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Effects of toxic metals and chemicals on biofilm and biocorrosion

Herbert H.P. Fang[a,*], Li-Chong Xu[a], Kwong-Yu Chan[b]

[a] *Department of Civil Engineering, Centre for Environmental Engineering Research, The University of Hong Kong, Pokfulam Road, Hong Kong*

[b] *Department of Chemistry, The University of Hong Kong, Pokfulam Road, Hong Kong*

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Abstract

Microbes in marine biofilms aggregated into clusters and increased the production of extracellular polymeric substances (EPS), by over 100% in some cases, when the seawater media containing toxic metals and chemicals, such as Cd(II), Cu(II), Pb(II), Zn(II), Al(III), Cr(III), glutaraldehyde, and phenol. The formation of microbial cluster and the increased production of EPS, which contained 84–92% proteins and 8–16% polysaccharides, accelerated the corrosion of the mild steel. However, there was no quantitative relationship between the degree of increased corrosion and the toxicity of metals/chemicals towards sulfate-reducing bacteria, or the increased EPS production.

Keywords: Biofilm; Biocorrosion; Extracellular polymeric substances (EPS); Steel; Sulfate-reducing bacteria (SRB); Toxicity

1. Introduction

Microorganisms tend to colonize on solid surface in natural environment. The biofilm forms a protective layer, reducing the exposure of the solid surface to the external environment. However, it could also result in localized corrosion and deterioration of the substratum materials, such as metals [34], polymers [19] and concrete [8].

The initial bacterial attachment to the metal surface and the subsequent formation of biofilm are dependent on the surface characteristics of the substratum, including metal surface free energy, roughness and hydrophobicity [27], as well as metallurgical features [36]. Bacteria tend to preferentially colonize onto the grain boundaries of steel. This could cause localized corrosion [17], and the resulting corrosion may further promote the patchy adsorption of microbes [25]. On the other hand, biochemical characteristics of the microbial surface and the extracellular polymeric substances (EPS) are equally crucial to the biofilm formation [22]. EPS are primarily composed of polysaccharides, uronic acid sugars and proteins, containing functional groups, such as carboxylic acid and amino acid groups, which could be acidic and capable of binding metal ions [16,29]. Thus, EPS can also affect the electrochemical characteristics of metal surface, and play an important role in the corrosion of metals [30].

Sulfate-reducing bacteria (SRB) are a group of microorganisms that are of interest to many microbiologists, material scientists and engineers. They are capable of using sulfate as electron acceptor, and often out-compete most other anaerobes for substrates in the presence of sulfate. SRB are highly efficient in the anaerobic degradation of many organic pollutants, as well as in the precipitation of heavy metals from wastewater as metal sulfides [6,33,37,31]. However, SRB are also commonly found in biofilm developed on the surfaces of ship hulls, heat exchangers, wastewater pipelines, resulting in biofouling and biocorrosion. Sulfide produced by SRB is known to cause cathodic hydrogen depolarization and may damage the passivity of stainless steel by accelerating anodic interaction [5].

*Corresponding author. Fax: 852-2559-5337.
E-mail address: hrechef@hkucc.hku.hk (H.H.P. Fang).

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On the other hand, the EPS secreted by SRB can complex metal ions and, thus, affect the corrosion [1,2].

Organic pollutants in coastal water provide carbon and energy sources for the proliferated growth of SRB due to the unlimited supply of sulfate from the seawater. However, industrial discharges of toxic metals and chemicals could affect the growth of SRB. Heavy metals used in electroplating are among the most significant industrial discharge in Hong Kong, as evidenced by the high metal contents in the local marine sediment [3]. It is thus of interest to examine the influence of toxic heavy metals and chemicals on the SRB-rich marine biofilm and on the resulting biocorrosion.

2. Materials and methods

2.1. Mild steel coupons

SRB biofilms were first developed on mild steel coupons ($10 \times 10 \times 1.5\,mm^3$). The mild steel compositions as analyzed by inductively coupled plasma optical emission spectrometry and spark optical emission spectrometry were (by %w/w): Fe 98.48%, C 0.06%, Si 0.13%, P 0.03%, S 0.045%, Mn 0.51%, Cr 0.10%, Mo 0.02%, Ni 0.14%, and Cu 0.49%. The coupons were wet polished with a series of grit SiC papers (grades 220, 400, 600, 800), followed by ethanol degreasing. After further polish using 0.3 μm alumina powder, the coupons were cleaned, dried and stored in a desiccator prior to use.

