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Review

Biofilms: an ESEM evaluation of artifacts introduced during SEM preparation

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SUMMARY

Descriptions of biofilms and their elemental compositions based on scanning electron micrographs and energy dispersive x-ray analysis cannot be related to the original condition of the biofilm on the surface. Solvent replacement of water removes extracellular polymeric material and reduces the concentration of elements bound within the biofilm. In the wet state, bacteria and microalgae are enmeshed in a gelatinous film that is either removed or dried to a thin inconspicuous residue during sample preparation for scanning electron microscopy. The environmental scanning electron microscope provides a fast, accurate image of biofilms, their spatial relationship to the substratum and elemental composition.

INTRODUCTION

Biofilms containing bacteria and associated extracellular polymers develop on all engineering materials placed in biologically active liquids. Biofilms can be either beneficial or detrimental in industrial processes. They remove dissolved and particulate contaminants in fixed film biological systems such as trickling filters, rotating biological contactors, and fluidized bed wastewater treatment plants. Biofilms determine water quality by influencing dissolved oxygen content and by serving as a sink for toxic and/or hazardous materials [16]. Biofilm reactors are used for commercial fermentation processes, including the manufacture of vinegar [35–37]. Microorganisms within biofilms are used to recover minerals [15,21] and to remove sulfur from coal [5,20]. Biofilms form undesirable deposits on industrial equipment causing reduced heat transfer [6], increased fluid frictional resistance [6], plugging [34], corrosion [26], and other types of deterioration [28].

Dental plaque, insulating biofilms on heat exchangers, drag-enhancing slime on ship hulls, and biofilms used in industrial processes are the result of similar developmental processes [1]. Biofilm formation has been evaluated as a function of substratum [25], liquid medium [25], carbon source [31], pH, and hydrodynamic parameters including flow rate [6]. Many of the conclusions about biofilm development, composition, distribution, and relationship to substratum have been derived from scanning electron microscopy (SEM) [7,14,29,30,32,33,43].

Preparation of biological material for SEM requires extensive manipulation, including fixation, dehydration, and either air drying or critical-point drying because the SEM operates at high vacuum. Non-conducting samples including biofilms must be coated with a conductive film of metal before the specimen can be viewed. Uncoated non-conductors build up local concentrations of electrons, referred to as 'charging', that prevent the formation of usable images. Energy dispersive x-ray spectroscopy (EDS) can be used to determine the elemental composition of surface films in the SEM, but EDS analyses must be completed prior to deposition of the thin metal coating. EDS data are typically collected from an area; the specimen must be removed from the specimen chamber and coated with a conductive layer, and returned to the SEM. The operator attempts to relocate and photograph the precise area from which the EDS data were collected.

The Electroscan Corporation (Wilmington, MA) recently introduced a new development in SEM technology: the environmental scanning electron microscope

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TABLE 1

Alloy compositions (weight %)

	UNS[a] No.	Cu	Ni	Zn	Fe	Sn	Mn	Mo	Cr	C	Si	P	S	N
Commercially pure copper	C11000	99.9 oxygen free												
90Cu : 10Ni	C70600	86.73–87.02	10.47–10.81		1.68–1.69		0.58–0.67							
Admiralty brass	C44300	72.10		26.96	0.04	0.9								
SS304	S30400		8.0–10.5		REM[b]		2.00		18.0–20.0	0.08	1.00	0.045	0.03	0.10
SSAL6X	N08366		23.5–25.5		REM[b]		2.00	6.0–7.0	20.0–22.0	0.03	1.00	0.03	0.03	

[a] Unified numbering system.
[b] REM, remainder.

(ESEM). This instrument uses a unique secondary electron detector capable of forming high resolution images at pressures in the range of 0.1 to 20 torr. At these relatively high pressures, specimen charging is dissipated into the gaseous environment of the specimen chamber, enabling direct observation of uncoated, non-conductive specimens. If water vapor is used as the specimen environment, wet samples can be observed. Wet biofilms can be observed directly, and EDS data can be collected at the same time sample morphology/topography is photographed. This paper compares images and elemental compositions of biofilms on metal surfaces obtained with ESEM with those from SEM.

