Form Approved
OBM No. 0704-0188



| 1. Agency Use Only *(Leave blank).* | 2. Report Date. | 3. Report Type and Dates Covered. |
|---|---|---|
| | 1993 | Final - Journal Article |

**4. Title and Subtitle.**
Advantages of Environmental Scanning Electron Microscopy in Studies of Microorganisms

**5. Funding Numbers.**
*Program Element No.* 0601153N
*Project No.* 03103
*Task No.* 320
*Accession No.* DN094463
*Work Unit No.* 13332A

**6. Author(s).**
Scott P. Collins*, Robert K. Pope*, Raymond W. Scheetz*, Richard I Ray, Patricia A. Wagner, and Brenda J. Little

**7. Performing Organization Name(s) and Address(es).**
Naval Research Laboratory
Ocean Science Branch
Stennis Space Center, MS 39529-5004

**8. Performing Organization Report Number.**
NRL/JA/7333--92-0004



**9. Sponsoring/Monitoring Agency Name(s) and Address(es).**
Naval Research Laboratory
Operations Research and Stragic Planning
Stennis Space Center, MS 39529-5004

**10. Sponsoring/Monitoring Agency Report Number.**
NRL/JA/7333--92-0004

**11. Supplementary Notes.**
*University of Southern Mississippi, Hattiesburg, MS
Published in Microscopy Research and Technique

**12a. Distribution/Availability Statement.**
Approved for public release; distribution is unlimited.

**12b. Distribution Code.**

**13. Abstract** *(Maximum 200 words).*

Microorganisms, including bacteria, fungi, protozoa, and microalgae, are composed predominantly of water which prohibits direct observation in a traditional scanning electron microscope (SEM). Preparation for SEM requires that microorganisms be fixed, frozen or dehydrated, and coated with a conductive film before observation in a high vacuum environment. Sample preparation may mechanically disturb delicate samples, compromise morphological information, and introduce other artifacts. The environmental scanning electron microscope (ESEM) provides a technology for imaging hydrated or dehydrated or dehydrated biological samples with minimal manipulation and without the need for conductive coatings.

Sporulating cultures of three fungi, Aspergillus sp., Cunninghamella sp., and Mucor sp., were imaged in the ESEM to assess usefulness of the instrument in the direct observation of delicate, uncoated, biological specimens. Asexual sporophores showed no evidence of conidial displacement or disruption of sporangia.



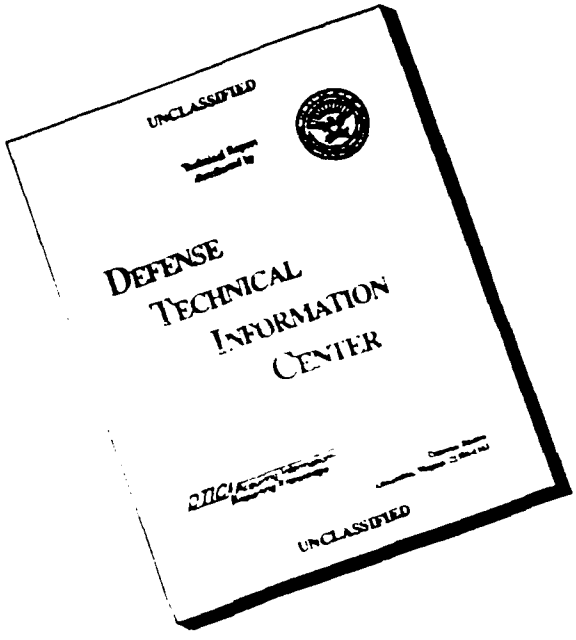
**14. Subject Terms.**
Biofouling, corrosion, biodeterioration, electrochemistry

**15. Number of Pages.**
8

**16. Price Code.**

| 17. Security Classification of Report. | 18. Security Classification of This Page. | 19. Security Classification of Abstract. | 20. Limitation of Abstract. |
|---|---|---|---|
| Unclassified | Unclassified | Unclassified | SAR |

# DISCLAIMER NOTICE




THIS DOCUMENT IS BEST QUALITY AVAILABLE. THE COPY FURNISHED TO DTIC CONTAINED A SIGNIFICANT NUMBER OF PAGES WHICH DO NOT REPRODUCE LEGIBLY.