2.2. SRB culture and growth conditions

The SRB seed was isolated from the local marine sediment and cultured in a complete-mix reactor using the modified Postgate's marine medium C [35] at 20–22°C for over 6 months. The medium was prepared by adding to each liter of seawater with 0.5 g KH_2PO_4, 1 g NH_4Cl, 0.06 g $CaCl_26H_2O$, 0.06 g $MgSO_47H_2O$, 6 ml sodium lactate (70%), 1 g yeast extract, 0.004 g $FeSO_47H_2O$, and 0.3 g sodium citrate. The pH was adjusted to 7.2 ± 0.1 using 1 M NaOH solution. Seawater was sampled from the Victoria Harbour, and sterilized by filtering through a 0.45 μm membrane before use.

2.3. SRB colonization and corrosion of steels

Experiments were carried out in parallel in 10 enclosed 1 l glass reactors containing the modified Postgate's marine medium C under anaerobic conditions. The medium was sterilized by filtering through a 0.22 μm membrane, and flushed with nitrogen to remove dissolved oxygen. The enriched SRB culture was used to seed reactors at the initial concentration of 2×10^6 cell/ml. Eight toxic metals and chemicals, including Cd(II) (10 mg/l), Cu(II) (20 mg/l), Pb(II) (50 mg/l), Zn(II) (20 mg/l), Al(III) (27 mg/l), Cr(III) (50 mg/l), glutaraldehyde (10 mg/l), and phenol (20 mg/l) were selected to test the toxic effect on biofilm growth at controlled concentrations. The initial concentrations of metals were chosen mainly based on their approximate concentrations in the marine sediment of Hong Kong [3]. Two reactors were served as controls. One was seeded with SRB, and the other without.

Three mild steel coupons were immersed in the test medium inside each reactor without any mixing. Sulfate concentration in each solution was analyzed daily by an ion chromatography to monitor the activity of SRB. Half of the medium in each reactor was replaced after 10 days with a fresh medium. Coupons were removed after 20 days for microscopic observation and for surface corrosion analysis.

2.4. Microscopic analysis

The biofilm structure in this study was examined by scanning electron microscopy (SEM, Steroscan 360, Cambridge, UK), and the pit corrosion on the mild steel surface was examined by atomic force microscopy (AFM, Nanoscope IIIA, Digital Instruments, USA). Each biofilm sample was fixed for 8 h in 2.5% glutaraldehyde, followed by dehydrating in a graded series of ethanol. The sample was then critical-point dried and coated with carbon for SEM examination [23]. To reveal the extent of corrosion, the biofilm was removed by immersing each coupon in an ultrasonic bath for 5 min and then in passive Clarke solution (36% HCl 1 l, Sb_2O_3 20 g and $SnCl_2$ 50 g) for 10–15 s to remove the corroded products and metal sulfide precipitates, if any. The exposed coupon surface was finally rinsed with distilled water, cleaned in 100% ethanol and dried under nitrogen flow. The surface was analyzed for pit corrosion by AFM in tapping mode with the standard etched silicon probe. The detailed procedures for the sample preparation and AFM operation were described in a previous study [40].

2.5. Characterization of EPS

The characteristics of biofilm EPS in each reactor were analyzed. Three biofilm-coated coupons were removed from each reactor after 20 days and immersed in 50 ml of a pH 7.5 TE buffer containing 10 mM Trizma base, 10 mM EDTA and 2.5% NaCl. Biofilm was scrapped from each coupon, and centrifuged at 4°C, 4×10^3 G for 20 min. The concentrated biomass was then re-suspended in a 10 ml of aqueous solution containing 0.85% NaCl and 0.22% formaldehyde at 80°C for 30 min for EPS extraction [9,21]. The EPS dissolved in the formaldehyde solution was recovered by further centrifugation at 4°C, 2×10^4 G for 30 min. The

carbohydrate content of EPS in the extracted solution was measured using the phenol/sulfuric-acid method [15] and the protein content using the Lowry method [26].

