat Perf 32: 65–68.
21 Walsh D, J Seagoe and L Williams. 1992. Microbiologically influenced corrosion of stainless steel weldments, attachment and film evolution. Corrosion, Paper No 165. NACE: Texas.
22 Walker JT, J Rogers and CW Keevil. 1994. An investigation of the efficacy of a bromine containing biocide on an aquatic consortium of planktonic and biofilm microorganisms including *Legionella pneumophila*. Biofouling 8: 47–54.