MICROSCOPY RESEARCH AND TECHNIQUE 25:398–405 (1993)

# Advantages of Environmental Scanning Electron Microscopy in Studies of Microorganisms

SCOTT P. COLLINS, ROBERT K. POPE, RAYMOND W. SCHEETZ, RICHARD I. RAY, PATRICIA A. WAGNER, AND BRENDA J. LITTLE

*Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, Mississippi 39406-5018 (S.P.C., R.K.P., R.W.S.); Naval Research Laboratory Detachment, Stennis Space Center, Mississippi 39529-5004 (R.I.R., P.A.W., B.J.L.)*

*KEY WORDS* Environmental scanning electron microscopy, Algae, Fungi

*ABSTRACT* Microorganisms, including bacteria, fungi, protozoa, and microalgae, are composed predominantly of water which prohibits direct observation in a traditional scanning electron microscope (SEM). Preparation for SEM requires that microorganisms be fixed, frozen or dehydrated, and coated with a conductive film before observation in a high vacuum environment. Sample preparation may mechanically disturb delicate samples, compromise morphological information, and introduce other artifacts. The environmental scanning electron microscope (ESEM) provides a technology for imaging hydrated or dehydrated biological samples with minimal manipulation and without the need for conductive coatings.

Sporulating cultures of three fungi, *Aspergillus* sp., *Cunninghamella* sp., and *Mucor* sp., were imaged in the ESEM to assess usefulness of the instrument in the direct observation of delicate, uncoated, biological specimens. Asexual sporophores showed no evidence of conidial displacement or disruption of sporangia.

Uncoated algal cells of *Euglena gracilis* and *Spirogyra* sp. were examined using the backscatter electron detector (BSE) and the environmental secondary electron detector (ESD) of the ESEM. BSE images had more clearly defined intracellular structures, whereas ESD gave a clearer view of the surface. *E. gracilis* cells fixed with potassium permanganate, *Spirogyra* sp. stained with Lugol's solution, and *Saprolegnia* sp. fixed with osmium tetroxide were compared using BSE and ESD to demonstrate that cellular details could be enhanced by the introduction of heavy metals. The effect of cellular water on signal quality was evaluated by comparing hydrated to critical point dried specimens. 

## INTRODUCTION

Traditional SEM uses either low energy secondary electrons ejected from the specimen surface or higher energy backscatter electrons reflected from the specimen to reproduce specimen topography. Better resolution is generally achieved in the secondary mode since electrons emanate from the upper 25 angstroms of the specimen surface. Both signals are typically collected by a scintillator detector (Everhart and Thornley, 1960) requiring a high vacuum environment.

The ESEM uses a vacuum gradient to maintain high vacuum conditions at the filament while permitting chamber pressures to be varied up to approximately 20 torr. The gaseous environment of the ESEM chamber is ionized by secondary electrons derived from the specimen. Electrons from ionizing gas molecules precipitate a cascade multiplication of the imaging current that is monitored by the ESD. (Danilatos, 1983, 1988). The positive ions produced upon ionization of gas in the specimen chamber neutralize charges on the specimen surface. Elimination of charging allows even nonconductive specimens to be observed without the need for metal coating. Use of water vapor for the imaging gas and cooling the specimen enables viewing of uncoated hydrated samples.

ESEM imaging of uncoated biological specimens frequently provides useful information about internal or overlapping structures. The intrinsic low atomic number of biological samples provides little resistance to transmission of signal from underlying materials when heavy metal coatings are absent. The number of backscatter electrons originating from a specimen increases with the atomic number of elements in the sample. Heavier elements generate more backscatter signal and appear brighter than elements of low atomic number. This work documents the subsurface signal generated in the ESEM and examines methods for potentiating it.

## MATERIALS AND METHODS

### Organisms

*E. gracilis, Cunninghamella* sp., and *Saprolegnia* sp. were obtained from Carolina Biological Supply Co. (Burlington, NC). *Spirogyra* sp. was collected from Lake Byron (Hattiesburg, MS) and *Aspergillus* sp. was

Received December 11, 1992; accepted in revised form March 13, 1993.

Address reprint requests to Dr. Raymond W. Scheetz, Department of Biological Sciences, University of Southern Mississippi, Hattiesburg, MS 39406-5018.

a local isolate. Algae were maintained at 23°C in pond water illuminated with sunlight. *Saprolegnia* sp. was maintained in pond water on hemp seeds at 23°C and cultures of *Aspergillus* sp. and *Cunninghamella* sp. were maintained on corn meal agar.