METHODS AND MATERIALS

The impact of SEM sample preparation on biofilms was evaluated using areal coverage, elemental composition, numbers and types of cells within the biofilm and morphology of individual cells.

Biofilms

Two types of biofilms were evaluated. In one series of experiments, naturally occurring estuarine biofilms formed over a 4-month period on types 304 and AL6X stainless steels and commercially pure copper coupons (1 cm^2) in an aquarium maintained with flowing Gulf of Mexico estuarine water. The turnover time for the aquarium was 63 h. Elemental composition of metal substrata are listed in Table 1.

In a second series of experiments, a mixed culture of obligate and facultative anaerobic bacteria, including sulfate-reducing bacteria [27], was used to inoculate copper-containing foils (90Cu : 10Ni, commercially pure copper, and admiralty brass) in an enrichment medium using lactate as the electron donor and carbon source for growth [40]. Table 1 lists the elemental composition of the foils. The medium was supplemented with 2.5% (w/v) NaCl for enrichment of marine microbes. Cultures were grown anaerobically at room temperature in sealed bottles for 4 months.

Biofilm preparation for SEM

Biofilms on copper surfaces were removed from the growth medium and fixed in 4% glutaraldehyde buffered with sodium cacodylate (0.1 M, pH 7.2) at 4 °C for a minimum of 4 h. Estuarine biofilms fixed in 4% glutaraldehyde in filtered seawater at 4 °C for a minimum of 4 h were dipped through a series of filtered seawater/distilled water washes to remove residual salts. Distilled water was removed from biofilms through a graded series of distilled water/acetone washes to acetone. Acetone was removed through a series of acetone/xylene washes to xylene and air dried. Other samples were taken from distilled water through a series of distilled water/ethanol washes to ethanol. Specimens were taken from ethanol to amyl acetate and critical-point dried from liquid CO_2. Dried specimens were sputter-coated with gold (20 nm thickness) using a Polaron (Line Lexington, PA) Series II Sputtering System, Type E5100.

Operating procedures for SEM

SEM images were collected with an AMRAY (Bedford, MA) 1000A scanning electron microscope operated at an accelerating voltage of 30 kev and a vacuum of 10^{-5} torr.

Biofilm preparation for ESEM

Biofilms on metal surfaces were fixed in 4% glutaraldehyde in filtered seawater or cacodylate buffer as previously described, rinsed to distilled water, and examined in the ESEM. Micrographs and EDS data were collected for the wet biofilms. Specimens were removed from the ESEM, dehydrated through a series of distilled water/acetone washes to acetone and reexamined. Specimens were returned to acetone and rinsed through a series of ace-

tone/xylene washes to xylene before final examination. Areal coverage was evaluated directly from the distilled water and after water removal with acetone and xylene. In this way, areal coverage could be evaluated independent of drying technique. EDS data were collected from the biofilms at each stage of sample manipulation. Bacteria on the copper-containing foils were visualized in the ESEM after fixation and washing in distilled water.

Operation procedures for ESEM/EDS

ESEM examination was performed in an Electroscan (Wilmington, MA) Type II microscope. Specimens were attached to a Peltier stage maintained at 4 °C and imaged in an environment of water vapor at 2–5 torr to maintain samples in a hydrated state. At the acetone or xylene stage, specimens were imaged using acetone or xylene vapor. The ESEM was operated at 20 kev using the environmental secondary detector. EDS data were obtained with a Tracor Northern (Middleton, WI) System II analyzer equipped with a diamond window light element detector. Samples were held at a 37.4 ° tilt during spectrum acquisition. A program correcting for atomic number (Z) [13], absorption (A) [18], and fluorescence (F) [19] was used for semiquantitative analysis during data acquisition. A complete review of ZAF correction procedures has been prepared by Beaman and Isasi [3].

A reference mark was etched on the edge of each metal specimen to facilitate relocation of precise positions on the specimen surface. The reference mark was centered on the display screen and the x–y coordinates recorded. Coordinates were also recorded for each subarea examined in detail. Differences in coordinates were established for subareas in relation to the reference mark. After removal of the specimen from the ESEM, experimental treatment, and replacement in the specimen chamber, subareas could be precisely relocated using x–y coordinates and the reference mark.

