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Influence of Substrate Composition on Marine Microfouling†

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Glass and metal substrates exposed to subtropical seawater exhibit a characteristic succession of periphytic microorganisms. Substrates which are biologically and physically inert (glass and stainless steel) fouled rapidly and produced a complex, two-tier microfouling layer. Characteristic microorganisms were bacteria, fungi, choanoflagellates, diatoms, ciliates, and microalgae. Active substrates (brass and copper-nickel alloys) were selective for bacteria which secrete extracellular mucoid material, fouled at a slower rate, and were characterized by a less diverse microfouling community. Substrate appears to influence microfouling at all stages of development, especially in the early stages of substrate conditioning, and primary film formation. Primary films may insulate periphytic microorganisms from toxic substrates.

Materials submerged in seawater typically develop a layer of attached organisms, a condition generally referred to as biofouling. Although organisms and their remains usually make up the most conspicuous component of fouling deposits, varying amounts of organic secretions, trapped detritus, inorganic precipitates, and substrate-derived corrosion products may also contribute significantly to the accumulation of a fouling layer.

Bell (2) calculated that a fouling film only 55 μm thick would result in an unacceptable reduction in the heat transfer coefficient of heat exchanger tubes in an Ocean Thermal Energy Conversion (OTEC) power plant, which utilizes the temperature difference between surface and subsurface waters as an energy source. Thus the ability to control biofouling in its earliest phases may ultimately determine the operational feasibility of OTEC and other ocean-based energy systems. Recognition of this, coupled with increasing concern over the effects of classical control measures (heavy metals and other toxic substances in antifouling coatings) on the environment, has spurred a renewed interest in understanding the early stages of biofouling.

Substantial evidence suggests that a discrete sequence of events occurs which eventually results in the formation of a complex fouling layer (11, 16), when a solid substrate is exposed to seawater. Baier (1) and Loeb and Neihof (10) presented evidence that dissolved organic compounds in seawater are adsorbed to clean solid surfaces immediately after exposure. This "molecular fouling" imparts a negative surface charge to the "fouled" substrate which may alter the wettability (6) and other physical and chemical characteristics of the surface. Corpe (4) and others observed that motile rod-shaped bacteria are the first organisms to colonize conditioned substrates. The bacteria and their organic secretions are the main constituents of the primary film or slime layer. Mechanisms of bacterial attachment were examined by Mitchell and colleagues (12, 13, 17) and Marshall (11); they determined that the physical and chemical parameters at the substrate/seawater interface influence the attraction and sorption of cells. Other microorganisms including diatoms, fungi, and protozoa appear in increasing abundance, and eventually a complex assemblage of microorganisms and other constituents is formed (9, 15).

This report examines the development of primary films and microbial fouling layers on several substrates exposed to natural seawater. The primary objective of our study was to examine the influence of substrate per se on microfouling film characteristics and to relate the observed differences in film characteristics to substrate toxicity. With that in mind, our experiments were designed to eliminate or greatly reduce the effects of other variables. Quantitative and semiquantitative data are presented for some samples, although the reliability of such data decreases with increasing exposure time and film complexity. The taxonomy of fouling microorganisms is discussed in general rather than specific terms, since the species involved vary with exposure site location and season.

† Contribution from the University of Miami, Rosenstiel School Marine and Atmospheric Science.

MATERIALS AND METHODS

Metal and glass samples were exposed near Miami, Fla. in a fiberglass tank containing ca. 15 m^3 of seawater. Water depth was maintained at 1.2 m over 35 cm of locally derived carbonate sediments. The exposure tank was located at Bear Cut, a tidal pass between Biscayne Bay and the Atlantic Ocean, and received water from the ocean or the Bay, depending upon the phase of the semidiurnal tides. A once-through flow of seawater was continuously maintained with a turnover time in the tank of approximately 18 h. The tank was under a natural light cycle. Macroalgae and invertebrates which colonized the tank indicated that the tank environment resembled that of the adjacent Biscayne Bay.

Coupons of stainless steel 304, 60/40 copper-zinc brass, copper-nickel 90/10 and 70/30 alloys were polished by 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 redistilled nanograde 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 monofilament line.

Caution was taken in placing and removing specimens to prevent them from contacting the air-water interface. Contact was prevented by sealing the specimens in plastic bags and breaking the seal after the bags had crossed the air-water interface. This procedure was necessary to eliminate contamination by bacterioneuston. DiSalvo (7) 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 collected after 4, 10, 16, 40, and 96 h of exposure time and then at weekly intervals.

