

SCANNING ELECTRON MICROSCOPE
OBSERVATIONS OF MARINE MICRO-
ORGANISMS ON SURFACES COATED
WITH ANTIFOULING PAINTS

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NAVAL POSTGRADUATE SCHOOL

Monterey, California



THESIS

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OF MARINE MICROORGANISMS ON SURFACES
COATED WITH ANTIFOULING PAINTS

by

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June 1981

Thesis Advisor:

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Scanning Electron Microscope Observations
of Marine Microorganisms on Surfaces
Coated with Antifouling Paints

by

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Lieutenant Commander, United States Navy
B.S., United States Naval Academy, 1971

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ABSTRACT

Scanning electron microscopy was used to observe microbiological primary fouling of glass slides and slides coated with U. S. Navy antifouling paints exposed in Monterey harbor. Four paints were tested, three of which contained copper or tin as their toxic ingredient and one which used a chlorinated pesticide, an organic compound, as the anti-fouling ingredient. Samples removed at regular intervals, of days up to several weeks, showed that bacterial slimes populated the glass and heavy-metal based paints early and in great numbers throughout the study, but the surfaces painted with the organic compound toxicant were free of all microfouling organisms. A succession of periphytic microorganisms was observed on glass and the heavy-metal based painted surfaces which began with bacteria followed by diatoms and later by protozoans.

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I. INTRODUCTION

A. GENERAL DISCUSSION OF MICROFOULING

Marine microfouling is a process which involves the interaction of living and non-living materials with solid surfaces submerged in seawater resulting in the establishment of a complex film (Corpe, 1977). This film, composed mainly of bacteria and diatoms plus secreted extracellular materials and accumulated debris, has been referred to as the "primary film", "bacterial fouling film", or "slime layer" (Horbund and Freiburger, 1970). The latter name has been applied because the film ultimately becomes thick enough to feel slippery or slimy (Haderlie, 1977). The composition of this film may also include yeasts, fungi, and protozoans. The settlement sequence of these constituents and the specific make-up of the film depends on several factors including location, season and year, depth, and proximity to previously fouled surfaces (O'Neill and Wilcox, 1971) and other physico-chemical parameters.

Early investigators noted that the presence of a slime film seemed to facilitate the attachment of larger fouling organisms such as bryozoans and barnacles (Woods Hole Oceanographic Institution, 1952; Horbund and Freiburger, 1970; Zobell, 1939). If the nature of the film was understood and if its characteristics controlled, fouling by macroscopic organisms might be substantially reduced. Aside from the

obvious benefits that control of macrofouling would have to shipping and other areas of marine engineering, control of the growth of primary slime film itself has become an area of concern with regard to Ocean Thermal Energy Conversion (OTEC) Systems and long term oceanographic instrumentation (Dexter, 1977).

Corpe (1977) stated that fouling by microbial films have two broad functions, (a) to provide a surface favoring the settlement and adhesion of animal larvae and algal cells, and (b) to provide a rich source of both particulate and soluble food material that could sustain and/or enhance the development of fouling populations. Further, Zobell (1939) wrote that bacteria might promote the fouling of submerged surfaces by:

1. Affording the larval forms of larger fouling organisms a foothold or otherwise mechanically facilitating their attachment.
2. Serving as food.
3. Discoloring bright or glazed surfaces.
4. Increasing the alkalinity of the film-surface interface thereby favoring the deposition of calcareous cements.
5. Influencing the e.m.f. potential of the surface.
6. Increasing the concentration of plant nutrients at the expense of the organic matter which the bacteria decompose.

The study of microfouling organisms, therefore, is an important contribution to the complete understanding of marine fouling.

1. Brief History of Microfouling Research

The study of marine microfouling has its roots in the 1930's and 1940's with research primarily conducted by Zobell and his colleagues. Zobell's research focused primarily on the early stages of settlement and growth of microorganisms, particularly bacteria on solid substrates immersed in the sea. From that time until the early 1960's only sporadic studies were conducted because there were very few investigators interested in the bacteriology of the ocean. Since the mid-1960's and especially since 1970 research has increased tremendously in marine bacteriology and the broad problems of bioadhesion in general and specifically in the initial stages of microfouling on solid substances (Haderlie, 1977).

Notable contributions to our fundamental understanding of marine periphytic bacteria and primary bacterial films have been made by Corpe and his colleagues at Columbia University, and Mitchell and his co-workers at Harvard. In addition, Baier of Cornell and Neihof and Loeb at the Naval Research Laboratory have helped explain the nature of the "molecular fouling" layer that precedes the attachment of bacteria to solid surfaces in the sea. The work of these investigators and many others will be drawn upon in discussing the results of the experiments conducted in the present study.

B. MOLECULAR FOULING

Molecular fouling or "surface conditioning" is the sorption of organic matter dissolved or suspended in sea water to solid

surfaces. This was first demonstrated by Zobell (1943) and since has been confirmed by many other investigators including Loeb and Neihof (1975, 1977). The sorption of dissolved material creates changes in the surface of the substrate (conditioning) which are favorable for subsequent biological settlement.

Organic materials dissolved in sea water originate as the end-products of bacterial decay, excretory products, dissolution from seaweeds, etc., and consist principally of sugars, amino acids, urea, and fatty acids (Taylor, 1977). Much of this non-living organic matter in the sea occurs in the form of small aggregates which are formed by the sorption of dissolved organic matter upon bubbles and other naturally-occurring surfaces (Riley, 1963).

Loef and Neihof (1975, 1977) have determined that molecular films may form within minutes after the substrate enters the water and continue to grow in thickness, leveling off after about 20 hours. They confirmed that molecular films were organic, electronegative, and composed of humic materials.

Baier (1973) determined that the initial events in biological adhesion are influenced by the texture, chemistry, and charge of the substrate surface. He reported that the adhesion of organisms to surfaces submerged in sea water depended in large measure on the "critical surface tension" or wettability of the substrate. When any clean solid object, whether it is glass, metal, wood, stone, or plastic, is immersed in natural

seawater, a layer of non-living organic matter immediately sorbes to the surface (Loeb and Neihof, 1975, 1977). This initial non-living film is a monolayer of glycoprotein which in a few hours may develop to a thickness of 200 Å (Baier, 1973). As molecules are absorbed they are changed from a 3-dimensional to a 2-dimensional form thus modifying their reactivity. The "critical surface tension" of the coated surface may be modified so that strong bonding is possible with the mucopolysaccharides exuded by the film forming bacteria (Baier, 1973).

C. SORPTION OF MICROFOULING ORGANISMS

1. Bacterial Film Formation

Bacteria have been found securely attached to substrates immersed in seawater after just a few hours (Corpe, 1972; Dempsey, 1981; Dexter, 1977; Gerchakov et al., 1976; O'Neill and Wilcox, 1971; Zobell and Allen, 1935). Two stages have been identified in the development of bacterial fouling communities. Initial colonization by rod-shaped bacteria, followed by stalked forms within 24 hours (Marshall et al., 1971b) to 48 or 72 hours (Corpe, 1973; Dempsey, 1981).

The initial colonizers have been identified as psuedomonads, principally species of Psuedomonas, Flavobacterium, and Achromobacter (Corpe, 1973; Corpe and Winters, 1972; O'Neill and Wilcox, 1971). Gram-negative species dominated the populations with only 10 to 15 percent being gram-positive (Dempsey, 1981). The secondary colonizers, in general, were

stalked, budding, or filamentous types identified as species of Caulobacter, Hypomicrobium and Saprospira (Dempsey, 1981; Marshall et al., 1971b). Zobell and Allen (1935) reported that between 40 and 50 species of marine bacteria could be isolated from the surface of glass slides immersed in seawater for a few days.

Marshall et al., (1971a) has defined two stages of sorption of bacteria to solid surfaces. Reversible sorption is an essentially instantaneous attraction of bacteria to a surface. Such bacteria are held weakly near the surface; they still exhibit Brownian motion and are readily removed by washing the surface. Irreversible sorption involves the firm adhesion of bacteria to the surface; they no longer exhibit Brownian motion and are not removed by washing.

The first stage, reversible sorption, involves physical forces which attract the cell to the surface. These forces include Van Der Waals forces of mass attraction and electrostatic forces due to the interaction between ionic groups on, or surrounding, the approaching cell and substrate surfaces (Dempsey, 1981). There is an apparent equilibrium condition between the electrostatic repulsion forces and the attractive influences of the Van Der Waals forces.

In irreversible sorption the bacteria, especially rods, produce extracellular bridging material called acid mucopolysaccharides (Corpe, 1970b). These high molecular weight polymers are believed to be important in the firm adhesion of the

organism to solid surfaces (Corpe, 1975). Adhesive extracellular polysaccharides have been observed with the aid of the transmission electron microscope (TEM) (Marshall et al., 1971a) and the scanning electron microscope (SEM) (DiSalvo and Daniels, 1975; Gerchakov et al., 1977). These polymers are quite resistant to dislodgement and appear to be sticky in nature as evidenced by the accumulation of algae and bits of debris on their surfaces (Corpe, 1970b). The tackiness of this secretion may encourage the settlement of other fouling species by providing a base to which organisms may readily attach and obtain nourishment.

Although investigations of the role of surface and extracellular polymers in bacterial aggregation has generally implicated polysaccharides, other polymeric material, notably nucleic acids and proteins typically excreted or introduced to the medium by cellular lysis, frequently have been shown to play a significant role in bacterial aggregation (Harris and Mitchell, 1973).

After attachment the bacteria reproduce by binary fission; each half grows to an average size of 1 to 2 μm and divides again. Bacterial counts on developing colonies indicate that at 20 degrees centigrade a population can double every 4 hours (Woods Hole Oceanographic Institution, 1952).

2. Sorption of Diatoms and Other Microfoulers

Following the establishment of the initial film of bacteria and their secreted extracellular polymers on a solid

substrate, additional bacteria and other microorganisms may attach. Debris, organic material, and other particular matter may also adhere to the surface creating an environment of intense biochemical activity. The other microfoulers which may now settle include benthic diatoms, filamentous microorganisms, and protozoans. These organisms, especially the diatoms, may contribute to the so-called primary film and eventually become a distinctive part of the microfouling community (Corpe, 1972; Woods Hole Oceanographic Institution, 1952). These diatoms, which can also live in suspension in the sea, are unicellular plants encased in siliceous shells. Although the presence of a bacterial film may facilitate the attachment of diatoms it is not essential. Like bacteria, reproduction and colonization by diatoms is a rapid process (Woods Hole Oceanographic Institution, 1952). The colonial diatom Lichmophora and the colonial ciliate protozoan Zoothamnium are often conspicuous as microfoulers (Haderlie, 1977).

D. PRIMARY FILM AND SUBSEQUENT MACROFOULING

Many studies have reported on the existence of some kind of ecological succession of fouling communities beginning with film forming bacteria, diatoms, and protozoans, and ending with barnacles, tunicates, mussels, and seaweeds (Haderlie, 1974; Horbund and Freiberger, 1970; O'Neill, 1975; Woods Hole Oceanographic Institution, 1952). There seems to be a general agreement among investigators in this area that one group of

organisms in some way changes or conditions the surface so that a second community can develop, and so on to the climax fouling community (Haderlie, 1977). However, it has not yet been proven that microfouling is a necessary prerequisite of heavy, destructive fouling, since barnacles and other such organisms have not been cultivated in the complete absence of microorganisms (Corpe, 1977).

II. OBJECTIVES

The primary objective of this study was to determine how well four U. S. Navy antifouling paints function in preventing or limiting the attachment of microfouling organisms in Monterey Bay. Three of the four paints tested were experimental and as such an evaluation of their ability to prohibit the formation of "slime film" has not yet been made. The project was proposed by the David W. Taylor Naval Ship Research and Development Center, Annapolis, Maryland to fulfill a Navy requirement to evaluate these paints.¹

One of the four paints, Formula 121, also known as Copper Oxide, was not an experimental paint but has been in general use by the Navy for many years. This paint's effectiveness against micro and macrofoulers has been determined by other researchers and was tested here again for comparison with the other experimental paints.

Microscope slides were coated with the paints and exposed to the harbor waters in Monterey for varying time intervals. A scanning electron microscope (SEM) was used to view the samples. The slides were examined to identify the attached organisms to genus and species where possible and to get a rough quantitative estimation of density of settlement of

¹V. J. Costelli, phone interview, June 1980. Mr. Costelli is the senior task scientist in charge of controlled release polymer formulations for antifouling paints.

some of them. From the types and numbers of organisms found on the test surfaces, a determination was made as to how effective each paint was in preventing microfouling.

Secondary objectives included verifying the sequence of microfouling organisms on uncoated glass slides in Monterey Harbor, developing field test procedures for exposing anti-fouling paints, and evaluating techniques for the employment of the SEM in observing microorganisms on such surfaces.

III. MATERIALS AND METHODS

A. GENERAL

Two experiments were carried out. Experiment 1 tested two antifouling paints and ran from 18 November 1980 to 17 February 1981. Experiment 2 tested four antifouling paints and was conducted from 18 February to 20 March 1981. The experiments consisted of suspending glass microscope slides coated with antifouling paints in the waters of Monterey Bay for periods ranging from 24 hours to several weeks. After recovery from the water the slides were chemically fixed and then prepared for viewing under the Scanning Electron Microscope (SEM). Observations and photographs of the slide surfaces made with the SEM were used in determining general numbers and kinds of organisms present for each immersion period.

Prior to Experiment 1, tests were conducted to determine the best laboratory and field test methods to be followed. As expected, some difficulties developed resulting in the elimination of two antifouling paints in the first experiment. These problems, however, were resolved and all four paints were tested in the second experiment.

1. Antifouling Paints

The four antifouling paints evaluated in this study included:

1. Navy Standard Formula 121 Red Vinyl Antifouling Paint. This paint incorporates cuprous oxide as the antifouling

ingredient and is intended for use on shipbottom exterior surfaces (Appendix A).

2. Navy Standard Formula 170 Black Camouflage Vinyl Antifouling Paint. The antifouling ingredients of this paint include tributyltin oxide and tributyltin fluoride (Appendix B).

3. DTNSRDC¹ Experimental Antifouling Paint Formula 2844-1114. This is a two component, lead peroxide cured polysulfide antifouling paint. It is intended for use primarily on rubber, incorporating Nopocide N-96 (2,4,5,6,-tetrachloroisophthalonitrile) as the antifouling ingredient (Appendix C).

4. Organotin Epoxy Gel Formula 196D DTNSRDC Experimental Antifouling Paint. This is a two component paint intended for use on glass reinforced plastic (GRP), incorporating Tributyltin as the antifouling ingredient (Appendix D).

B. EXPERIMENT 1

Experiment 1 tested two experimental antifouling paints, Formula 1114 and Formula 196D.

1. Experimental Substrates

Standard glass microscope slides one inch by three inches were used as the surfaces upon which the paints were applied and exposed to seawater. Glass slides were used because they were easy to manipulate throughout all phases of testing and because results, especially from the uncoated

¹David Taylor Naval Ship Research and Development Center.

glass slides, could be compared with similar work performed by other investigators including Corpe (e.g., 1970a, 1970b, 1972, 1975, 1977), Dempsey (1981), Dexter (1976), DiSalvo and Daniels (1975), Gerchakov et al., (1976), Tosteson and Corpe (1975), Winters and Corpe (1972), and Zobel (1939). Corpe (1970a) noted that glass slides have been used by many investigators as the traditional method for study of aquatic bacteria because they can be examined by microscope and cultures can be isolated from the surfaces.

Common nylon paint brushes 2.5 centimeters wide were used to apply the paints to the slide surfaces. Care was taken to ensure that no oil or grease contaminated the slides and interfered with paint adhesion. Two coats of paint were applied directly over the smooth glass surfaces. The slides were air-dried at a temperature of about 18.0 degrees centigrade for a minimum of five days before exposure in the water.

Microscope slide boxes of 100 slide capacity were used to hold the slides during field exposure. To ensure a free flow of water around the slides two windows 18.0 centimeters by 4.0 centimeters were cut out of the front and back of the boxes (Figure 1). Two slide boxes were assigned to hold the coated slides for each paint. A maximum of six slides were placed in each box. Limiting the capacity of each slide box allowed sufficient horizontal separation of approximately 8.0 centimeters between samples to avoid concentration of anti-fouling toxicant between adjacent slides.

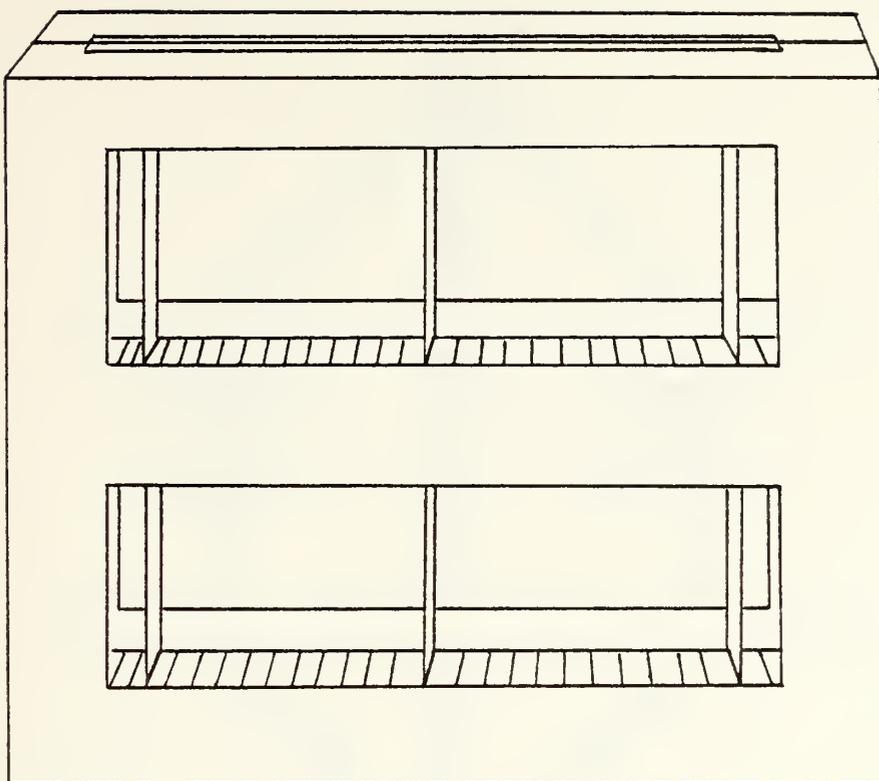


Figure 1. Microscope slide box showing positions of painted slides

Two boxes assigned to each paint were attached to a polyvinylchloride (PVC) pipe by nylon cord so that the boxes were fixed about 31.0 centimeters apart. The entire assembly was suspended by polyethylene rope which had a 14.0 kg weight on the lower end (Figure 2).

All of the uncoated control glass slides were held in one slide box, each slide separated from one another by about 2.5 centimeters. The control slide box was attached directly to the polyethylene rope and anchored with a 14.0 kg weight. The arrangement of the slide box(s) with the rope, weight,

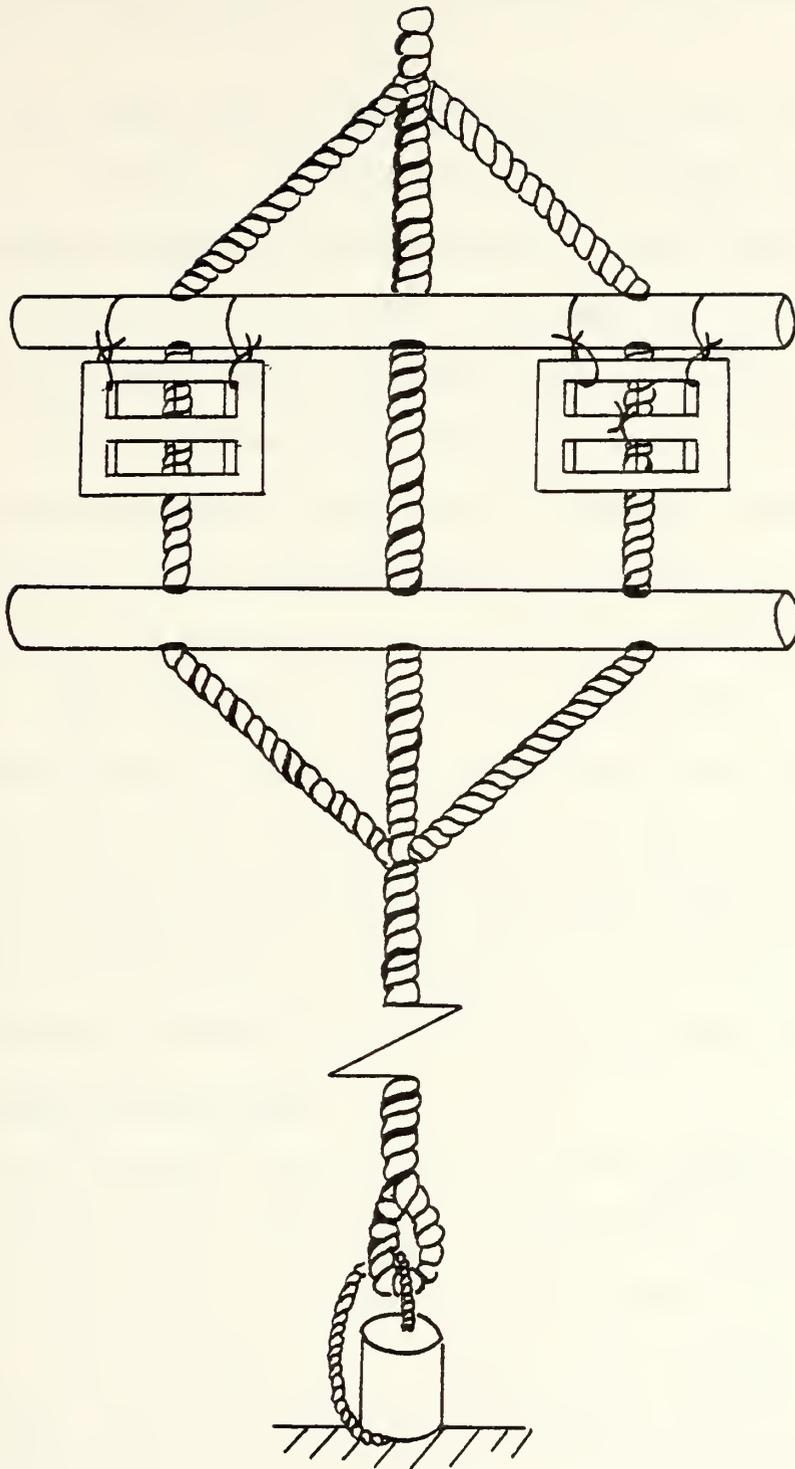


Figure 2. Exposure array showing the arrangement of slide boxes

with or without PVC pipe, will hereafter be referred to as an array.

The boxes with slides were exposed with the slides oriented vertically. A horizontal arrangement of slides was not considered necessary because other investigators, especially O'Neill (1975), have shown that the number of microfoulers on horizontally placed slide surfaces did not differ from vertically arranged slides having the same immersion periods.

The arrays were suspended in Monterey harbor beneath the tide station of Municipal Wharf No. 2 (Figure 3). This location was in a shaded site which seldom received direct sunlight. The arrays were anchored in the water so that the slide boxes were 3.0 meters from the bottom and approximately 3.5 meters below mean lower low water. The control array and the arrays containing painted slides were separated by a minimum horizontal distance of 1.5 meters to limit the possibility of contamination between adjacent sets of arrays (Figure 4).

2. Sample Collection

One uncoated control slide and one sample from each of the two paints under study were removed after immersion periods of: (1) 24 hours, (2) 48 hours, (3) 4 days, (4) 6 days, (5) 8 days, (6) 10 days, (7) 14 days, (8) 18 days, (9) 22 days, (10) 26 days, (11) 30 days, (12) 49 days, and (13) 83 days. Because initially the total number of slides exposed for each paint was limited to twelve, the long-range test of 83 days did not begin on 18 November 1980. Slides for this test were

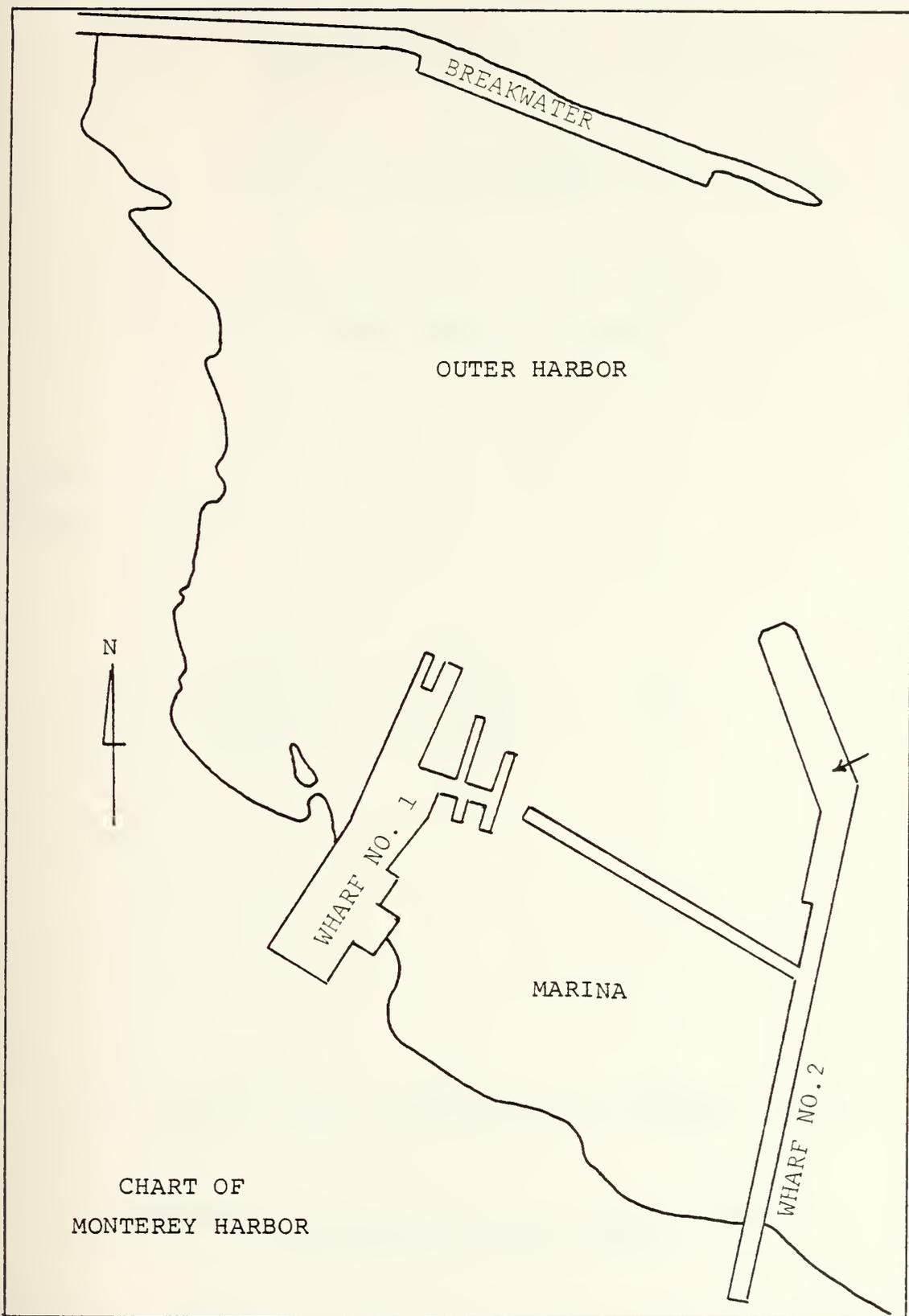


Figure 3. Location of test site (denoted by arrow)

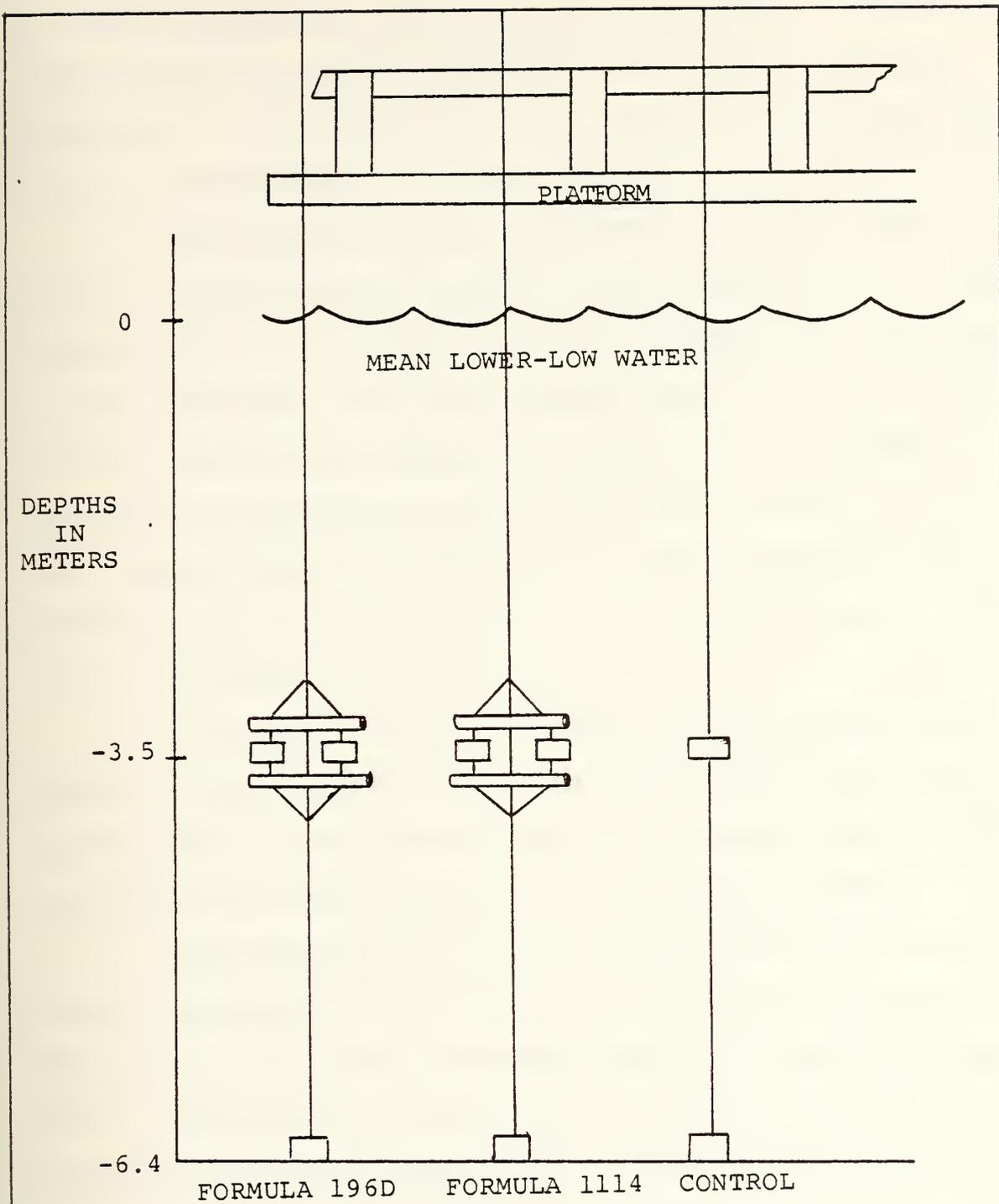


Figure 4. Relationship of arrays to sea surface and each other

put into the exposure boxes on 26 November 1980. Since four sample recovery periods had passed, the addition of these slides still allowed for more than sufficient horizontal separation between samples to avoid the concentration of toxicant.