TABLE 2

Weight percent of elements found on 304 stainless steel surfaces after exposure to estuarine water and after treatment with acetone and xylene

Element	Base metal	After exposure to estuarine water	After xylene
Fe	72.55	11.24	72.43
Cr	18.10	4.71	18.17
Ni	8.02	34.00	7.78
Si	0.93	7.35	1.06
Al	0.40	3.33	0.57
Cl		0.66	
S		0.99	
K		1.88	
Na		2.12	
Mg		4.98	
Ti		28.74	

RESULTS

Figure 1a is an ESEM image of a wet estuarine biofilm on 304 stainless steel. The distribution is patchy. There is no visible corrosion and machining marks are evident on the surface. Figure 1b is an ESEM image of the same area after the biofilm was treated with acetone and xylene. Areal coverage by the biofilm was reduced. Table 2 summarizes the elemental composition of the biofilm before and after solvent extraction of water. The wet biofilm contained elevated levels of Ni, Si, Al, and Ti. After solvent removal of water, the elemental composition of the biofilm was similar to base metal before colonization.

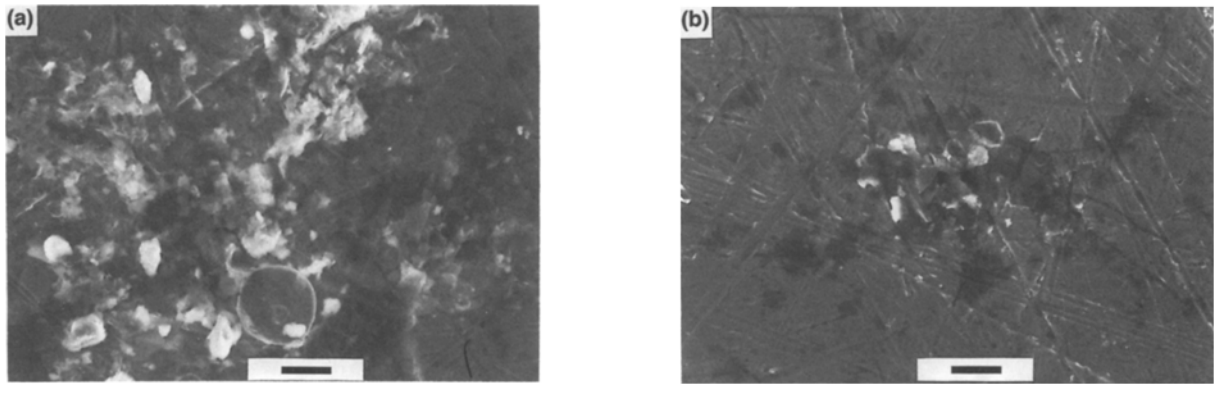

Fig. 1. (**a**) ESEM image of wet estuarine biofilm on 304 stainless steel surface (marker = 10 μm). (**b**) ESEM image of estuarine biofilm on 304 stainless steel surface after treatment with acetone and xylene (marker = 10 μm).

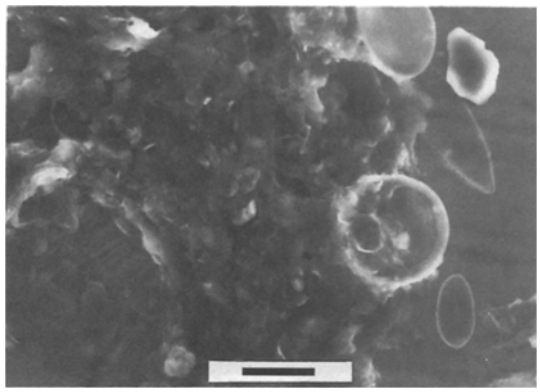

Fig. 2. ESEM image of wet estuarine biofilm on AL6X stainless steel surface showing gelatinous structure and accumulation of diatoms (marker = 10 μm).

Figure 2 shows the gelatinous appearance of the wet biofilm on the AL6X stainless steel surface. Individual bacteria could not be distinguished within the wet biofilm, but numerous diatoms were enmeshed within the gel-like matrix. After preparation for SEM, bacteria could be observed as a single layer of cells (Fig. 3a,b). The bacterial cells in Fig. 3a appear to be partially covered with a surface film, while the extracellular polymer surrounding the cells in Fig. 3b has been reduced to fine filaments connecting the cells.