There was no cell lysis during EPS extraction, as confirmed by comparing the DNA content in the control biofilm and in the extracted EPS following the established procedures [18]. Results show that although the dry mass of the control biofilm samples contained $0.69 \pm 0.05\%$ DNA, the extracted EPS contained only $0.022 \pm 0.002\%$ DNA.

2.6. Analytical methods

Sulfate concentration in each reactor was analyzed by an ion chromatograph (Shimadzu HPLC 10A) equipped with a CDD-6A conductivity detector and an Allsep Anion column (Alltech). A solution containing 4.0 mM of *o*-phthalic acid with pH 4.2 adjusted by lithium hydroxide was used as the mobile phase. The flow rate of the mobile phase was 1.0 ml/min, the oven was kept at 40°C, and the detector at 43°C.

3. Results and discussion

3.1. Influence of metals and chemicals on SRB activity

Fig. 1 illustrates the reduction of sulfate concentration over time in each reactor. In the control reactor with SRB, 85% of sulfate was reduced after 9 days, from the initial 2090 to 305 mg/l. However, the SRB activity was severely impaired in the presence of heavy metals and toxic chemicals. Only 52% of sulfate was reduced in the presence of Cd(II) with the initial concentration of 10 mg/l in seawater medium. The corresponding figures were 5% for Cu(II) (20 mg/l), 73% for Pb(II) (50 mg/l), 17% for Zn(II) (20 mg/l), 77% for Al(III) (27 mg/l), 38% for Cr(III) (50 mg/l), 25% for glutaraldehyde (10 mg/l) and 58% for phenol (20 mg/l). Results showed that these

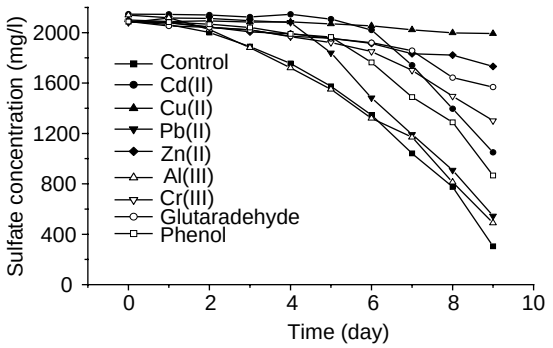

Fig. 1. Reduction of sulfate concentration over time in the reactors.

toxic metals and chemicals had various degrees of toxic effect towards the SRB activity. However, reduction of SRB activity did not inhibit biofilm growth on the test coupons in all reactors.

Most of the metal ions reacted with sulfide, forming precipitated as insoluble metal sulfides. Some metal ions might also complex with yeast extract and citrate in the modified Postgate's marine medium C. The effects of sulfide precipitates and possible metal–organic complexes were not examined in this study.

3.2. Influence on biofilm structure

Fig. 2 illustrates the SEM images of biofilm developed in all reactors after 20 days. Fig. 2a illustrates that there was no detectable biofilm on the steel coupon in the control reactor without SRB, as expected. Fig. 2b, on the other hand, illustrates that the presence of a biofilm on the steel coupon in the other control reactor with SRB. Microbes were distributed rather uniformly over the biofilm. Figs. 2c–j illustrate, respectively, the SEM images of biofilms in the presence of Cd(II) (10 mg/l), Cu(II) (20 mg/l), Pb(II) (50 mg/l), Zn(II) (20 mg/l), Al(III) (27 mg/l), Cr(III) (50 mg/l), glutaraldehyde (10 mg/l) and phenol (20 mg/l). Figs. 2c–j show that microbes in biofilm clustered into patches on the steel surface when the seawater medium contained toxic pollutants. This phenomenon was observed in a preliminary study [11], which showed that microbes were uniformly scattered over the biofilm in Cr(III)-free and dilute (10 mg/l) Cr(III) seawater solutions, but aggregated to form clusters as Cr(III) concentration increased to 50 mg/l or higher. Results in Figs. 2c–j illustrate that the cluster formation is a common phenomenon when microbes in biofilm are exposed to a toxic environment, regardless the toxicity is of either organic or inorganic nature.