These exposure periods were chosen because the early periods matched exposure times of other investigators. Immersion periods from 24 hours up to and including the 14th day of this experiment match the exposure times of O'Neill (1975) in his research into primary film formation. This permitted comparisons to be made with O'Neill's work as well as that of other investigators including Corpe (1972), Dempsey (1981), Gerchakov et al., (1976), O'Neill (1971), O'Neill and Wilcox (1971), and Marshall et al., (1971).

To avoid artificially increasing the bacterial populations of the exposed slides, the sample boxes were recovered and returned to the water sealed within plastic bags, avoiding contact with the neuston at the air-sea interface.

Water samples for salinity determination and surface bucket temperatures were taken at the exposure site every two days up to and including exposure period 11 (day 30) of Experiment 1. The seawater samples were tested for salinity using a Plessey Salinometer Model 6230N. Temperatures were determined using a standard bucket thermometer.

3. Sample Preparation

After the slide samples were removed from the water they were chemically preserved and prepared for study under the SEM. The steps followed in sample preparation included:

- a. Fixation
- b. Dehydration
- c. Freeze-drying
- d. Gold plating

- a. Fixation

Immediately after recovery from the water the slides were put into a coplin jar containing two percent gluteraldehyde and fixed for two hours. The gluteraldehyde was diluted with filtered seawater from the exposure site. A 0.2 μm milipore filter was used to filter the water. Dilution with this seawater was done to ensure an isotonic environment for fixation.

- b. Dehydration

The dehydration process was employed in order to preserve the shape of the microorganisms through the freeze-drying process. Following fixation the samples were washed in distilled water several times and then immersed in a graded series of aqueous acetone (dimethyl ketone) solutions, for five minutes in each concentration. The acetone was diluted with distilled water. The dehydration sequence followed was consistent with experimental procedures of other researches Hayat (1978) and Taylor (1977). The dilutions used were as follows:

- 1. 10 percent acetone
 - 2. 30 percent acetone
 - 3. 50 percent acetone

4. 70 percent acetone
5. 90 percent acetone
6. 100 percent acetone (2 changes)

Slides coated with Formula 1114 and Formula 196D showed varying degrees of blistering and cracking after dehydration. This effect became more pronounced as the immersion time in the water increased. This problem, however, did not exclude any slides from being studied under the SEM. The other two antifouling paints which were to be tested in Experiment 1, Formula 170 and Formula 121, were eliminated from this test because dehydration in either acetone or another similar solution, methyl alcohol, severely affected the painted surfaces. Formula 170 was completely dissolved in both dehydration agents while the entire surface of slides painted with Formula 121 cracked and peeled when dipped into liquid nitrogen following dehydration.

c. Freeze Drying

Following the dehydration step, slides were plunged into liquid nitrogen and freeze-dried at -50°C for four hours in a model 10-141 Unicool, manufactured by the Virtis Company, Gardiner, New York. After freeze drying the samples were stored in a desiccator until final preparation for viewing under the SEM.

d. Gold Coating

Nonconductive specimens, like those examined in this study, cannot rapidly channel the excess primary electrons

away from the scanned area and a local charge may build up on the specimen's surface. This increases abnormally the secondary electrons emitted to the collector and creates a localized glow which destroys the imaging of the microscope (Hayat, 1978). To avoid this situation, an extremely thin conductive coating of gold, approximately 100 Å thick, was applied to the slides by vaporizing a gold disc in an evacuated chamber.

The plating process was accomplished by a DSM-5 Cold Sputtering Module mounted inside a bell jar vacuum evaporator known as a Denton DV-502. This equipment was manufactured by the Denton Vacuum Corporation, Cherry Hill, New Jersey. The size of the pedestal upon which the plating was done permitted only one slide to be coated at a time. Due to the expense associated with the process, all slides were cut in half in order to plate one side of two different samples at a time.

C. EXPERIMENT 2

Experiment 2 tested all four paints of interest:

1. Formula 170
2. Formula 1114
3. Formula 196D
4. Formula 121

1. Experimental Substrates

Because of paint adhesion problems experienced in Experiment 1, the surfaces of all the glass slides to be painted were roughened prior to coating. This was accomplished

by passing them across a belt sander. The texture of the painted slide surfaces, however, showed no evidence of the slightly roughened surface below and had the same texture as the paint applied to smooth glass. Paint on the surfaces of the slides in Experiment 2 adhered well, showing no signs of the cracking which occurred in Experiment 1.

The slides were painted and exposed to the water in the same manner as in Experiment 1, with the exception that there were now a total of five arrays involved in the test.

2. Sample Collection

Sample collection steps followed exactly those used in the first experiment except that instead of thirteen collection periods only eight were used: (1) 24 hours, (2) 48 hours, (3) 4 days, (4) 6 days, (5) 8 days, (6) 10 days, (7) 14 days, and (8) 30 days.

Sea-surface bucket temperatures and water samples were taken with each slide sample recovered instead of every two days as earlier.

3. Sample Preparation

All preparation steps followed in the first experiment were used in the second except that the dehydration step was eliminated. In morphological studies the dehydration step is important in preserving the shape of microorganisms. Deleting this step in sample preparation may cause some of the microorganisms to appear flattened, but they retain their basic shape Hayat (1978). Since the aim of this study was to simply

examine the numbers and types of organisms present and not their morphology, elimination of this step still permitted the goals of the study to be achieved. There was no noticeable change, however, in the shape of the organisms viewed under the SEM between the two experiments. A similar result regarding the effects of dehydration was recorded by Dempsey (1981).

A summary of the steps followed in sample preparation in Experiment 2 included:

1. Fixation
2. Freeze drying
3. Gold plating

D. EXAMINATION USING THE SEM

The gold-coated slide samples were viewed with an Hitachi S-450 SEM using an accelerating voltage of 15KV. Initially, a quick look over most of the slide surface was made using magnifications ranging from 63X to 5000X and various SEM scans. This was done to get a view of the types of organisms present and whether or not they were uniformly distributed. Areas near the edge of the slides were avoided because fouling populations may be anomalously high there.

In both experiments the only microorganisms which appeared to be uniformly distributed were rod-shaped bacteria. This uniform distribution agrees with studies conducted by DiSalvo and Daniels (1975) and O'Neill (1975) who stated that bacterial

cells appeared to be uniformly spaced over the test surfaces rather than grouped in aggregates.

After this general scan, two or more locations were selected as being representative of the surface and photographs taken. Polaroid Type 55 positive-negative 4X5 inch land film was used for all photographic work. This is a fine-grain panchromatic film, yielding a positive print and a negative of high resolution. Photographs were also taken of any unusual or interesting organisms.

Magnifications of 1,500X or 3,000X were employed extensively in photographing bacteria and other organisms. The number of photos taken of each slide surface were limited due to cost and the uniform distribution of bacteria.

A quantitative estimation of the numbers of bacteria was made for each immersion period of both experiments using SEM photographs. The bacterial count was an average for the photo(s) of that exposure period.

Where possible, identification of organisms to genus and species was made. When this was not possible, identification was limited to general groups of organisms (bacteria, filamentous microorganisms, hydroids, bryozoans, diatoms, fungi, protozoans, etc.).

IV. RESULTS

A. EXPERIMENT 1

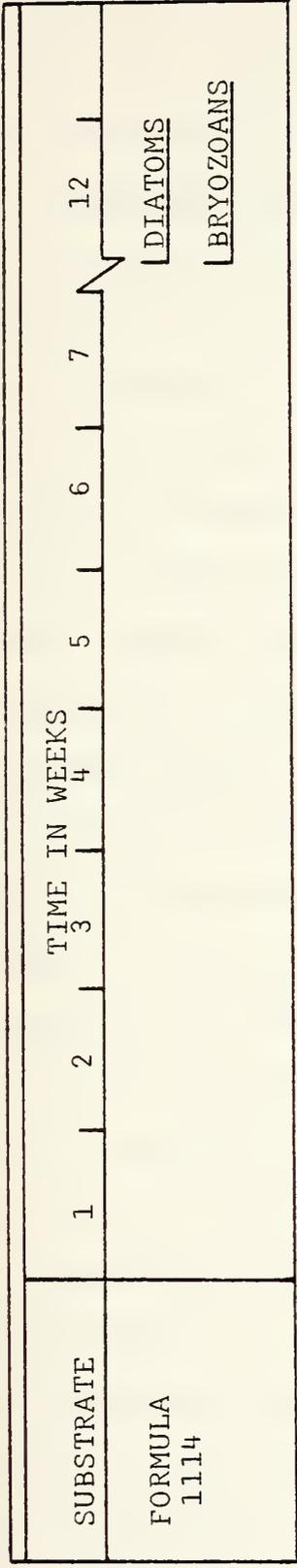
1. General

A succession of periphytic microorganisms was observed on uncoated glass slides during Experiment 1 (Table 1). This succession included rod-shaped bacteria followed by diatoms, filamentous microorganisms, and finally protozoans. Slides coated with Formula 196D or Formula 1114 did not exhibit a settlement sequence. Formula 196D surfaces were colonized early and throughout the experimental period by bacteria. No other organisms appeared on this surface until diatoms were seen after 83 days of exposure. Glass slides coated with Formula 1114 were free of all fouling organisms until after 83 days of immersion when diatoms and macrofouling organisms such as bryozoans became evident.

When bacteria were seen on the test surfaces in both Experiments 1 and 2 they appeared to be uniformly distributed which permitted an estimate of their populations to be made from two or three SEM photographs. The word sorbed will be used to refer to the adhesion or attachment of bacteria and other microorganisms to solid surfaces, a term used by other researchers in this area including Corpe (1977) and Marshall et al., (1971a).

Throughout the presentation of results from both experiments, attention has been directed to photographic

TABLE 1 - Continued



plates to help clarify the text material. Each photographic plate contains one or more references, indicated in parenthesis, which were used to identify the organisms. The scale on each photograph is in microns.

2. Organisms Sorbed to Glass

The major changes on the glass surfaces with respect to the microfouling organisms occurred during the first four weeks of exposure, a result which agrees with similar observations by Gerchakov et al., (1976). The first organisms to appear were rod-shaped bacteria approximately 1.0--2.0 μm long. They were evident following the first immersion period of 24 hours. Plate 1 presents a good example of the size and the appearance of these organisms although the picture is from a coated slide. The bacteria increased up to 48 hours then decreased slightly, leveling off until the 14th day (Figure 5). No bacterial counts on any surfaces in either Experiment 1 or 2 were made beyond 14 days. This was because the presence of so many organisms and so much organic debris after 14 days of immersion negated the possibility of counting bacteria, a condition which agrees with research results of O'Neill (1971) and O'Neill and Wilcox (1971).

The numbers of bacteria sorbed to the glass surfaces as illustrated in Figure 5 must be considered an approximation of low confidence because of the few SEM pictures used for counting in each exposure period. The graph of the results, however, does show a characteristic accelerated growth period

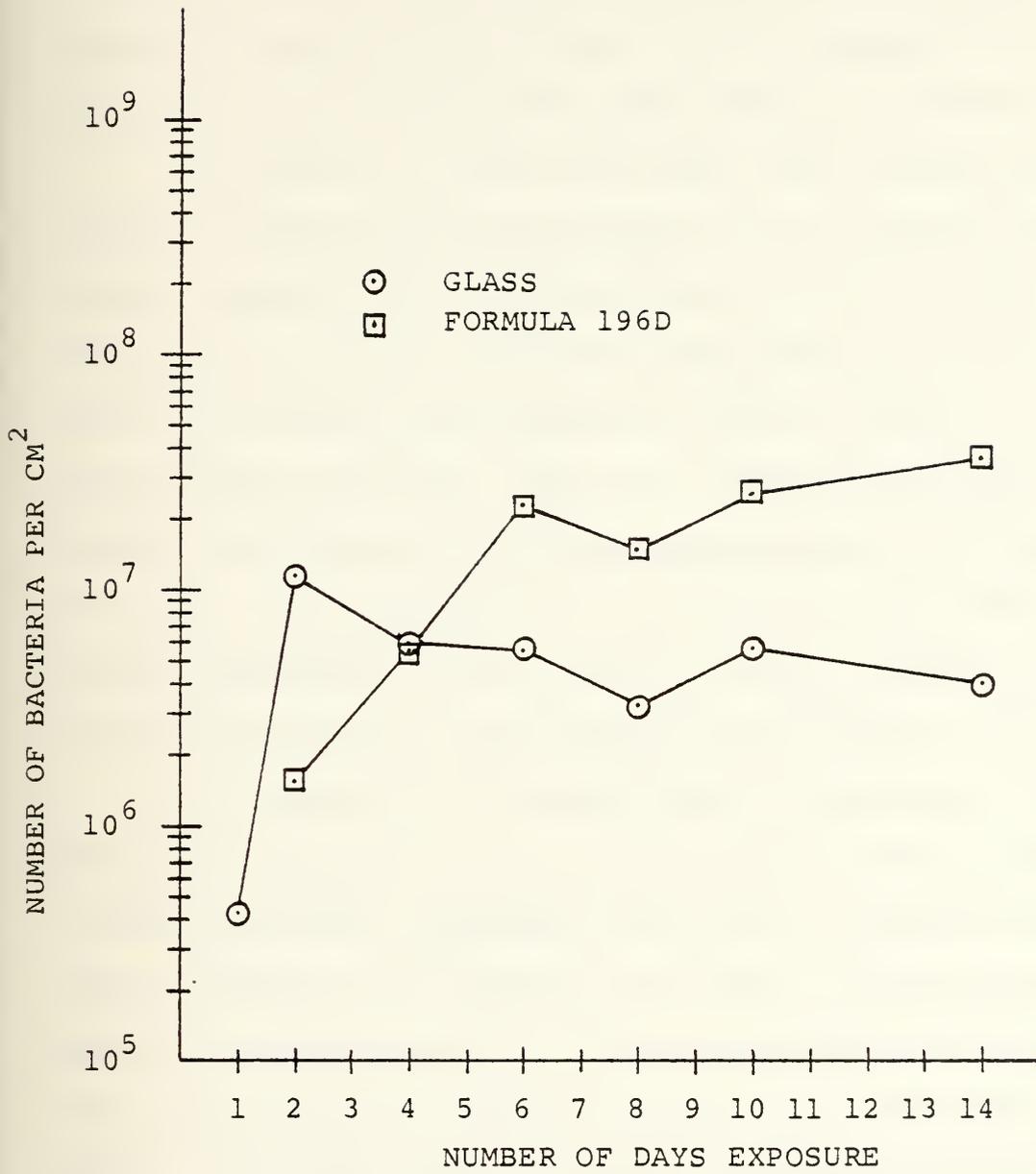


Figure 5. Numbers of bacteria sorbed to surfaces in Experiment 1

during the initial 24 to 48 hours of exposure, followed by a leveling off with a slightly decreasing population. This kind of growth profile agrees with research and observations by Bott and Pinherio (1976), Dexter (1976), Marshall et al., (1971a), O'Neill (1971,1975), and O'Neill and Wilcox (1971).

By the end of the first week ring-forming bacteria, unknown filamentous microorganisms, and solitary diatoms became evident on the glass surface (Plate 2). These organisms, although present throughout the remaining exposure periods, were not uniformly distributed and so no attempt at estimating their numbers was made. The most conspicuous of the diatoms present were the centric forms Thalassiosira and Coscinodiscus (Plate 3). Identification of bacteria to genus and species was not attempted. Ring forming bacteria, however, had the size and appearance of Flectobacillus (Sieburth, 1975).

At the end of the second week of exposure a greater number and variety of diatoms were seen. Several pennate diatoms especially Cocconeis populated the surface along with centric types. The surface also became littered with a large amount of organic debris. It was around these organic deposits that the greatest diatom density was seen. Bacteria in the form of rods and rings as well as filamentous microorganisms continued to sorb to the glass throughout the remaining exposure periods of Experiment 1.

At 18 days exposure the surface was dotted with various kinds of protozoa, most notably the ciliated protozoan,

Cilophoran (possibly C. carchesium or C. ephelota) (Plate 4). Small numbers of planktonic algae known as coccolithophoroids were also seen. A worm tube casing was observed firmly attached to the surface giving evidence to the possible beginning of fouling by macroscopic organisms. Organic debris continued to increase over the surface and provided areas of active bacterial and diatomaceous activity.

After 22 and 26 days of immersion the population of protozoa, like the vorticelled ciliate shown in plate 5, increased, while the number of bacteria and diatoms appeared to decrease slightly. The surface landscape was a mixture of diatoms, protozoa, filamentous microorganisms, and debris (Plate 6). An unusual mass of very small cylindrical objects, believed to be a group of protozoans was seen for the first and only time after 22 days exposure (Plate 7).

Following 30 days of exposure there was a dramatic change in the numbers and types of organisms seen on glass. The entire surface was covered with many algal filaments and protozoans as well as several bryozoan colonies. The protozoans included many Zoothamnium colonies (Plate 8) and a few foraminifera (Plate 9). About eight bryozoan colonies were observed attached to the slide surface (Plates 10 & 11). The development of the bryozoan colonies may be looked upon as the real beginning of macrofouling. There was a noticeable decrease in the number of bacteria and diatoms sorbing to glass. Generally, many of the diatoms in evidence were fragmented and appeared

to be decaying. Only a few diatoms were complete like the centric form seen in Plate 12.

After 49 days of immersion the number and variety of protozoa had increased noticeably. Zoothamnium colonies and peritrichous ciliates, some housed within loricas, continued to be the dominant organisms of the surface population (Plate 13), as well as the suctorian Acineta (possibly A. tuberosa) (Plates 14 & 15). Another organism which had not been observed before on glass was identified as the hydroid Obelia (Plates 16 & 17). These organisms were seen scattered across the glass surface in small numbers. The number of bacteria and diatoms sorbed to the surface continued to be few in number. One unidentified organism was also observed (Plate 18).

After 83 days of exposure the glass surface was a densely-layered environment which included both micro and macrofouling organisms as well as large amounts of debris. Algae filaments and protozoa were the dominant organisms present along with some hydroids and a few bryozoan colonies. Again the number of bacteria and diatoms seen remained few in number.

Three organisms which were seen for the first time included the planktonic diatom Nitzschia (possible N. closterium) (Plate 19), a skeleton of a flagellated plant known as a silicoflagellate (Plate 20), and many unknown ciliated bodies (Plates 21 & 22).

3. Organisms Sorbed to Formula 196D

The first microfouling organisms to appear were the ubiquitous rod-shaped bacteria, which were not seen until after 48 hours of exposure (Table 1). These organisms seemed to thrive on the Formula 196D paint surface. Like the bacteria sorbed on glass, these rod-shaped bacteria exhibited an initial rapid growth period followed by a slower but steadily increasing population (Figure 5). However, after four days of exposure, there was a significant increase in the numbers of bacteria seen on the Formula 196D surfaces as compared with glass immersed in seawater for the same period of time. These results indicate that the bacteria may have a preferential attraction for or be stimulated by this paint. This condition was noted through the second week of exposure testing until bacterial counts were discontinued.

The bacteria remained the major colonizers of this painted surface until the 18th day of exposure when filamentous microorganisms joined them (Plate 23). Diatoms were not seen until the 12th week of exposure and included planktonic types Chaetoceros (Plate 24), pennate forms Cocconeis, and centric forms Thalassiosira (Plate 25). Several unknown bulbous-like organisms also became visible after 83 days of immersion (Plate 26) along with a few sponge spicules (Plate 27).

4. Organisms Sorbed to Formula 1114

No organisms of any kind were seen attached to the surface of paint Formula 1114 until after 83 days of exposure.

For the entire experimental period this painted surface had a very "rocky" appearance, composed of paint artifacts, salt crystals, and some debris (Plate 28). At this time several bryozoan colonies were observed without the aid of a microscope. The SEM also revealed a great number and variety of diatoms, including a small number of a species not seen before, such as Biddulphia longicururis (Plates 29 & 30), along with many familiar centric forms, Thalassiosira (Plate 31). No bacteria, however, were seen on this paint during Experiment 1.

5. Temperatures and Salinities

Temperature and salinity values collected for the first 30 days of Experiment 1 have been graphed in Figure 6. The average surface water temperature for the period was 12.6°C and the average salinity was 33.56 ‰.

B. EXPERIMENT 2

1. General

A succession of periphytic microorganisms was observed on four out of the five test surfaces exposed in Experiment 2 (Table 2). The types of organisms and the times of their initial settlement on glass slides matched almost exactly the initial settlement time and succession of microfoulers seen on glass surfaces in Experiment 1. The settlement sequence on slides painted with Formula 196D, Formula 170, and Formula 121 consisted of bacteria followed by diatoms and finally protozoa. Slides coated with Formula 1114, on the other hand, became colonized early in the second week and throughout Experiment 2

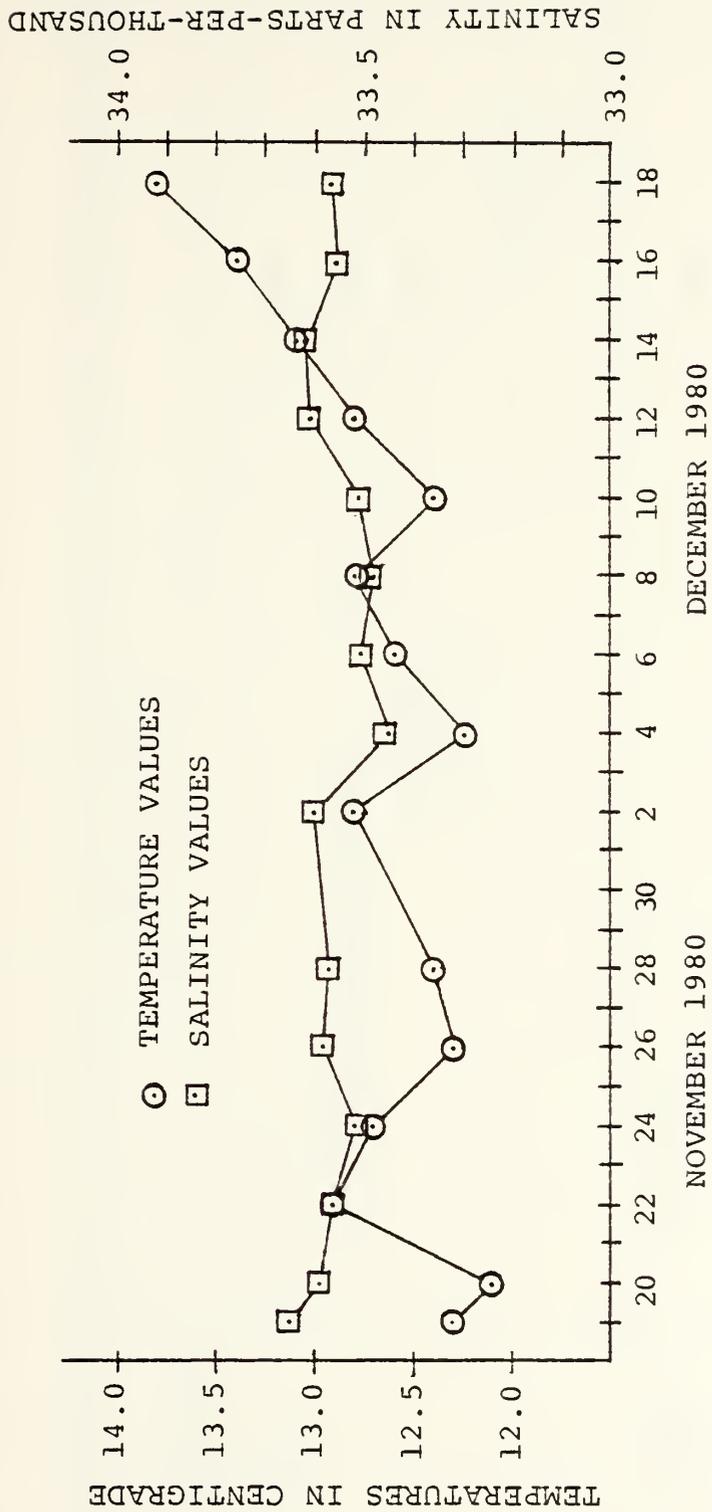


Figure 6. Temperature and salinity values recorded during Experiment 1

TABLE 2

SETTLEMENT SEQUENCE OF DOMINANT FOULING ORGANISMS
OBSERVED IN EXPERIMENT 2

SUBSTRATE	TIME IN WEEKS				
	1	2	3	4	5
GLASS	<u>BACTERIA</u> ————	————	————	————	————
	<u>FILAMENTOUS MICROORGANISMS</u> ————	————	————	————	————
	<u>DIATOMS</u> ————	————	————	————	————
	<u>PROTOZOANS</u> ————	————	————	————	————
	<u>BRYOZOANS</u> ————	————	————	————	————
FORMULA 196D	<u>BACTERIA</u> ————	————	————	————	————
	<u>DIATOMS</u> ————	————	————	————	————
	<u>PROTOZOANS</u> ————	————	————	————	————

exclusively by solitary diatoms. In general, a greater number and variety of diatoms were seen in this experiment than in Experiment 1.

This section includes detailed results from exposure testing of glass slides coated with Formula 170 and Formula 121, two paints which were not examined in Experiment 1. For the test surfaces of uncoated glass, Formula 196D, and Formula 1114, a detailed listing of microorganisms and their settlement sequence will be made only when these results differ from those of Experiment 1. Similar results from the two experiments will be noted with general comments.

2. Organisms Sorbed to Glass

As in Experiment 1, the first organisms to appear were rod-shaped bacteria. These microfoulers were seen throughout all exposure periods of this experiment. An accelerated growth period, similar to the bacterial growth observed in Experiment 1, was again seen in the initial 24 to 48 hours (Figure 7). Unlike the results of Experiment 1, the number of bacteria continued to increase slowly beyond the initial growth period until counting was no longer possible. The number of bacteria graphed in each immersion period agrees well with the bacterial counts of corresponding periods in Experiment 1.

Diatoms became evident on glass after 14 days of immersion. In general, the same centric diatoms Thalassiosira and Coscinodiscus as well as pennate forms Cocconeis, were seen. Two additional diatoms were identified which had not

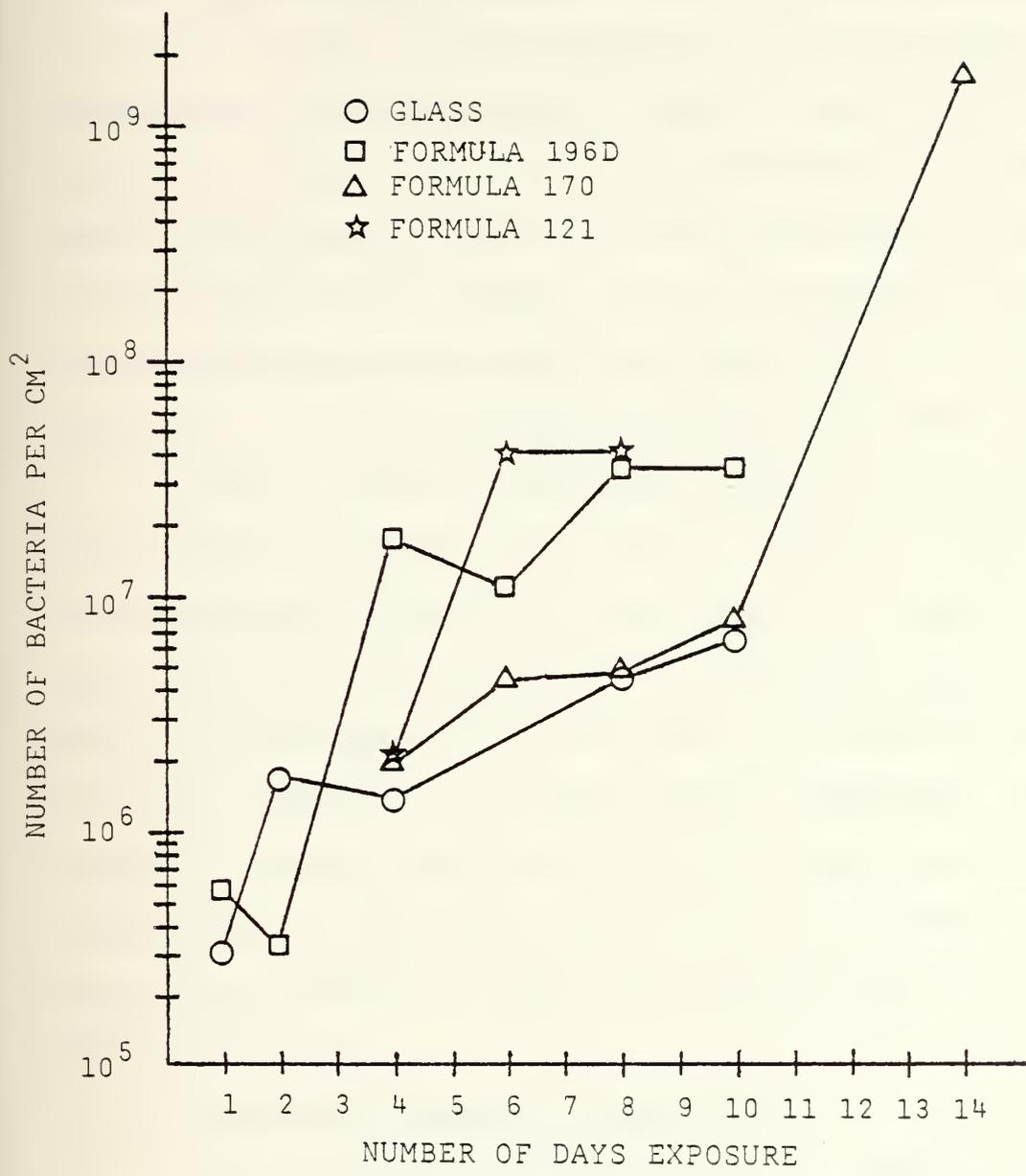


Figure 7. Numbers of bacteria sorbed to various surfaces in Experiment 2.

been observed in Experiment 1. These included several groups of rectangular diatoms, Thalassionema (Plate 32), and centric diatoms, Skeletonema costatum (Plate 33), seen scattered about the glass surface. As in Experiment 1, diatom density was concentrated around deposits of debris. Also, a variety of ciliated protozoa, the same seen in Experiment 1, began to populate the glass. These organisms were joined by two unidentified forms not seen before. Several clusters of what appeared to be transparent sacks containing eggs dotted the surface (Plate 34), along with several attached organisms (Plate 35).

After 30 days of immersion in Monterey Bay the glass slide surface was again populated by colonies of bryozoans and Zoothamnium as well as a large number of vorticelled ciliates (Plate 36). The surface also contained many protozoans known as Choanoflagellates which were not seen before in either experiment (Plate 37). Several unknown organisms, possibly ciliates, were also seen for the first time (Plate 38). As in Experiment 1, the number of bacteria and diatoms sorbed to the surface appeared to have decreased noticeably at this exposure period.

3. Organisms Sorbed to Formula 196D

After 24 hours of exposure unusual geometric formations, not seen before, were observed scattered about the painted surface (Plate 39). They did not appear to be organic but rather crystalline in nature.