Cells were enumerated either by direct counting in a scanning electron microscope or by epifluorescence light microscopy. Duplicate samples prepared for fluorescence microscopy were fixed in 4% glutaraldehyde in seawater and stained for 15 min in 0.01% acridine orange (basic orange 14, Matheson, Coleman and Bell, Norwood, Ohio). Direct counting was done with a Leitz Ortholux I microscope with a Plöem illuminator system consisting of an HPO-220 lamp, heat filter, BG-38 exciter filter, one or two KP-490 exciter filters, a TK-150 beam splitter, and a K-510 barrier filter. Thirty fields of view were counted for each sample.

Sample coupons for scanning electron microscopy were fixed in 4% glutaraldehyde in 0.45-μm membrane-filtered (Millipore Corp., Bedford, Mass.) seawater immediately after their retrieval and either critical-point dried or air dried. Air-dried samples were washed in distilled water, dehydrated in acetone, immersed in xylene and air dried. For critical-point drying, samples were washed in distilled water, 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 vacuum evaporator and examined in an AMR-900 scanning electron microscope. All samples illustrated in this report were critical-point dried.

For filamentous fungi and yeasts, substrates were placed on Difco marine agar 2216 modified by the addition of 50 mg of gentamicin per liter, which allowed fungi and yeasts to grow but inhibited most bacteria. The modified agar also contained 10 g of glucose per liter and 0.05 g of phenol red per liter to elucidate acid-producing organisms. This technique did not permit quantitation of fungi, but did provide an excellent means to detect the first appearance of fungi on the substrates and to catalog the various genera which appeared during the course of this study.

RESULTS

Glass and stainless-steel substrates were initially exposed during the spring of 1975; subsequent exposure periods between 1975 and September 1978 included brass and copper-nickel alloys as well as glass and stainless steel. Data presented here are based on the several exposure periods during which similar fouling events were observed for each substrate type.

Glass and stainless steel 304. Glass and stainless steel 304 showed remarkably similar sequences of fouling events during the first 5 weeks of exposure. In general stainless steel and glass fouled at approximately the same rate and supported similar successions of microorganisms.

Rod-shaped bacteria were the first organisms to appear on glass and stainless steel. Samples exposed for as little as 4 h bore isolated bacterial cells. Mechanical agitation in various solutions during specimen preparation for light and scanning electron microscopy did not dislodge the cells, indicating that they were firmly attached to the substrate. Direct counting with fluorescence microscopy indicated a density of ca. 1.1×10^3 bacteria/mm^2 on glass after 4 h of exposure. After day 1, small colonies of dividing cells were observed (Fig. 1). Some of the bacteria may have attached as colonies on particulate debris (14). Fungi appeared after 2 days of exposure. Nine genera were isolated from glass and stainless steel on modified Difco marine agar 2216. *Aspergillus, Penicillium,* and *Nigrospora* were found on both metal and glass. Genera found only on metal were *Cephalosporium, Alternaria,* and *Dactylaria,* whereas *Torula, Pestalotia,* and *Humicola* (syn., *Monotospora*) were found only on glass. Although fungi were present, bacteria were the dominant organisms during week 1. Direct counting of bacteria on glass exposed for 6 days indicated 7.3×10^4 cells/mm^2.

The next group of microorganisms to appear in significant numbers were colonial choanoflagellates, illustrated in Gerchakov et al. (9). These microorganisms reached their peak abun-

dance (ca. 1.3×10^3 cells/mm^2) during week 2 of exposure and then rapidly diminished in abundance. At least two species of the family Acanthoecidae were observed.

An unusual group of cells also appeared during week 2 of exposure and are assumed to be a type of bacteria. The cells are small (0.4 to 0.6 μm in diameter) and hemispherical in shape (Fig. 5 and 6 in Gerchakov et al. [9]). They typically occurred in clusters of up to 100 cells. Attempts to determine whether the cells were autofluorescent were unsuccessful because of their small size. 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. They were firmly attached to the stainless steel or glass surface and left behind a characteristic circular deposit after death of the cell.

Organisms seen during weeks 1 and 2 of exposure were characteristically bacteria, fungi, and choanoflagellates, with other microorganisms occurring in relatively insignificant numbers. At about 15 days, fungi decreased in relative abundance; bacteria continued to increase, and pennate diatoms, filamentous algae, and other organisms became more conspicuous. Accompanying that change, a thin fouling layer became visible to the naked eye and the surface lost its luster. The scanning electron microscope revealed that the assemblage of fouling microorganisms changed significantly between 2 and 3 weeks of exposure. Pennate diatoms and bacteria were the dominant organisms during week 3 (Fig. 2).