### Specimen Preparation for ESEM

Hydrated cells of *E. gracilis* were observed directly in the ESEM at 3–5 torr water vapor, 4°C. *E. gracilis* cells were collected on 0.2 μm polycarbonate filters, *Spirogyra* sp. filaments were placed on carbon planchets, and *Saprolegnia* sp. mycelia were teased from hemp seed cultures.

Hydrated cells of *E. gracilis* were observed in the ESEM following fixation in 1% formaldehyde for 10 min. Fixation of freeze-dried or critical point dried cells was accomplished with either 2% glutaraldehyde in 0.1 M pH 7.2 cacodylate buffer, followed by 2% osmium tetroxide, or with 1% potassium permanganate. Cells treated with iodine were fixed in 2% cacodylate buffered glutaraldehyde, followed by a buffer wash, and then immersed in Lugol's solution (Gautier, 1960) diluted 1:10 for 1 h. Destaining of iodine treated cells was achieved by distilled water washes, monitored by light microscopy.

Filter paper containing fixed cells was placed on a copper block previously immersed in liquid nitrogen. The cold copper block and frozen specimens were quickly transferred to a Denton DV-502 (Denton Vacuum, Inc., Cherry Hill, NJ) high vacuum evaporator and maintained at reduced pressure. Gold coatings were applied with a Polaron E5100 sputter coater (Fisons Instruments, San Carlos, CA).

### Operating Parameters for ESEM

Operating parameters were varied for each micrograph. Specific information is included in the figure caption as detector type, accelerating voltage, chamber pressure, chamber temperature, and working distance.

## RESULTS

Agar plugs from sporulating cultures of *Cunninghamella* sp., *Mucor* sp., and *Aspergillus* sp. were maintained in a hydrated state and directly imaged with ESD. The expanded apical region of *Cunninghamella* sp. conidiophores were covered with ornamented conidia (Figs. 1, 2). Recently formed sporangia of *Mucor* sp. possessed an intact transparent peridial membrane studded with crystals through which sporangiophores were visible (Figs. 3, 4). Scanning with the electron beam caused the fragile peridia to break into discoidal platelets, most of which contained central crystals. Long undisturbed chains of conidia were evident on *Aspergillus* sp. conidiophores (Fig. 5). The three fungal specimens showed little evidence of mechanical disruption.

ESEM images of uncoated, hydrated *E. gracilis* cells showed spiral ridges and grooves of the periplast through which cytoplasmic structures were visible (Fig. 6). *E. gracilis* cells are spindle shaped with spirally arranged ridges and grooves covering the cell surface. Superimposed on the ridges and grooves are spherical raised areas resulting from apposition of internal structures and the cell periplast. Gold-coated, freeze-dried cells clearly revealed external structures, but details of cytoplasmic organization were lacking (Fig. 7). Uncoated, freeze-dried *Euglena* sp. cells examined with either ESD or BSE detectors did not exhibit spiral grooves of the periplast as distinctly as gold-coated cells, but cytoplasmic structures were revealed (Figs. 8, 9). Internal membranous structures including chloroplasts and vacuoles were evident in permanganate-fixed *E. gracilis* cells. The ESD detector provided more signal from the periplast while the BSE detector more clearly defined internal structures (Figs. 10, 11).

Gold-coated, freeze-dried *Spirogyra* preparations defined the cylindrical nature of the cells that compose filaments and also depicted an indistinct spiral band associated with each cell (Fig. 12). Details of internal structures were lacking. Uncoated, freeze-dried cells of *Spirogyra* sp. examined with BSE or ESD detectors showed distinct spiral chloroplasts dotted as intervals with starch grains. BSE and ESD images were similar, with ESD providing a slight enhancement of surface features and BSE revealing more internal information as to distribution of particulates (Figs. 13, 14). Dilute Lugol's solution applied to *Spirogyra* sp. cells resulted in intensification of signals arising from circular areas spaced at intervals along spiral chloroplasts (Figs. 15–18). Comparison of critical point dried, iodine-treated cells with freeze-dried cells demonstrated superior preservation of chloroplast morphology in freeze-dried preparations.

Branched filamentous thalli of the Oomycete *Saprolegnia* sp. were evident in gold-coated, freeze-dried cells, as were elongate sporangia and the globose oogonia reproductive structures. However, no cytoplasmic structures were evident (Figs. 19, 20). *Saprolegnia* sp. cells fixed in permanganate yielded a strong BSE signal from forming zoospores (Figs. 21) and oospores (Fig. 22), while the bounding sporangial and oogonial walls, respectively, appeared transparent. Osmium tetroxide-fixed hyphal cells of *Saprolegnia* sp. showed large numbers of spherical cytoplasmic vesicles approximately 1.0 μm in diameter (Fig. 23). Many of the vesicles appear linearly aligned with cytoplasmic filaments. Osmium tetroxide fixation revealed cleaved zoospores within sporangia as well as the distribution of vesicles and vacuoles within individual zoospores (Fig. 24).