Rod-shaped bacteria became visible after 48 hours, colonizing the surface until the conclusion of the experiment.

The bacteria again exhibited a rapid growth period followed by a slower but steadily increasing population (Figure 7). As in Experiment 1, after four days of exposure, there was a significant increase in the number of bacteria seen on the Formula 196D surface as compared with corresponding glass samples.

Diatoms settled on Formula 196D after only 6 days as compared to 83 days in Experiment 1. The diatom population, however, was sparse, composed mainly of centric forms, Thalassiosira. These organisms were joined after 8 days and throughout the remaining experimental period by planktonic diatoms, Chaetoceros.

After 14 days of exposure the centric diatom Skeletonema costatum joined the developing population along with several silicoflagellates.

On the 30th day of the experiment, the Formula 196D surface was littered with a large number of shelled protozoans identified as foraminifera (Plate 40). Several worm tube casings were also seen (Plate 41). Unlike uncoated glass, the number of bacteria and diatoms continued to populate the Formula 196D surface in large numbers.

4. Organisms Sorbed to Formula 1114

Solitary diatoms were sparsely settled across the Formula 1114 surface after 8 days of immersion (Table 2). They continued to populate the surface in increasing numbers throughout the remaining exposure periods. Diatoms identified

included pennate forms Amphora and centric types Thalassiosira, Cosinodiscus, and Skeletonema costatum. Diatoms were the only organisms which settled on paint Formula 1114 in Experiment 2.

5. Organisms Sorbed to Formula 170

No organisms were seen until after 4 days of immersion (Table 2). The first microfoulers which appeared at this time were identified as rod-shaped bacteria (Plate 42). Like the bacteria sorbed to glass and Formula 196D, the bacteria on this surface exhibited an early rapid growth period followed by a slight decrease in population for a few days (Figure 7). From day 4 through day 10 of the bacteria counted on the surface of Formula 170, agreed well with the number of bacteria counted on Formula 196D and glass for the same exposure times. However, by day 14 a significantly greater number of bacteria were counted, giving evidence of another rapid growth period.

The bacteria were joined after 8 days of immersion by a sparse population of solitary diatoms including pennate forms and centric types Skeletonema costatum (Plate 43). The diatoms which were seen throughout the remainder of this experiment continued to be solitary and few in number.

After 14 days a few unidentified stalked organisms, believed to be protozoa were seen.

Following 30 days of exposure the Formula 170 surface contained a dense population of bacteria and organic debris. Diatoms and protozoa, although few in number, were also seen along with some coccolithophorids (Plate 44).

6. Organisms Sorbed to Formula 121

Rod-shaped bacteria became visible on this painted surface after 4 days of exposure (Table 2). The bacteria increased significantly from day 4 to day 6 and then leveled off through to day 8. Beyond the 8th day the bacteria appeared to be grouped in layers covered by a film which made counting them impossible.

After 6 days of exposure a large number and variety of diatoms were also seen. The forms identified included pennate types (possibly Amphora) (Plate 45), centric types Skeletonema costatum (Plate 46), and some other planktonic diatoms like Chaetoceros (Plate 47). As noted in Experiment 1, diatom density was most intense around organic debris deposits.

The number of diatoms, bacteria, and debris continued to increase through days 8, 10, and 14. After day 14 the surface was littered with debris, bacteria, diatoms, and some protozoans (Plate 48). This condition was again seen following 30 days of immersion with the exception that the debris and organisms mentioned were more numerous and two additional organisms were identified. Many solitary stalked microfoulers believed to be ciliated protozoans were seen (Plate 49), along with several coccolithophorids (Plates 50, 51, & 52). The three coccolithophorids pictured in these plates, although probably of different species, may be exhibiting three different stages of decay. Plate 50 may be a healthy coccolithophorid

that was killed at the time the sample was fixed, while Plates 51 and 52 show increasing stages of decay.

7. Temperatures and Salinities

Temperatures and salinities recorded for Experiment 2 have been graphed in Figure 8. The average surface water temperature for the test was 13.3°C and the average salinity was 33.37 ‰.

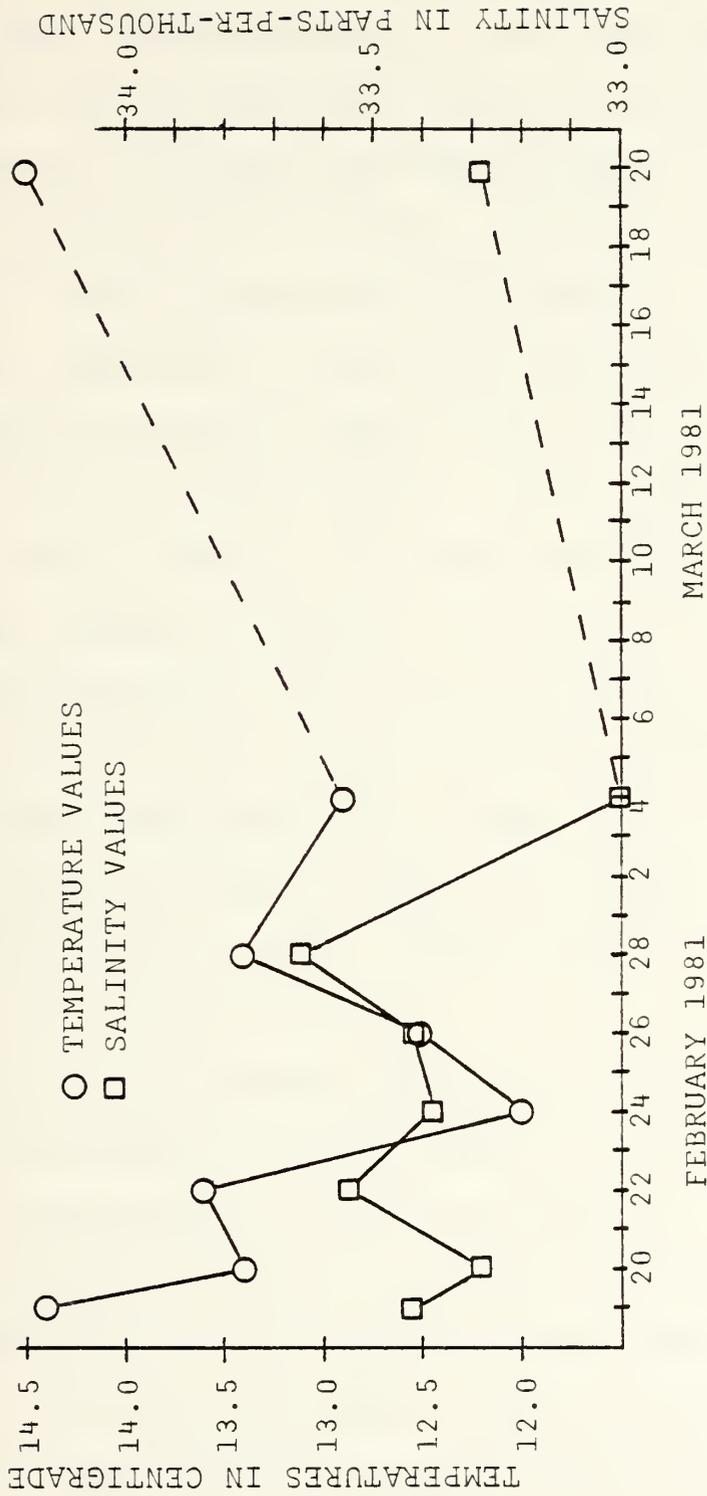


Figure 8. Temperature and salinity values recorded during Experiment 2

V. DISCUSSION

The glass surfaces tested in both Experiments 1 and 2 showed what may be considered typical microfouling sequences. The succession included rod-shaped bacteria followed by filamentous microorganisms, diatoms, protozoa, and finally bryozoans. Similar sequences and times of initial settlement of fouling organisms on glass have been reported by other investigators including Corpe (1970a, 1972), Dempsey (1981), Dexter (1976), Gerchakov et al., (1976), Marshall et al., (1971), O'Neill (1971), and O'Neill and Wilcox (1971).

It was desired to compare the number of bacteria seen on glass in Experiments 1 and 2 with the results of similar work by other researchers. Figure 9 displays bacterial counts made on glass from Experiments 1 and 2 and the results of counts of bacteria made on glass or plexiglass from research conducted by Dexter (1976), O'Neill (1977, unpublished), and O'Neill and Wilcox (1971).

O'Neill's unpublished research was conducted in Monterey harbor from December 1976 to January 1977 using polymethylmethacrylate (plexiglass) as the test substrate. Dexter conducted his tests using a variety of substrates including glass immersed in Woods Hole harbor, Massachusetts, from July through August 1975, during the peak of the fouling season at that location. The average sea surface temperature was 22.6°C. O'Neill and Wilcox conducted their bacterial counts using a

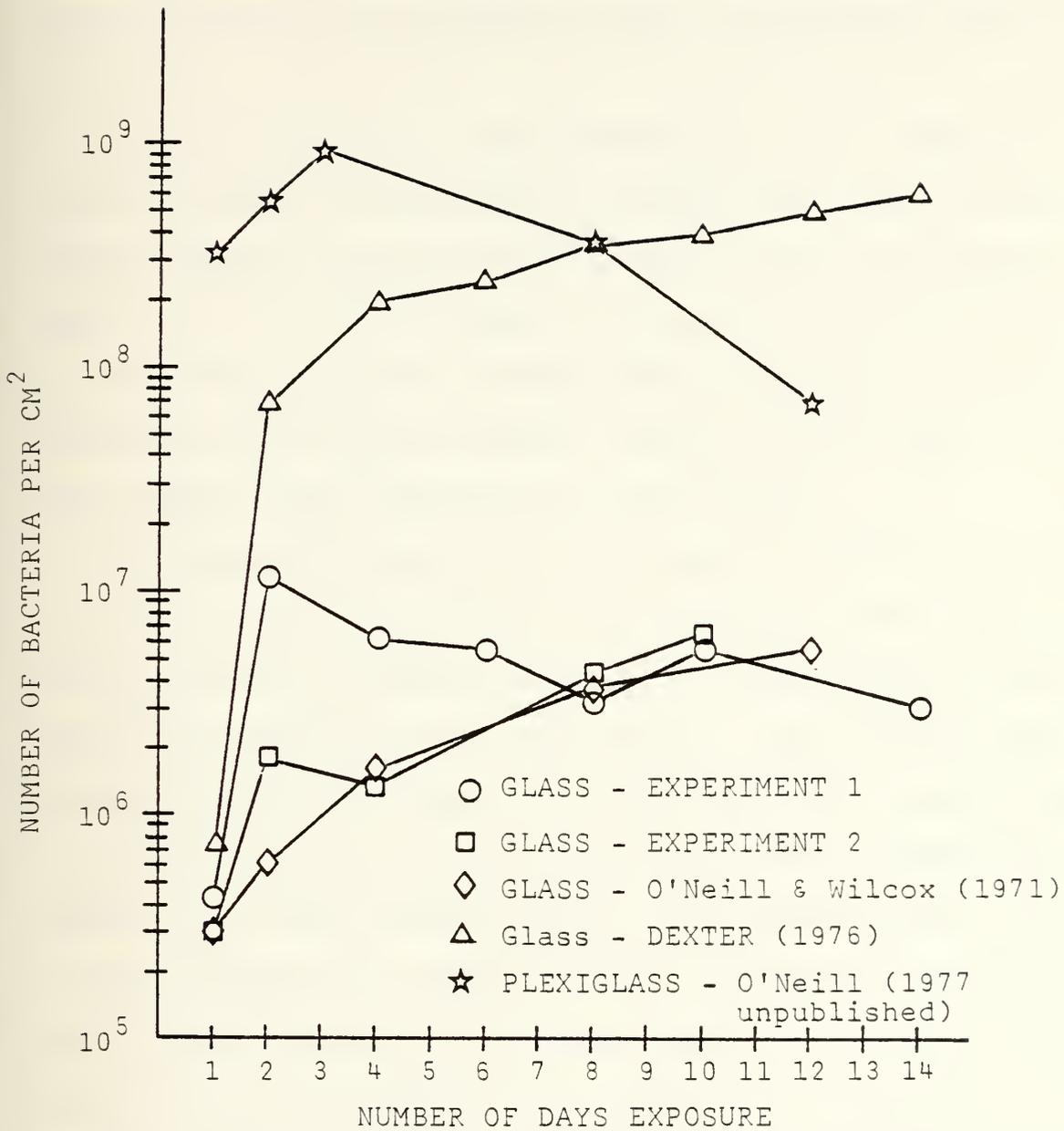


Figure 9. Results of bacterial attachment seen on glass in Experiments 1 and 2 and results of similar tests made on glass or plexiglass by three different research groups.

variety of substrates including glass immersed in the harbor at Port Hueneme, California from July to September 1965. The water temperature at the time of their test ranged from 13.0 to 18.0 °C.

Although there is a large difference in the number of bacteria noted in corresponding periods, all three research results showed an initial rapid growth period followed by a leveling off and then a slowly increasing or decreasing population. The growth trend exhibited by Experiments 1 and 2 agrees well with these research results. This trend is the only common factor between all these curves.

The bacterial counts of Dexter, made in Woods Hole harbor, and O'Neill and Wilcox made in Port Hueneme harbor agree more with the results of Experiments 1 and 2 than that of O'Neill which was conducted in Monterey harbor. The factors influencing bacteria settlement are many. It is not possible here to evaluate these factors in explaining the agreement or disagreement of these research results with Experiments 1 and 2. The graphed data from the three researchers was provided only to give a general view of the number of bacteria which may sorb to glass surfaces, the variability of bacterial populations with location and time of year, and some typical growth profiles.

Microfouling seen on all but one of the antifouling paints followed a similar succession sequence to that observed on glass. Formula 196D, tested in both experiments, along with Formula 170 and Formula 121, tested in Experiment 2, exhibited

a fouling sequence which included bacteria, followed by diatoms, and finally protozoa. These coatings appeared to have altered the times for the initial settlement of these organisms as well as their abundance. Although bacteria were not seen until 48 hours of immersion on the Formula 196D surface and after 4 days on the slides painted with Formula 170 and Formula 121, their numbers quickly increased to the point where they were significantly more abundant than the bacteria on glass slides. Paint Formula 1114, however, showed no sequence of organisms and was free of bacteria throughout both experiments. The only microfoulers observed on this paint were diatoms.

To explain the bacterial numbers and the microfouling sequences it was necessary to discuss the toxicants used in each paint formulation and their effects on microorganisms. The paints tested and their toxic ingredients are summarized below:

Formula 196D - Tributyltin

Formula 170 - Tributyltin oxide and Tributyltin fluoride

Formula 121 - Cuprous oxide

Formula 1114 - Nopcocide N-96

There has been some discussion by various researchers concerning the effects that paints containing tributyltin and copper have on microfouling. O'Neill (1975) tested an anti-fouling paint containing tributyltin oxide (TBTO) against the settlement of bacteria, diatoms, and barnacles under controlled

laboratory conditions. He concluded that the presence of TBTO appears to inhibit the development of a primary film of bacteria and diatoms and does hinder later barnacle attachment.

A different result was reported by Dempsey (1981) who said that tolerance to heavy metal poisons is a common phenomenon in microorganisms. He stated that organotin antifouling paints are not effective against gram-negative bacteria, although they are highly toxic to gram-positive species. Furthermore, in laboratory tests of another tin-based antifouling paint containing triphenyl tin fluoride (TPTF), Dempsey found that extensive bacterial communities developed on the TPTF paint after 4 weeks of exposure.

Corpe (1977) also reported that primary film forming bacteria are little affected by metallic paints or other toxic coatings such as organotin. In tests conducted on slides submerged in the sea for 24 and 96 hours, Corpe indicated that the same kinds and numbers of bacteria were isolated from the test surfaces whether they were coated with copper and mercury paint, tributyltin, or uncoated.

The sorption of microorganisms observed on Formula 196D, Formula 170, and Formula 121, seem to agree with the results of Corpe and Dempsey. Not only did these paints appear to have very little effect on these microfoulers in general, but they appear to actually stimulate the growth or attraction of bacteria. An explanation of these results is provided by Corpe (1975) who reported that copper or lead in a concentration of

4×10^{-4} M actually stimulated growth of bacteria when the nutrient concentration was high.

Approximately 85 to 95 percent of fouling bacteria are gram-negative (Corpe, 1973). A major characteristic of these bacteria is the presence of a lipopolysaccharide (LPS) outer cell layer which may act as a penetration barrier, especially to hydrophobic compounds (Dempsey, 1981; Corpe, 1977). Therefore, tolerance to antifouling paints containing metals such as copper, tin, lead, or mercury probably results from the LPS layer acting as a penetration barrier. Evaluation of the presence of such an outer cell layer was beyond the laboratory capabilities used in Experiments 1 and 2. However, many of the bacteria, on glass and painted surfaces, exhibited holdfast structures known as polymeric fibrils (Marshall et al., 1971). The extracellular appendages were more evident from bacteria on the heavy metal paints than from those sorbed to glass.

No macrofouling organisms (i.e., bryozoans) were seen on any of the antifouling paints for the first 30 days in either experiment. However, after 83 days of exposure, Formula 1114 had many well developed bryozoan colonies scattered across the painted surface. The toxicant used in Formula 1114 was Nopocide N-96. This compound is a chlorinated pesticide and is considered to be organic in nature containing no heavy metals. The results of Experiments 1 and 2 suggest that this substance may be effective in controlling or preventing

marine bacterial settlement and/or growth. However, the establishment of the bryozoan colonies on this paint may indicate that Nopocide is not as effective in preventing macrofouling.

VI. CONCLUSIONS

Of the four paints tested in this study only Formula 1114 can be said to be effective in preventing and limiting the early development of microfouling organisms. This paint, however, may not be as effective in limiting the sorption of macroorganisms such as bryozoans. The results of Experiments 1 and 2 also suggest that the establishment of a complete microfouling community which includes bacteria, diatoms, and protozoa is not a necessary precursor to the settlement of macrofoulers.

The other three paints with copper or tin as their toxic ingredient only delayed the onset of microfouling by a few days. The bacterial populations seemed to thrive on these heavy metal paints, achieving populations greater than those observed on glass. Much circumstantial evidence has been gathered to support the possibility that bacterial fouling layers with their extracellular mucilage secretions may render antifouling paints less efficient. It has been recognized that Formula 121, for example, is effective in preventing the settlement of barnacles and other macrofouling organisms. However, the effective life of the paint may be shortened by microfoulers. If the experimental paints, Formula 170 and Formula 196D prove to be effective in combating macrofouling, their surfaces will most likely be covered with bacteria and

other microfouling organisms, a condition which may also shorten their service life.

This study also established that there is a definite sequence to the sorption of marine microfoulers on glass and even on some antifouling paints. This sequence begins with the settlement of bacteria and is followed by filamentous microorganisms, diatoms, and finally protozoa.

Future research in this area should concentrate on longer exposure periods ranging from 6 months to several years. Test substrates should include hull steel and glass reinforced plastic and any other surfaces for which the end application is intended.

APPENDIX A

NAVY STANDARD FORMULA 121
RED VINYL ANTIFOULING PAINT

INGREDIENTS	AMOUNT IN POUNDS ¹
Cuprous oxide	1440
Rosin	215
Vinyl resin ²	55
Tricresyl phosphate	50
Methyl isobutyl ketone	165
Xylene	115
Antisettling Agent	5 to 9

Source: Department of the Navy Military Specification Mil-P-15931C, Paint, Antifouling, Vinyl (Formula Numbers 121 and 129)

Notes: 1. The formula, given slightly in excess of 100 gallons to allow for manufacturing loss, may be proportioned to the size batch desired.

2. The resin shall be a vinyl chloride-vinyl acetate copolymer. It shall contain 85 to 88 percent vinyl chloride and 12 to 15 percent vinyl acetate. The resin shall have a specific gravity of 1.35 to 1.37.

APPENDIX B

NAVY STANDARD FORMULA 170
BLACK CAMOUFLAGE VINYL ANTIFOULING PAINT

INGREDIENTS	AMOUNT (parts by mass) ¹
Vinyl resin ²	150
Bis (tributyltin) oxide (TBTO) ³	36
Tributyltin fluoride (TBTF) ³	155
Carbon black	18
Titanium dioxide	6
Ethylene glycol monoethyl ether acetate	26
Normal propanol ⁴	95
Normal butyl acetate	<u>370</u>
	856

Source: Department of the Navy Military Specification DOD-P-24588, Paint, Antifouling Vinyl, Camouflage (Formula numbers 170, 171, 172, and 173), 2 May 1979.

Notes: 1. Use of kilograms as mass units results in a volume slightly in excess of 833 liters. Use of pounds as mass units results in a volume slightly in excess of 100 gallons.

2. The resin shall be a copolymer of vinyl acetate and another monomer which contains carboxyl groups. It is manufactured by Air Products Chemical Company, Allentown, Pennsylvania under the name VINAC ASB-516.

3. Manufactured by M&T Chemicals Incorporated, Rahway, New Jersey, or by Cincinnati Milacron Chemicals Incorporated, 500 Jersey Avenue, New Brunswick, New Jersey.

4. This material contains a minimum of 97 percent normal propanol with a minimum boiling point of 95 degrees centigrade.

APPENDIX C

DTNSRDC EXPERIMENTAL ANTIFOULING PAINT
FORMULA 2844-1114

INGREDIENTS	POUNDS PER 103 GALLONS	GALLON PER 103 GALLONS
<u>COMPONENT A</u>		
Liquid polysulfide polymer (Thiokol LP-2) ¹	385	35.9
Toxic (Nopcocide N-96) (2,4,5,6- tetrachloroisophthalonitrile) ²	385	26.7
Carbon black	11.6	0.8
Adhesion promoter (Durez 10694) ³	19.2	1.8
Thixotrope (Cabosil M-5) ⁴	11.6	0.6
Solvent (Xylene)	269.4	37.2
	<u>1081.6</u>	<u>102.9</u>

COMPONENT B

Lead paste Mixture for 103 gallons of component A ⁵	80.83	5.29
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Source: David Taylor Naval Ship Research and Development Center Purchase Description (draft), Antifouling Paint, DTNSRDC Experimental Formula 2844-1114, December 1980.

Notes: 1. Thiokol LP-2, manufactured by Thiokol Chemical Company, 930 Lower Ferry Road, P. O. Box 1296, Trenton, New Jersey.

2. Nopcocide N-96 is manufactured by the Diamond Shamrock Chemical Company, process chemicals division, 350 Kemble Avenue, Morristown, New Jersey.

3. Durez 10694 is manufactured by the Hooker Chemical Corporation, durez plastics division, 14120 Walck Road, North Tonawanda, New Jersey.

4. CAB-O-SIL M-5 is manufactured by Cabot Corporation, 125 High Street, Boston, Massachusetts.

5. Lead Paste Mixture:

	Parts by wt.	Lbs. to make 1 Gal.	Gal. to make 1 Gal.
Lead Peroxide 90-95% PBO ₂	112.5	7.64	0.10
Plasticizer (Thiokol TP-680)	109.5	7.44	0.87
Stearic acid	3.0	0.20	0.03
	<u>215.1</u>	<u>15.28</u>	<u>1.00</u>

APPENDIX D

ORGANOTIN EPOXY GEL FORMULA 196D
DTNSRDC EXPERIMENTAL ANTIFOULING PAINT

INGREDIENTS	AMOUNT (parts by weight)
<u>COMPONENT A</u>	
EPON 828 ²	30
EPI-REZ 505 ³	50
60% Tributyltin ester of SMA 1000 ⁴	160
Lampblack	2.26
Titanium dioxide	0.52
<u>COMPONENT B</u>	
DMP-30 ⁵	8

Source: David W. Taylor Naval Ship Research and Development Center Technical Manual TM-28-80-105, Investigation of the Sprayability of Organotin Epoxy Gel Coats, by J. A. Montemarano, S. A. Cohen, A. M. Ross, and A. R. Parks, 27 June 1980.

Notes: 1. Use of kilograms as mass units results in a volume of approximately 232 liters. Use of pounds as mass units results in a volume of approximately 28 gallons of paint.

2. EPON 828 is an epoxy resin with a density of 1.15 g/cc. It is manufactured by the Shell Chemical Company.

3. EPI-REZ 505 is an epoxy resin with a density of 1.01 g/cc. It is manufactured by the Celanese Corporation.

4. The organotin component of this formulation is the 60 percent tributyltin ester of SMA 1000. The SMA 1000 is a copolymer of styrene: maleic anhydride (1:1 molar ratio), manufactured by the Atlantic Richfield Corporation.

5. This is a curing agent known as TRIS (dimethylaminomethyl) Phenol, distributed by Miller-Stephenson Company.

APPENDIX E



Plate 1. Glass coated with paint Formula 196D immersed 6 days, 3000X. Rod-shaped bacteria, some showing hold-fast structures.

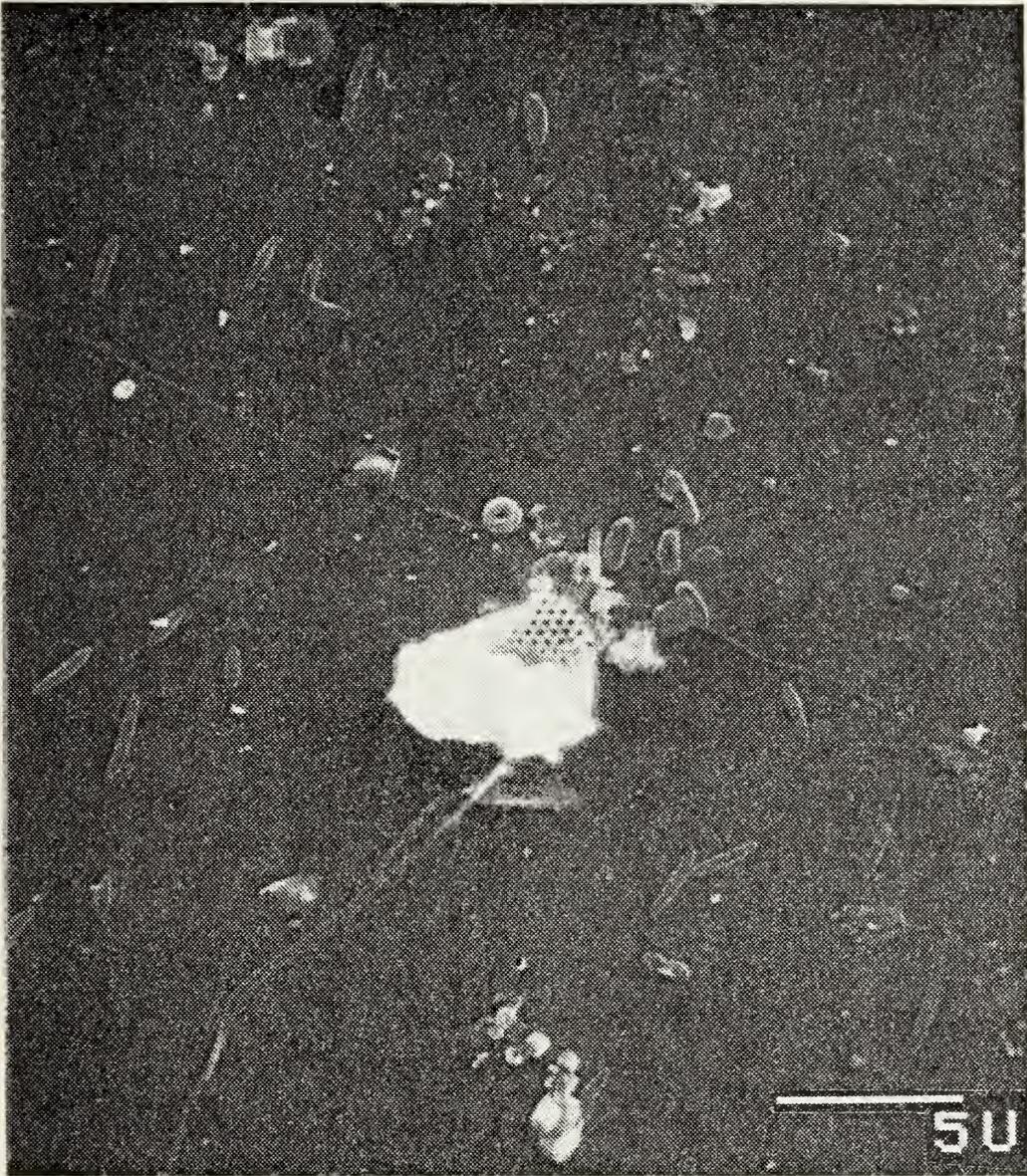
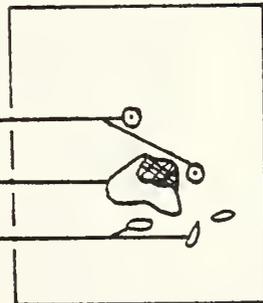


Plate 2. Glass immersed 6 days, 3000X. Rod-shaped and ring-forming bacteria.

Ring-forming bacteria (possibly Flectobacillus) (Sieburth, 1975)
 Diatom frustle fragment
 Rod-shaped bacteria



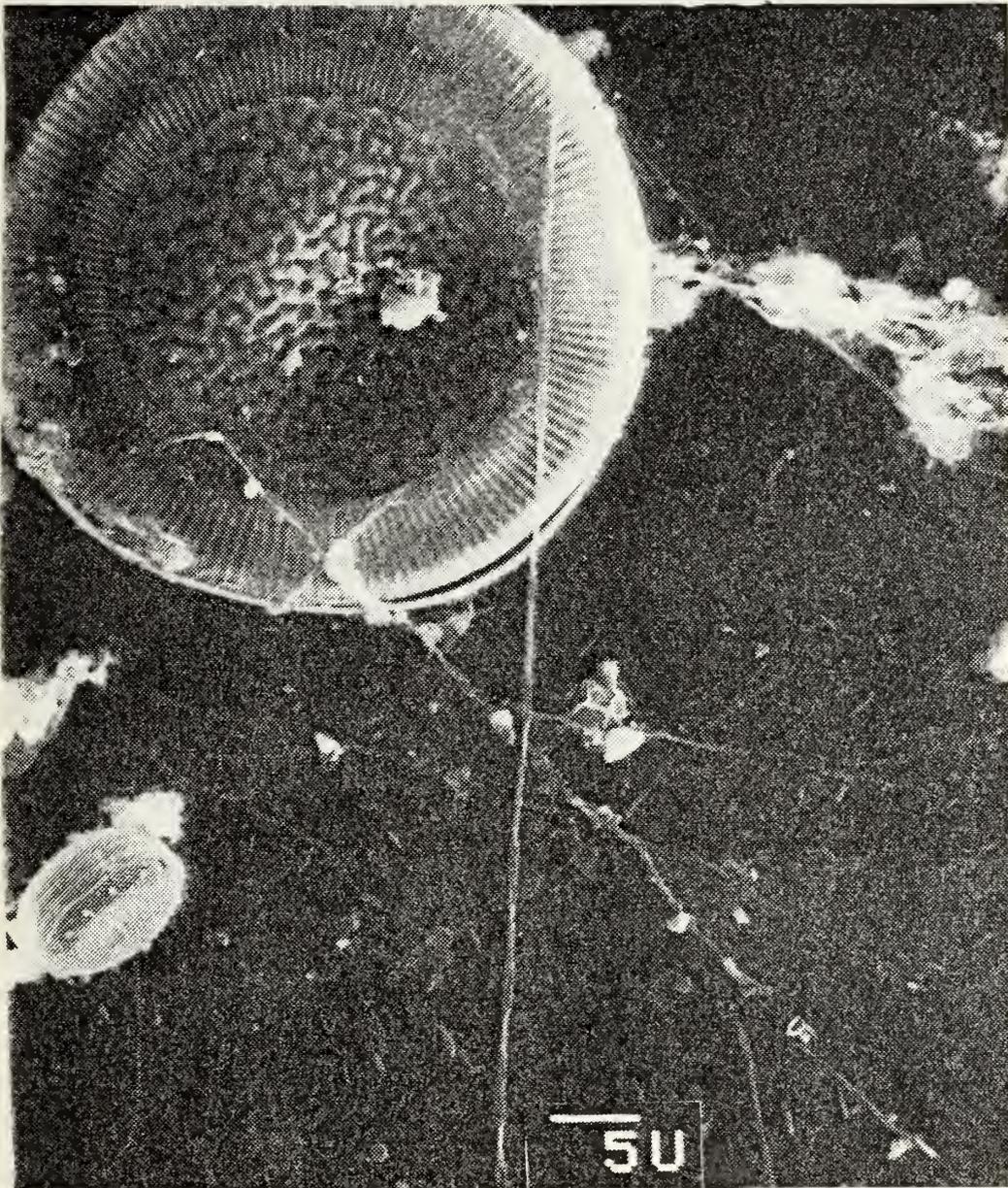


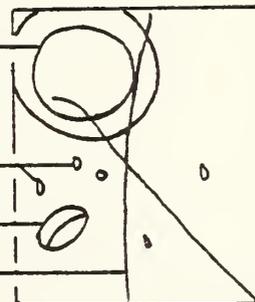
Plate 3. Glass immersed 8 days, 1500X. Two centric diatoms, filamentous microorganisms, and rod-shaped bacteria.