Wet estuarine biofilms on commercially pure copper surfaces appeared to be uniform with prominent diatom frustules (Fig. 4a). After specimen dehydration through acetone, diatoms were removed from the biofilm (Fig. 4b). After treatment with xylene, the same specimen area exhibited extensive cracking (Fig. 4c). The elemental composition of the wet biofilm on the copper surface (Table 3) reflects the presence of diatom frustules with an elevated proportion of silicon. Aluminium and iron were also concentrated in the wet biofilm. After removal of the diatoms, silicon and aluminum concentrations decreased. After solvent removal of the water, only chlorides remained on the copper surface.

TABLE 3

Weight percent of elements found on commercially pure copper surfaces after exposure to estuarine water for 4 months and after sequential treatment with acetone and xylene

Element	Base metal	Cu in estuarine water	Cu after acetone	Cu after xylene
Al		9.49	1.22	0.74
Si		21.38	1.89	1.27
Cl		0.93	15.9	15.93
Cu	99.9	59.62	80.99	82.06
Mg		1.96		
P		0.98		
S		0.95		
Ca		0.49		
K		0.67		
Fe		3.52		

Surfaces of the copper-containing foils colonized by the mixed culture of anaerobic bacteria containing SRB immediately after removal from the culture medium were uniformly covered with a black gelatinous film and localized deposits. Figure 5a is a photograph of black, wet deposits on 90Cu : 10Ni foil taken directly from the growth medium. Figure 5b shows the same foil after fixation, dehydration, and critical-point drying. Much of the surface material was removed from the specimen. Nontenacious corrosion layers sloughed from the surface during fixation and drying, exposing bare metal. Adherent layers ranging in color from gray to yellowish brown were

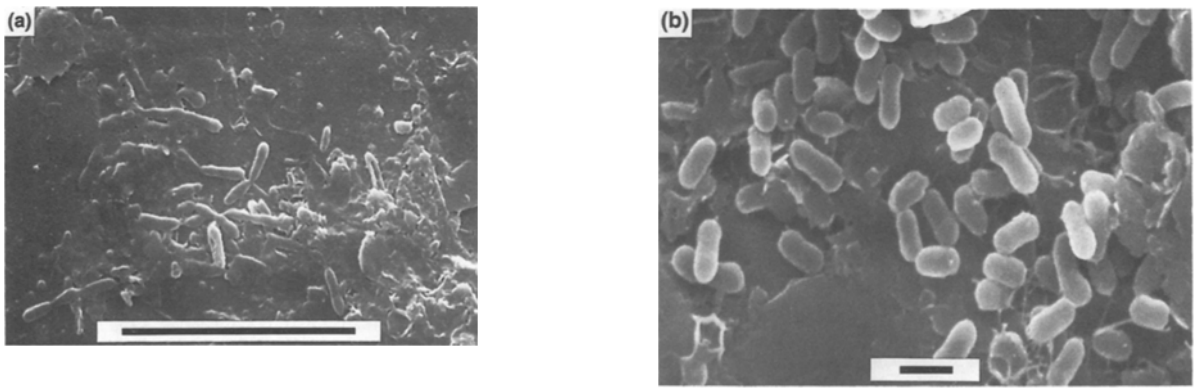

Fig. 3. (**a**) SEM of bacterial cells on AL6X stainless steel surface. Cells appear to be embedded in residual polymeric matrix (marker = 10 μm). (**b**) SEM of bacterial cells on AL6X. Extracellular polymer appears as thin strands connecting cells (marker = 1 μm).