Diatoms which colonized the substrates during the early phases of fouling were typically nonmotile and firmly attached to the substrate. Although more than 20 taxa of *Diatomaceae* were observed in primary films, one pennate species was always clearly dominant. The dominant species, however, was not the same during the several exposure periods. Especially common on glass and stainless-steel substrates were *Nitzschia* spp. and representatives of *Naviculaceae*. The siliceous frustules (shells) of living diatoms are covered with a thin layer of cytoplasm and therefore appeared relatively smooth when viewed by scanning electron microscopy; dead diatoms were recognized by their empty frustules. A monolayer of small diatoms (~10 μm in length) typically covered the substrate. As many as 1.8×10^4 cells/mm^2 were present during maximum abundance between 2 and 5 weeks exposure.

Another important group of microorganisms to appear during week 3 of exposure were the peritrichous ciliates (*Zoothamnion* spp.), which are colonial, possess a contractile stalk, and utilize bacteria as a food source. *Zoothamnion* spp. were abundant during weeks 3 and 4, reached a maximum density of ca. 1.1 colonies/mm^2, and were rare on later samples. Peritrichous ciliates are illustrated in Fig. 16 in Gerchakov et al. (9).

At about 5 weeks of exposure, the fouling layer had developed into a two-tiered structure. 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 nonmotile diatoms. Above that layer was the second tier consisting of: large, colonial, motile diatoms which occurred in sheets of 50 or more cells (Fig. 3); other diatoms; ciliates and flagellates; bacteria and fungi growing on fecal pellets and other organic detritus; mucoid material; and a variety of other organisms in lesser abundance (Fig. 4). Mechanical removal of the outer fouling layer revealed that the substrate was covered with the remains of first-tier organisms, especially siliceous diatom frustules cemented to the specimen suface (Fig. 5 and 6). In general, diatoms, bacteria, and filamentous algae were the most conspicuous microorganisms of the second-tier fouling layer.

Attempts to quantify periphytes comprising fouling layers which developed on glass and stainless steel 304 exposed for 5 weeks or longer were unsuccessful because of the complex assemblage of microorganisms and the three-dimensional character of the fouling layer(s).

Samples exposed for more than 5 weeks developed a thick fouling layer consisting of the microorganisms previously described, as well as numerous invertebrates, including worms, gastropods, sponges, and the unidentified larvae and juveniles of these and other organisms. These macroscopic fouling organisms are beyond the scope of this report.

Prolonged exposure of stainless steel produced various amounts of corrosion products which then affected the composition of the fouling layer. During the early phases of microfouling, however, both glass and stainless steel behaved similarly.

Brass. No microorganisms were observed on brass after 4 h of exposure, although as described earlier isolated rod-shaped bacteria were observed on the glass control. Isolated bacteria were observed on brass after 1 day of exposure. Small colonies of 10 to 30 rod-shaped bacteria were present at 2 days of exposure; by 6 days of exposure, microcolonies consisting of several hundred cells each were common. Between 1 and 2 weeks of exposure, areas of the brass surface colonized by bacteria became covered with a slime-like material, whereas noncolonized areas appeared free of slime. At three weeks of exposure, 25 to 30% of the brass surface was