Centric diatom Coscinodiscus (possibly C. marginatus) (Cupp, 1943)

Rod-shaped bacteria

Centric diatom Thalassiosira (Cupp, 1943)

Filamentous microorganism (Sieburth, 1975)



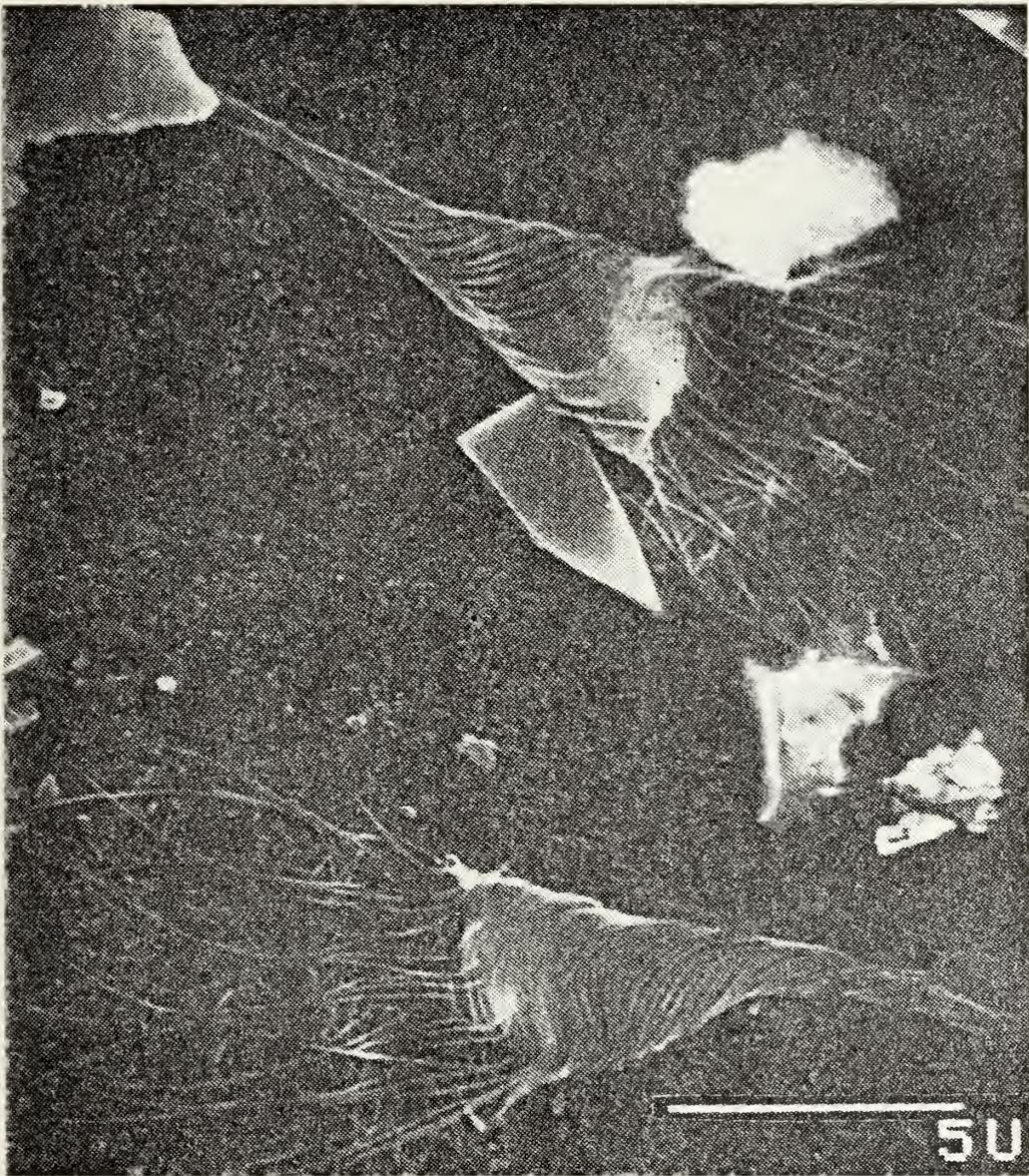


Plate 4. Glass immersed 14 days, 5000X. Two vorticellid protozoans (I. Abbott, personal communication).

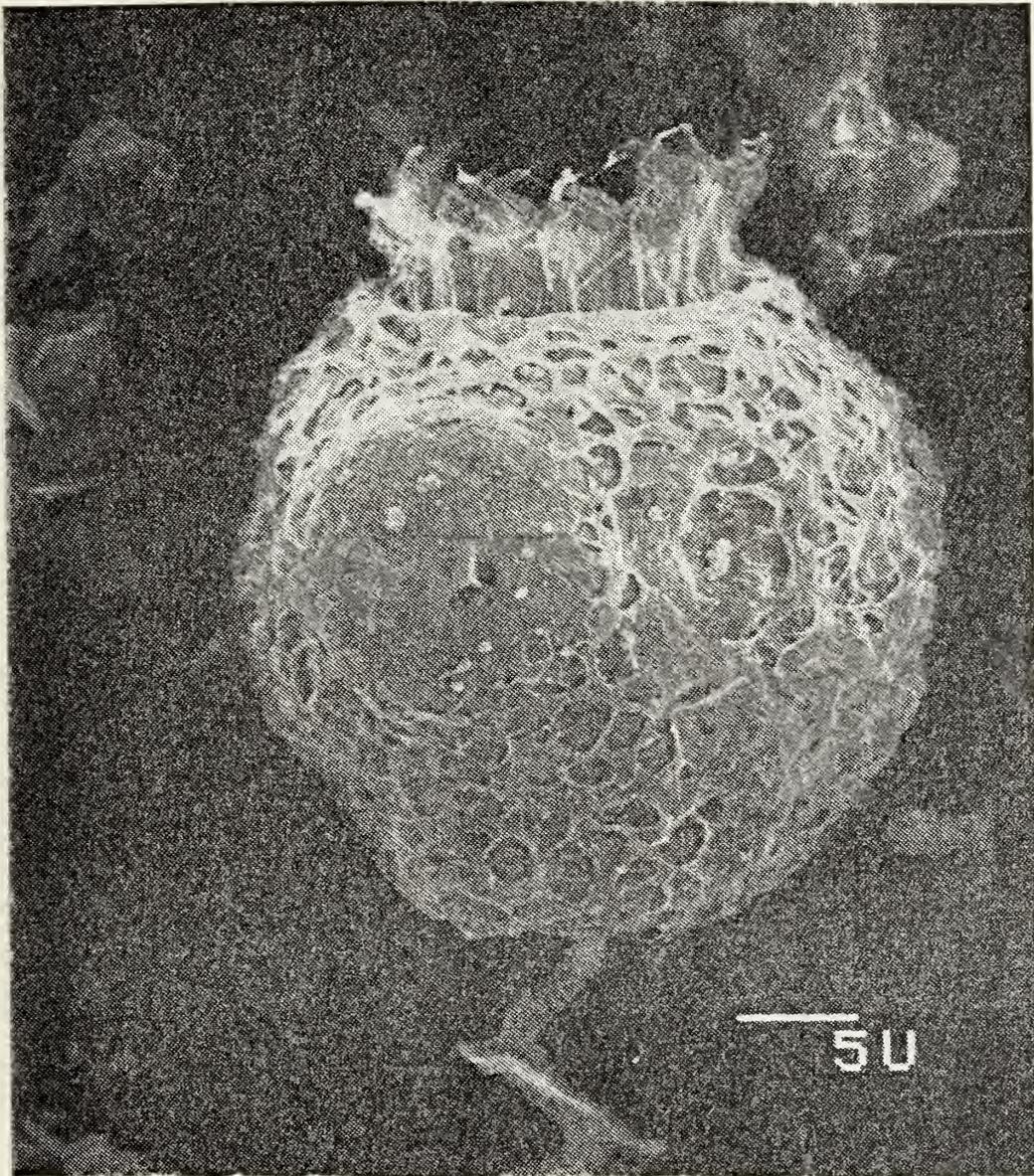


Plate 5. Glass immersed 83 days, 2000X. Close-up of a spiral-stalked protozoan. Note the cilia extended from the top of the organism. It moves these fine hair-like structures in a rhythmic manner creating a vortex thus drawing food into itself (Cupp, 1943).

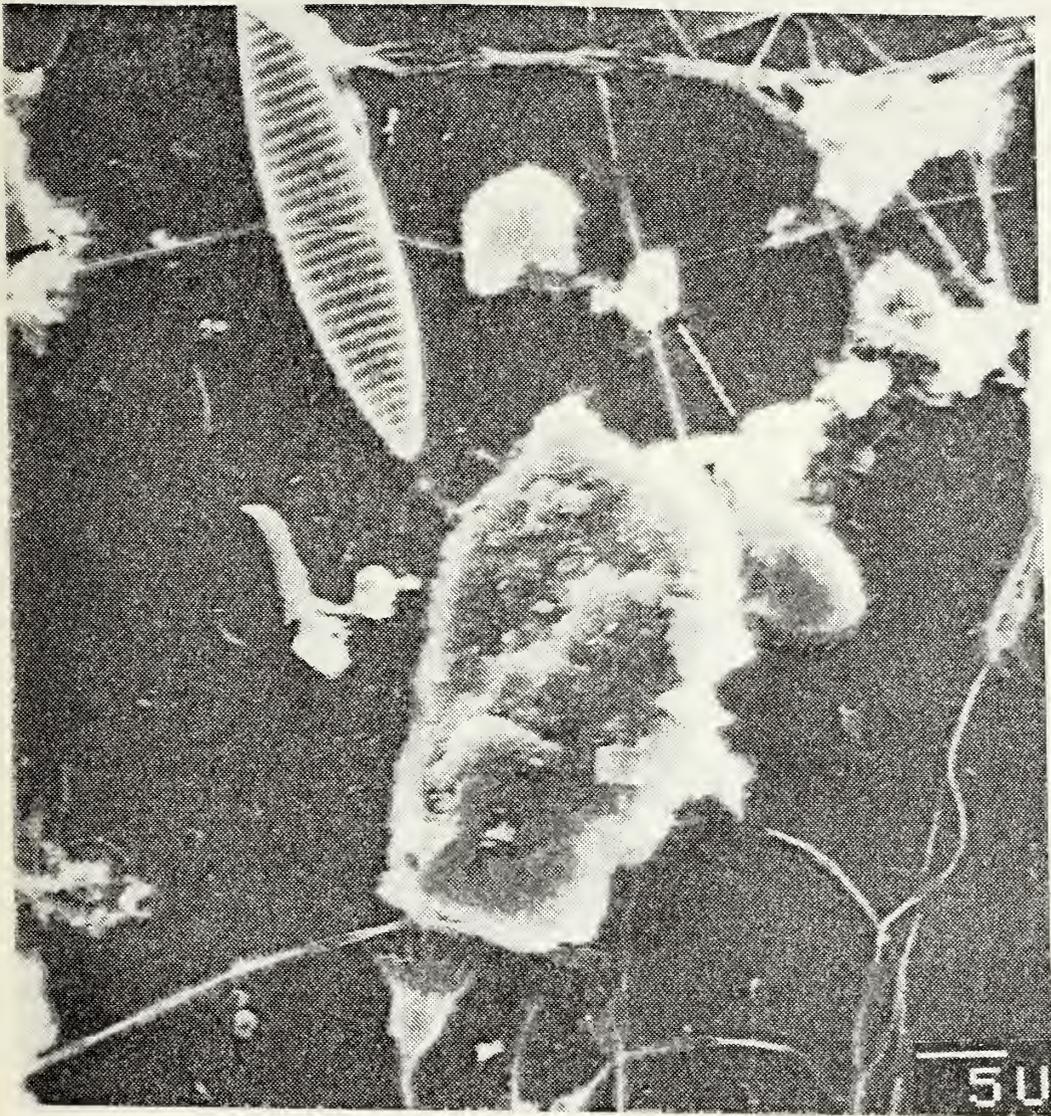
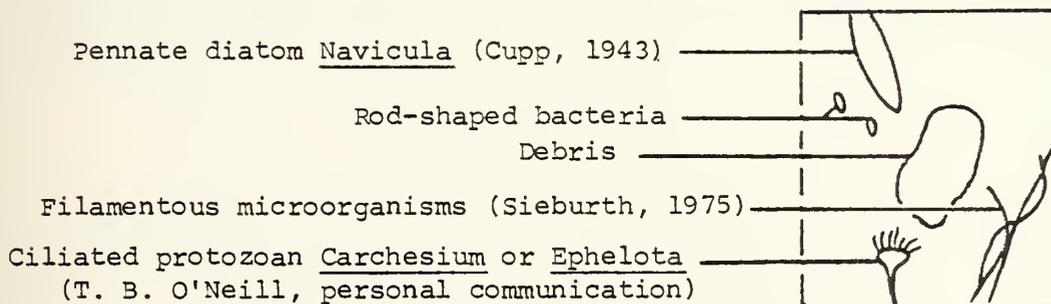


Plate 6. Glass immersed 26 days, 1500X. Pennate diatom, protozoan, filamentous microorganisms, bacteria and debris.



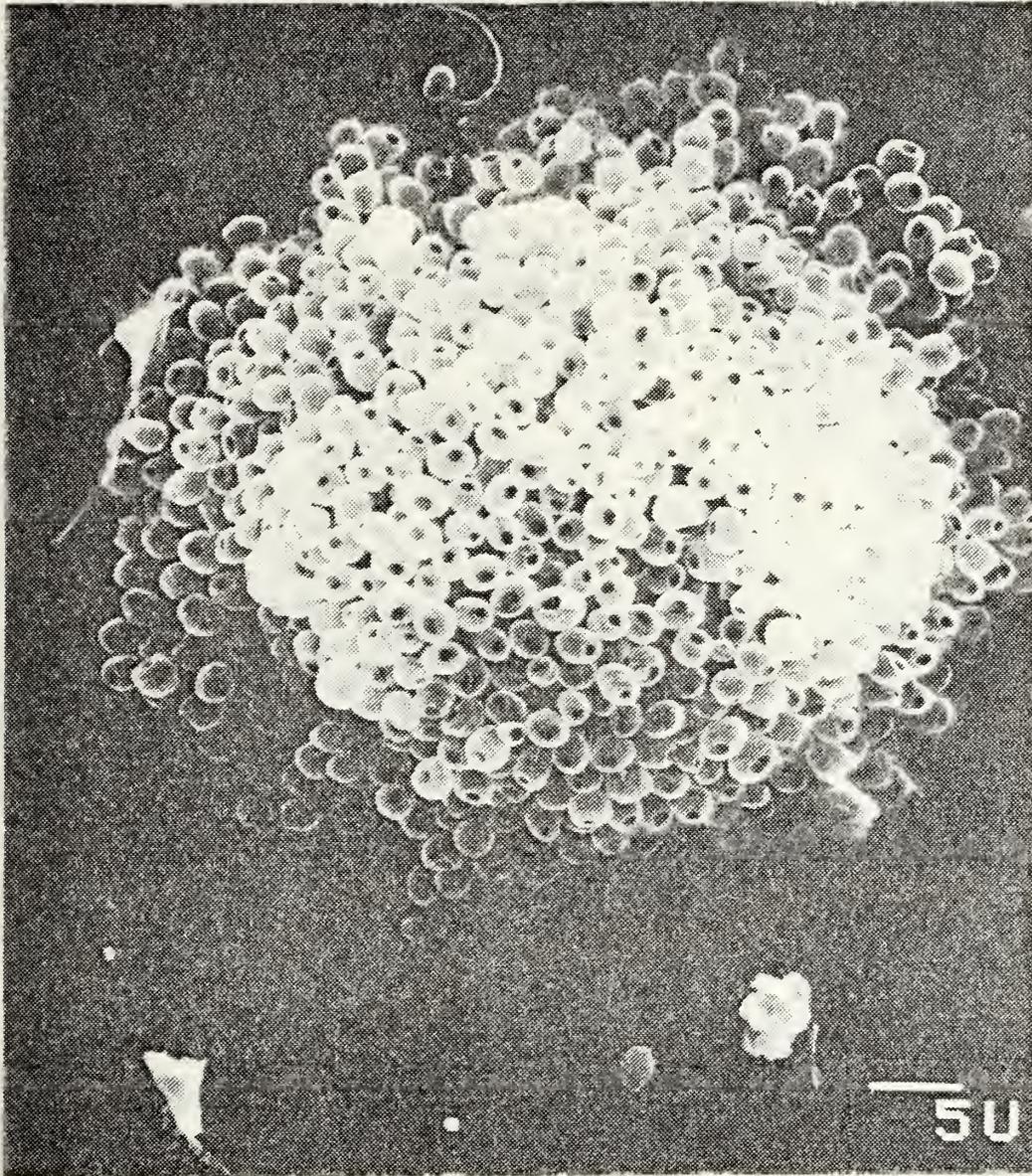


Plate 7. Glass immersed 22 days, 1500X. This unusual mass of cylindrical objects was identified as possibly being the exogenous buds of a ciliated protozoan, Ephelota gemminifera (T. B. O'Neill, personal communication).

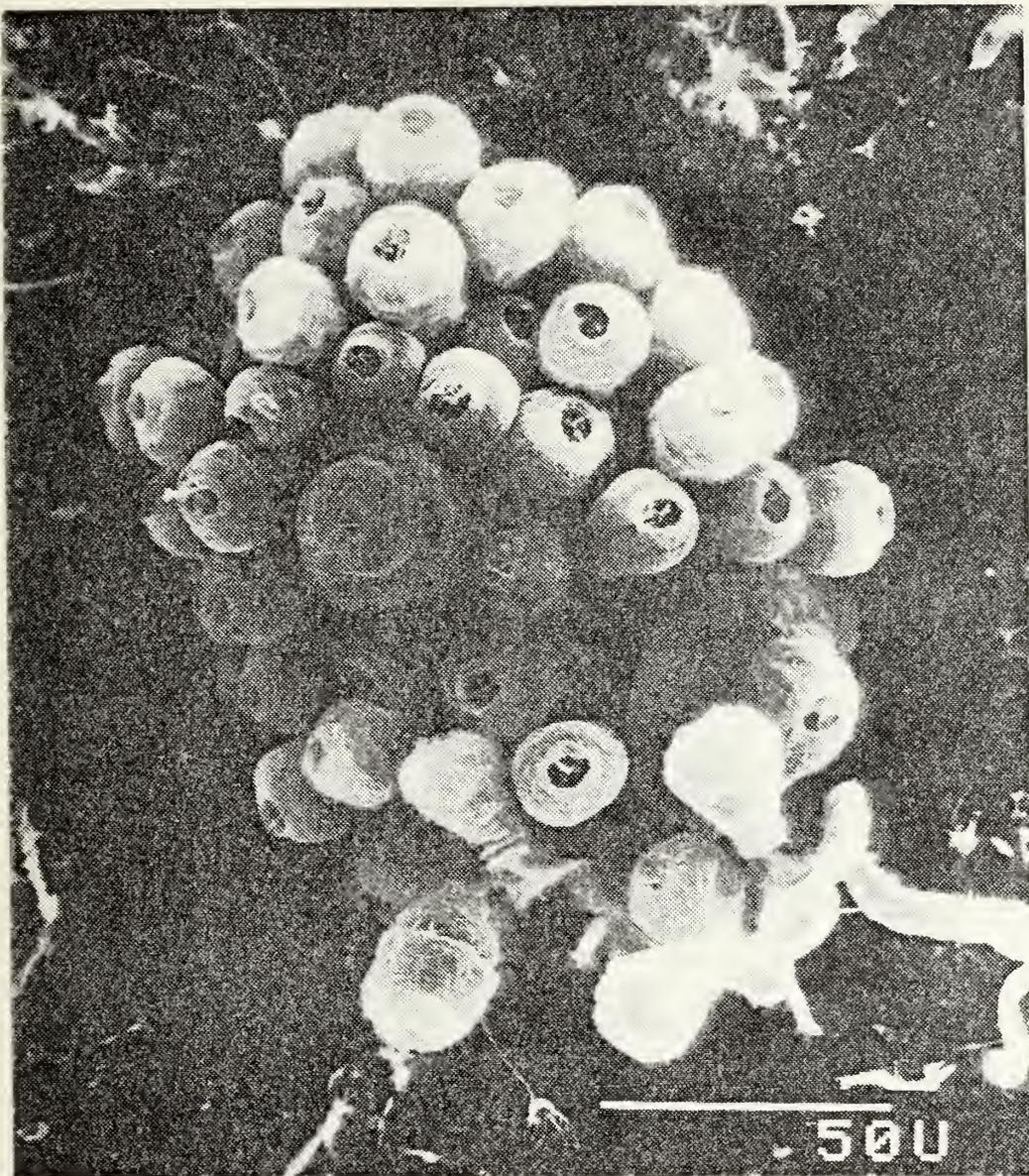


Plate 8. Glass immersed 49 days, 500X. The colonial protozoan, Zoothamnium (Sieburth, 1975, 1979).

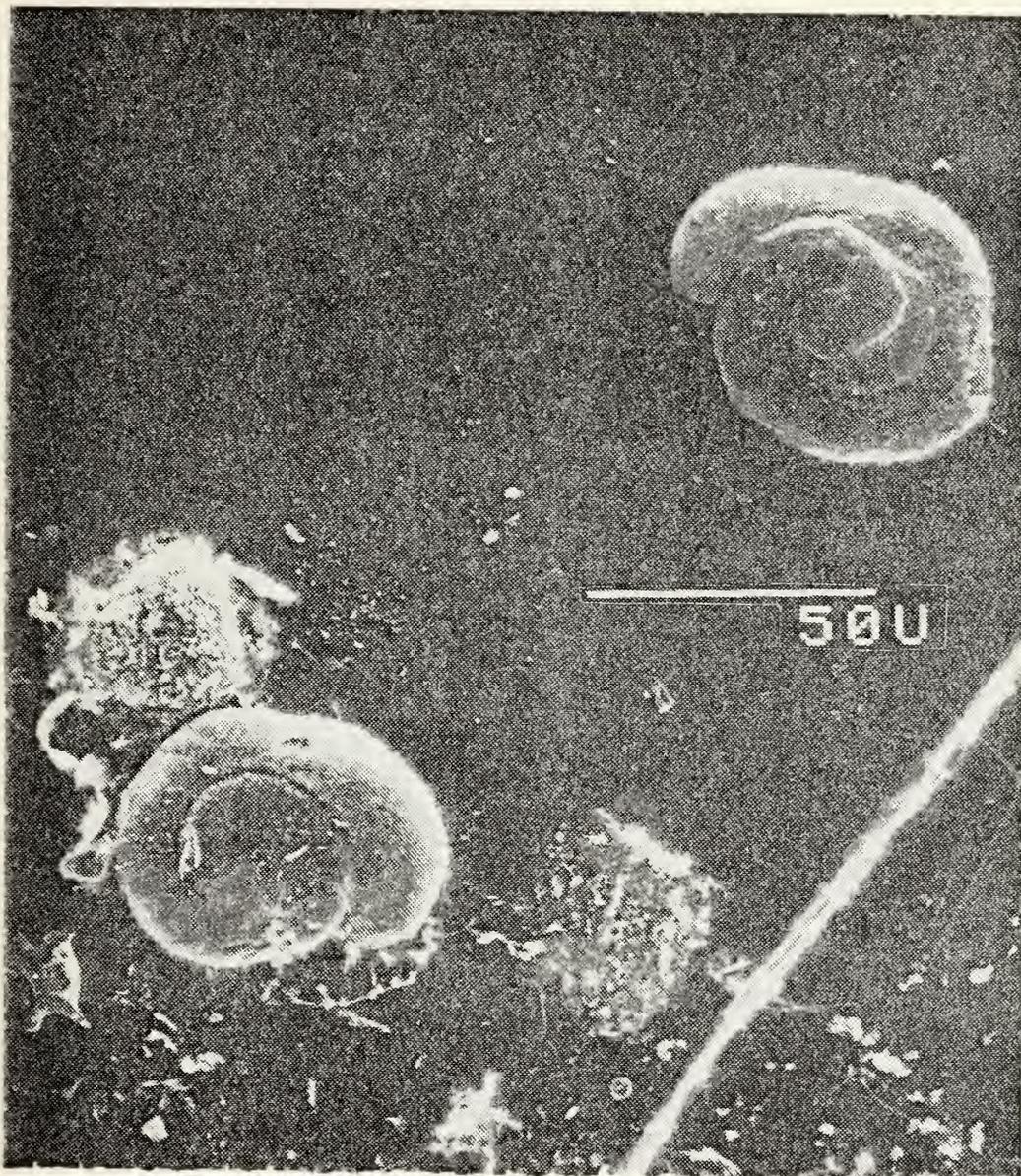


Plate 9. Glass immersed 83 days, 500X. Two shelled protozoans called foraminifera (Stinemeyer and Reiter, 1958).



Plate 10. Glass immersed 49 days, 30X. Bryozoan colony surrounded by many microscopic protozoans (Morris et. al., 1980).



Plate 11. Glass immersed 49 days, 150X. Close-up of a bryozoan colony showing four opercula.

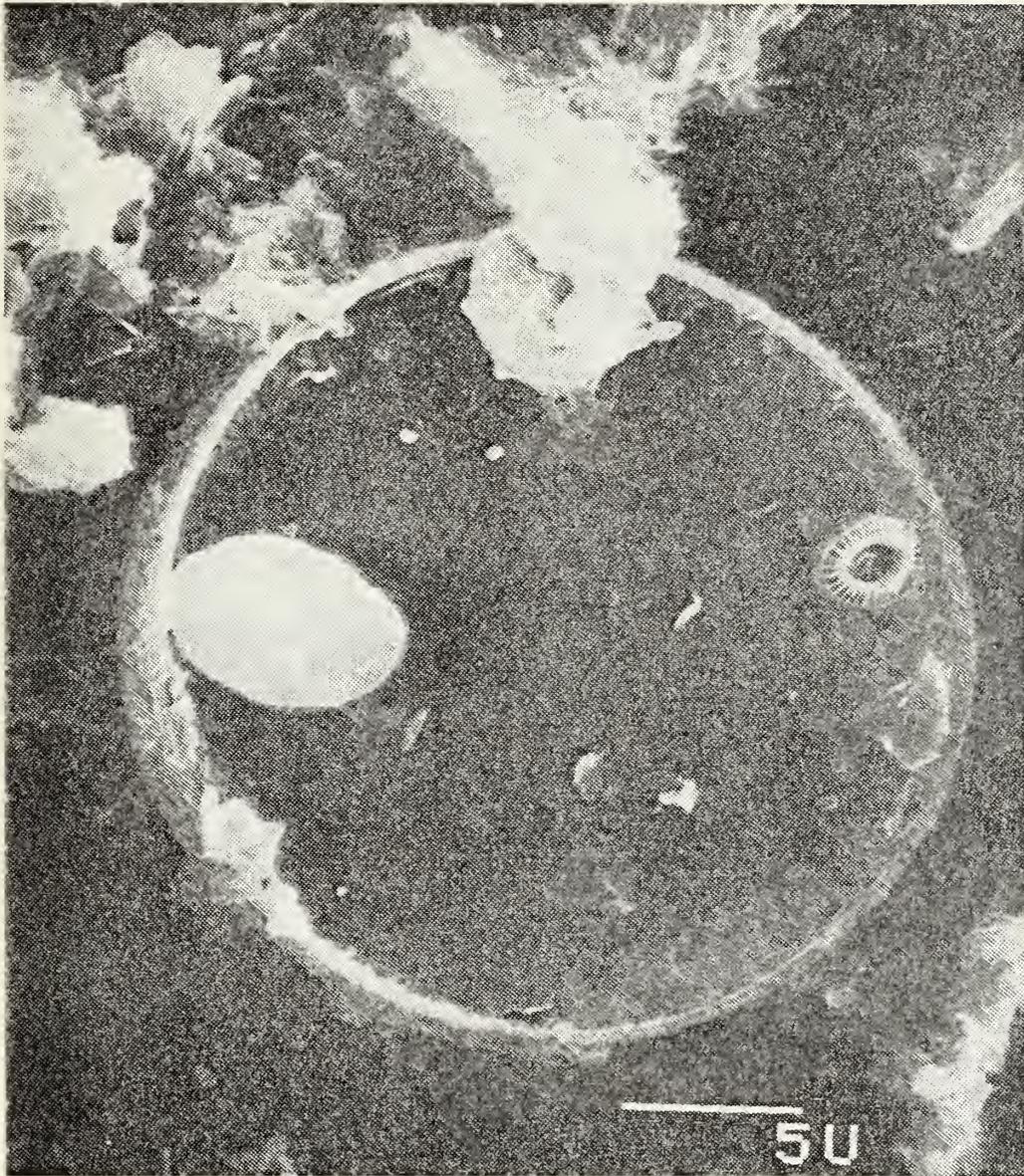


Plate 12. Glass immersed 83 days, 3000X. Centric diatom with a coccolith plate on the surface (Cupp, 1943).