## DISCUSSION

The obvious advantage of using the ESEM to image microbiological specimens is the ability to view samples with minimum specimen preparation. Specimens can be viewed directly, without drying and without conductive coatings. More than simply reducing preparation time, the ability to directly scan biological specimens enables delicate specimens to be viewed with minimal mechanical disruption. The simple act of placing specimens in fixative solutions or dehydration agents prior to critical point drying may alter morphology (Little et al., 1991). Pressure changes in drying units can also disrupt and distort fragile biological material.

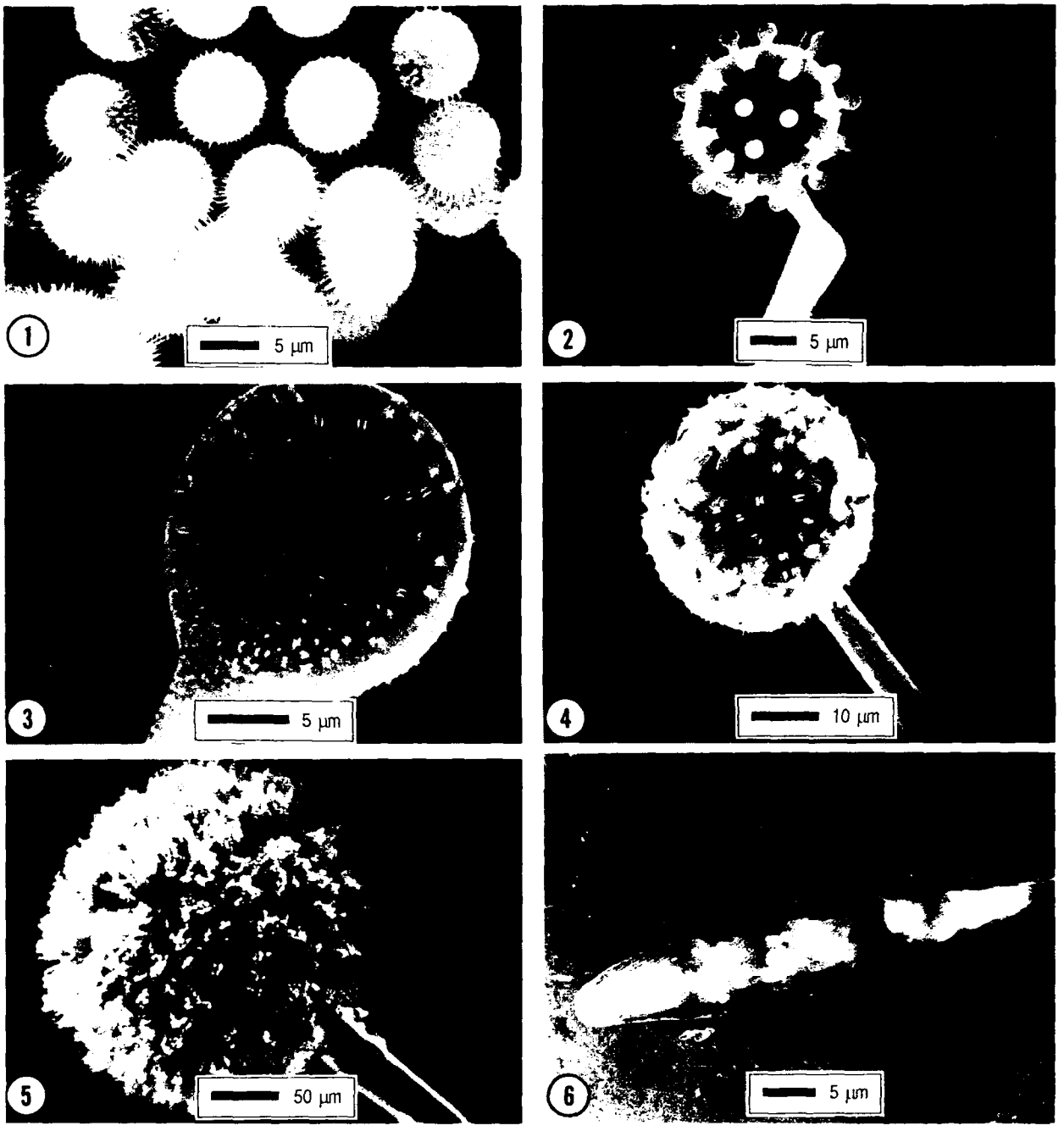



Fig. 1. Uncoated, hydrated *Cunninghamella* sp., mature conidia (ESD, 20 kv, 4.4 torr, 4 C, 7.3 mm).