The cluster formation is likely due to the natural response of the microbes. To avoid exposure to toxicity, microbes tend to aggregate in order to reduce the total surface area in contact with the environment. Similar observations were reported [28] that the aggregated bacteria were less sensitive to toxicants in solution containing biocide than the same bacteria growing in dispersion.

3.3. Influence on EPS production

Another natural response of microbes upon exposure to a toxic environment is to stimulate the production of EPS. EPS are mainly composed of polysaccharides (EPS_c) and proteins (EPS_p). The former often carries functional groups, such as acetyl, succinyl, pyruvyl and sulfonate. The latter can be glycosylated with oligosaccharides to form glycoproteins or can be substituted with fatty acids to form lipoproteins [38]. EPS is the

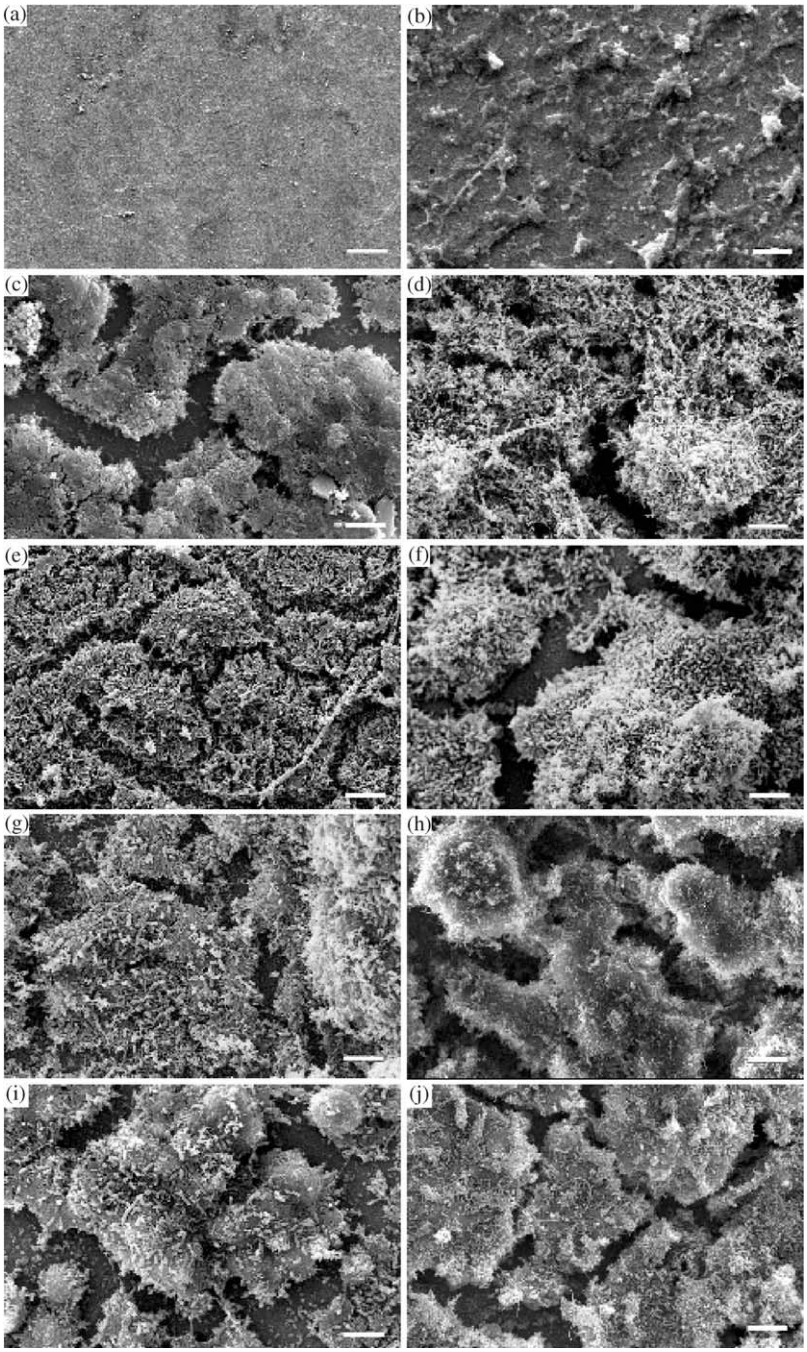

Fig. 2. SEM images of mile steel coupons from: (a) control reactor without SRB seeding, (b) control reactor seeded with SRB, and reactors containing (c) 10 mg/l Cd(II), (d) 20 mg/l Cu(II), (e) 50 mg/l Pb(II), (f) 20 mg/l Zn(II), (g) 27 mg/l Al(III), (h) 50 mg/l Cr(III), (i) 10 mg/l glutaraldehyde, and (j) 20 mg/l phenol. (bar = 10 μm).