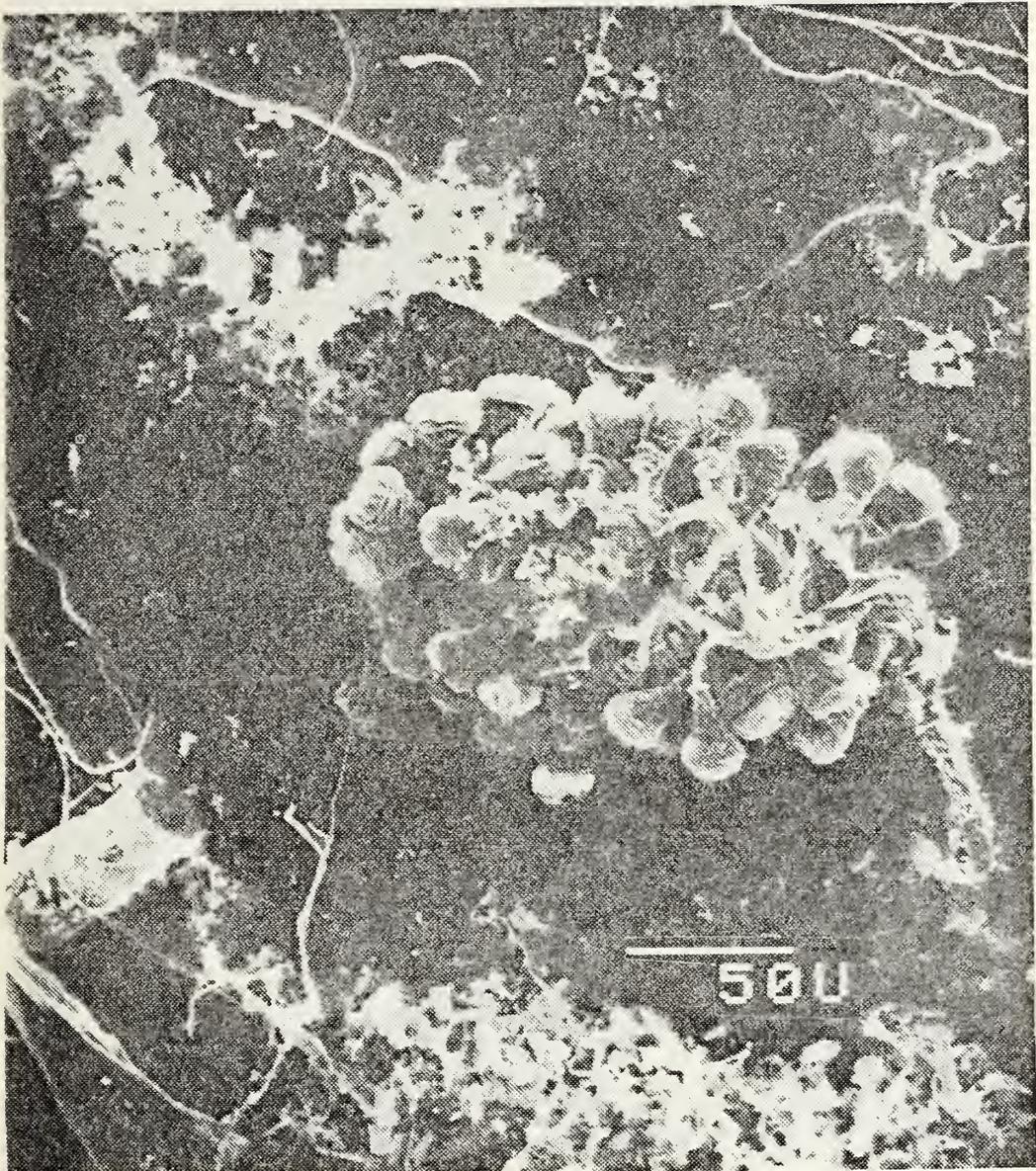
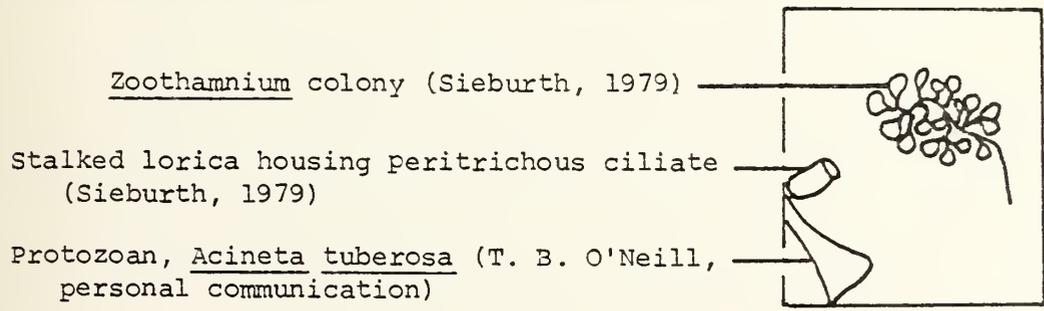


Plate 13. Glass immersed 49 days, 280X. Zoothamnium colony and some singular protozoans.



Zoothamnium colony (Sieburth, 1979)

Stalked lorica housing peritrichous ciliate (Sieburth, 1979)

Protozoan, Acineta tuberosa (T. B. O'Neill, personal communication)



Plate 14. Glass immersed 49 days, 700X. The protozoan *Acineta tuberosa* (T. B. O'Neill, personal communication; Sieburth, 1979).

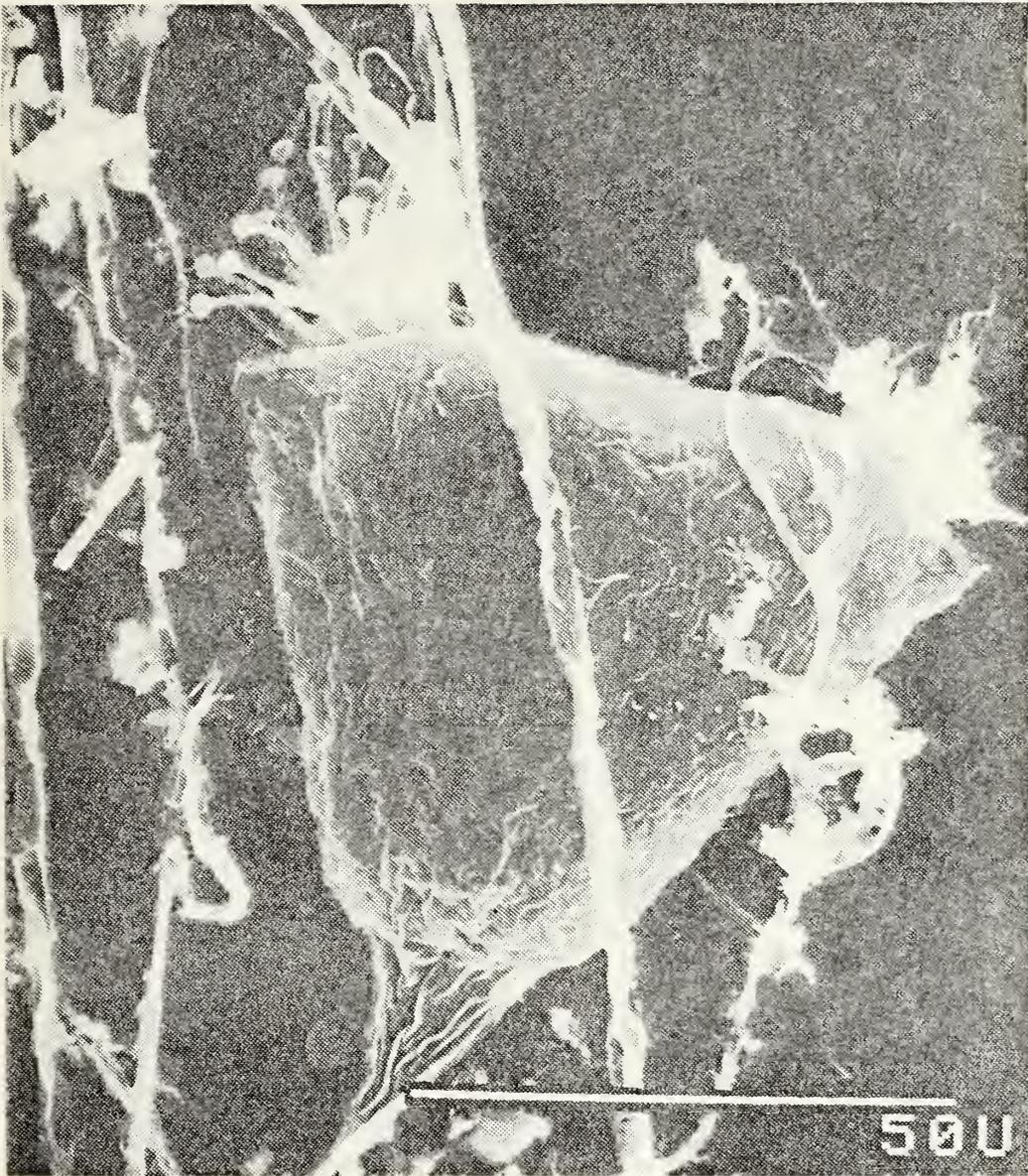
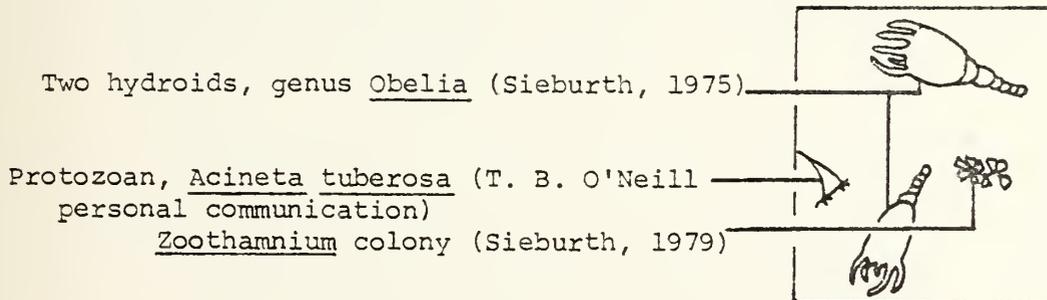


Plate 15. Glass immersed 49 days, 940X. A solitary protozoan, possibly Acineta (T. B. O'Neill, personal communication; Sieburth, 1979).



Plate 16. Glass immersed 49 days, 110X. Two hydroids of the genus *Obelia* surrounded by protozoans.



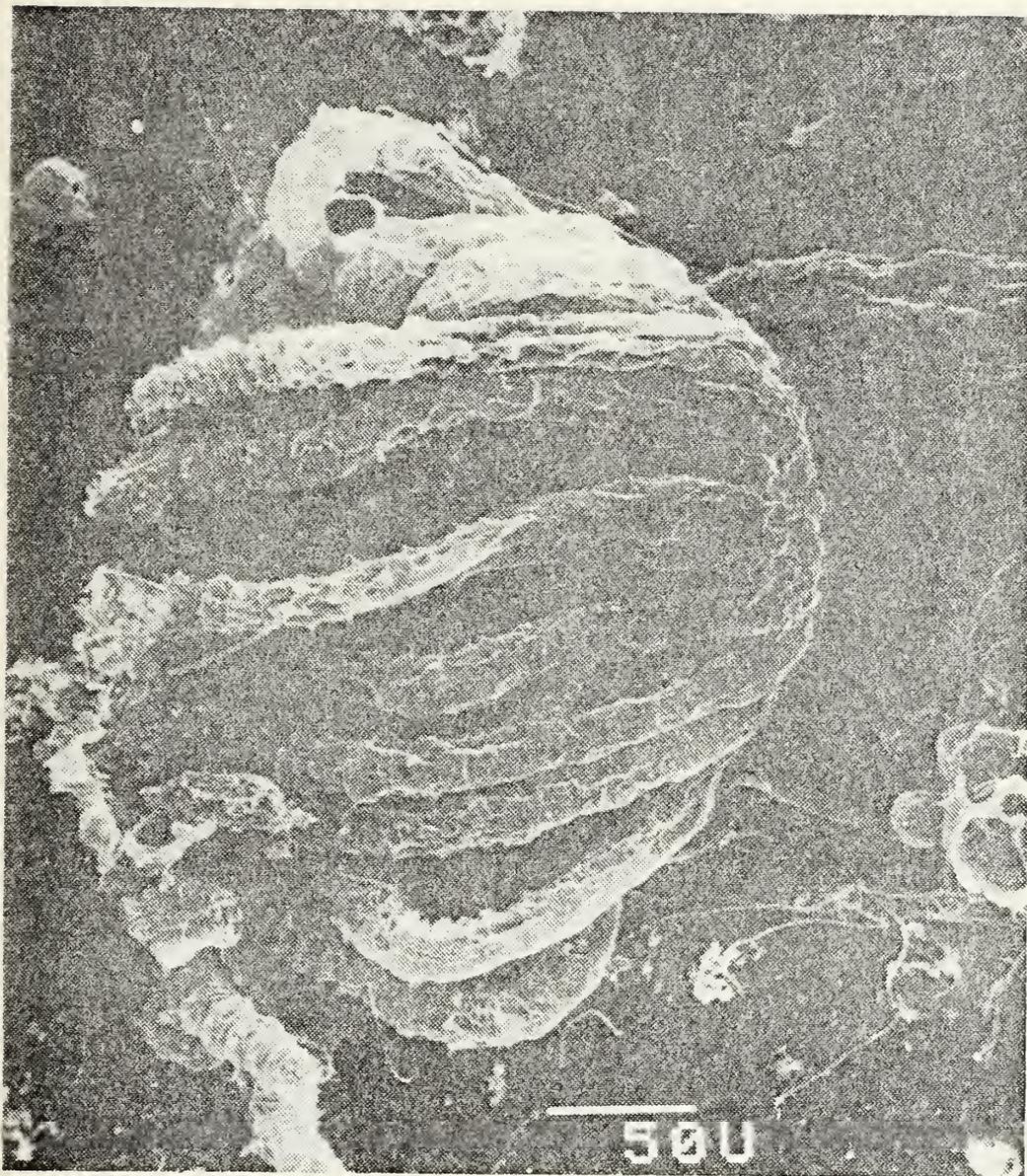


Plate 17. Glass immersed 49 days, 250X. Close-up of the hydroid Obelia. Note what appear to be spikes which cover the surface of the tentacles. These are the triggering mechanisms for the stinging cells or the nematocysts (Sieburth, 1975).



Plate 18. Glass immersed 49 days, 130X. Large unknown organism surrounded by protozoans.

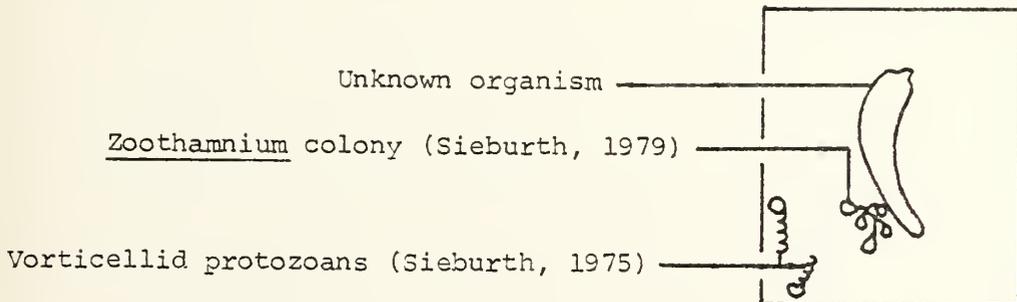




Plate 19. Glass immersed 83 days, 1600X. The planktonic diatom Nitzschia closterium (Cupp, 1943).

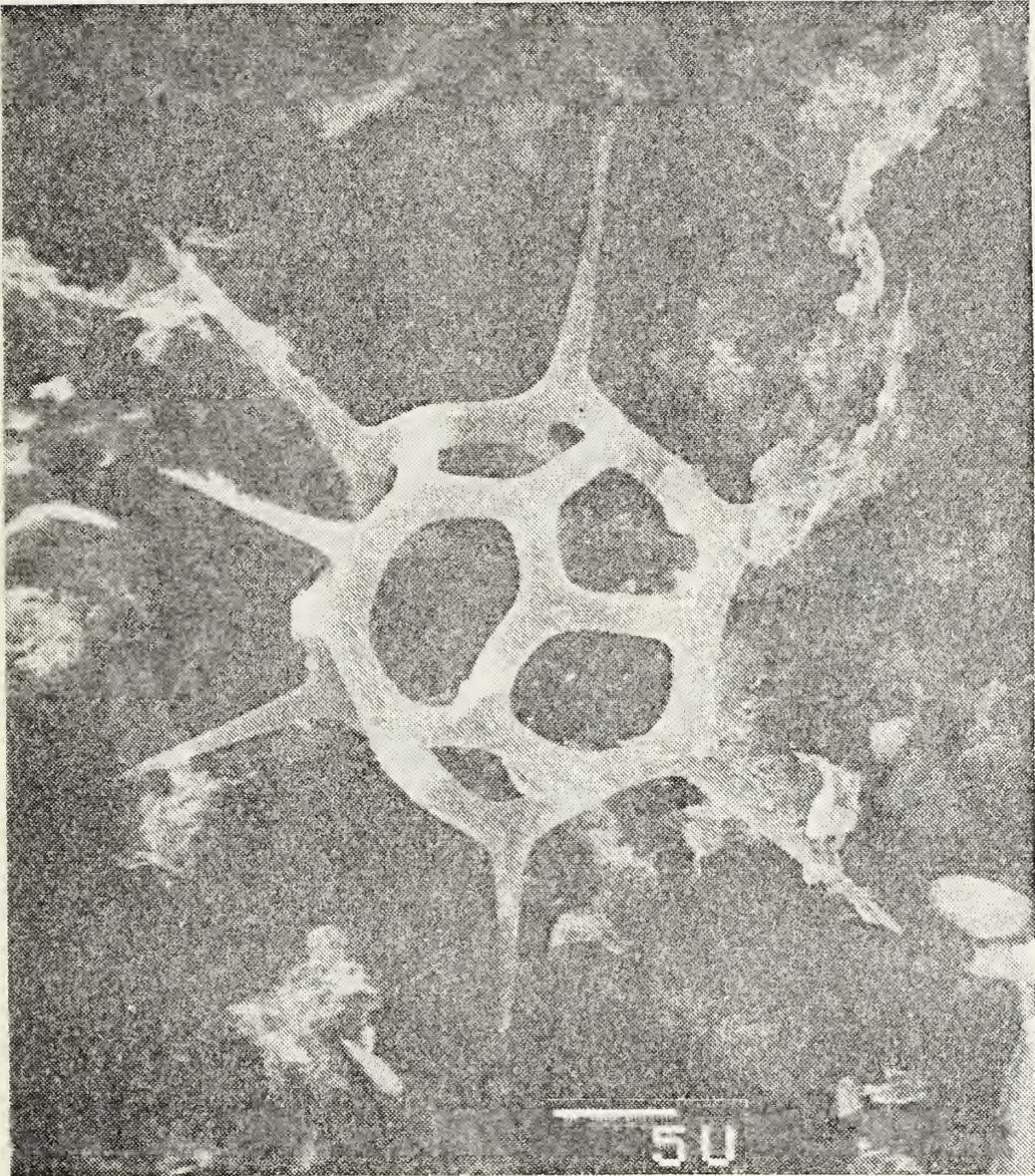


Plate 20. Glass immersed 83 days, 2000X. The skeleton of a single-celled flagellated plant known as a silicoflagellate (I. Abbott, personal communication; Sieburth, 1975).

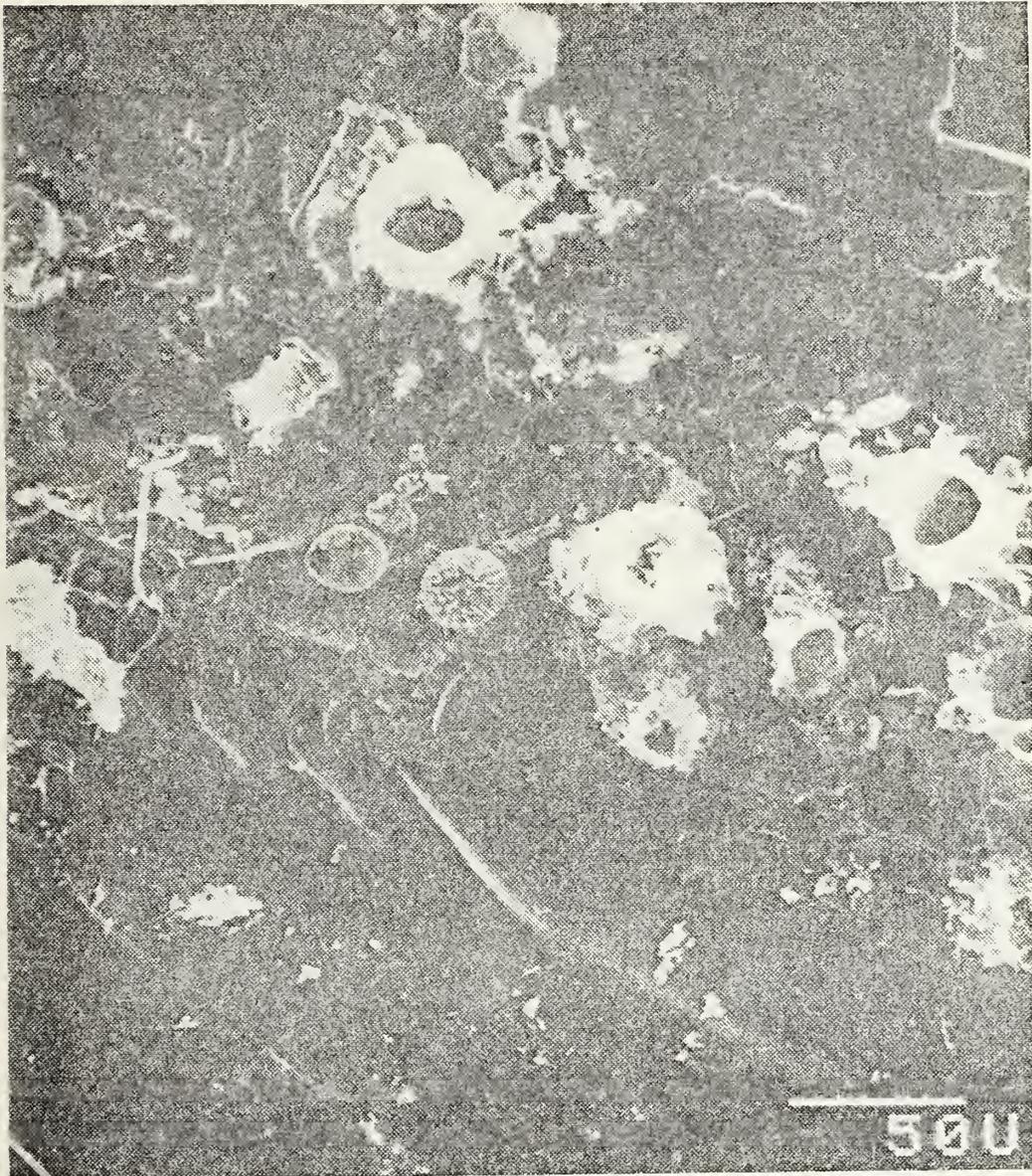


Plate 21. Glass immersed 83 days, 300X. Several peritrichous ciliates within protective loricas or houses and two unknown ciliated bodies.

Loricas housing peritrichous ciliates
(Sieburth, 1979)

Unknown ciliated organisms

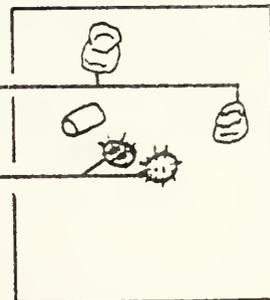




Plate 22. Glass immersed 83 days, 2700X. Close-up of an unknown ciliated body.



Plate 23. Glass coated with paint Formula 196D immersed 30 days, 1500X. Rod-shaped bacteria, debris, and a filamentous microorganism.

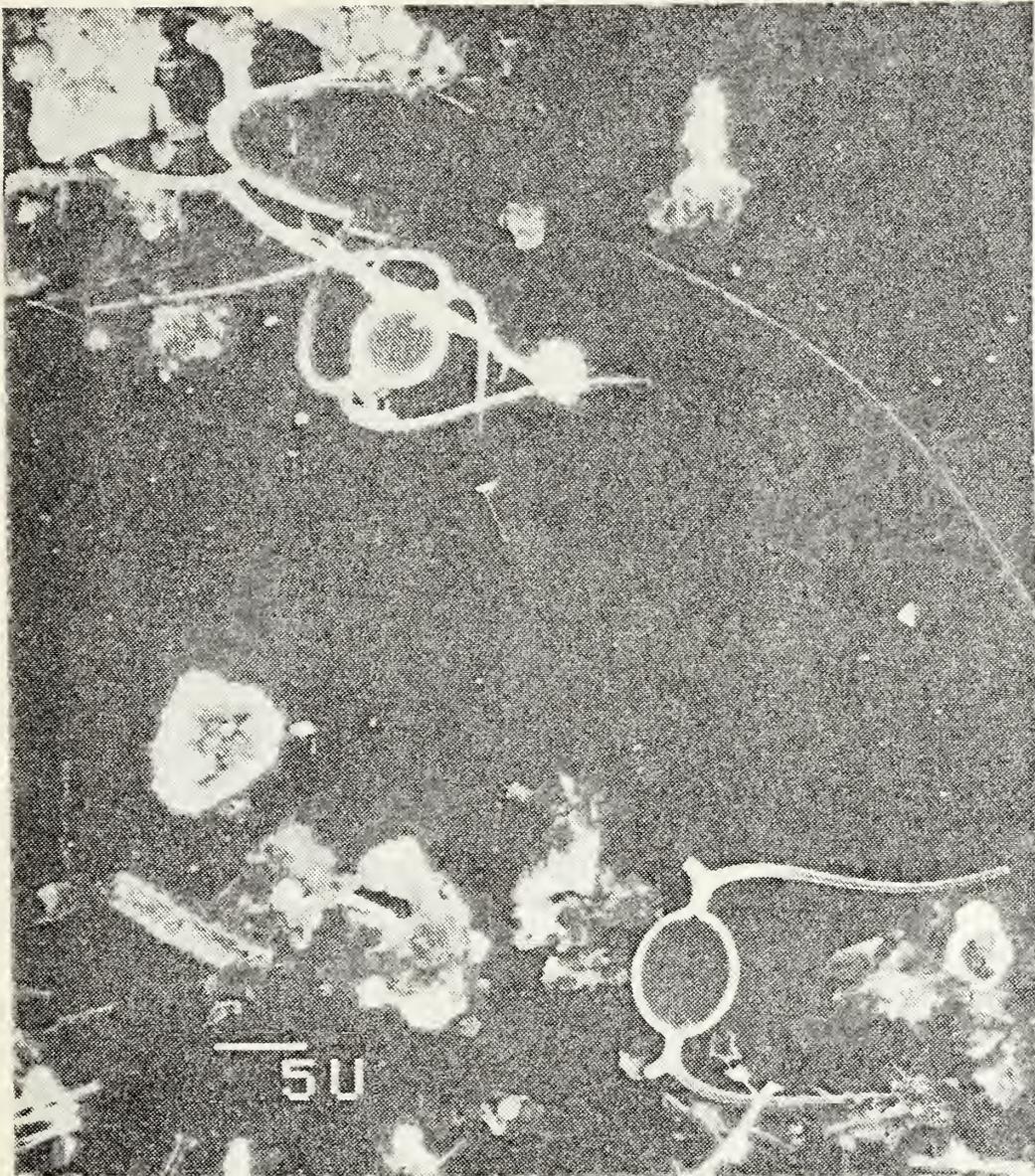
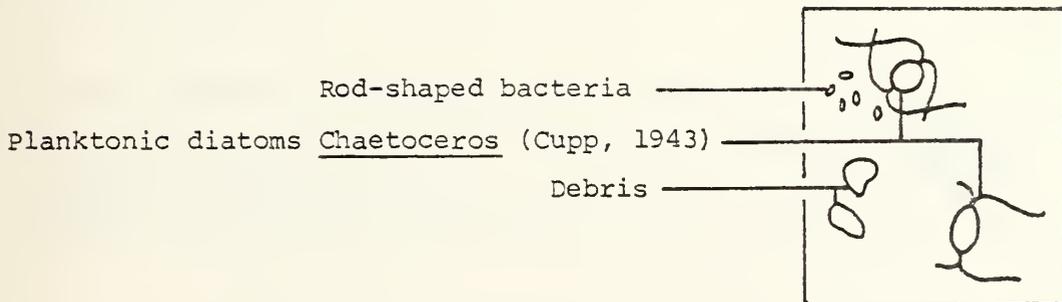


Plate 24. Glass coated with paint Formula 196D immersed 10 days, 1500X. Two planktonic diatoms, bacteria, and debris.



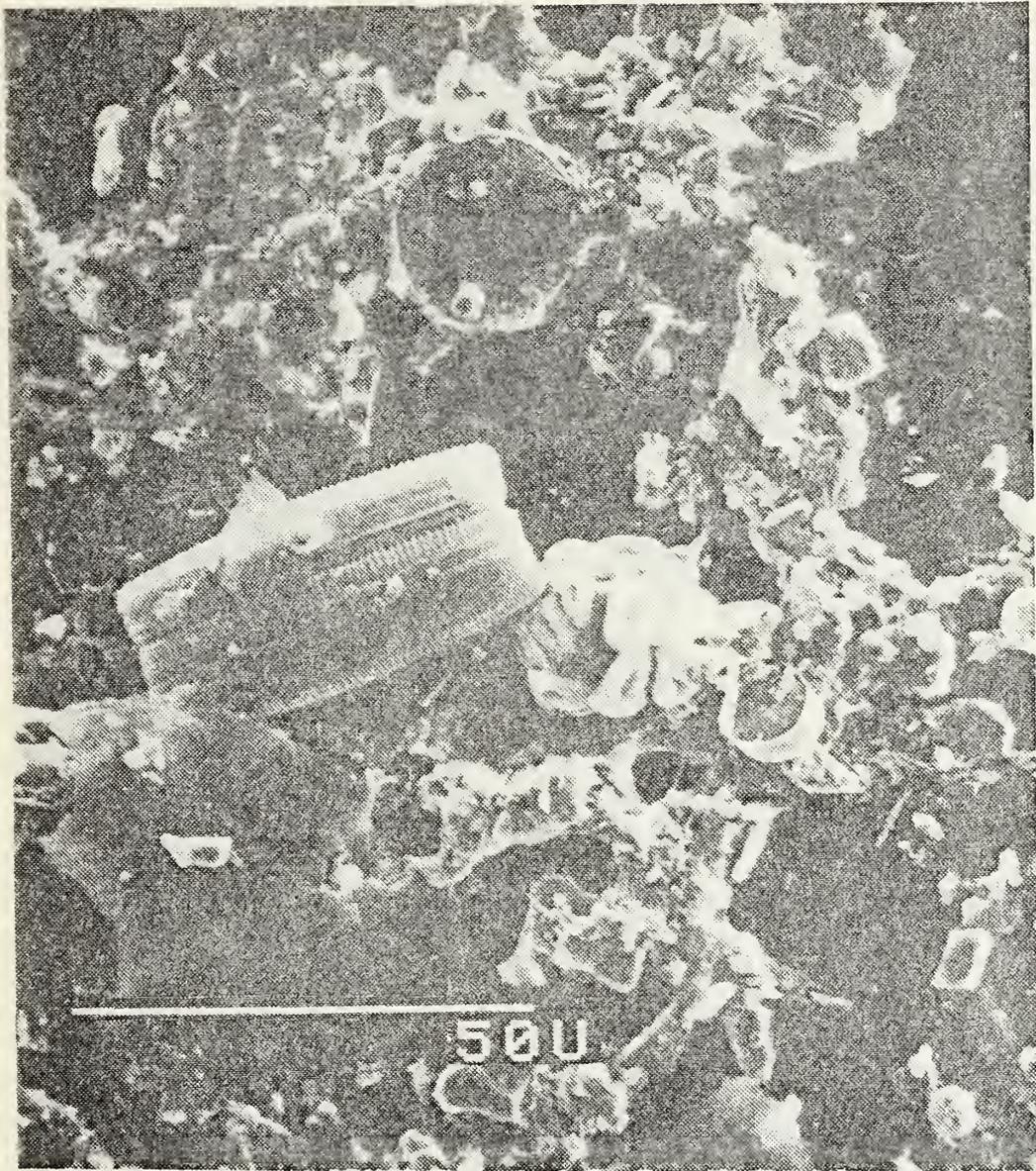
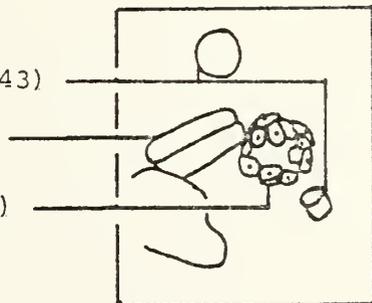


Plate 25. Glass coated with paint Formula 196D immersed 83 days, 800X. Several centric diatoms, a broken coccolithophorid, and debris.

