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AD-A025 592

SUCCESSION OF PERIPHYTIC MICROORGANISMS ON
METAL AND GLASS SURFACES IN NATURAL SEAWATER

MIAMI UNIVERSITY

PREPARED FOR
OFFICE OF NAVAL RESEARCH

JUNE 1976

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ADA 025592

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO.	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Succession of Periphytic Microorganisms on Metal and Glass Surfaces in Natural Seawater		5. TYPE OF REPORT & PERIOD COVERED Scientific paper
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Gerchakov, S.M., D. S. Marszalek, F. J. Roth, and L. R. Udey		8. CONTRACT OR GRANT NUMBER(s) A-0201-0026 N00014-67-021-0026
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of Miami, Department of Microbiology P. O. Box 520875 Miami, Florida 33152		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research, Ocean Science and Technology code 480, Arlington, Va. 22217		12. REPORT DATE June, 1976
		13. NUMBER OF PAGES 27 pp.
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Distribution of this document is unlimited.		
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)		
18. SUPPLEMENTARY NOTES Presented at the 4th International Congress on Marine Corrosion and Fouling, Juan-les-Pins - Antibes (France), June, 1976.		
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) Fouling Corrosion Periphytic-microorganisms		
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Stainless steel and glass substrates were exposed to Biscayne Bay (Florida) seawater for several months. Samples were removed at regular intervals and examined for periphytic microorganisms by scanning electron microscopy and by microbiological culture techniques. The purpose of this study is to search for a possible relationship between microbial fouling and corrosion in the marine environment.		

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SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

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SUCCESSION OF PERIPHYTIC MICROORGANISMS ON METAL AND GLASS
SURFACES IN NATURAL SEAWATER

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ABSTRACT

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To be presented at the 4th INTERNATIONAL CONGRESS ON MARINE
CORROSION AND FOULING, Juan-les-Pins - Antibes (France), 14-19 June, 1976.

INTRODUCTION

The implication of microorganisms in metal corrosion processes was suggested as early as 1891 when Garrett postulated that the corrosion of lead could be due to ammonia, nitrites, and nitrates produced by bacterial action. Gaines (1910) concluded that underground corrosion of iron and steel structures was partially due to bacterial activity. In fact, the iron bacterium Gallionella ferruginea was isolated from corrosion products on buried steel conduits, and high concentrations of sulfur and organic matter found in the products suggested the presence of sulfur bacteria. Von Wolzogen Kühr and van der Vlugt (1934) postulated in their classical paper that certain organisms, primarily those of the bacterial genus Desulfovibrio, depolarize the cathodic sites of the corrosion cells by removing hydrogen that accumulates on the surface of iron and thus promote corrosion under anaerobic conditions. Iverson (1966) presented direct evidence for this depolarization process.

Not all investigators agree on the importance of the depolarization mechanism (Nelson, 1962), but an increasing volume of information has been gathered implicating microorganisms in corrosion processes in various environments. For example, Thiobacillus concretivorus was isolated in large numbers from corroded concrete structures at widely separate localities (Parker, 1945a). Furthermore, he found that in an environment similar to sewer conditions, rapid corrosion occurred only after conditions became favorable to Th. concretivorus growth and proliferation (Parker, 1945b). Wacks, et al. (1964) found Desulfovibrio desulfuricans to be responsible for the corrosion of aviation gasoline stored in tanks containing small amounts of water. Hendey (1964), investigating Cladosporium rosinae as a fuel contaminant in kerosene-type fuel storage tanks and

fuel tanks of aircraft, suggested that the fungal metabolites are capable of attacking aluminum.

Kalinenko (1959) found bacterial colonies on aluminum, brass and bronze plates immersed in natural seawater, and suggested that the bacterial colonies accelerate the electrochemical processes of metal corrosion. Rozenberg and Ulanovskii (1960) stated that bacteria may enhance corrosion of stainless steel in seawater by decreasing the protective effects of cathode polarization; conversely, bacteria may retard corrosion by sedimentation of CaCO_3 and Mg(OH)_2 from seawater on the steel surface. Tawadse and Keinchadse (1972) have shown that the corrosion losses of several steels were lower in sterile seawater than in the same medium to which bacteria were added.

Compton (1970) pointed out the naiveté of some scientists who consider seawater a simple solution of sodium chloride contaminated with a few additional salts, and called attention to the so-called "biological factors" which are so often mentioned with regard to the uniqueness of seawater. LaQue (1972) reported that the longer a polluted sample of seawater is kept, the more corrosive it appears to be.

Discussion of the various mechanisms that may be operating in biological corrosion is outside the scope of this paper and may be found in, among others, papers such as Costello (1969) and Iverson (1972). It is apparent that whatever mechanism may prevail, periphytes affect corrosion processes more profoundly than planktonic organisms.

Preliminary results of a systematic study of the effects of dissolved organic matter and surface-associated microorganisms on metal corrosion in the marine environment indicate not only the ubiquitous nature of periphytic microorganisms, but that stainless steel surfaces enrich or select for potentially corrosion-enhancing bacteria.

MATERIALS AND METHODS

An experimental tank (4.6 m long, 2.5 m wide, and 1.7 m deep) with about 35 cm of marine carbonate sediments overlaid with 1.2 m deep seawater (Biscayne Bay, Florida) was utilized for this study (winters of 1974-5 and 1975-6). A once-through flow of natural seawater was continuously maintained with a turnover time of approximately 18 hours. The tank was under a diurnal light cycle, and its tank environment very closely resembled that of the bay.

Specimen Preparation and handling

Stainless steel 304 coupons (2.7 cm x 2.7 cm x 0.5 cm) were polished using successively finer grades of emory paper (220, 320, 400 and 600), followed by jeweler's rouge. The specimens were then washed in detergent, rinsed with distilled water, dehydrated with methanol, weighed and stored in a desiccator until used. Each specimen was suspended at a depth of 30 cm below the water surface by means of a monofilament nylon line. Glass microscope slides were similarly cleaned and placed in slots made in rubber stoppers which were also suspended by means of a monofilament line.

Caution was taken in placing and harvesting specimens so as to prevent them from contacting the air/water interface. This was achieved by sealing the specimens in plastic bags and breaking the seal after the bags had crossed the air/water interface. This procedure was necessary in order to eliminate contamination by bacterioneuston. In fact, DiSalvo (1973) has demonstrated that surfaces coming in contact with the air/water interface held up to three orders of magnitude more attached bacteria per unit area than surfaces kept from contact

with it.