fundamental element of biofilm. They form a stable structural network mediated by either covalent interactions between adjacent polymeric chains or by multivalent cation bridges [20]. EPS could also serve as a nutrient reserve to ensure survival under famine conditions [39]. In addition, they could also form a protective shield for the cells against the adverse influences from the external environment [38]. They either delay or prevent toxicants from reaching microbes by diffusion limitation and/or by chemical reactions. Furthermore, a

recent study [10] demonstrated that the EPS was highly adhesive in nature, based on the force curves measured by AFM. They may act as an adhesive binding adjacent cells and thus further enhance the aggregation of microbes during cluster formation [4].

Table 1 summarizes the EPS contents in the biofilm in each reactor after 20 days. Results show that the production of EPS was indeed stimulated by the presence of toxic metals and chemicals. On each cm^2 of steel surface, a total of 40.1 μg of EPS were produced in the control biofilm, of which 4.9 μg were EPS_c and 35.2 μg were EPS_p. Productions of both EPS_c and EPS_p increased when biofilms were exposed to the toxic metals and chemicals. However, there was no quantitative relationship between the EPS production and the relative toxicity of the metals and chemicals towards SRB based on sulfate reduction. For example, Cu(II) at the initial concentration of 20 mg/l was most toxic to SRB among all tested metals and chemicals; only 5% of sulfate was reduced after 9 days, as compared to 85% in the control medium. However, despite its toxicity towards SRB, Cu(II) stimulated the production of EPS only slightly, from 40.1 $\mu g/cm^2$ in the control to 42.6 $\mu g/cm^2$, most of the increased production was EPS_c. On the other hand, Cr(III) (50 mg/l) and Zn(II) (20 mg/l) were less inhibitory to SRB activity, and yet they stimulated the EPS productions by over 100% to 88.5 $\mu g/cm^2$ and 86.5 $\mu g/cm^2$, respectively. In general, the presence of toxic metals and chemicals stimulated EPS_p production more than that of EPS_c.

Most of EPS produced in pure culture studies were polysaccharides [32]. Table 1 however shows that all the biofilms produced in this study were primarily composed of proteins; the EPS_p/EPS_c ratios ranged from 5.3 to 11.3. The preferential production of EPS_p over EPS_c was common in mixed-culture systems, so as activated sludge [14] and anaerobic granular sludge [21].

The EPS not only are crucial to the structural integrity of the biofilm, but may also be directly involved in metal dissolution from the corroding metal surface [7]. This is mainly due to the acidic and metal-binding nature of the EPS. EPS contain functional groups, such as carboxylic and amino acids. Using a pH microelectrode, Lewandowski et al. [24] and Roe et al. [30] found that the biofilm pH at the corroded metal surface covered with EPS was only 4.5. Furthermore, the binding capacity between individual metal ions and specific EPS vary considerably. This could result in the formation of ion concentration cells, causing further corrosion on the metal surface [12,13]. It is thus natural for one to speculate that the increased production of EPS when the biofilm was exposed to toxic metals/chemicals would also accelerate the corrosion of mild steel. This was indeed the case, as discussed in the next section.