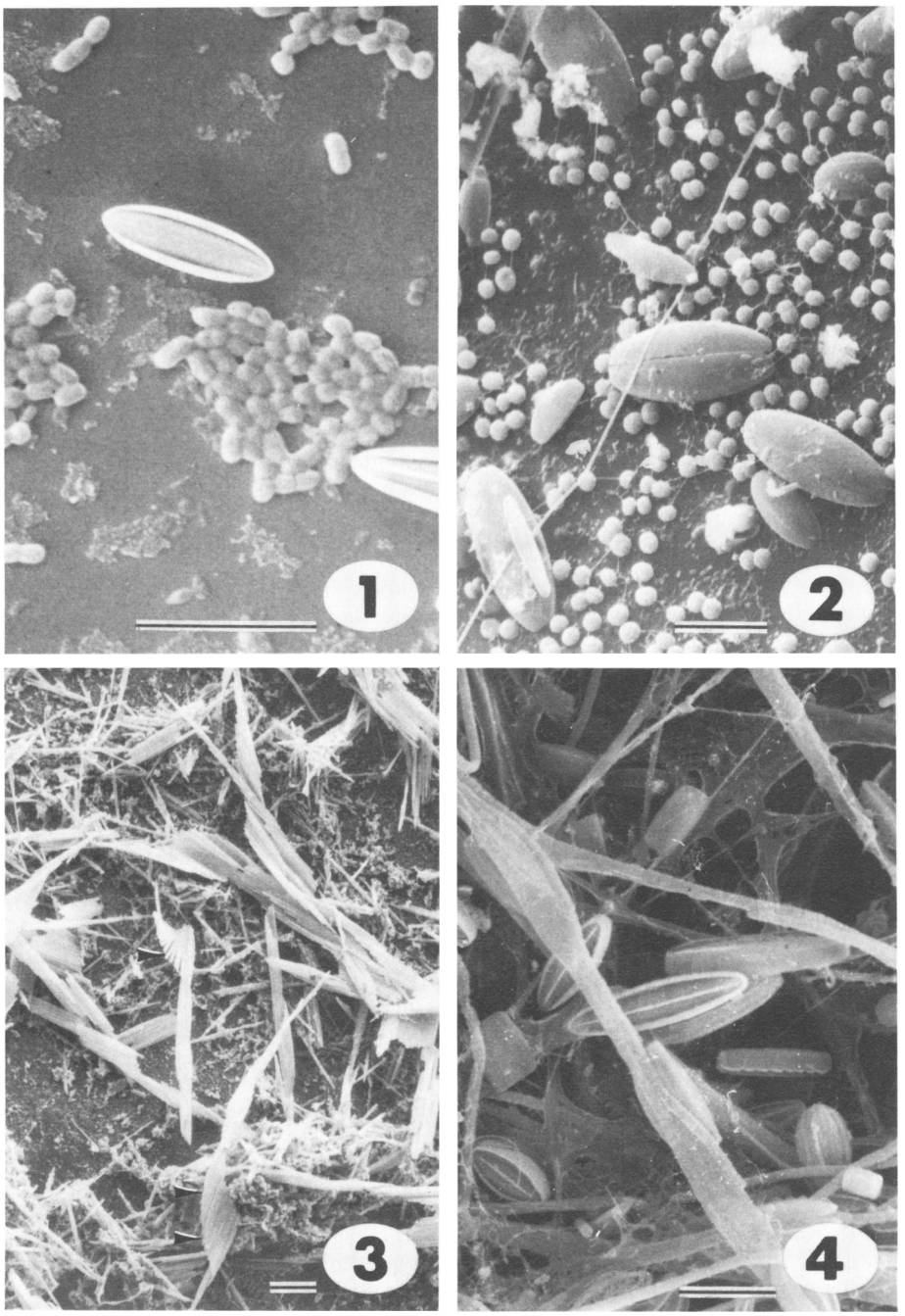

FIG. 1 to 4. *Bar, 10 μm.*

FIG. 1. *Glass. Rod-shaped bacteria were the initial microorganisms to colonize glass and metal substrates. Note colony formation and dividing cells. Seven days of exposure.*

FIG. 2. *Diatoms and bacteria are dominant first-tier microorganisms in intimate contact with glass and stainless-steel 304 substrates. Glass at 45 days of exposure.*

FIG. 3. *Glass and stainless steel begin to develop a two-tier fouling layer after about 5 weeks of exposure. Sheet-like colonies of large motile diatoms are characteristic of the second-tier layer. Glass, 56 days of exposure.*

FIG. 4. *Detail of surface of second-tier layer seen in upper portion of Fig. 5. Note organic slime between diatoms and filamentous bacteria. Glass, 84 days of exposure.*

covered by slime; ca. 3.5×10^5 rod-shaped bacteria/mm^2 (total surface) were associated with the slime deposits. At 4 weeks of exposure the surface area covered by slime had increased to ca. 45% without a corresponding increase in the bacterial population.

Spherical cells 2 to 4 μm in diameter with a single filamentous extension (flagellum, stalk, or hypha) 10 to 35 μm long were observed after 1 day of exposure. Maximum abundance of ca. 6.0×10^2 cells/mm^2 occurred between 1 and 3 weeks of exposure and did not change significantly with continued exposure. The taxonomic identity of these cells is presently unknown.

Choanoflagellates were observed during most but not all exposure periods. When present, choanoflagellates appeared between 3 and 6 days of exposure, rapidly increased in abundance (up to 3.2×10^2 cells/mm^2) during week 2, and became rare or absent after 2 weeks of exposure. Two species of *Acanthoecidae* were observed which were identical to those on glass and stainless steel.

Pennate diatoms and filamentous microalgae were occasionally observed on brass exposed for more than 2 weeks; these microorganisms remained numerically insignificant throughout the exposure period.