Three specimens each of metal coupons and of glass slides were initially harvested after 4, 10, 16, 40 and 96 hours exposure time, followed by semiweekly and then weekly sampling intervals. Water samples were taken concurrently. The triplicates were assigned for bacterial, fungal and yeast analyses, and for scanning electron microscopic studies. Following the microbial sampling, the metal coupons were cleaned and weighed to determine weight-loss.

Scanning Electron Microscopy

All samples were fixed for one hour in 4% glutaraldehyde in seawater and washed with several changes of distilled water. Metal samples were then dehydrated in acetone, immersed in xylene and air dried. Glass samples were dehydrated in ethanol and critical point dried in Freon 13 using Freon TF as an intermediate fluid. Glass and metal samples were then coated with Au-Pd in a Denton vacuum evaporator and examined in an AMR-900 scanning electron microscope operated at 21 kV accelerating voltage.

Microbiological Analyses

For bacteria, specimens were transported to the laboratory and aseptically removed from the bags. Their surfaces (14 cm^2) were then scraped with sterile carbon steel razor blades and then with sterile swabs to remove the bacteria. The material thus removed was resuspended in 2% saline containing 0.1% peptone, and appropriate dilutions made in the same buffer. Water samples were also diluted with saline-peptone.

To enumerate total heterotrophic bacteria and total acid-producing heterotrophs, samples were plated in duplicate on a modification of Difco Marine Agar 2216 (MMA). This modification consisted of the addition of 0.5 g/l cycloheximide (to inhibit fungi) as well as 10 g/l glucose and 0.05 g/l phenol red to enumerate

those organisms which produce acid during the dissimilation of glucose. Total and acid-producing colonies growing on this medium were counted after one day, and again after 14 days of incubation.

Baar's medium (Skerman, 1959) with 3% Na_2SO_3 and 2.5% NaCl was used to enrich and select for halophilic Desulfovibrio spp. from metal, glass and water.

For filamentous fungi and yeasts, the specimens were placed on another modified Difco Marine Agar 2216 which allowed fungi and yeasts to grow but not bacteria. This modification (Fungal Marine Agar: FMA) was made by adding the antibiotic, gentamicin at 50 mg/l and by adding 10 g/l glucose and 0.05 g/l phenol red to elucidate acid-producing organisms. The technique of placing the metal and glass specimens on agar surfaces did not permit accurate quantitation of the number of fungi on these surfaces. It did, however, provide an excellent means by which to detect the first appearance of fungi on the samples and a means of cataloguing the various genera which arose during the time course of this study. Water samples collected at each sampling period were filtered through Millipore membranes (0.45 μ) and, subsequently, the membranes were placed on MMA and FMA plates to delineate the number and type of bacteria and fungi, respectively.

RESULTS

Scanning Electron Microscopy (SEM)

A succession of periphytic microorganisms was observed on both the glass and stainless steel substrates, with the major changes occurring during the first five weeks of exposure. In general, the stainless steel was fouled at approximately the same rate and with similar microorganisms as the glass control substrate; brass substrates exposed under identical conditions produced a wholly different succession of periphytes (results will be published elsewhere).

The first organisms to appear on stainless steel and glass were rod-shaped bacteria. Colonization by bacteria occurred within the first few hours of exposure; samples exposed for as little as four hours contained isolated bacterial cells. After the first day, small colonies and dividing cells were observed (Fig. 1). Fungi appeared between two and three days exposure. These early appearing fungi were all similar in appearance and typically consisted of a holdfast, stalk and bulbous sporangium (Fig. 2). The number of fungal cells increased rapidly and, with the bacteria, were the most abundant microorganisms during the first week.

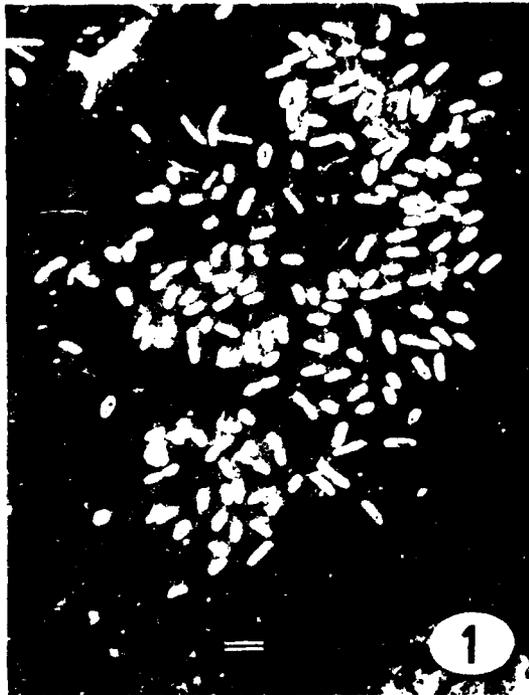
Soon after the appearance of the fungi illustrated in Fig. 2, filamentous microorganisms of unknown taxonomy were observed (Figs. 3 and 4). These organisms were formed of minute filaments approximately $0.1 \mu\text{m}$ diameter which arose from a holdfast attached to the substrate. The filaments coalesced to form an elongate stalk and eventually parted to form a basket-like terminal structure. During the first week, most specimens were solitary and had a single terminal structure, but at about ten days, most were colonial (Fig. 3) and had as many as 15 terminal structures, each colony sharing a common holdfast. These filamentous organisms might arise from a structure visible in Fig. 4.

Figures 5 and 6 illustrate a group of bacteria (?) which appeared during the second week of exposure. These cells are of unusually small size (0.4 to $0.6 \mu\text{m}$ diameter), are hemispherical in shape, and are characterized by a central pore (see Fig. 6). They typically occurred in clusters of up to 100 cells. The "hemispheres" were observed on most samples exposed more than 10 days, but because of their small size were easily masked by larger bacteria, diatoms, and other fouling organisms. Hemispherical bacteria were firmly attached to the stainless steel or glass surface and left behind a characteristic circular deposit after death of the cell (see Figs. 5 and 13).

The first two weeks of exposure were characterized by bacteria and fungi, with other organisms occurring in relatively insignificant numbers. At about 15 days, fungi decreased in relative abundance, bacteria continued to increase, and diatoms, filamentous algae, and other organisms became more conspicuous. Accompanying that change was a loss of surface luster of the specimens and a thin fouling layer became visible to the naked eye. The SEM revealed that the assemblage of fouling microorganisms changed significantly between two and three weeks exposure, with diatoms and bacteria becoming the dominant organisms during the third week. Figures 7, 8, and 9 illustrate the progressive increase in diatom abundance, and the appearance of colonies of coccoid bacteria.