Fig. 2. Uncoated, hydrated *Cunninghamella* sp., expanded conidiophore with conidia forming on surface (ESD, 20 kv 4.9 torr, 4 C, 8.4 mm).

Fig. 3. Uncoated, hydrated *Mucor* sp., immature sporangium with crystals on surface (ESD, 20 kv, 3.2 torr, 4 C, 8.4 mm).

Fig. 4. Uncoated, hydrated *Mucor* sp., mature sporangium with spores visible through the peridium (ESD, 20 kv, 4.2 torr, 4 C, 8.1 mm).

Fig. 5. Uncoated, hydrated *Aspergillus* sp., conidial chains (ESD, 20 kv, 3.6 torr, 4 C, 6.4 mm).

Fig. 6. Uncoated, hydrated *E. gracilis* (ESD, 20 kv, 4.4 torr, 4 C, 7.4 mm).

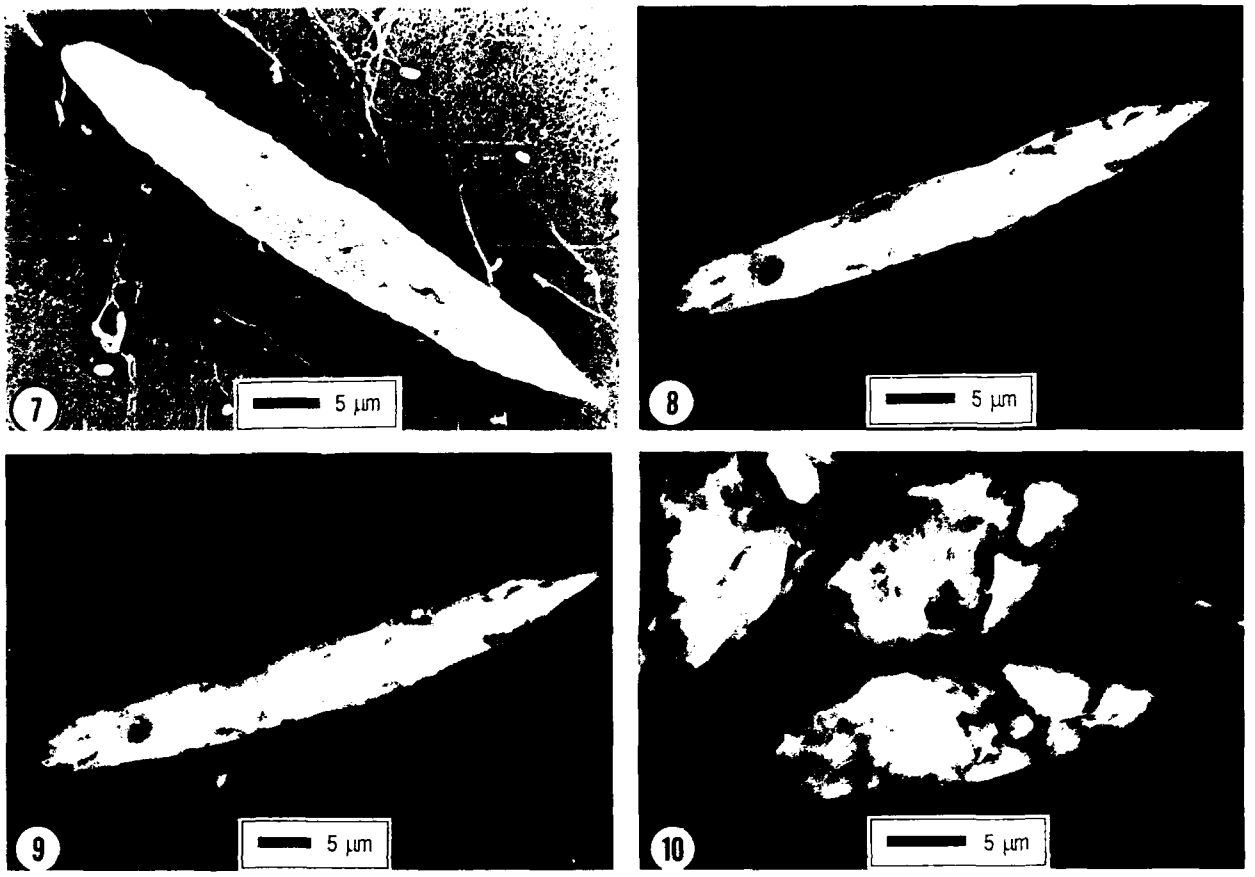



Fig. 7. Gold-coated, freeze-dried *E. gracilis* (BSE, 20 kv, 4.4 torr, 23 C, 7.0 mm).

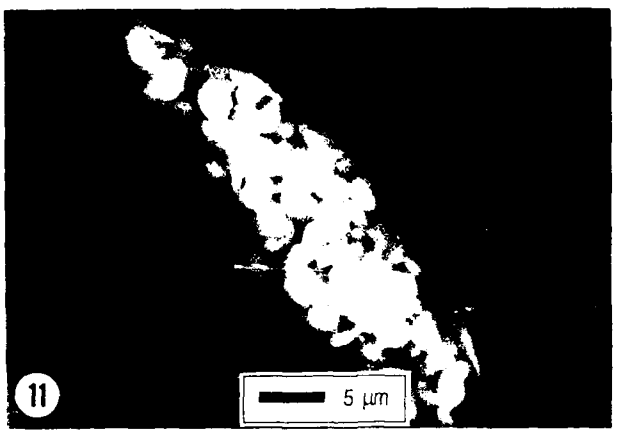
Fig. 8. Uncoated, freeze-dried *E. gracilis* (ESD, 20 kv, 4.4 torr, 23 C, 7.4 mm).