Table 1
EPS contents in biofilms after 20 days

Toxicant	EPS_c (μg/cm^2)	EPS_p (μg/cm^2)	Total EPS (μg/cm^2)
Control	4.9±0.2	35.2±12.1	40.1
Cd(II)	5.0±2.3	56.3±12.0	61.3
Cu(II)	6.8±2.5	35.8±9.4	42.6
Pb(II)	6.6±3.8	47.6±25.0	54.2
Zn(II)	9.0±2.0	77.5±23.5	86.5
Al(III)	7.0±3.9	67.8±8.5	74.8
Cr(III)	8.9±2.1	79.6±21.7	88.5
Glutaraldehyde	5.9±0.8	52.1±15.1	58.0
Phenol	7.3±1.4	48.9±9.6	56.2

3.4. Influence on corrosion of mild steel

Fig. 3 illustrates the AFM topographic images of the steel surfaces, after removing the biofilm, in each reactor for 20 days. The degrees of corrosion are indicated in a gray scale—the darker the site the deeper the corrosion. Fig. 3a illustrates the absence of corrosion, as expected, on the coupon from the control reactor without SRB. Fig. 3b illustrates that there was only a minor corrosion on coupons from the control reactor with SRB. Figs. 3c–j illustrate, however, that the degree of corrosion increased significantly when the biofilms were exposed to toxic metals and chemicals.

AFM has higher resolution and more accurate measurement in vertical dimension than most of other microscopic techniques. Many surface corrosion characteristics may be quantified from the AFM images. Table 2 summarizes that surface roughness, depth of pit corrosion and total corroded volume of all the tested steel coupons. Based on the AFM images, the surface roughness of the original mild steel coupons averaged 5.8 nm. Results in Table 2 show that, after immersed for 20 days in the toxicity-free seawater, for every 10^4 μm^2 of surface area, the coupons from the control reactor seeded with SRB had on average a roughness of 30 nm, a pit depth of 183 nm and a corroded volume of 70 μm^3. Table 2 also shows that surface roughness, pit depth and corroded volume increased when the steel coupons were exposed to toxic metals and chemicals. However, there was no quantitative relationship between the degrees of corrosion and the reduction of SRB activity or the increase production of EPS. For example, Pb(II) (50 mg/l) caused the highest degree of corrosion, increasing the roughness to 196 nm, pit depth to 839 nm and corroded volume to 685 μm^3 for every 10^4 μm^2 of steel surface; but as compared to the control it reduced the SRB activity by only 14% and increased the EPS production by only 35%.

It is also interesting to note that the corrosion patterns in Figs. 3c–j appear to match the microbial cluster patterns of the corresponding biofilms in Figs. 2c–j.