Early stages of formation of an inorganic "oxide" layer (Fig. 7) characteristically developed on brass substrates during week 1 of exposure to seawater. The oxide layer was composed of acicular crystallites ca. 0.5 to 1 μm in length and when fully developed was of relatively uniform thickness. Microorganisms were in contact with the oxide layer rather than with the original polished surface (Fig. 8). Colonies of rod-shaped bacteria on brass produced more extracellular secretions (Fig. 8) than on either glass or stainless steel. Continued exposure of brass for up to 8 weeks produced a thicker slime film which eventually obscured the oxide layer (Fig. 9). At that time the brass surface appeared dull in reflected light, but relatively clean to the unaided eye. Glass and stainless steel, in contrast, had developed visible fouling films.

Copper-nickel alloys. Microbial successions observed on copper-nickel 90/10 and 70/30 alloys were similar. Isolated rod-shaped bacteria were present on the substrates after 4 h of exposure, and microcolonies were observed at 1 week. Direct counts of acridine orange stained cells at 4 h of exposure indicated that there were 6.0×10^2 cells/mm^2 on 90/10 alloy and 6.5×10^2 cells/mm^2 on 70/30 alloy. At 6 days of exposure direct counts were 4.8×10^3 cells/mm^2 and 2.2×10^3 cells/mm^2, respectively. During week 2 the abundance of bacteria increased rapidly and slime-like deposits appeared on the metal surfaces in association with the bacteria. Attempts to count cells on substrates exposed for more than 1 week were unsuccessful, impeded by the tendency of the bacteria to occur in clumps consisting of cells, slime, corrosion products, and surface debris.

Of the various substrates investigated, copper-nickel alloys exhibited the most variability in fouling layer characteristics. Only the bacteria were consistently present as dominant members of the fouling community. The colonial and stalked peritrichous ciliate *Zoothamnion* spp. was observed on ca. 60% of the samples. When present, it appeared during week 3 of exposure, reached a maximum density of 18 colonies/mm^2, and then rapidly diminished in abundance after a few days. Fungi and pennate diatoms were observed after 3 weeks of exposure, were more consistent in their appearance as members of the fouling community, but were highly variable in abundance. Fungi of the genera *Cephalosporium, Alternaria,* and *Dactylaria* were cultured from copper-nickel substrates exposed for more than 16 days, but were absent from these substrates sampled earlier. Routine analysis of the exposure tank seawater always revealed the presence of one or more of the yeast-like fungi *Candida, Rhodotorula,* and *Cryptococcus;* these fungi, however, were never isolated from the copper-based substrates.

The surfaces of copper-nickel 90/10 and 70/30 alloys were similar during the early stages of primary film formation (Fig. 10). A later stage primary film of copper-nickle 70/30 alloy is illustrated in Fig. 11. At this stage, several types of bacteria were present, occasional diatoms and fungi were observed, and a slime layer (visible by desiccation cracks) covered the metal surface.

Copper-nickel 90/10 alloy exposed for more than 6 weeks developed a second-tier layer which in some respects differed significantly from that which developed on glass and stainless steel. Characteristics unique to the copper-nickel 90/10 second-tier layer were: (i) organic slime was significantly more abundant than on either glass or stainless steel. (ii) the second-tier layer on copper-nickel 90/10 alloy was very loosely attached to the primary film.

The second-tier film was easily detached and examined independent of the metal substrate and attached primary film. The fractured film viewed in cross-section (Fig. 12) reveals primarily slime, diatoms, bacteria, and filamentous algae. The abundance of organic slime was also apparent by the relatively smooth appearance of