Centric diatoms, Thalassiosira (Cupp, 1943)

Centric diatom dividing (E. C. Haderlie, personal communication)

Broken coccolithophorid (Sieburth, 1979)



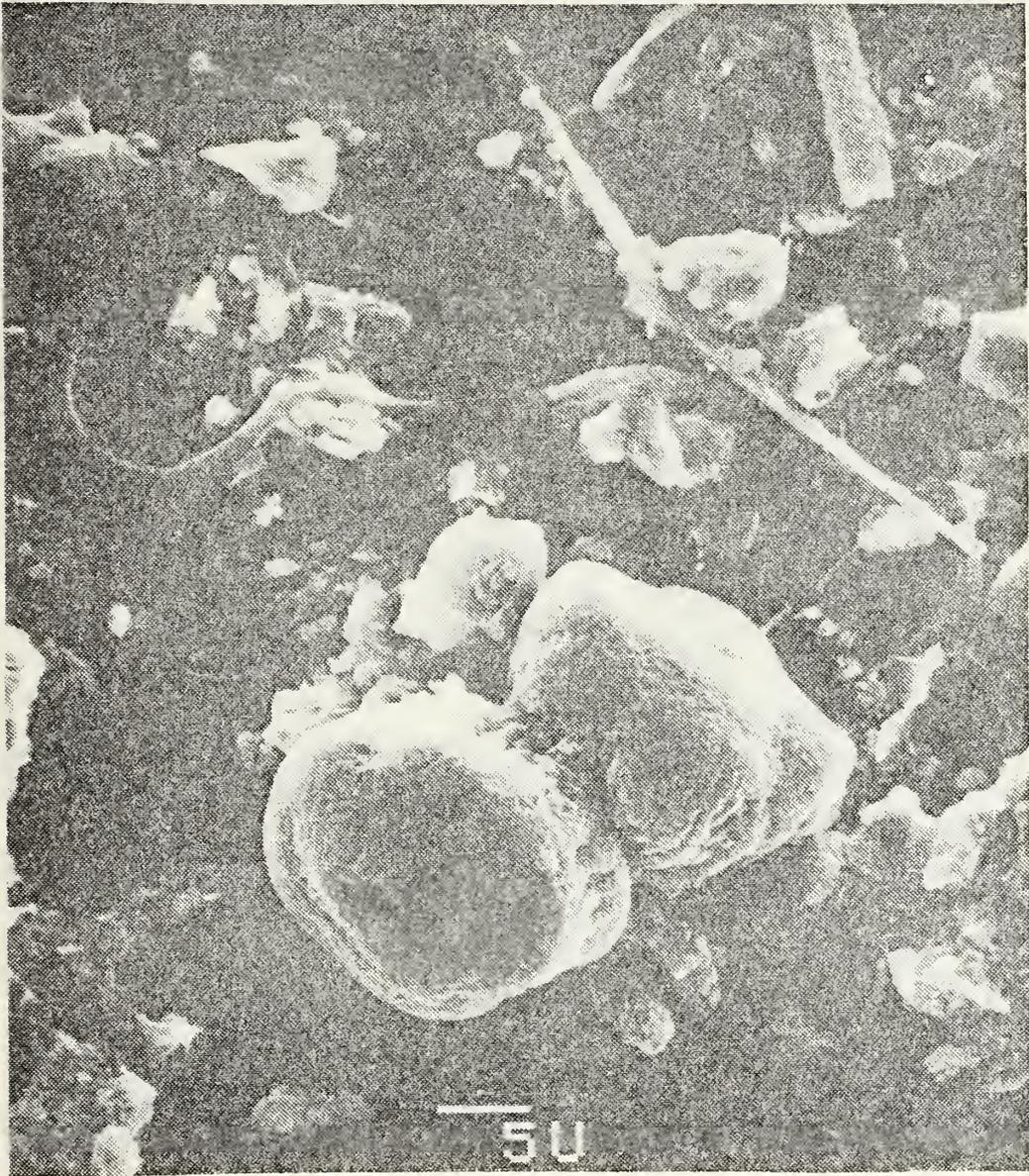


Plate 26. Glass coated with paint Formula 196D immersed 83 days, 1500X. Unknown bulbous-like organism.

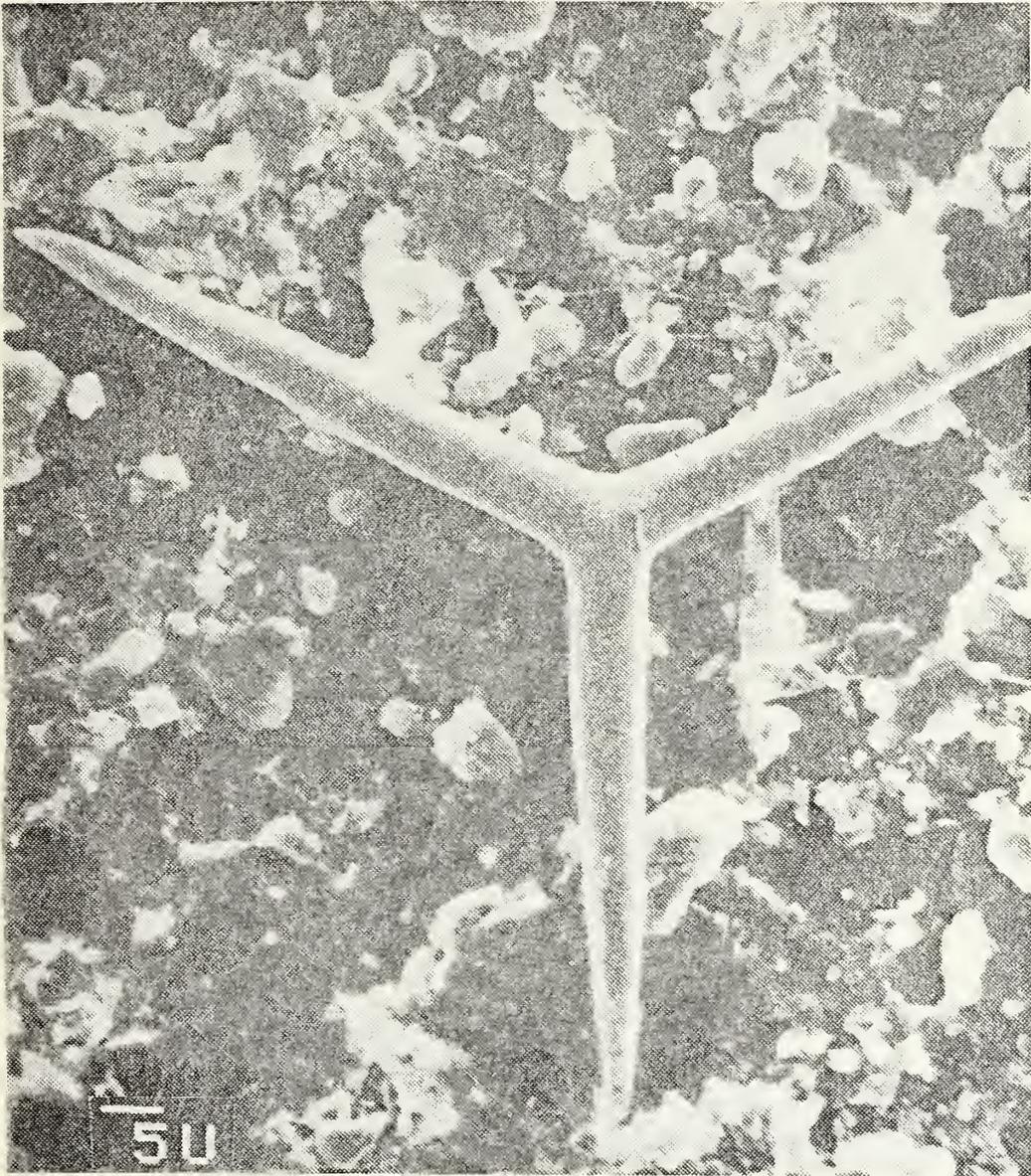


Plate 27. Glass coated with paint Formula 196D immersed 83 days, 1000X. Sponge spicule possibly from the genus Leucosolenia (Light, 1975).

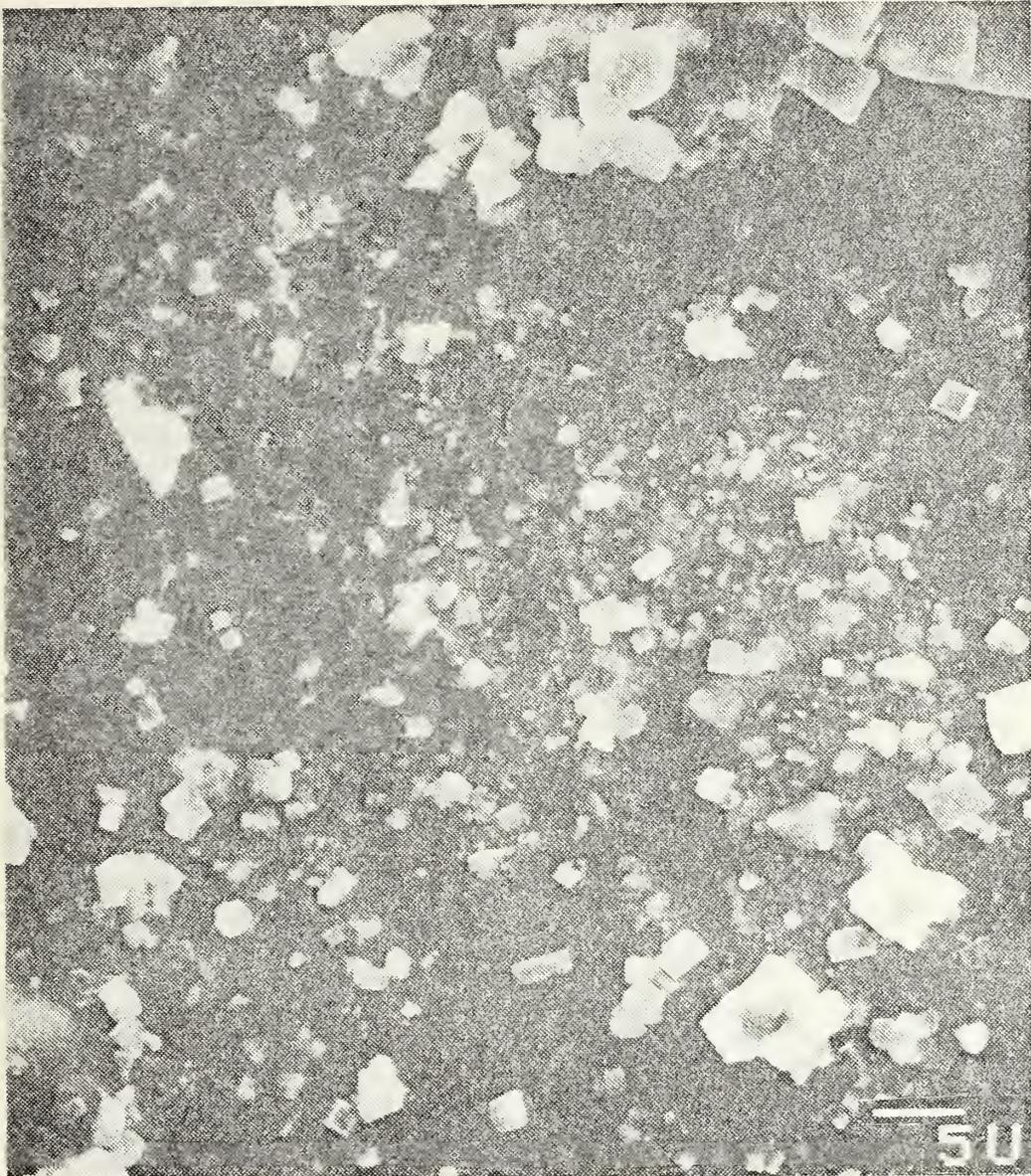


Plate 28. Glass coated with paint Formula 1114 immersed 4 days, 1500X. Surface composed of paint artifacts, salt crystals, and some debris.



Plate 29. Glass coated with paint Formula 1114 immersed 83 days, 1000X. The planktonic diatom, Biddulphia longricuris (Cupp, 1943).

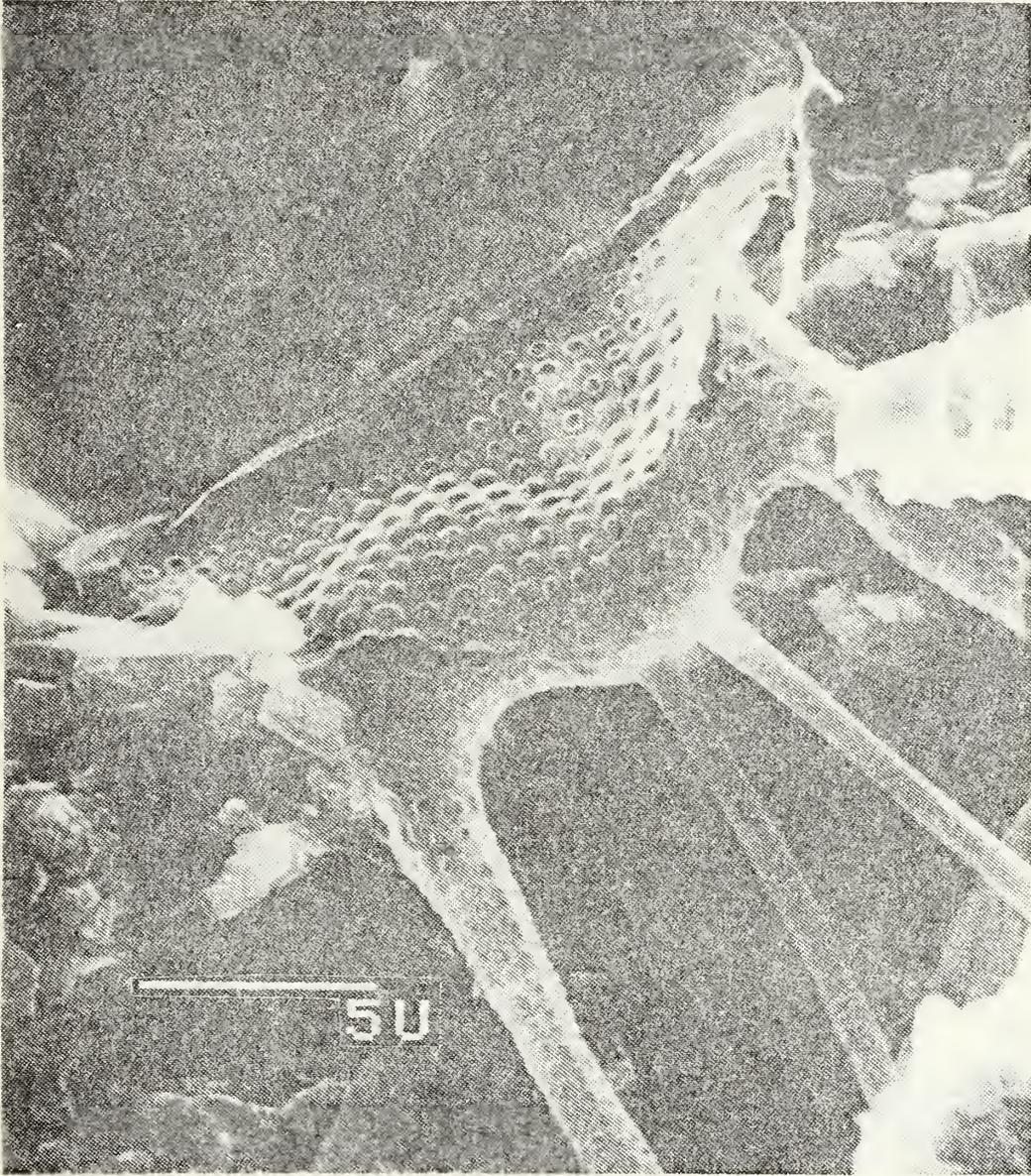


Plate 30. Glass coated with paint Formula 1114 immersed 83 days, 4000X. Close-up of the diatom Biddulphia longicruris (Cupp, 1943).



Plate 31. Glass coated with paint Formula 1114 immersed 83 days, 1500X. Several broken diatoms of the genus Thalassiosira (Cupp, 1943).

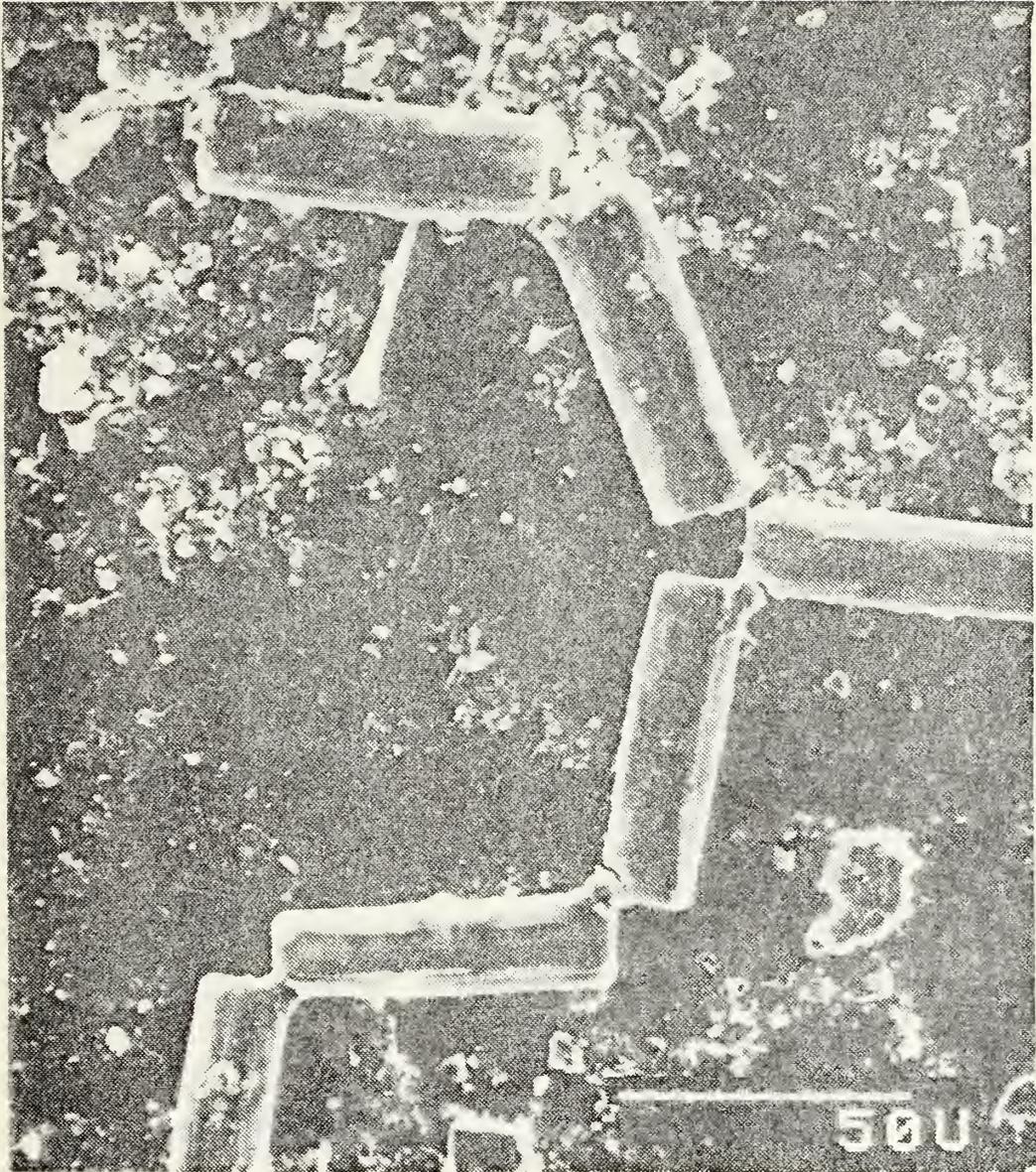


Plate 32. Glass immersed 14 days, 500X. Linked rectangular diatoms of the genus Thalassionema (I. Abbott, personal communication; Cupp, 1943).

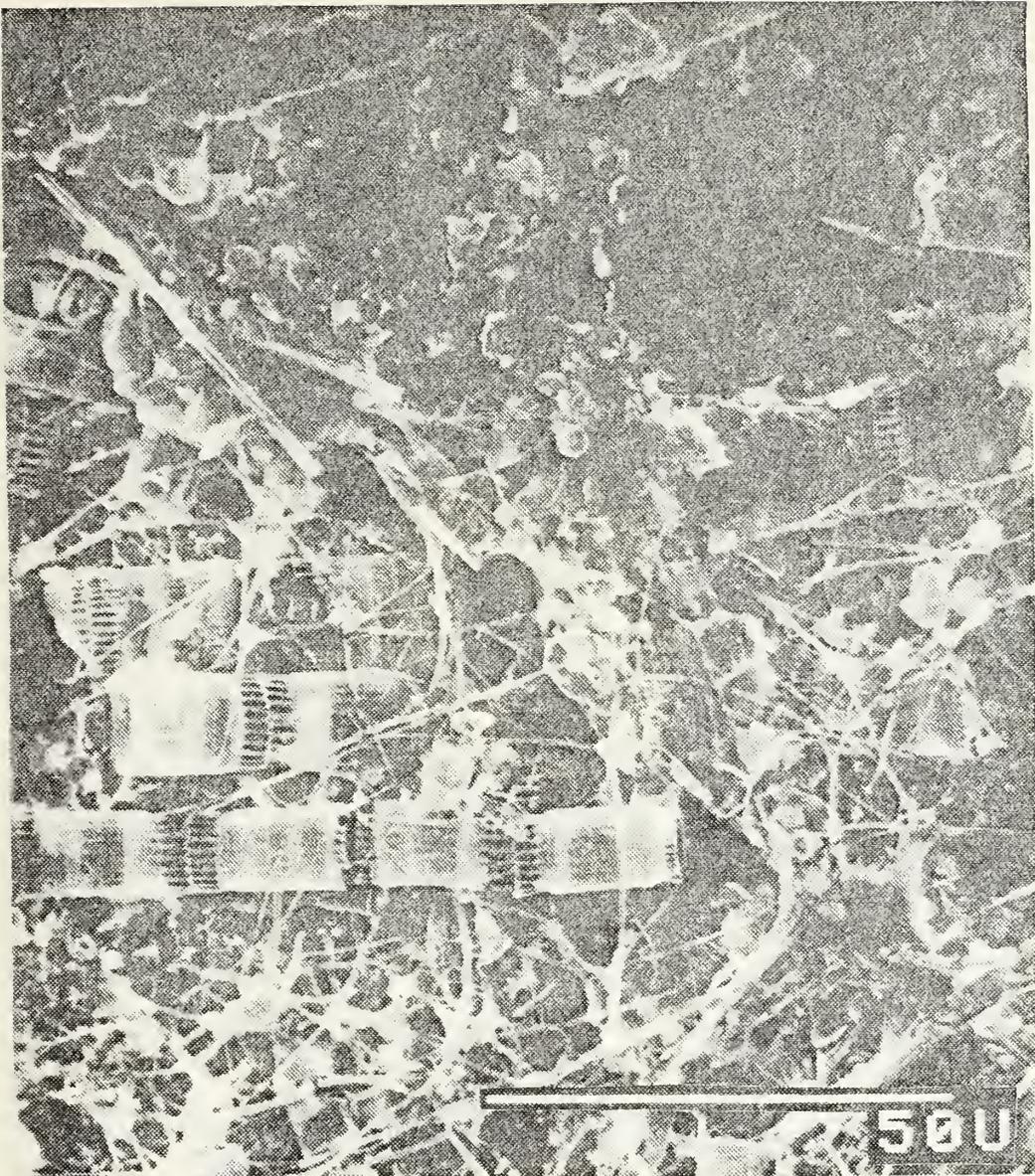
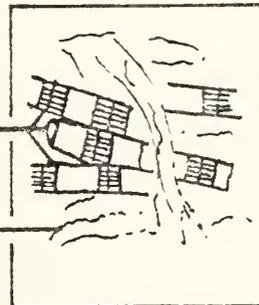


Plate 33. Glass immersed 14 days, 800X. Centric diatoms, entangled with debris on the surface of a worm casing.

Centric diatoms, Skeletonema costatum
(Cupp, 1943)

Worm tube casing



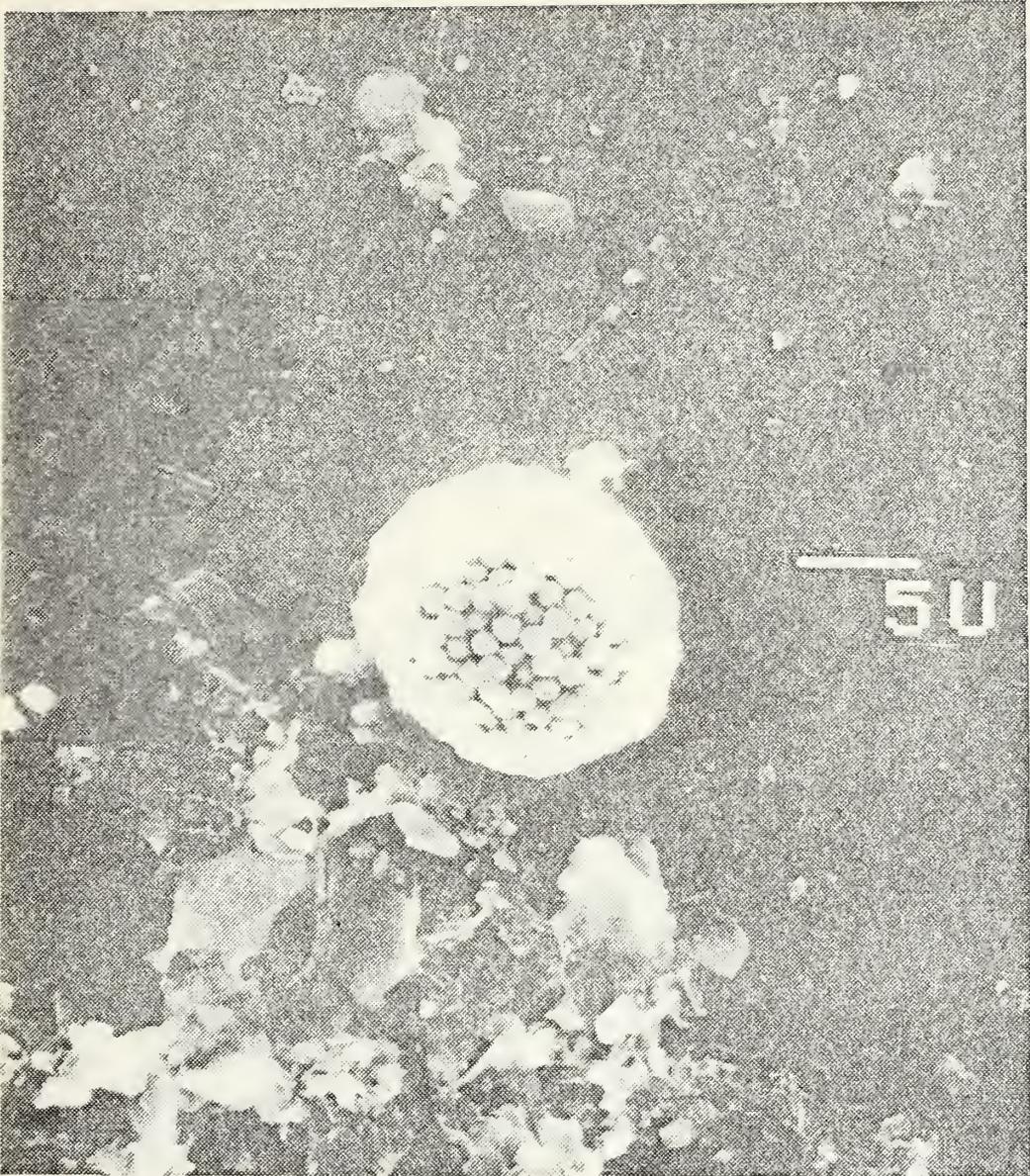


Plate 34. Glass immersed 14 days, 1500X. Unknown object, possibly a transparent sack containing eggs

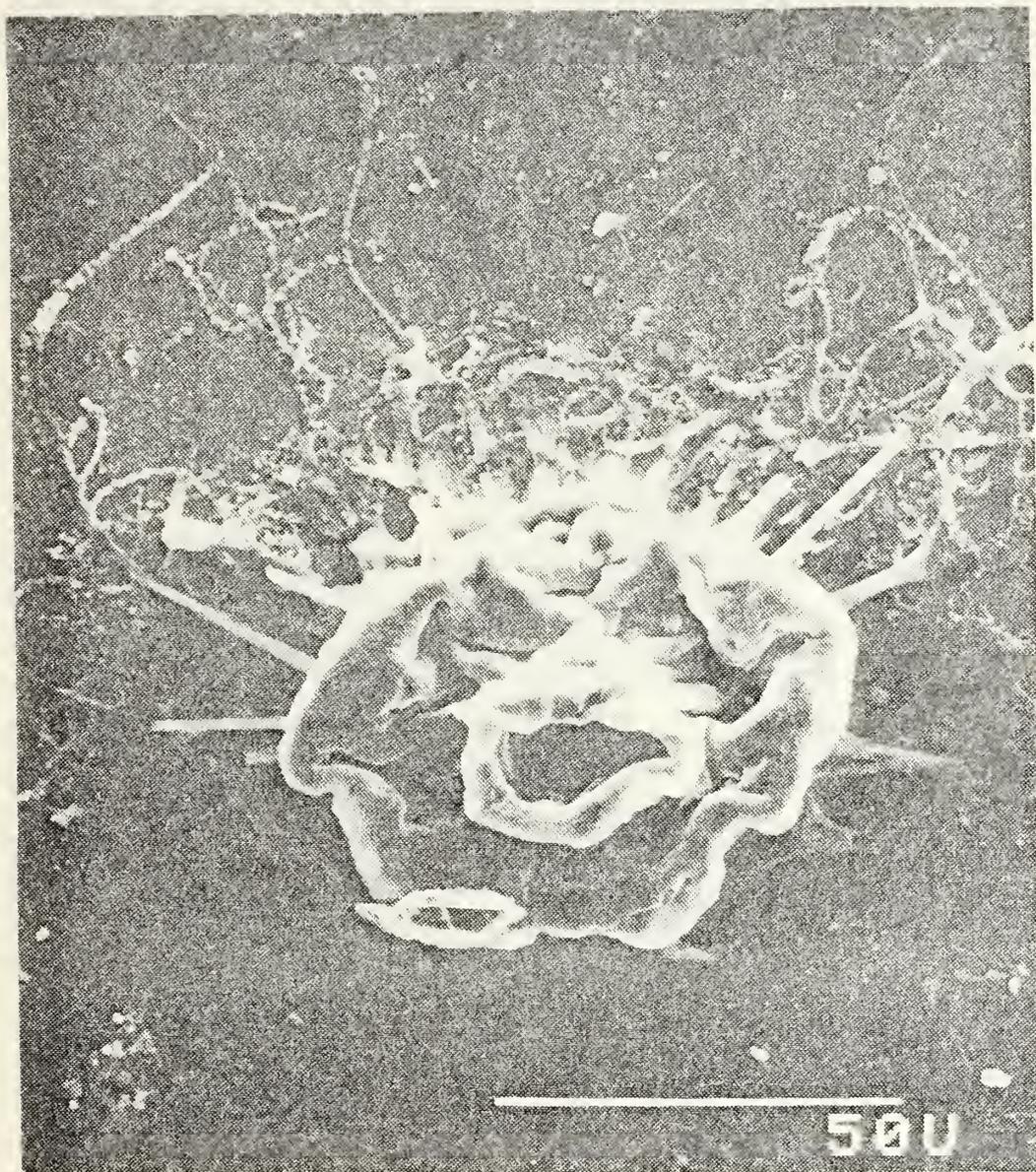


Plate 35. Glass immersed 14 days, 700X. Unknown attached organism. Note the hold-fast structures.