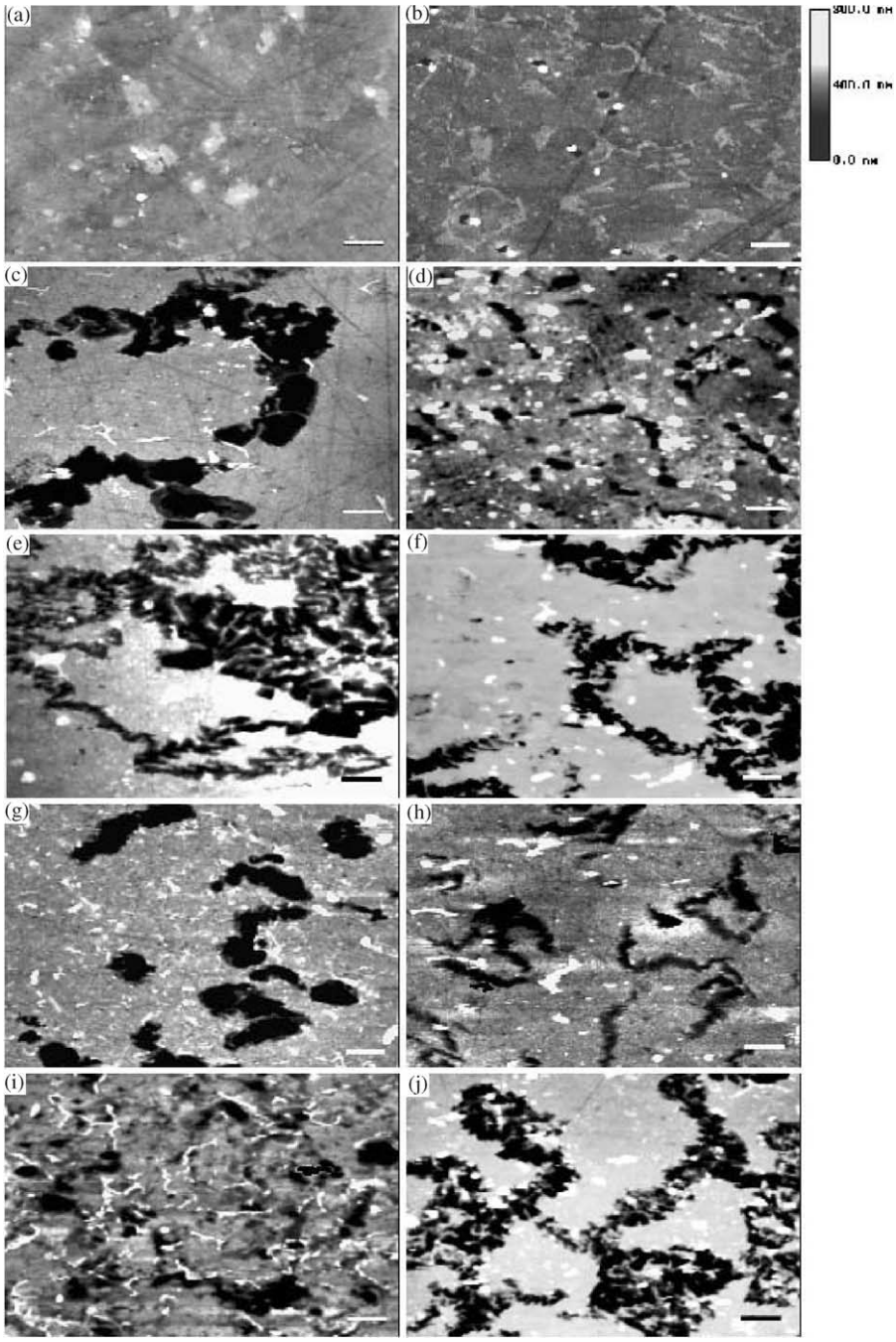

Fig. 3. AFM images of mild steel coupons from: (a) control reactor without SRB seeding, (b) control reactor seeded with SRB, and reactors containing (c) 10 mg/l Cd(II), (d) 20 mg/l Cu(II), (e) 50 mg/l Pb(II), (f) 20 mg/l Zn(II), (g) 27 mg/l Al(III), (h) 50 mg/l Cr(III), (i) 10 mg/l glutaraldehyde, and (j) 20 mg/l phenol The AFM images show corrosion patterns resembling those of microbial clusters in Fig. 2. (bar = 10 μm; the pit depth is indicated by the degree of darkness).

Corrosion appeared to occur mostly in the regions between microbial clusters. The aggregation of microbial cells leads to the gradient in electrochemical activity. The microbial clusters became barriers to diffusion and the area under which became the cathode; whereas the regions between microbial clusters allow the surface to have greater access to chloride and sulfate in the seawater medium, and to act as the anode,

Table 2
Pit depth, total corroded volume and roughness of mild steel after 20 days

Toxicant	Pit depth (nm)	Corroded volume ($\mu m^3/10^4 \mu m^2$)	Roughness (nm)
Control	183±60	70±26	30±10
Cd(II)	522±214	265±134	89±27
Cu(II)	641±178	355±125	125±10
Pb(II)	839±486	685±193	196±51
Zn(II)	797±395	534±233	174±11
Al(III)	818±259	607±150	158±5
Cr(III)	297±113	252±123	72±19
Glutaraldehyde	428±364	199±146	91±32
Phenol	593±289	604±182	180±41

resulting in accelerating the electrochemical corrosion reactions.

Furthermore, the regions between microbial clusters were likely to have higher EPS concentration [10]. This would cause increased surface corrosion due to the acidic [24] and iron-binding nature of the EPS [2].

4. Conclusion

When exposed to seawater media containing toxic metals and chemicals, the SRB in the biofilm aggregated into clusters, and increased the production of EPS. The EPS, which contained 84–92% proteins and 8–16% polysaccharides, were responsible for the increased corrosion of the mild steel, due to the acidic and iron-binding nature of the EPS. However, there was no quantitative relationship between the degree of increased corrosion and the toxicity of metals/chemicals towards SRB, based on sulfate reduction, or the increased EPS production.

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