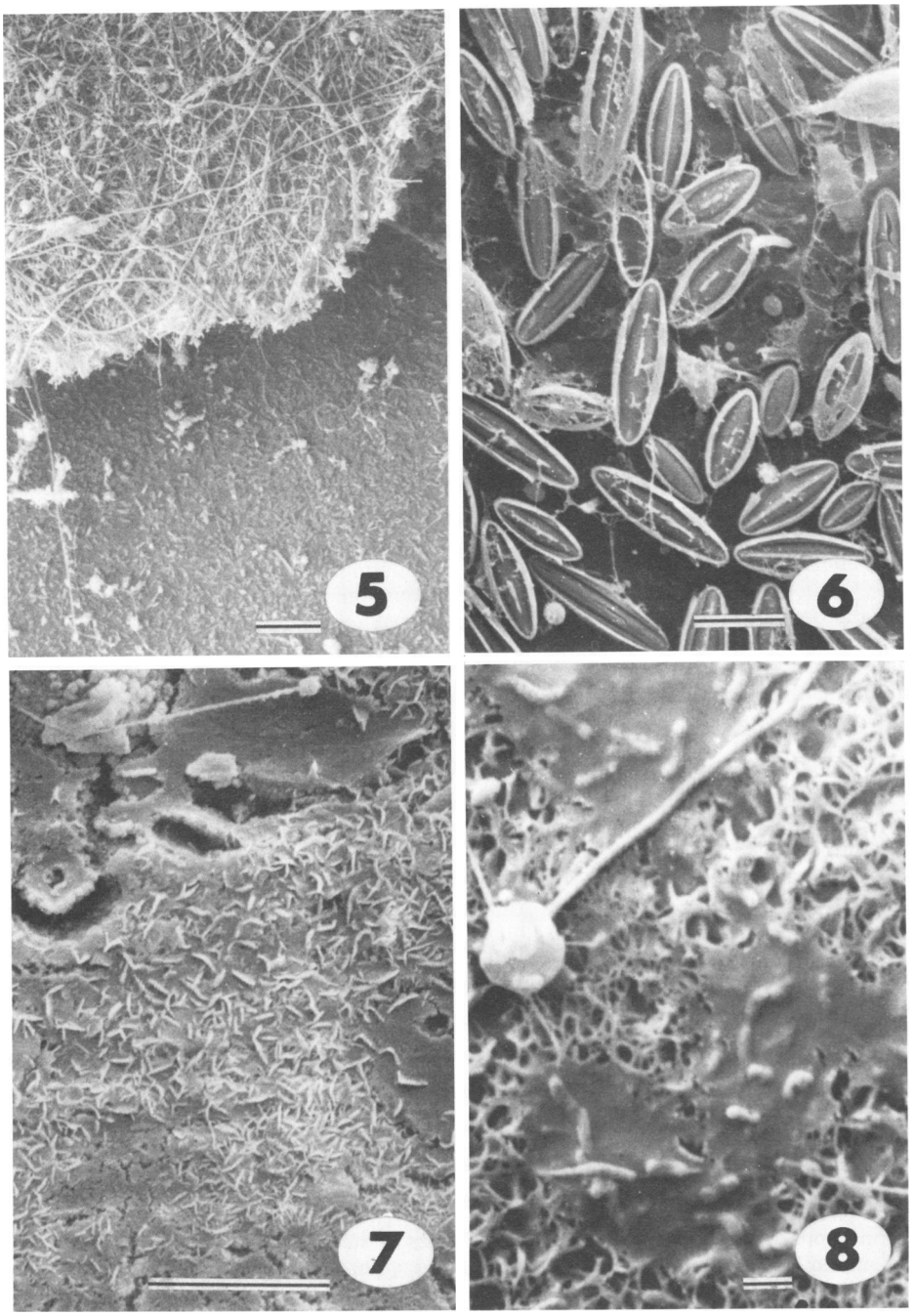

FIG. 5 to 8. *Bar, 10 μm.*

FIG. 5. *Removal of second-tier fouling layer reveals remains of first-tier layer visible in lower portion of micrograph. Small organisms in first-tier layer are diatoms; bacteria are also present but not visible at this magnification. Glass, 84 days of exposure.*

FIG. 6. *Detail of lower portion of Fig. 5. Bacteria, organic sline, and diatom frustules are visible after removal of second-tier layer.*

FIG. 7. *Brass. Crystallites beginning to form oxide layer after exposure for 1 day.*

FIG. 8. *Brass. Oxide layer covers entire surface. Colonies of rod-shaped bacteria and extracellular secretions cover portions of the oxide layer. Bacteria appear indistinct in the micrograph because cells are embedded within slime. Sixteen days of exposure.*