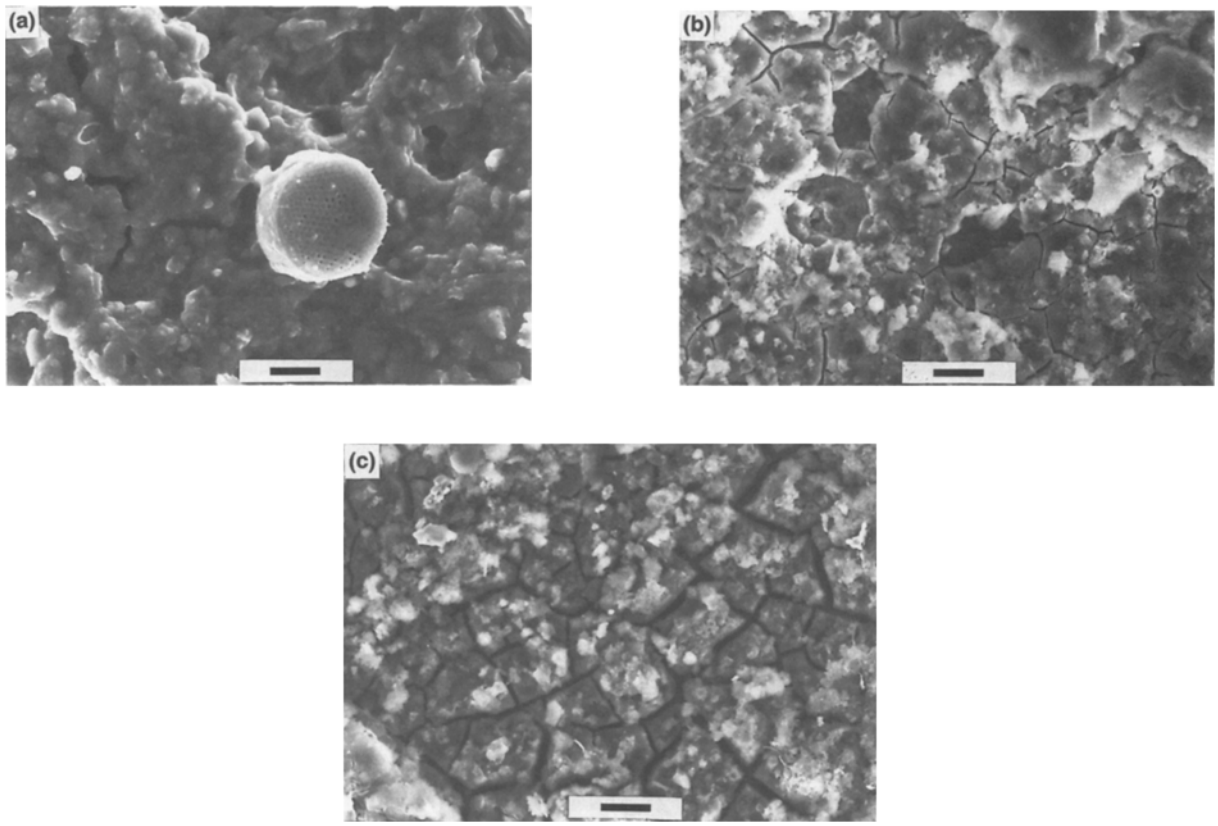

Fig. 4. (**a**) ESEM image of wet estuarine biofilm containing diatom on copper surface (marker = 10 μm). (**b**) ESEM image of estuarine biofilm after treatment with acetone (marker = 10 μm). (**c**) ESEM image of estuarine biofilm after treatment with acetone and xylene (marker = 10 μm).