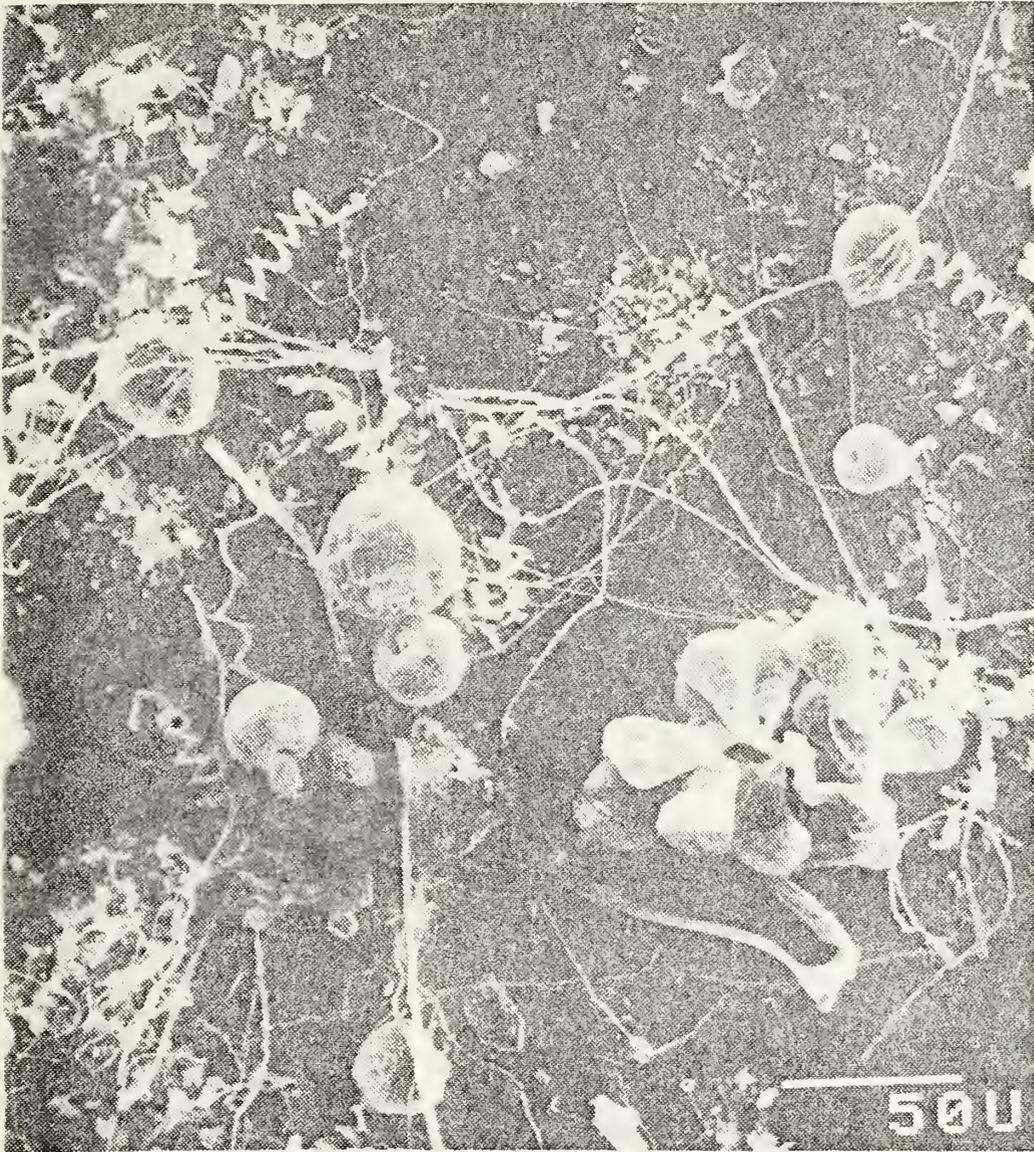
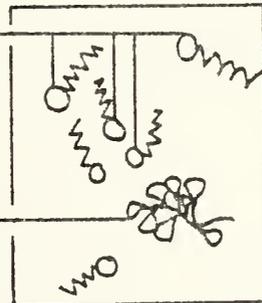


Plate 36. Glass immersed 30 days, 300X. One colonial protozoan surrounded by several solitary protozoans.

Vorticellid protozoans (Sieburth, 1975)

Zoothamnium colony (Sieburth, 1979)



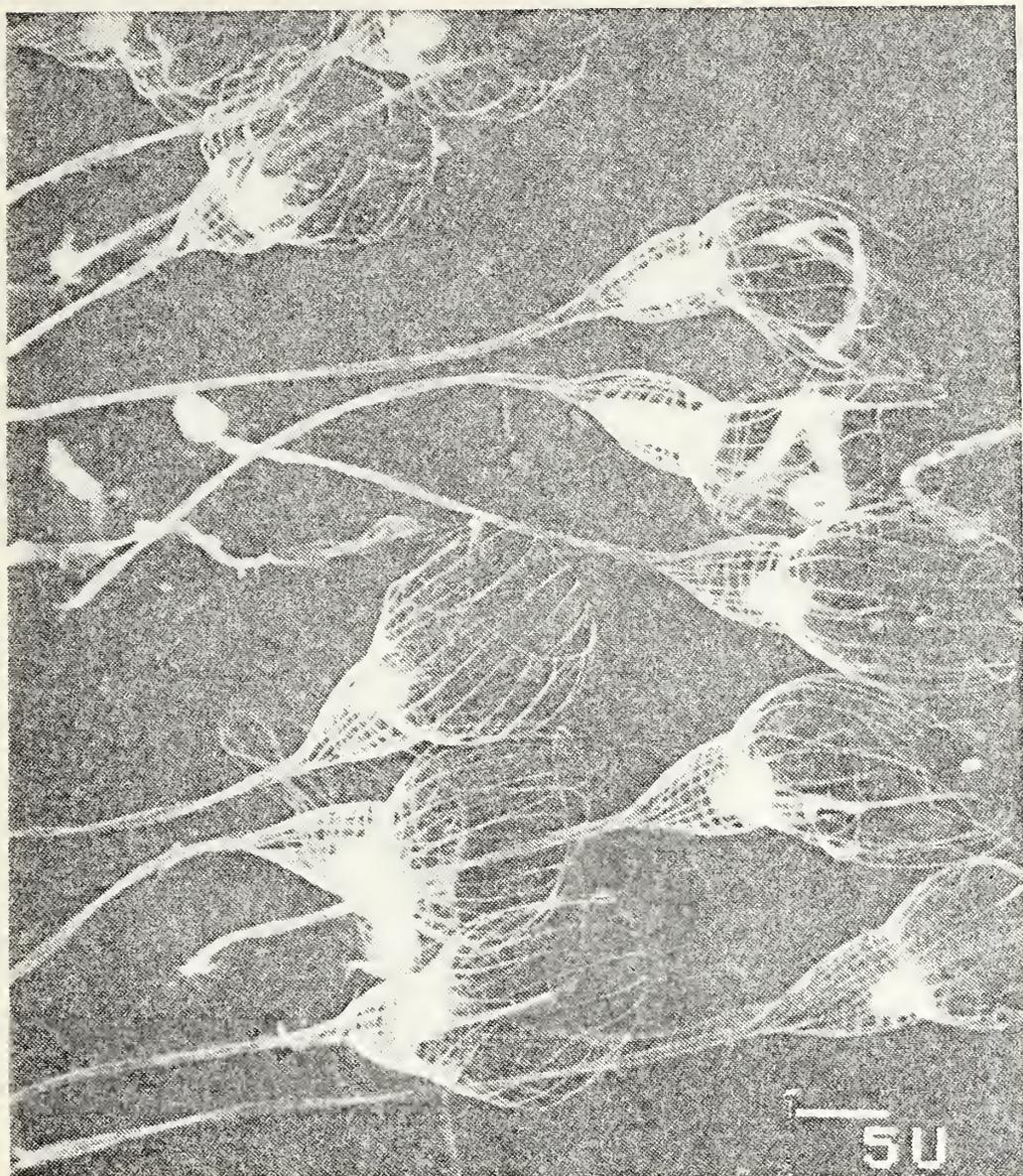


Plate 37. Glass immersed 30 days, 1500X. Several stalked protozoans known as choanoflagellates (Sieburth, 1979).

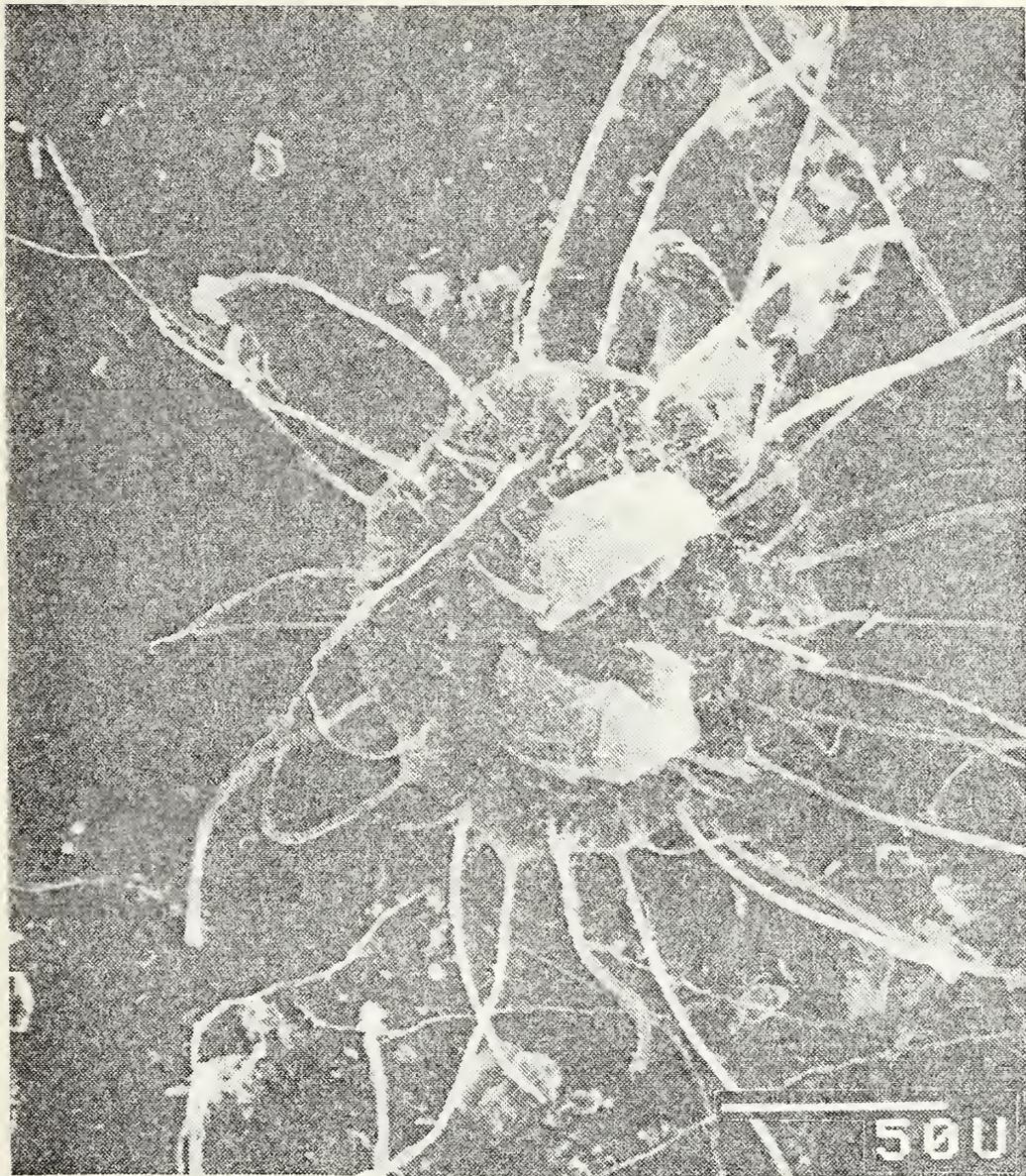


Plate 38. Glass immersed 30 days, 380X. Unknown ciliated organism.

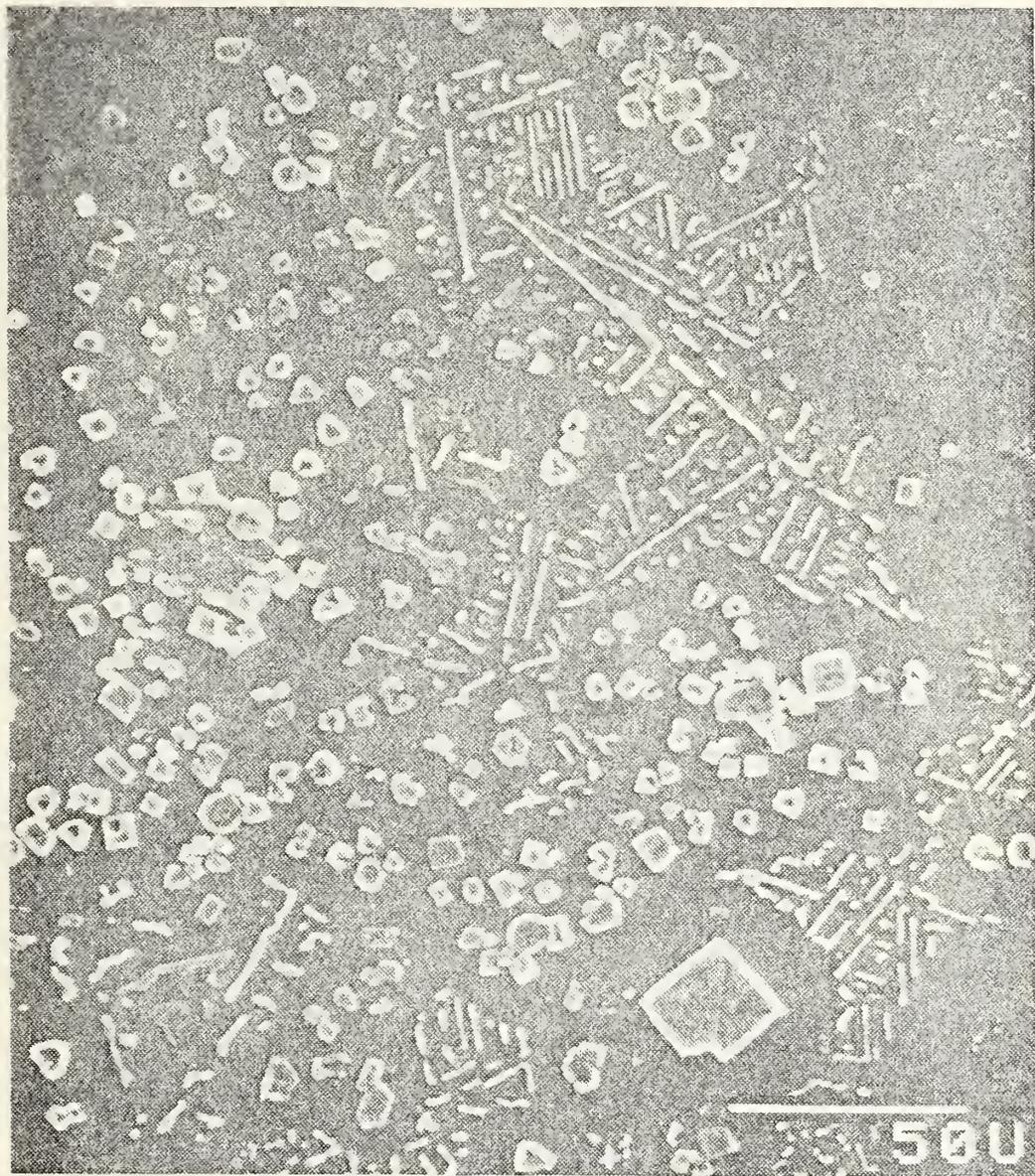


Plate 39. Glass coated with paint Formula 196D immersed 24 hours, 400X. Salt and other crystals forming an unusual geometric pattern.

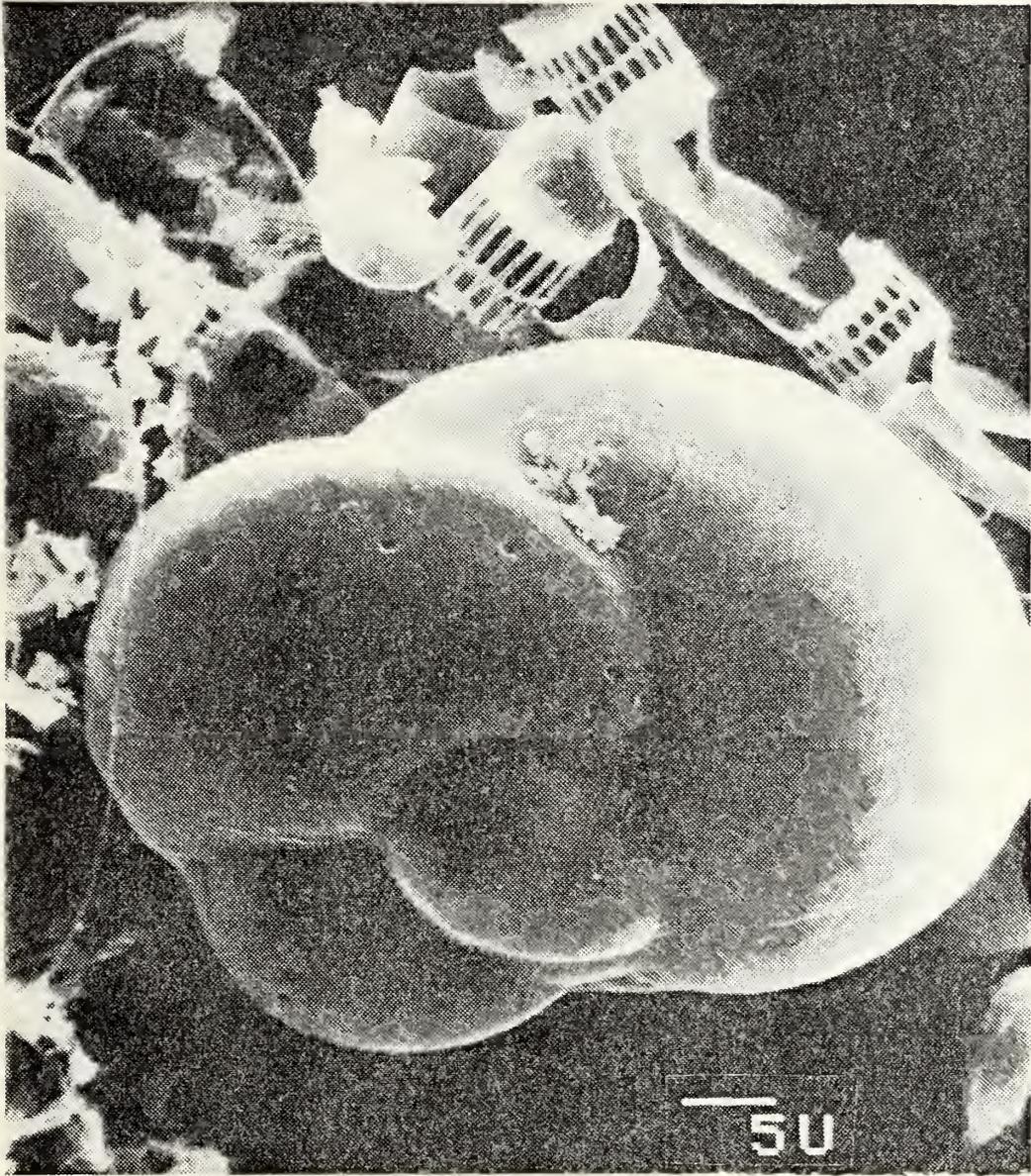


Plate 40. Glass coated with paint Formula 196D immersed 30 days, 1500X. A foraminifera surrounded by Broken diatoms (Stinemeyer and Reiter, 1958).

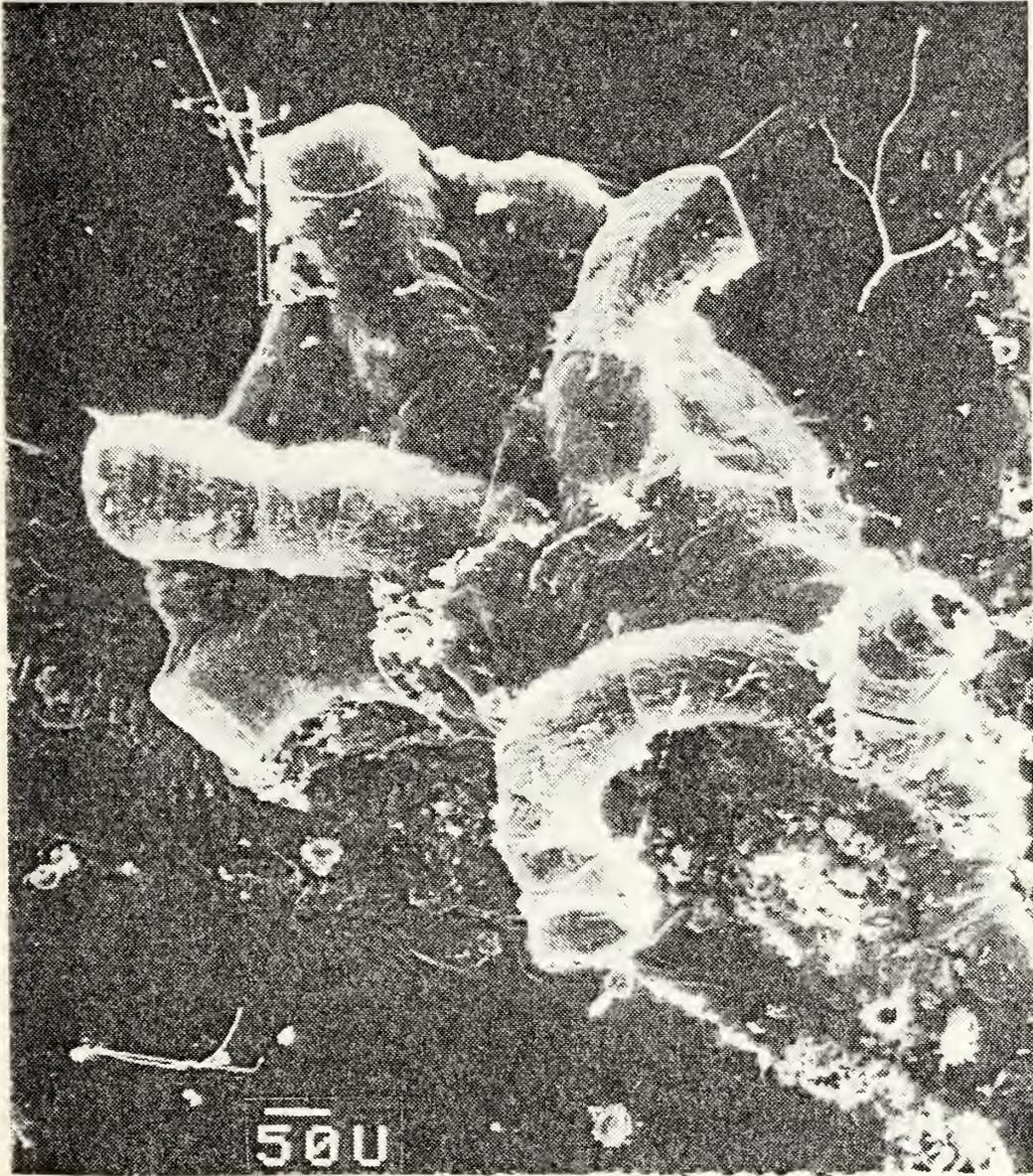


Plate 41. Glass coated with paint Formula 196D immersed 30 days, 100X. Worm tubes (Haderlie, personal communication).

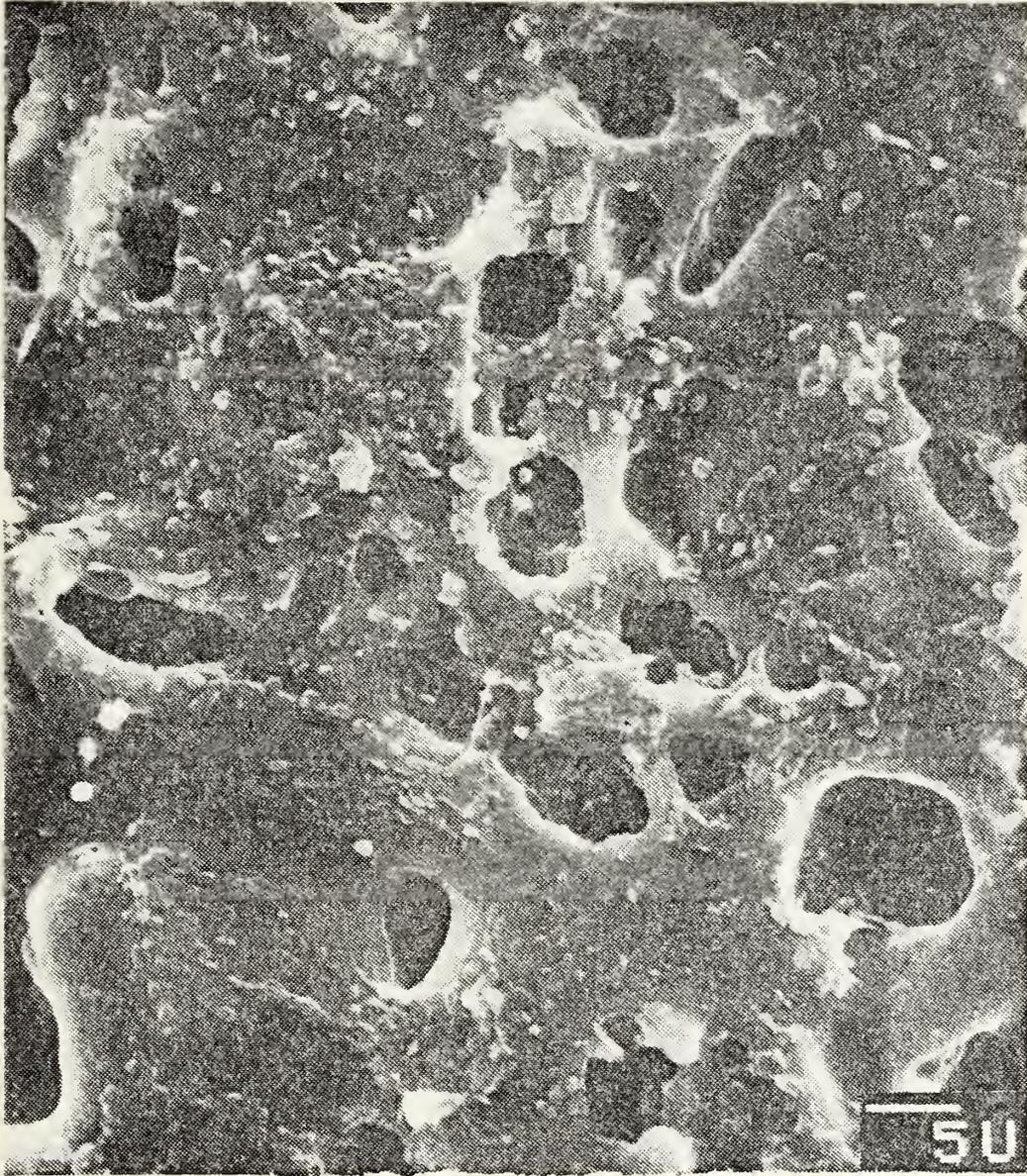


Plate 42. Glass coated with paint Formula 170 immersed 4 days, 1500X. The irregular surface is the texture of the paint itself. The paint is completely covered by rod-shaped bacteria.