Diatoms which had initially colonized the samples were typically non-motile, firmly attached to the substrate, and were represented by Mastoglia spp. (Fig. 8) and Nitzschia spp. (Fig. 9). The siliceous frustules (shells) of living diatoms are covered with a thin layer of cytoplasm and therefore appeared smooth in the SEM; dead diatoms were recognized by their empty frustules (Fig. 13, Cocconeis spp.). More than 20 taxa of Diatomaceae were observed as fouling organisms during the course of this study. Another important group of microorganisms to appear during the third week of exposure were the peritrichous ciliates (Fig. 16, Zoothamnion spp.), which are colonial, possess a contractile stalk, and utilize bacteria as a food source. Zoothamnion spp. were abundant during the third and fourth weeks, and were rare on later samples.

At about five weeks exposure, the fouling layer had developed into a two-tiered structure seen in Figures 10 and 11. The first tier or initial layer of fouling organisms was in intimate contact with the stainless steel or glass substrate, and consisted mainly of bacteria, fungi, and non-motile diatoms. Above that layer was found the second tier consisting of: large, colonial, motile diatoms which occurred



- Figure 1 Microcolony of rod-shaped bacteria. Bacteria are the earliest observed periphytes (1 day exposure). Bar scale = 10 μ m
- Figure 2 Fungus. Note holdfast, stalk, and bulbous "sporangium" (1st week exposure) Bar scale = 10 μ m
- Figure 3 Unidentified filamentous, colonial microorganisms. (1st week exposure). Bar scale = 10 μ m
- Figure 4 A single unidentified filamentous microorganism developing from a spore-like structure (1st week exposure). Bar scale = 10 μ m

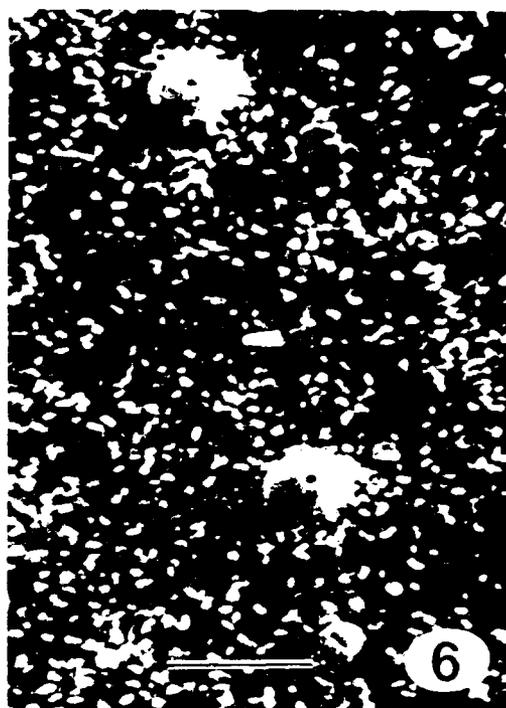
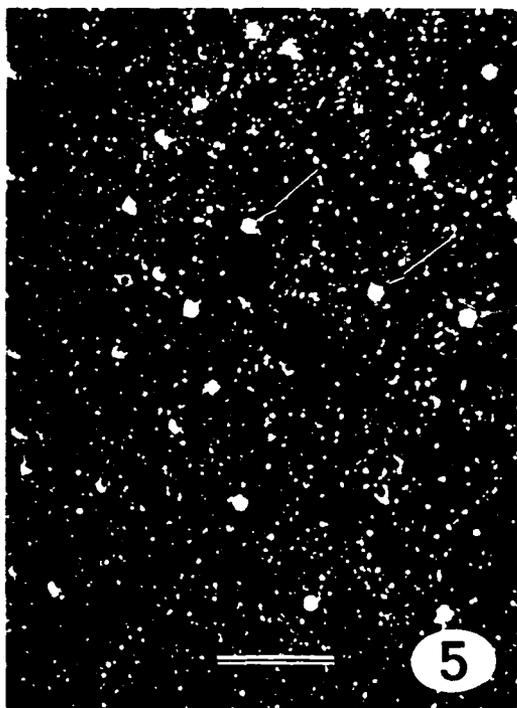


Figure 5 Microcolony of hemispherical bacteria (?). Arrows indicate individual cells (2nd week exposure). Bar scale = 5 μ m
 Figure 6 Detail of hemispherical bacteria. Note central pore and minute size. Bar scale = 1 μ m
 Figure 7 Diatoms and filamentous algae appear during the 3rd week of exposure. Bar scale = 10 μ m