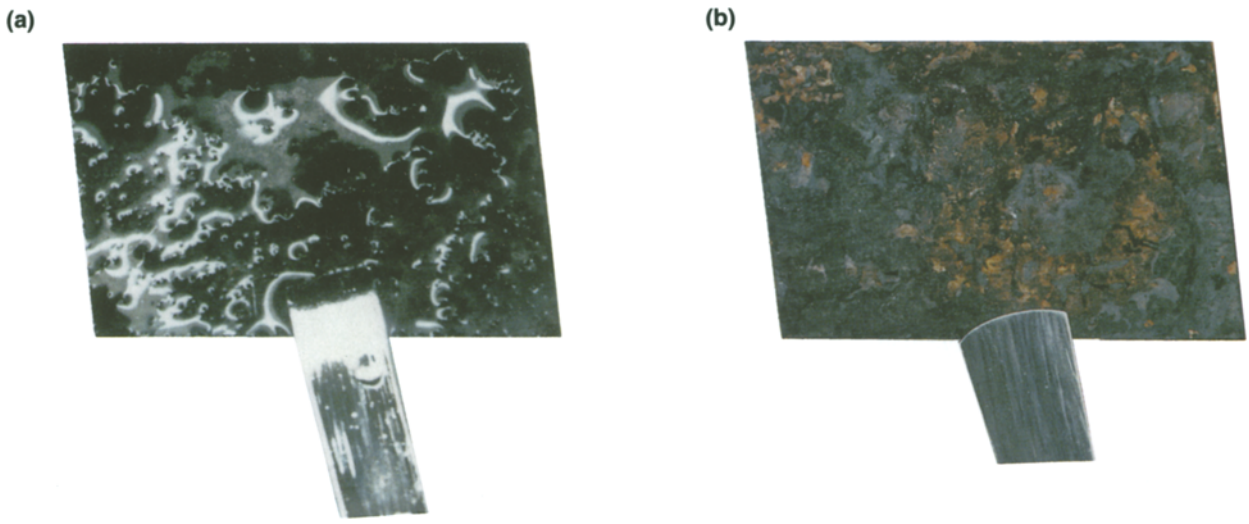

Fig. 5. (**a**) 90Cu : 10Ni surface colonized by SRB immediately after removal from culture medium. (**b**) 90Cu : 10Ni surface colonized by SRB after preparation for SEM.

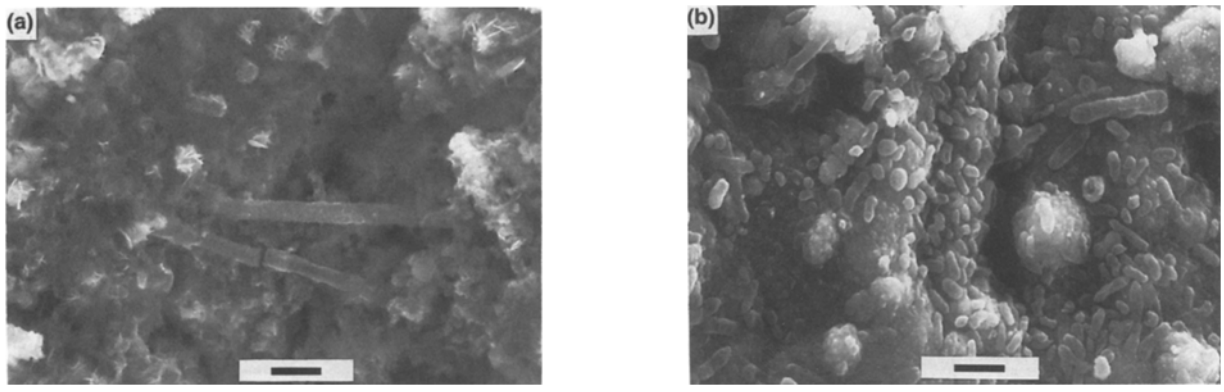

Fig. 6. (**a**) ESEM image of bacteria within sulfide corrosion layers on 90Cu : 10Ni foil (marker = 2 μm). (**b**) SEM image of bacteria on residual corrosion layers of 90Cu : 10Ni after fixation, dehydration, and critical-point drying (marker = 2 μm).

curled and easily dislodged from the surface. ESEM micrographs of the wet 90Cu:10Ni surface show bacteria within the corrosion layers, while an SEM image of the same area indicates the presence of monolayer of cells attached to a corrosion layer (Fig. 6a,b). This was typical of all copper-containing materials colonized by the SRB. ESEM images of the wet copper-containing surfaces document several bacterial morphologies within the copper sulfide corrosion layers (Fig. 7a,b). Bacterial cells attached to base metal under the corrosion layers could be located with both SEM and ESEM. The shape and dimension of the bacterial cells attached to the base metal were not altered by the SEM preparation.

DISCUSSION

Microorganisms that colonize surfaces produce polymers and form a gel matrix that is central to the structural integrity of the biofilm [10]. The gel immobilizes water at the metal/biofilm interface and traps metal species and corrosion products. In addition, some bacteria, including SRB, produce metabolites that react with metal surfaces to produce localized corrosion. Investigators using transmission electron microscopy have shown that biofilms on metal surfaces are three-dimensional and that bacterial cells are stratified within biofilms and corrosion layers [4]. However, most SEM images of biofilms indicate a monolayer of cells with inconspicuous extracellular material and corrosion products associated with the cells.