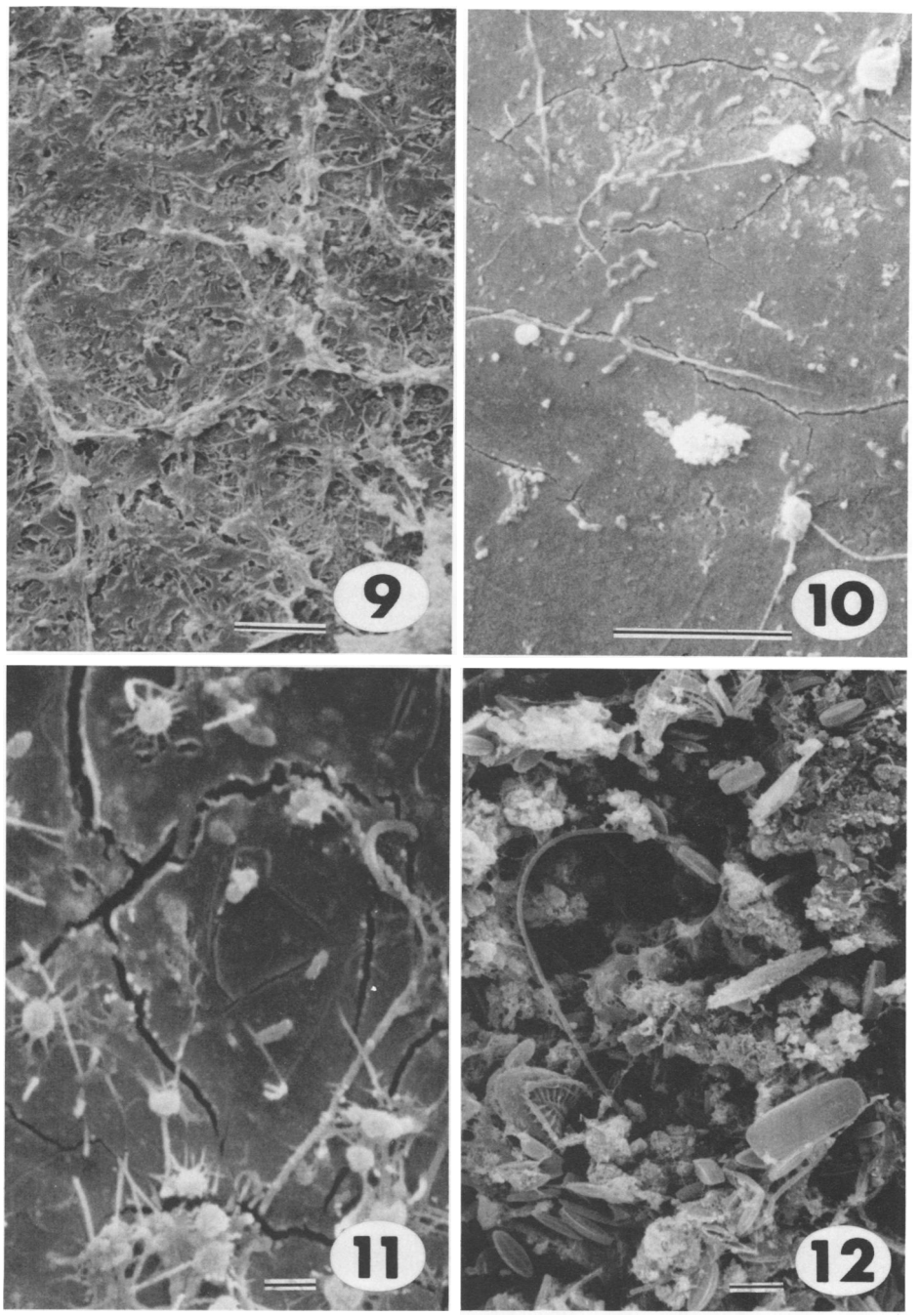

FIG. 9 to 12. *Bar, 10 μm.*

FIG. 9. *Brass exposed for 5 weeks was covered with a slime layer which completely obscured the underlying oxide layer.*

FIG. 10. *Copper-nickel 90/10 alloy is initially colonized by rod-shaped bacteria. Fouling of 70/30 alloy was similar. Seven days of exposure.*

FIG. 11. *Copper-nickel 70/30 alloy after 2 weeks of exposure reveals slime with dessication cracks, rod-shaped cells, spiral forms, and cocci.*

FIG. 12. *Copper-nickel 90/10 alloy. Cross-section of loosely attached second-tier layer reveals bacteria and diatoms embedded in organic slime. Surface and underside of this layer appeared smooth because of high slime content. Fifty-six days of exposure.*

the surface and underside (formerly in contact with the primary film surface) of the second-tier layer.

DISCUSSION

The formation of primary films and biofouling layers is undoubtedly influenced by several factors including seawater chemistry, turbulence, temperature, light, nutrient availability, biota in the water column available for colonization, substrate, and exposure time. Thus the data presented here document microfouling in subtropical, inshore, marine waters and do not necessarily apply to other marine habitats (e.g., colder, deeper, or open-ocean environments).