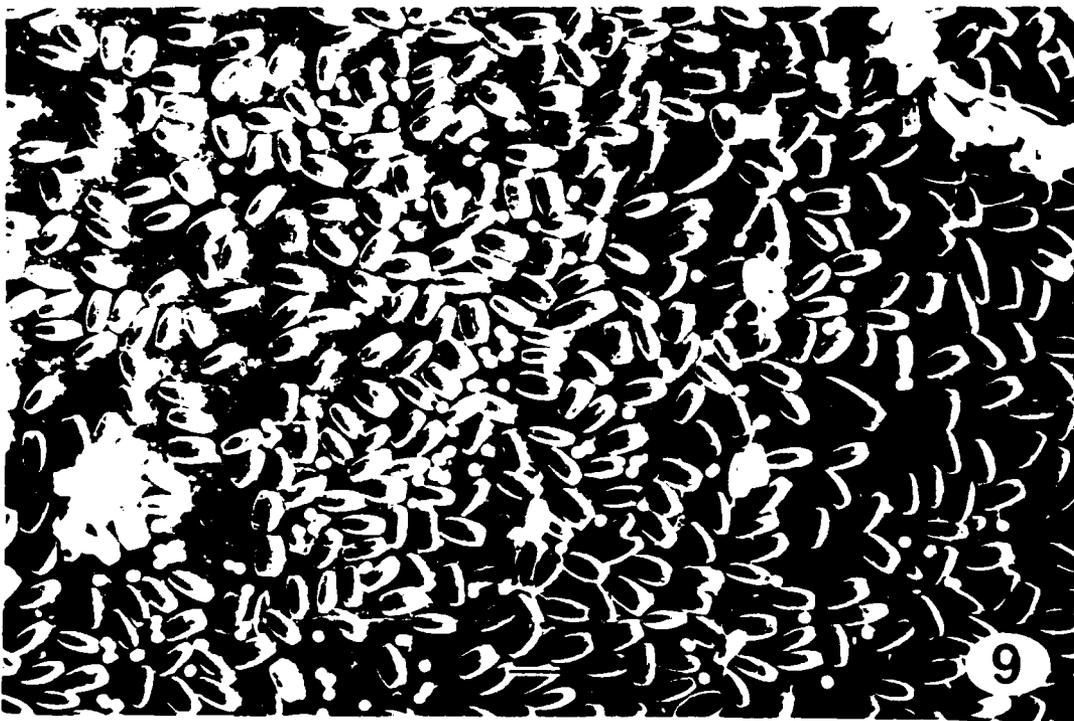
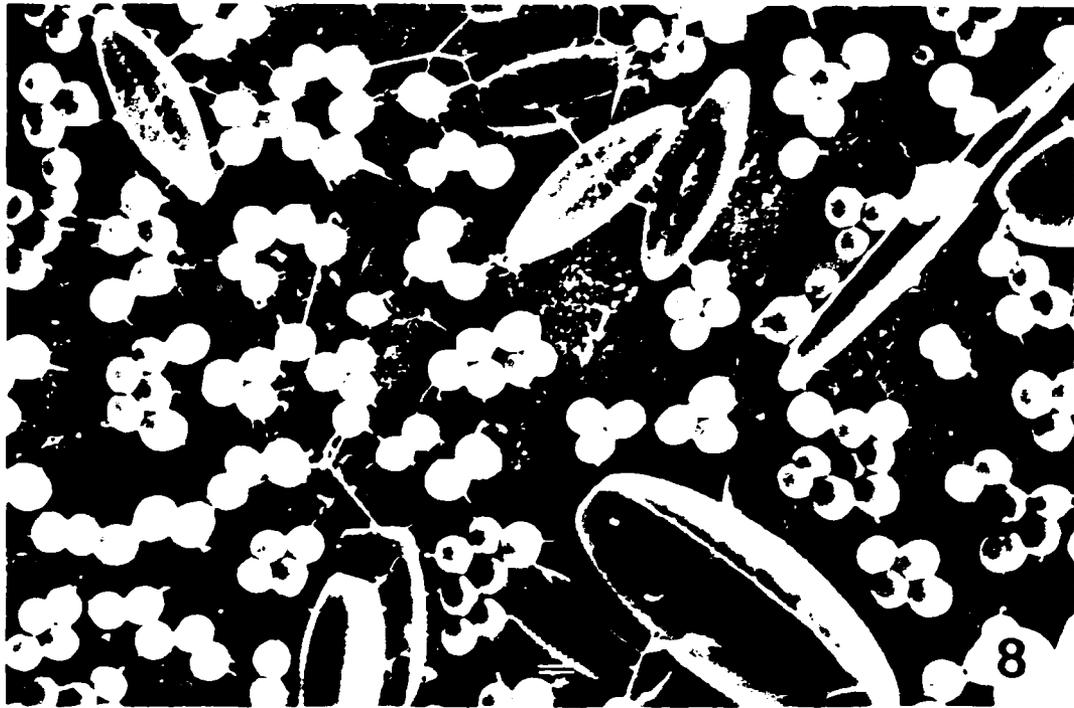


Figure 8 Diatoms and coccoidal bacteria are dominant first tier microorganisms (5th week exposure). Bar scale = 1 μ m

Figure 9 Diatoms (*Nitzschia* spp.) densely colonize the substrate surface, also seen are coccoid bacteria (5th week exposure). Bar scale = 10 μ m

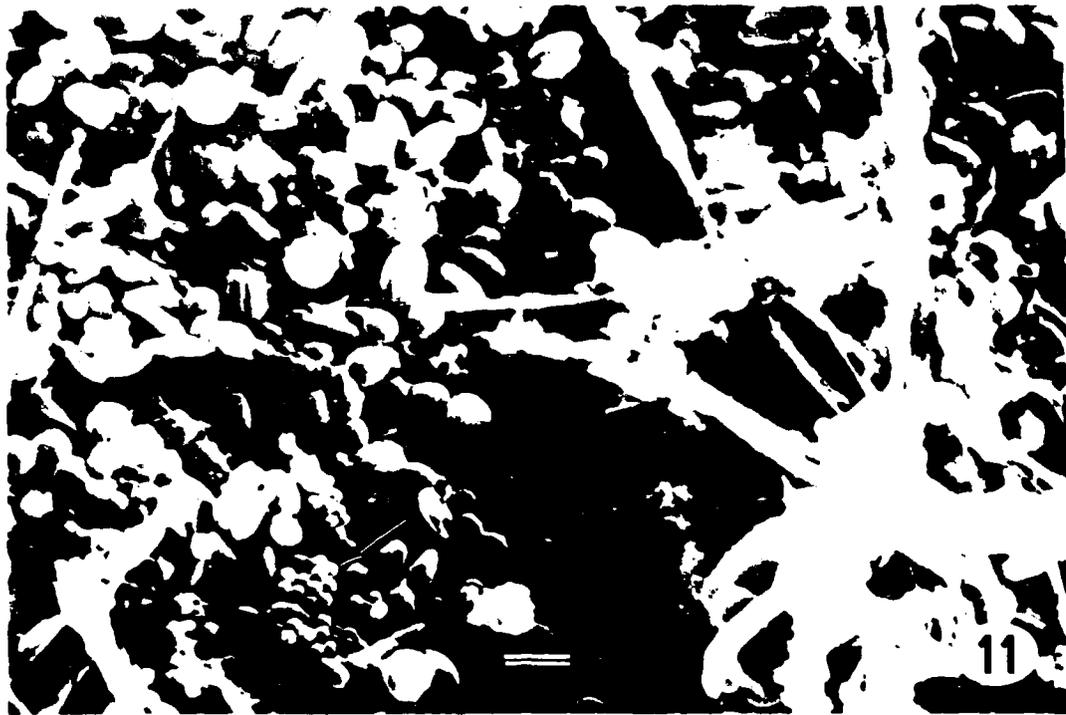
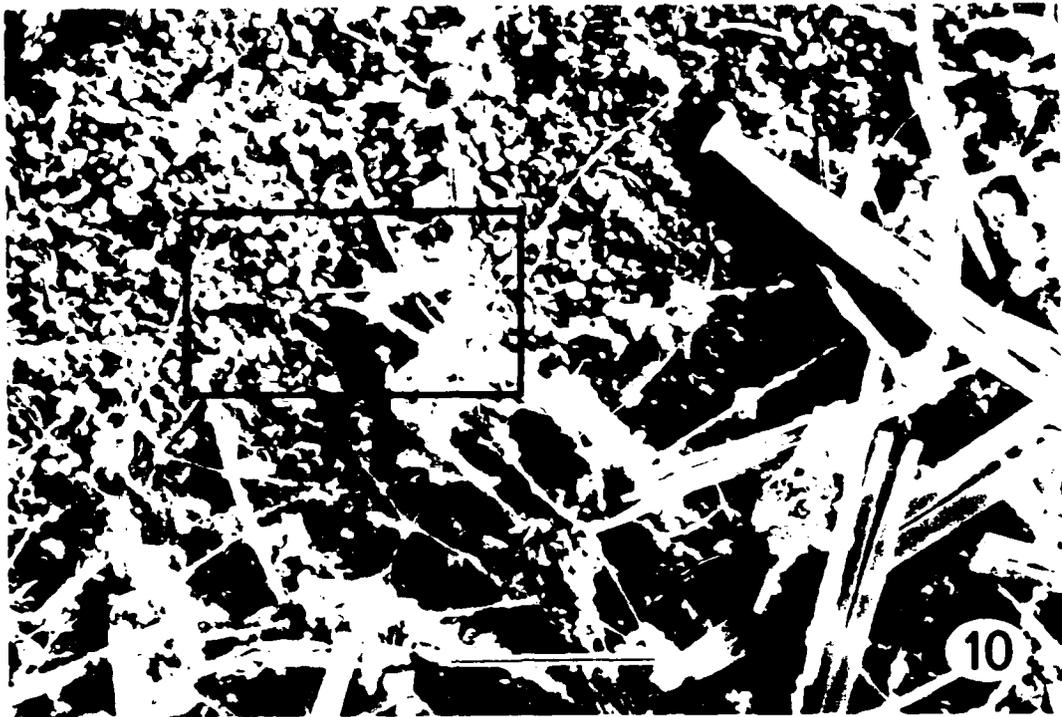