ESEM images of wet estuarine biofilms on stainless steel surfaces indicated a gelatinous layer in which numerous microalgae could be located. It was impossible to image individual bacterial cells within the gel. After solvent removal of water and drying, much of the extracellular polymeric material from the biofilm was also removed, decreasing the actual areal coverage by the biofilm of the surface and exposing bacterial cells (Fig. 3a,b). It is this monolayer of bacterial cells that is typically referred to as the biofilm.

Many microorganisms produce extracellular polymeric acidic polysaccharides that can bind and precipitate

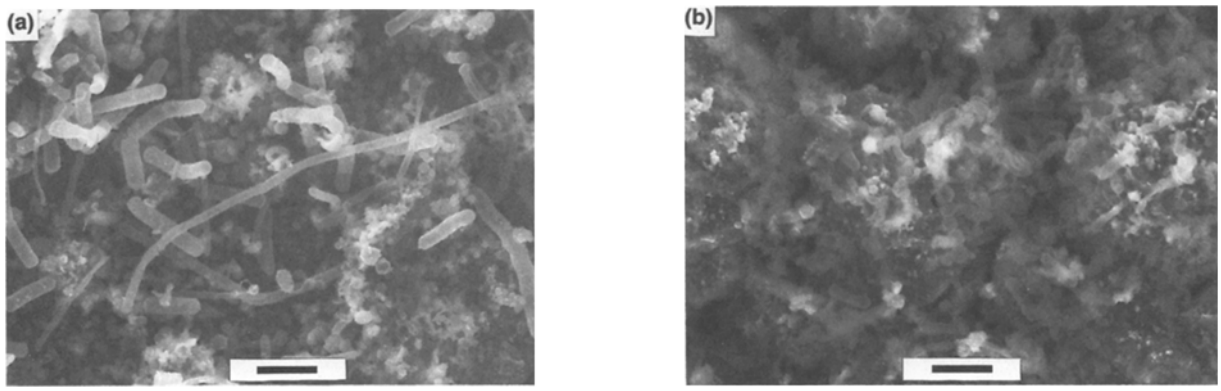

Fig. 7. (**a**) ESEM image of bacteria within sulfide corrosion layers on aluminum brass foil (marker = 5 μm). (**b**) ESEM image of bacteria within sulfide corrosion layers on commercially pure copper foil (marker = 5 μm).

heavy metals from media, including sewage sludge [11] and seawater [9]. In this study, the estuarine biofilm on the stainless steel surface accumulated local concentrations of heavy metals including Al, Ni and Ti (Table 1). The presence of dissolved Ti in estuarine water capable of being bound by extracellular polymers was noteworthy. The source of the dissolved Ti was a paint manufacturer that routinely dumped $TiCl_4$ upstream of the estuarine water collection site. Dehydration of the biofilm with acetone and xylene either extracts bound metals from the biofilm by ion exchange/solvent extraction, or removes the metals with the extracellular polymeric material. The solvent effects of acetone and xylene cannot be separated from the mechanical disturbance to the biofilm during sample manipulation.

Copper surfaces exposed in marine environments form a layer of $Cu_2(OH)_3Cl$, independent of alloy composition [38]. Copper alloys prevent or retard the settlement of macrofouling species, such as barnacles and mussels. However, bacteria, microalgae, protozoa, and their cellular exudates form a slime layer on copper-containing surfaces. Biofilm formation on copper surfaces is of particular importance because of the widespread use of copper alloys in heat exchangers and as cladding for gas platforms. Biofilm buildup leads to reduced heat transfer, increased fluid friction, and increased corrosion. The wet biofilm on the copper surface (Fig. 3a), seen in the ESEM, was uniform and contained diatoms. Diatoms were removed from the surface during the acetone wash. Cracks appeared in the copper chloride layer after treatment with xylene. The elemental composition of the original wet surface indicated the presence of the silicon-containing frustules and iron bound within the biofilm. After acetone and xylene washes, only the surface-bound chlorine persisted (Table 2).