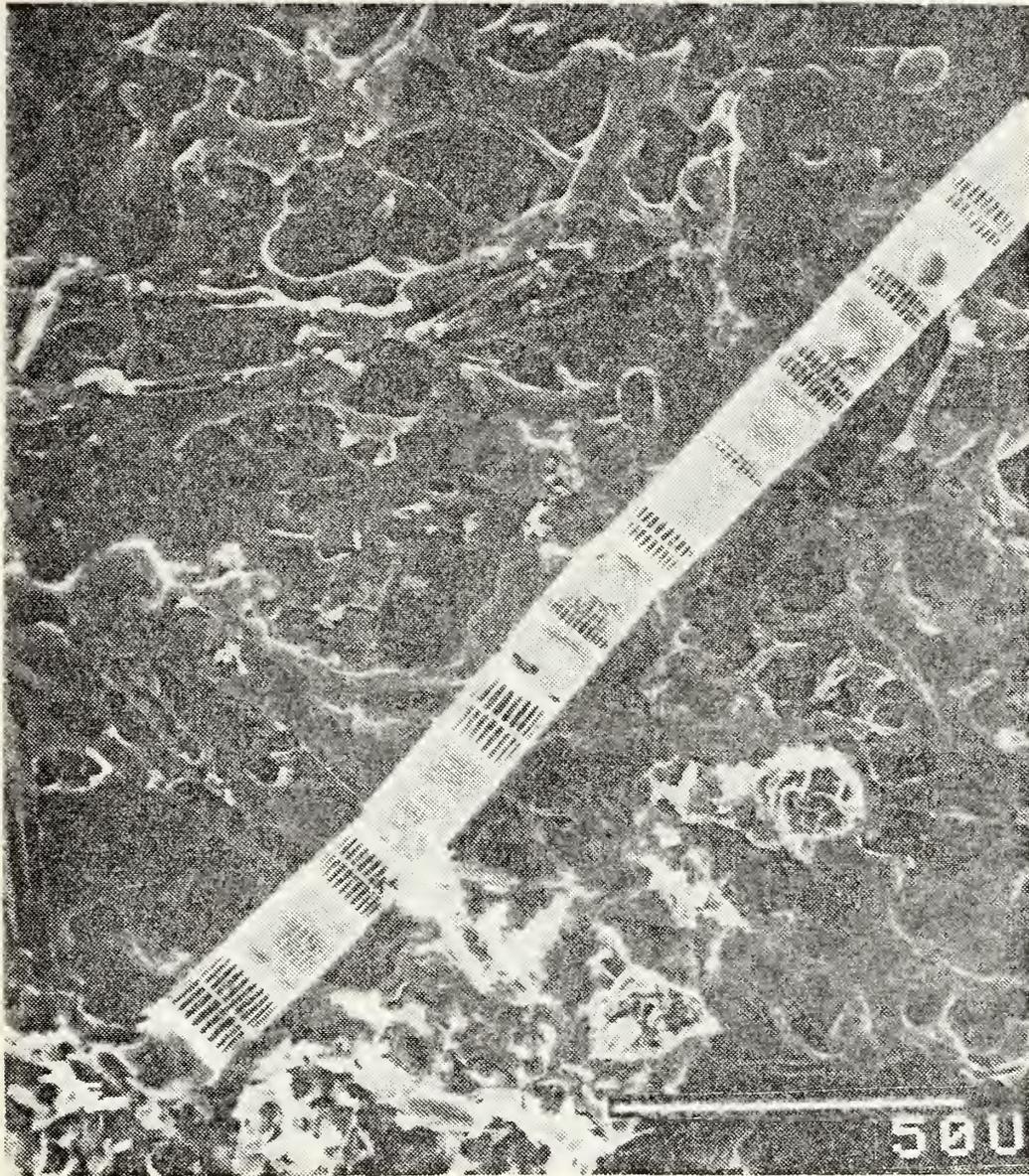
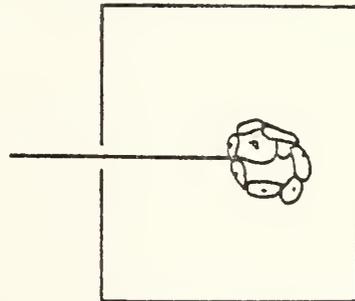


Plate 43. Glass coated with paint Formula 170 immersed 8 days, 600X. A long chain of the centric diatom *Skeletonema costatum* (Cupp, 1943)



Plate 44. Glass coated with paint Formula 170 immersed 30 days, 1500X. A coccolithophorid with a few plates missing.

Broken coccolithophorid (Sieburth, 1979)



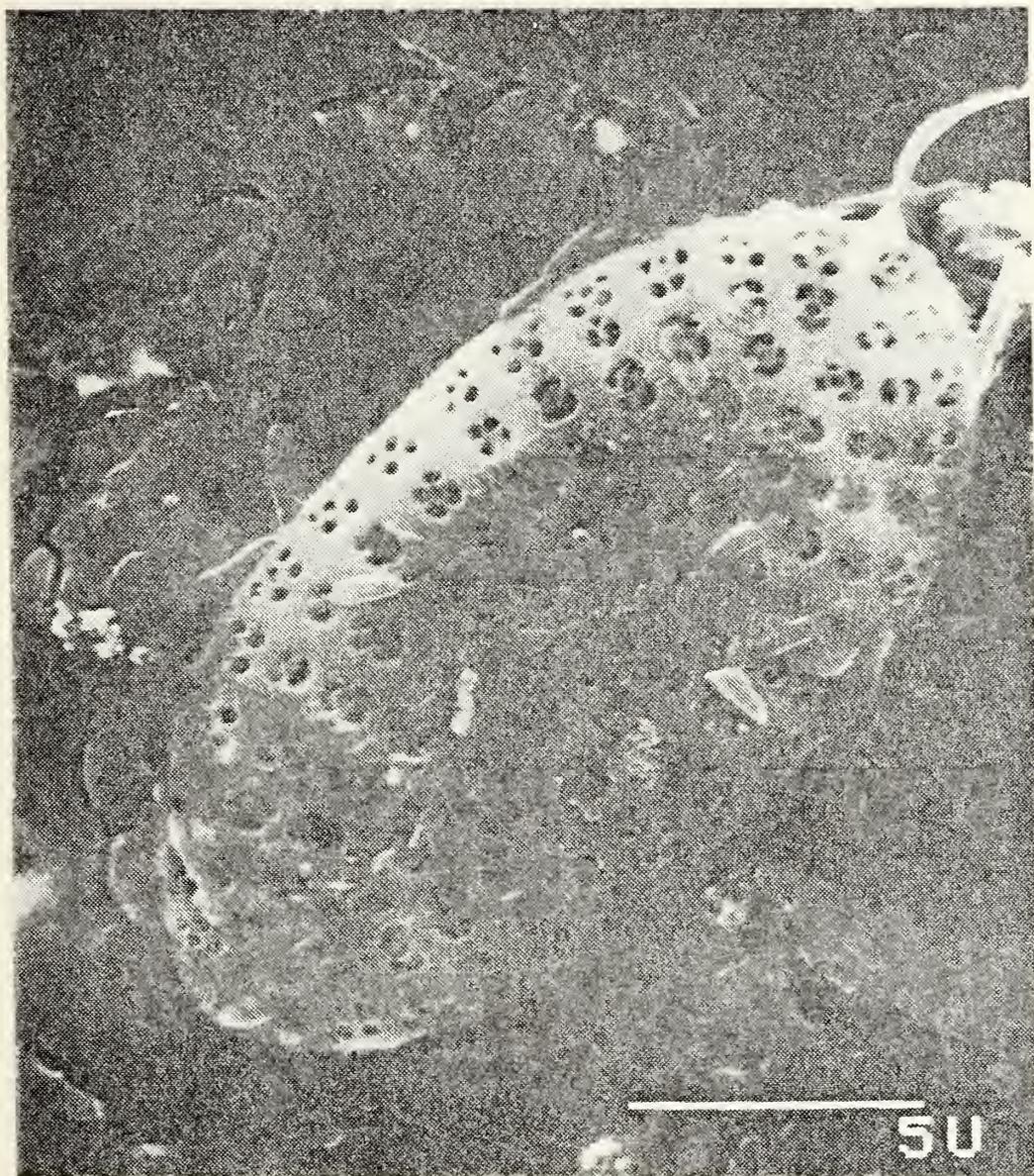


Plate 45. Glass coated with paint Formula 121 immersed 6 days, 5000X. Pennate diatom, possibly of the genus Amphora (I. Abbott, personal communication).

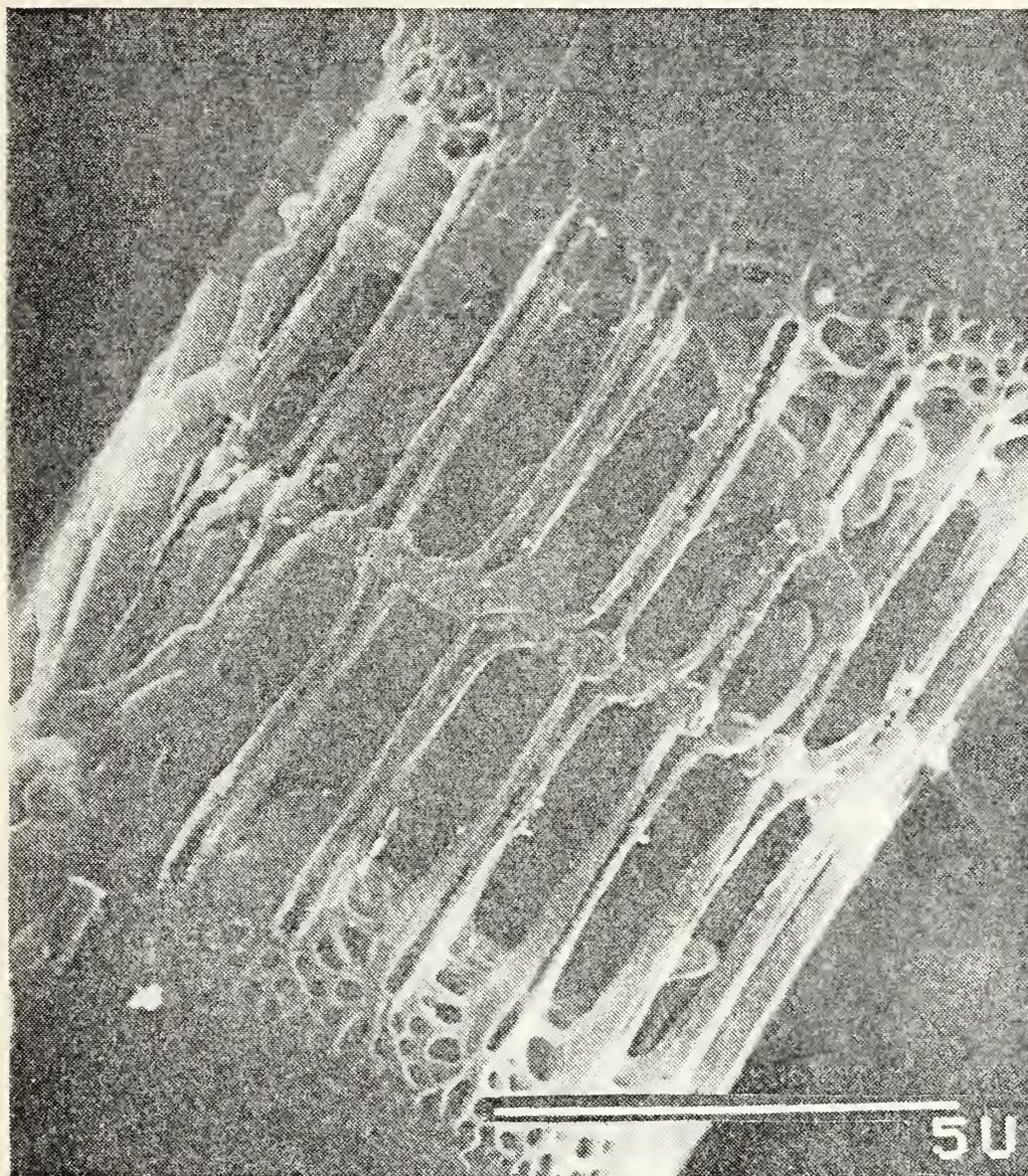


Plate 46. Glass coated with paint Formula 121 immersed 6 days, 8000X. Close-up of the centric diatom *Skeletonema costatum*. Note the rod-shaped Bacteria on the surface of the diatom.

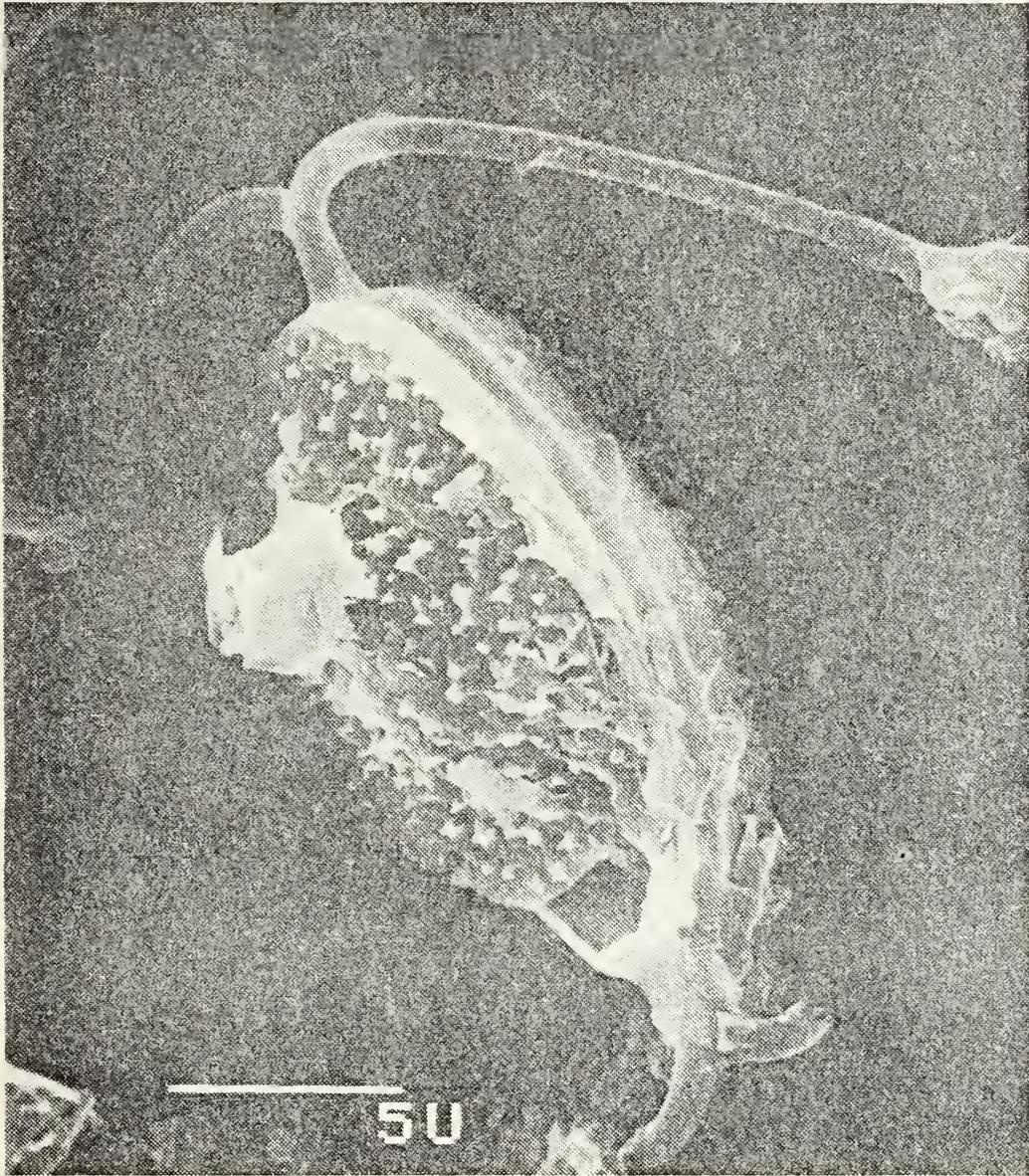


Plate 47. Glass coated with paint Formula 121 immersed 6 days, 4000X. The planktonic diatom Chaetoceros radicans (Cupp, 1943).

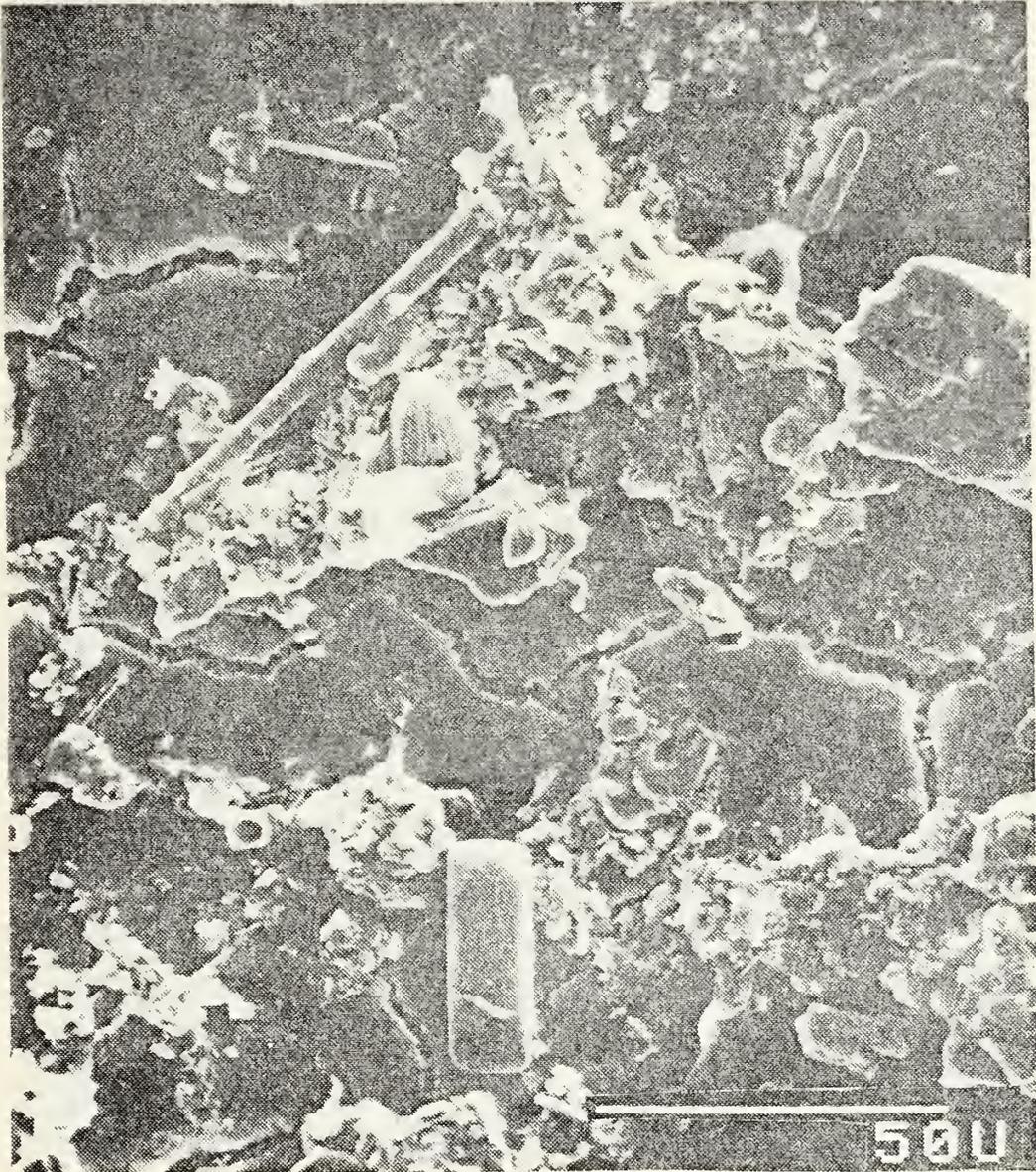


Plate 48. Glass coated with paint Formula 121 immersed 14 days, 600X. Diatoms, protozoans, and some debris.

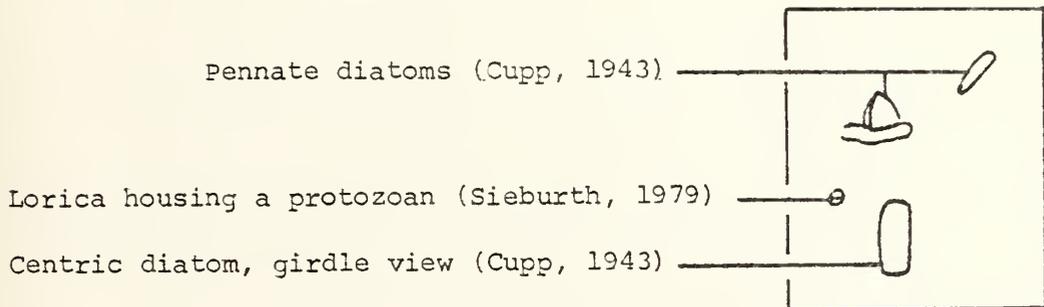




Plate 49. Glass coated with paint Formula 121 immersed 30 days, 4000X. Stalked, ciliated protozoan.

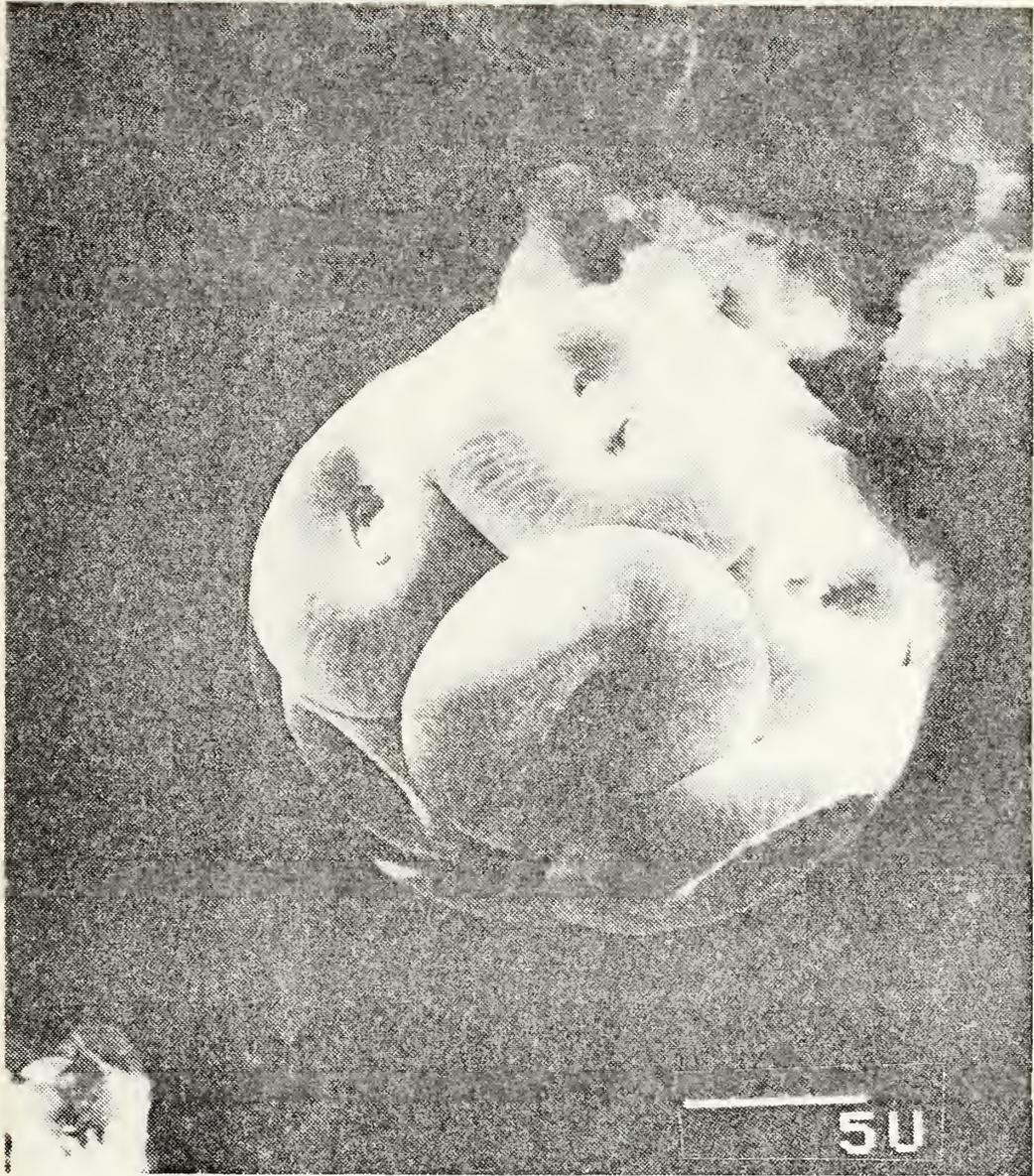


Plate 50. Glass coated with paint Formula 121 immersed 30 days, 3000X. Coccolithophorid (Sieburth, 1979).

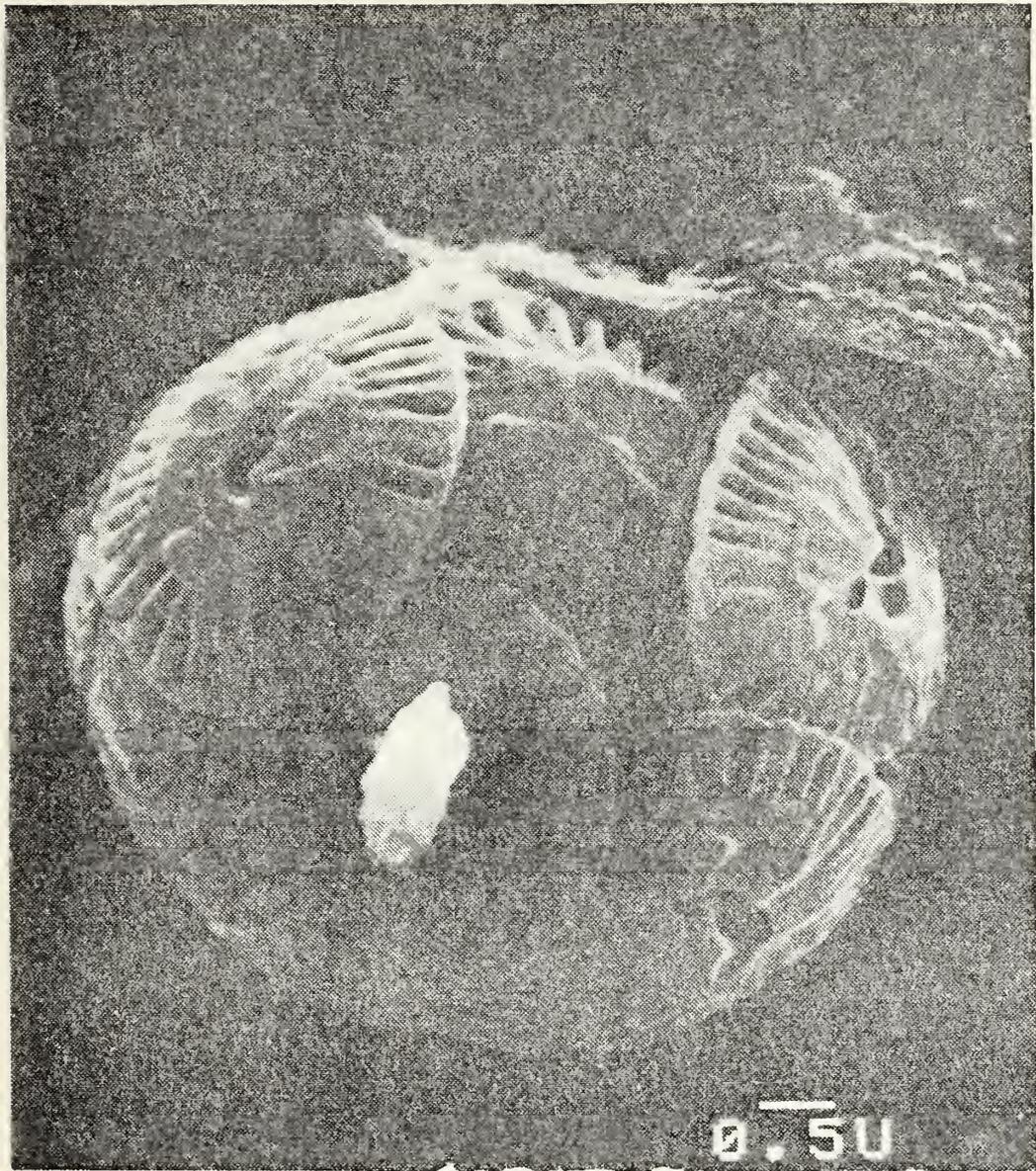


Plate 51. Glass coated with paint Formula 121 immersed 30 days, 12,000X. Coccolithophorid (Sieburth, 1979)

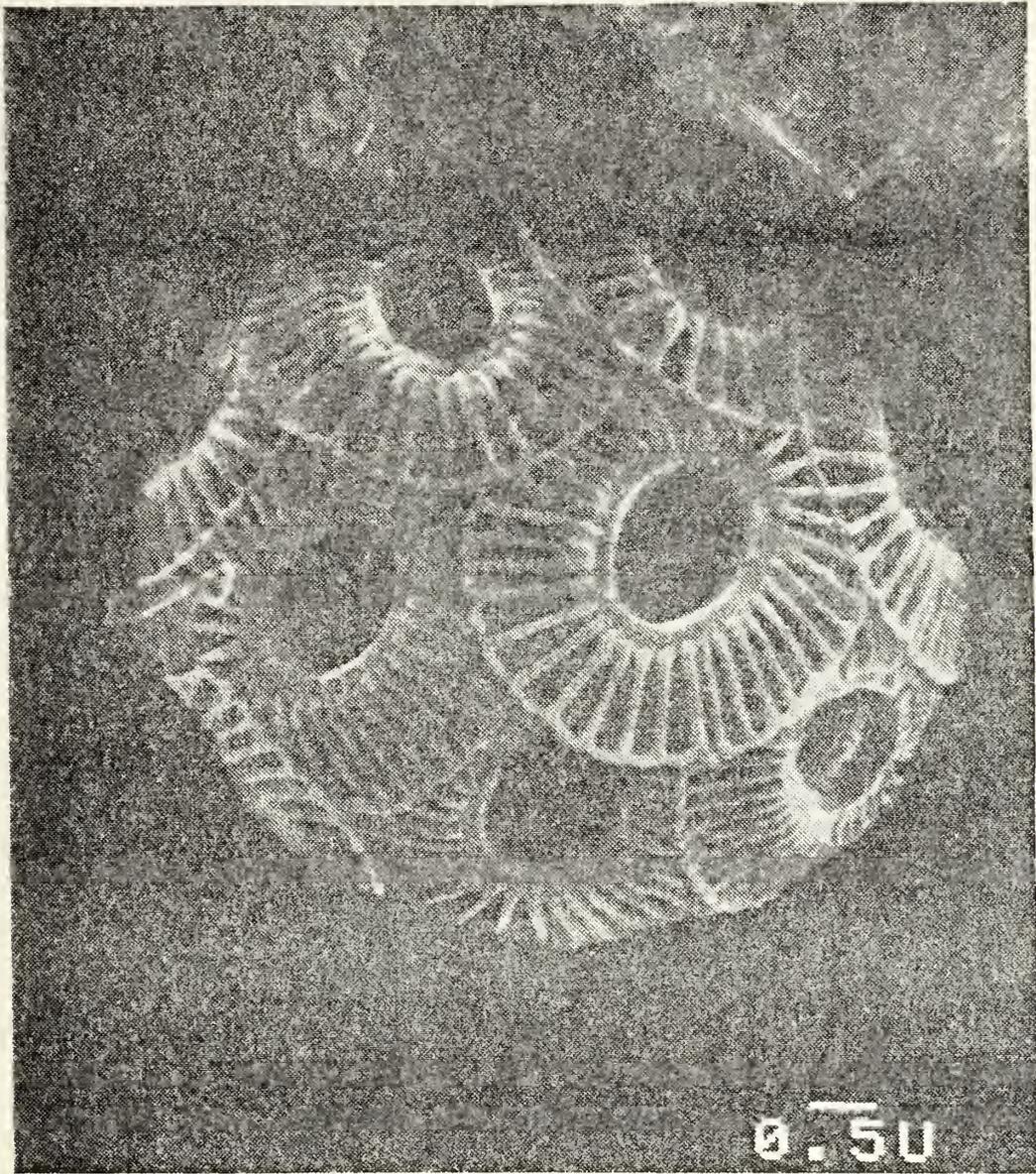


Plate 52. Glass coated with paint Formula 121 immersed 30 days, 10,000X. Coccolithophorid (Sieburth, 1979).

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