Figure 10 Fouling layer consists of two tiers. First tier organisms (upper left) are in direct contact with substrate; second tier organisms (lower right) lie on first tier layer (6th week exposure). Bar scale = 0.1 mm

Figure 11 Detail of Figure 10. Note minute diatoms and coccid (arrow). Bar scale = 10 μ m

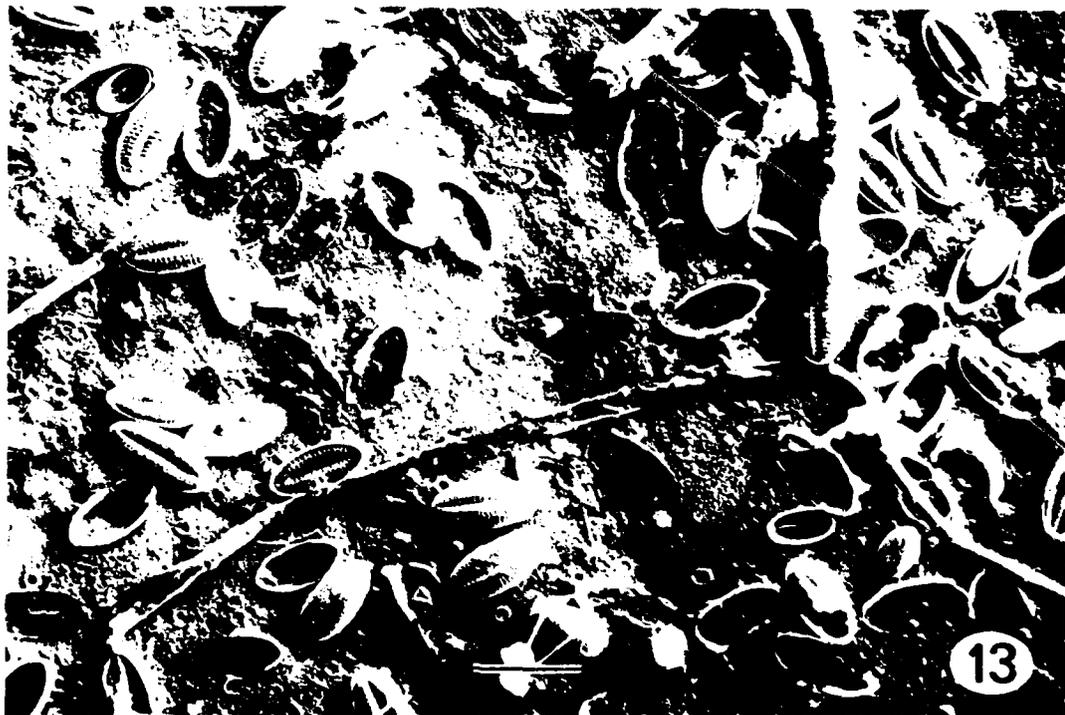
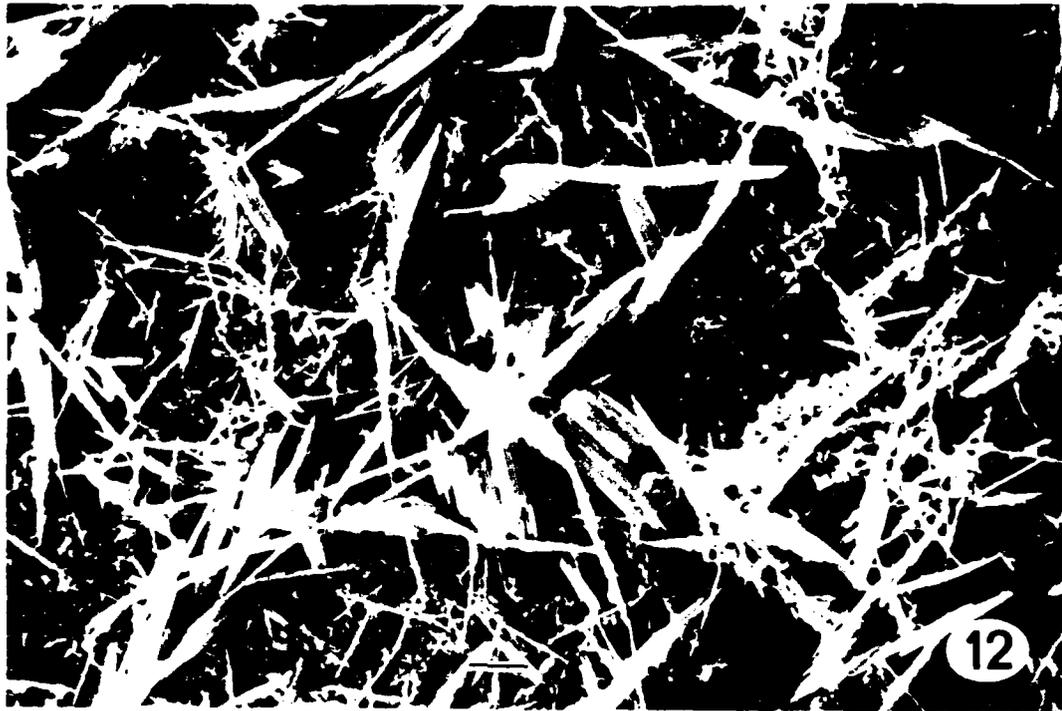
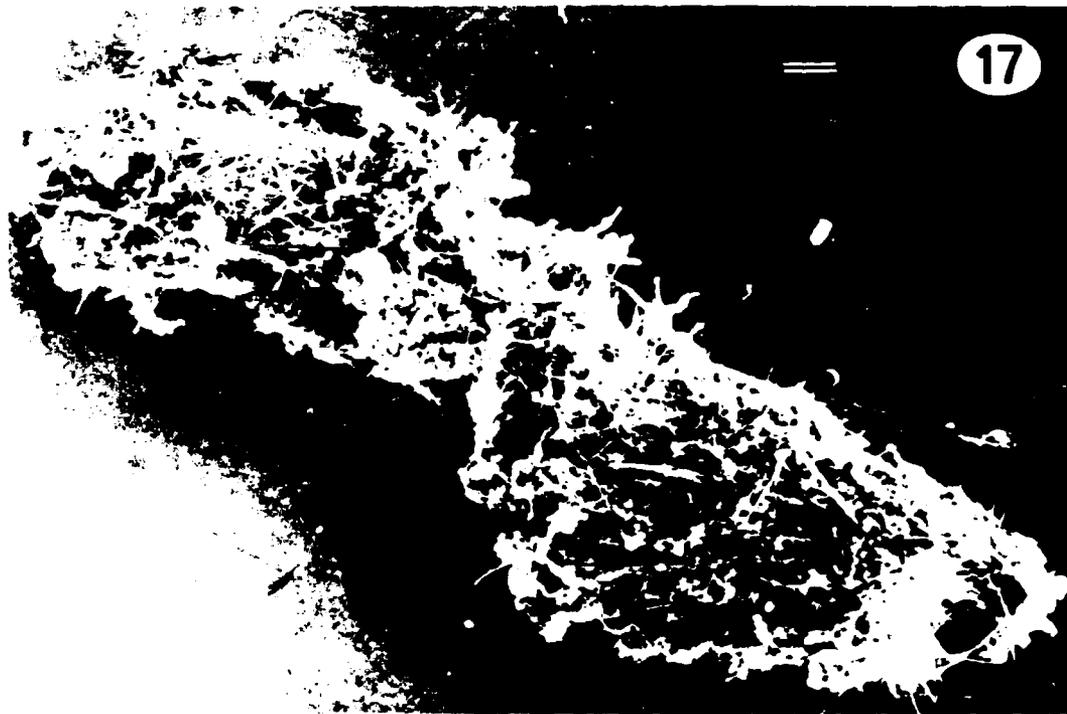