The most conspicuous change in the biological composition of the estuarine biofilms was the partial removal of diatoms from the stainless steel surface and the complete removal of diatoms from the copper surface. Diatoms contain siliceous shells. In the presence of light, they produce oxygen and can alter local oxygen concentrations within biofilms and at metal/biofilm interfaces [26]. Diatoms attach to surfaces by means of acid polysaccharides [12]. Glycoproteins may be involved in the synthesis of the adhesive [8]. Additionally, diatom frustules may be trapped within the layers of the gelatinous matrix that is of bacterial origin (Fig. 2). Solvent and mechanical disruption of the adhesives reduces the number of attached diatoms so that their numbers within biofilms are underestimated by traditional SEM techniques. It should be noted that not all diatoms were removed from stainless steel surfaces by SEM preparation.

Sulfate-reducing bacteria are a diverse group of anaerobic bacteria that can be isolated from a variety of environments [39,40] including seawater [2], where the concentration of sulfate is typically 2 g per liter. Even though the oxygen content of seawater ranges from 6 to 10 ppm, anaerobic microorganisms survive in anaerobic microniches until conditions are suitable for their growth [10,44]. If the aerobic respiration rate within a biofilm is greater than the oxygen diffusion rate, the metal/biofilm interface can become anaerobic and provide a niche for sulfide production by sulfate-reducing bacteria [22]. The metal under the biofilm and corrosion layers is called the base metal to differentiate it from the layers of metal ions that have been derivatized by corrosion reactions.

The impact of sulfides on the corrosion of copper alloys has received a considerable amount of attention. Little et al. [23,24] documented localized corrosion of copper alloys by sulfate-reducing bacteria in estuarine environments. Others reported the failure of copper alloys in polluted seawater containing waterborne sulfides that stimulate pitting and stress corrosion cracking [42]. Copper alloys suffer accelerated corrosion attack in seawater containing 0.01 ppm sulfide after 1-day exposure [17]. A porous layer of cuprous sulfide with the general stoichiometry $Cu_{2-x}S, 0 < x < 1$ forms in the presence of sulfide ions [45]. Copper ions migrate through the layer, react with more sulfide, and produce a thick, black scale.

It has been argued that if the copper sulfide layer were djureleite ($Cu_{1.96}S$), the sulfide layer would be protective [36]. Even if such a sulfide film were technically passivating, the film's mechanical stability is so poor that sulfide films are useless for corrosion protection. In the presence of turbulence, the loosely adherent sulfide film is removed, exposing a fresh copper surface to react with the sulfide ions. For these reasons, turbulence-induced corrosion and sulfide attack of copper alloys cannot easily be decoupled. In the presence of oxygen, the possible corrosion reactions in a copper sulfide system are extremely complex because of the large number of stable copper sulfides [41], their differing electrical conductivities, and catalytic effects. Transformations between sulfides or of sulfides to oxides result in changes in volume that weaken the attachment scale and oxide subscale, leading to spaling. The lack of tenacity of the copper sulfide corrosion products was evident during fixation, dehydration, and drying. Fragile surface layers on all copper-containing foils colonized by SRB were removed during sample manipulation.

Most SEM micrographs of SRB on copper surfaces indicate a monolayer of cells overlaying a sulfide layer. TEM has been used to demonstrate that bacteria were intimately associated with the corrosion products and that, on copper-containing surfaces, the bacteria were found between alternate layers of corrosion products and

attached to base metal [4]. ESEM images demonstrate that SRB were distributed through the sulfur-rich corrosion layers. Fixation, dehydration, and critical-point drying resulted in a loss of material from the surface so that many bacteria were removed with the surface deposits. Bacterial cells attached to the base metal were tenaciously attached to the surface and were not removed or distorted during the SEM preparation.

The impact of glutaraldehyde fixation and distilled water washes on areal coverage and chemical composition of biofilms was not specifically evaluated in this paper. Bacterial cells within the corrosion layers on copper surfaces that had not been treated with fixative could be imaged with the ESEM, but damage from the beam was evident after a few minutes. Additionally, biofilms from estuarine sources that had not been treated with glutaraldehyde and washed to distilled water were dominated by the sodium chloride from the medium. Any diffusional process that results in the fixation of the cells and removal of salts may remove elements bound within the biofilm and cause some distortion

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