Our experiments were designed so that all factors except substrate composition were mutually constant during the exposure period, i.e., that all substrates were affected by other factors in the same way. The observed variations in the succession of periphytic microorganisms and fouling film characteristics, therefore primarily reflect the influence of substrate. Many of the variables mentioned earlier are interrelated. The substrate, for example, has intrinsic chemical and physical properties which affect the seawater chemistry at the substrate-seawater interface; the surface charge will in part determine the species of organic molecules attracted to the surface (the chemistry of the conditioning layer) and therefore the quantity and quality of dissolved nutrients adsorbed to the conditioned specimen surface. Dexter et al. (6) showed that substrate wettability, as indicated by the critical surface tension for wetting, influenced the rate of attachment of marine bacteria to a variety of substrates. Chet and Mitchell (3) found that some organic compounds are capable of repelling motile bacteria, whereas Corpe et al. (5) were able to enhance the rate of settlement of bacteria to glass slides coated with bacterial mucopolysaccharides. A positive chemotactic response of motile marine bacteria to surfaces covered with a primary organic film was observed by Young and Mitchell (17). These and other studies strongly suggest that the substrate may influence the microbiota initially attracted to the conditioned surface and may continue to influence the formation of the primary film and subsequent fouling layer(s).

The data acquired in this study suggest that there is a general correlation between the degree of biological and physical "inertness" of the substrate and fouling. Glass and stainless-steel substrates are similar in that both are biologically relatively inert in seawater; they are not toxic to most microorganisms. Glass and stainless steel also have a high degree of surface physical stability (stainless steel corrodes relatively slowly during the early stages of exposure). The succession of periphytic microorganisms and rate of fouling were essentially identical for both substrates in our exposure series. The formation of iron oxide and other corrosion products occasionally observed on portions of the stainless-steel surface either prevented the formation of a "typical" biofouling film or destroyed the film at those sites. The loss of surface physical stability or the release of corrosion products which may be toxic at increased concentrations or both meant that the substrate was no longer physically inert, and perhaps no longer biologically inert, a condition reflected in the biofouling film.

All of the copper-based alloys examined (brass, copper-nickel 70/30 and 90/10 alloys) are biologically active. Copper is toxic to a variety of microorganisms and is extensively used in antifouling coatings. Surface instability due to dezincification of brass and to the formation of corrosion products on all the copper-based alloys was observed. The copper-based alloys appeared to be selective for certain microorganisms, especially bacteria associated with copious secretions of extracellular mucoid material. In contrast to glass and stainless steel, slime eventually covered the entire surface of the copper-based samples. It is tempting to speculate that these particular organisms were able to colonize the "toxic" surface because their organic secretions served as an insulator or buffer between the organisms and the metal surface. The scanning electron micrographs of bacteria embedded in slimes (Fig. 8 and 12) do seem to support this speculation. It is not known whether these substrates select for bacteria with unusually high rates of slime secretion or induce secretion in a normal flora.

A similar interpretation can be applied to the formation of a two-tier layer on the 90/10 copper-nickel alloy. The second-tier layer formed more than 2 weeks after a similar layer was observed on the glass control, after the surface of the copper-nickel was covered with a relatively thick primary film or slime layer. The microfouling community found in the second-tier layer (Fig. 12) appeared normal (i.e., similar to glass) except for the relatively larger amount of organic slime. It was also observed that on coupons of 90/10 alloy which corroded and developed inorganic crusts, bacteria and the associated slimes were restricted to those portions of the metal surface which were free of thick corrosion deposits. See especially Fig. 6 and 7 in Gerchakov et al. (8).

Compared to glass and stainless steel, the copper-based alloys fouled at a slower rate and appeared relatively clean to the unaided eye. Second-tier layers were firmly attached to glass

and stainless steel, and a visible surface residue was present after washing; in contrast, second-tier layers which developed on copper-nickel 90/10 alloy were easily removed by washing and most likely would not have formed in the presence of turbulence or higher flow rates. Visible alteration of copper-nickel, when observed, was limited to a greenish discoloration of the surface. Visible corrosion on steel often produced rust-like deposits up to 1 cm thick.

Our observations indicate that substrate exerts an important influence on microfouling at all stages of the development of fouling films and layers. This influence is greatest during events leading to the formation of a primary film, and decreases as film thickness increases. Primary films appear to insulate periphytic microorganisms from toxic substrates and may be prerequisite to the formation of second-tier layers and subsequent fouling on toxic substrates. Among substrates exposed under identical conditions, the species diversity of periphytic microfouling communities is highest on substrates which are physically and biologically inert; diversity is lowest on substrates which are physically or biologically active (e.g., corrosive or toxic), or both.

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