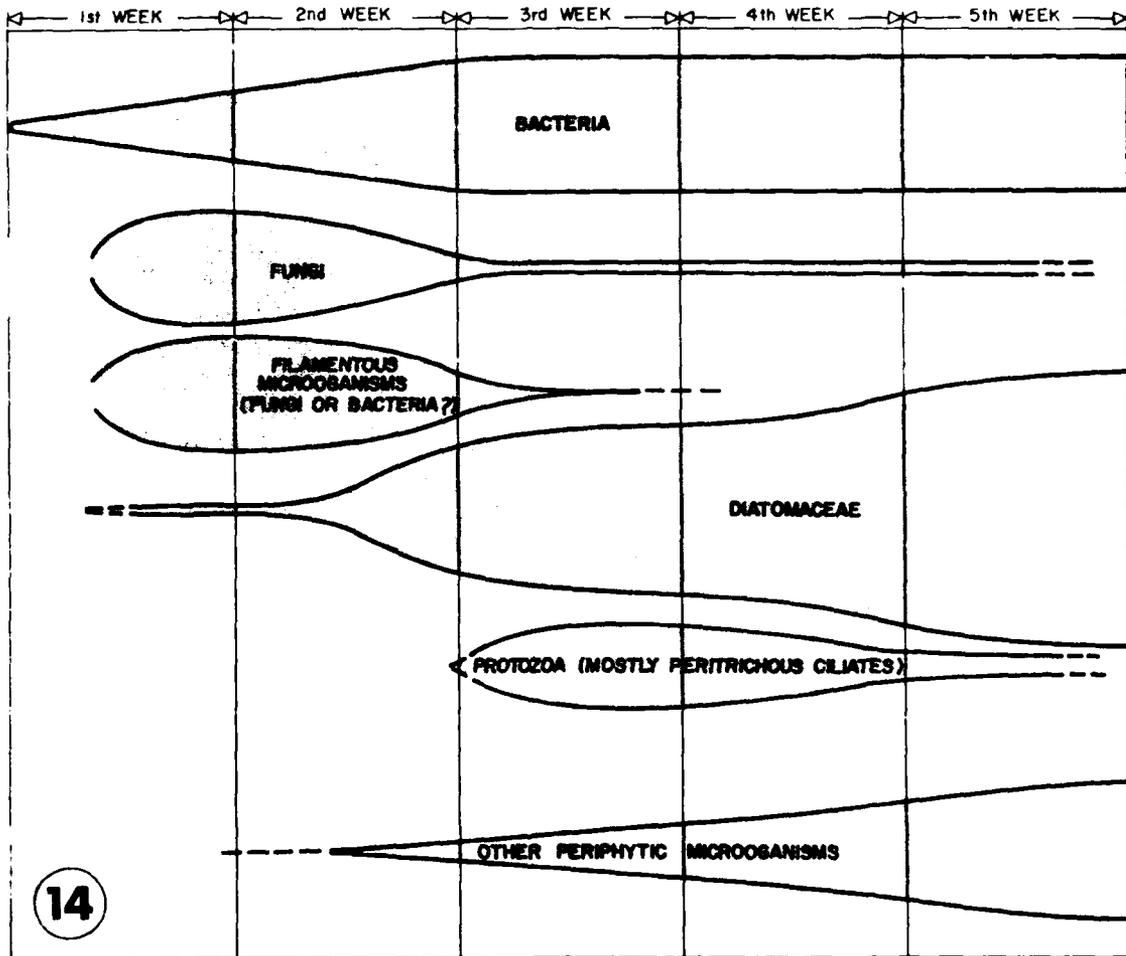
Figure 12 Colonial diatoms (*Thalassionima* spp.) characteristic of second tier layer are mostly large and motile (8th week exposure). Bar scale = 0.1 mm

Figure 13 Removal of second tier organisms reveals remains of first tier organisms, mostly siliceous diatom frustules (13th week exposure). Bar scale = 10 μ m



- Figure 15 Filamentous algae become important fouling organisms on substrate exposed for more than 5 weeks. Bar scale = 1 μ m
- Figure 16 Peritrichous ciliates, colonial protozoa which consumes bacteria. Note contractile stalk (arrow). Maximum abundance during 3rd and 4th week of exposure. Bar scale = 10 μ m
- Figure 17 Worm tube composed of detritus and living microorganisms. Worms and other macroscopic organisms become increasingly abundant as fouling organisms with prolonged exposure of substrates. Bar scale = 0.1 mm

DOMINANT PERIPHYTIC MICROORGANISMS ON STAINLESS STEEL AND GLASS SUBSTRATES
IN RELATION TO EXPOSURE TIME



in sheets of 50 or more cells (Fig. 12); other diatoms; ciliates and flagellates; bacteria and fungi growing on fecal pellets and other organic detritus; and a variety of other organisms in lesser abundance. Mechanical removal of the fouling layer revealed the substrate to be covered with the remains of first-tier organisms, especially siliceous diatom frustules cemented to the specimen surfaces (Fig. 13). In general, diatoms and filamentous algae (Fig. 15) were the most conspicuous microorganisms of the second-tier fouling layer.

The succession of periphytic microorganisms observed during the first five weeks exposure is graphically summarized in Fig. 14, which indicates the relative abundance of the major taxa leading to the development of a two-tier fouling layer.

Samples exposed for more than five weeks developed a thick fouling layer consisting of the microorganisms previously described, as well as numerous invertebrates including worms (Fig. 17), gastropods, sponges, and the larvae and juveniles of other organisms. These macroscopic fouling organisms are beyond the scope of this report.

Fungi and Yeasts

A list of genera that were found on the stainless steel and glass surfaces and in the surrounding seawater is presented in Table I. Nine genera of filamentous fungi were isolated from the surfaces. Three of these, Aspergillus, Penicillium and Nigrospora were found on both metal and glass. The first two were more abundant on the metal, which may be important because these genera produce the most acid from the dissimilation of glucose. Genera found only on metal were Cephalosporium, Alternaria and Dactylaria, while Torula, Pestalotia and Humicola (syn., Monotospora) were found only on glass. Although organisms resembling yeasts were seen on occasion by SEM, no yeasts were isolated from either metal or

TABLE 1

The Occurrence of Filamentous Fungi, and Yeast on Surfaces
of Stainless Steel and Glass and in Biscayne Bay water

Exposure Time (Days)	Organisms on Stainless Steel	Organisms on Glass Slides	Water Column Organisms	CFU/Liter
10	<u>Aspergillus</u> spp.		<u>Candida parapsilosis</u> <u>Rhodotorula</u> spp. <u>Aureobasidium pullulans</u>	32 44 62
22	<u>Aspergillus</u> spp.		(No sample taken)	
29	<u>Aspergillus</u> spp. <u>Penicillium</u> spp.		<u>Candida parapsilosis</u>	38
36	<u>Penicillium</u> spp.	<u>Aspergillus</u> spp.	<u>Penicillium</u> spp. <u>Aspergillus</u> spp.	4 6
45	<u>Nigrospora</u> spp.	<u>Torula</u> spp.		
			<u>Candida</u> spp. <u>Saccharomyces</u> spp.	12 2
52	<u>Aspergillus</u> spp.	<u>Nigrospora</u> spp.	<u>Candida parapsilosis</u>	30
70	<u>Penicillium</u> spp. <u>Nigrospora</u> spp. <u>Cephalosporium</u> spp.	<u>Penicillium</u> spp.	<u>Penicillium</u> spp.	10
77	<u>Alternaria</u> spp.	<u>Nigrospora</u> spp.	<u>Aspergillus</u> spp. <u>Penicillium</u> spp. <u>Curvularia</u> spp.	2 4 2
84	<u>Penicillium</u> spp.	<u>Nigrospora</u> spp.	<u>Aspergillus</u> spp.	2
91	<u>Alternaria</u> spp. <u>Dactylaria</u> spp.	<u>Montospora</u> spp.		
			<u>Rhodotorula glutinis</u>	50
98	(To be identified)	<u>Penicillium</u> spp.	<u>Candida</u> spp.	34

glass specimens. This may have been a result of the isolation method used. Yeasts were more plentiful in the surrounding seawater than were filamentous fungi. Members of the genera Candida, Rhodotorula and Saccharomyces were present in water samples. In addition, Aureobasidium spp. and Curvularia spp. were found in water but not on either glass or metal surfaces. The only genera found on surfaces which were also found in water were species of Aspergillus and Penicillium. This indicates that metal and glass surfaces are enriching for the other observed genera.

Bacteria

The number of heterotrophic bacteria colonizing the surfaces of metal and glass increased over a three-month period. Figure 18 represents the colony-forming units per cm^2 of surface (CFU/ cm^2) observed on MMA plates after 24-hour incubation, while Fig. 19 depicts the CFU/ cm^2 after 14-day incubation. In the first case, the fast-growing bacteria are enumerated, while at 14 days both fast and slower growing types were present. These data show that there was a steady increase in both fast and slow growing bacteria throughout this study.

The proportion of bacteria that produced acid from the dissimilation of glucose, relative to the total number of organisms, also increased with time. A marked difference between substrates, however, was observed after 50-day exposure time. The proportion of acid producers at that time began to significantly increase on the stainless steel surfaces, while that proportion for the glass and water samples stabilized and then started to decrease.

All bacteria isolated from the surfaces were gram negative; rod-shaped bacteria appeared first and then were joined by coccoidal forms. About half of the isolates were motile. It was also observed that the majority of the bacteria which initially colonized the surfaces produced large amounts of mucoidal matter. This

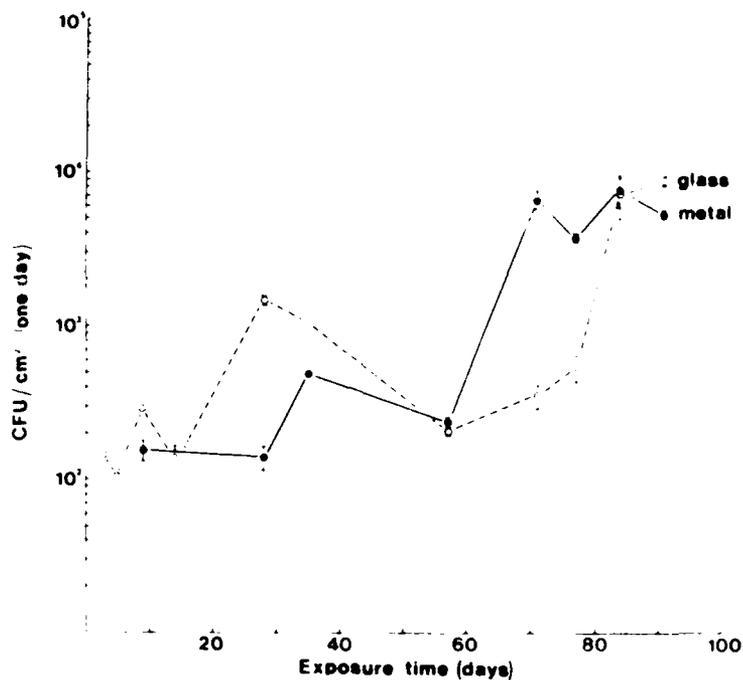


Figure 18. The number of fast growing heterotrophic bacteria cultured from the surfaces of stainless steel and glass as a function of exposure time to Biscayne Bay water.

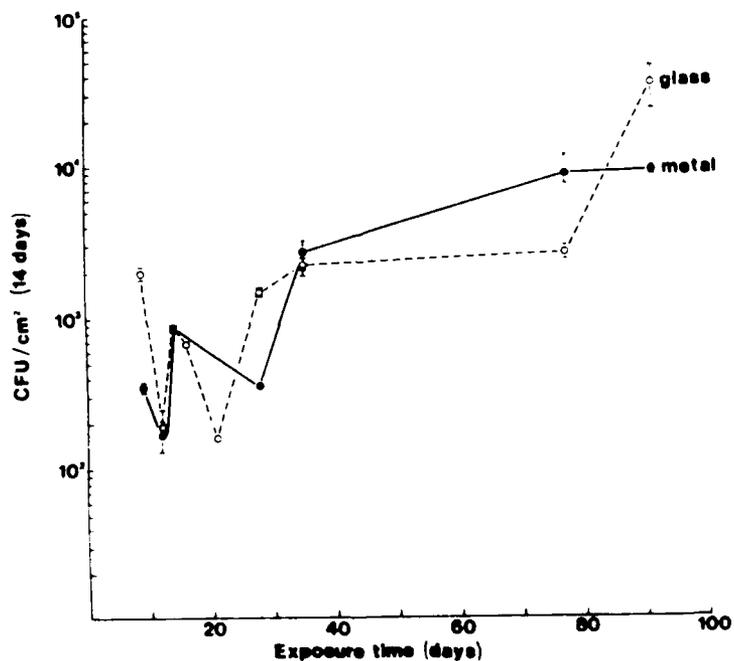


Figure 19. Total heterotrophic bacteria cultured from the surfaces of stainless steel and as a function of exposure time to Biscayne Bay water.

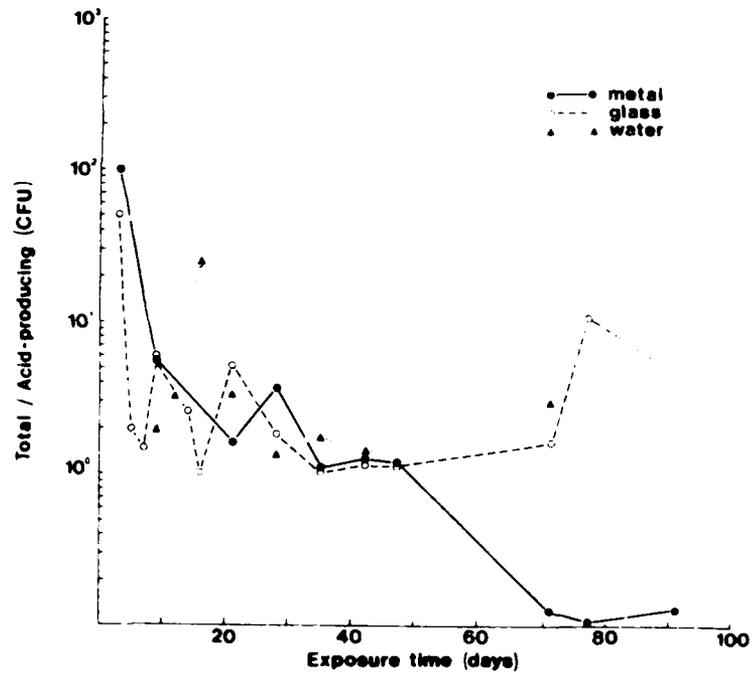


Figure 20. Ratios of total heterotrophic bacteria to acid-producers on stainless steel and glass, and in Biscayne Bay water.

material may be the uronic acid-containing carbohydrate described by Corpe (1970, 1974).

Desulfovibrio spp. were consistently isolated, though in small numbers, from the exposed surfaces.

DISCUSSION

Our observation that bacteria colonize metal or glass surfaces within several hours of exposure to seawater, with other microorganisms appearing only after continued exposure, is in agreement with other reports (Marshall, et al., 1971; Corpe, 1974).

Hendricks (1974) reported that bacteria sorbed to surfaces appear to be metabolically more active than suspended organisms based on respiration and enzymatic studies. Therefore, the intimate contact of the bacteria with the metal surfaces is of consequence in corrosion processes. Due to their metabolic activities, bacteria can give rise to differential-concentration cells which result in differences in potential and subsequent corrosion currents. The presence of acid producing bacteria on metal surfaces gives credence to the potential of microorganisms to locally dissolve passivating oxide films. We have demonstrated that increasing proportions of bacteria isolated from stainless steel were able to produce acid from glucose. We are now investigating if bacteria can produce acid from dissolved organic matter isolated from seawater. The reason that stainless steel selects or enriches for acid producers after 50-day exposure time is not known at this time.

Finally, we wish to draw attention to the presence of sulfate-reducing bacteria on metal surfaces suspended in well aerated seawater. This observation suggests that micro-anaerobic conditions exist at the metal/seawater interface.

Thus, bacterial corrosion mechanisms that have generally been ascribed to anaerobic environments may also be operating in cases where metals are exposed to aerated seawater.

ACKNOWLEDGMENTS

This investigation was supported by the U. S. Office of Naval Research contract N00014-67-A-0201-0026 under project NR 083-300. We would like to thank Dr. W.P. Iverson of the U.S. National Bureau of Standards for his helpful suggestions.

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