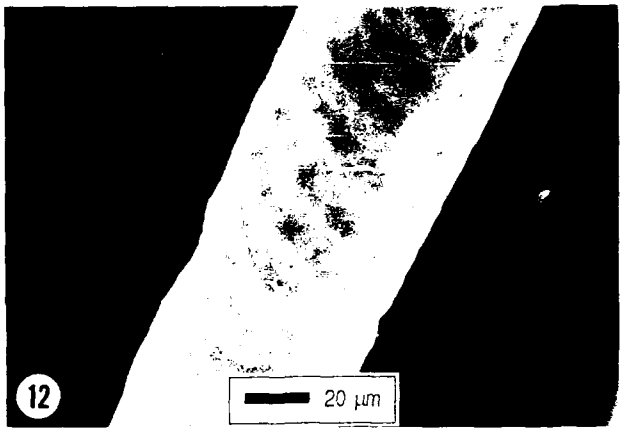
Fig. 9. Uncoated, freeze-dried *E. gracilis* (BSE, 20 kv, 4.4 torr, 23 C, 6.1 mm).

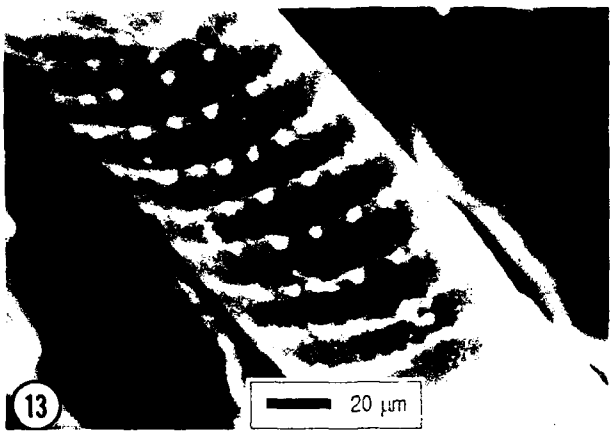
Fig. 10. Uncoated, freeze-dried, $KMnO_4$ fixed *E. gracilis* (ESD, 30 kv, 4.4 torr, 23 C, 8.1 mm).

Sporulating cultures of *Cunninghamella* sp., *Mucor* sp., and *Aspergillus* sp., were used to demonstrate that the ESEM can provide accurate images of uncoated, hydrated, sporulating fungi. The asexual fruitings of *Cunninghamella* sp. were found intact, with the expanded apex of the conidiophore covered with ornamented conidia (Figs. 1, 2). Immature sporangiophores of *Mucor* sp. with spores in the process of forming were imaged without collapse (Fig. 3), underscoring the advantage of viewing specimens with minimum handling. The delicate peridial membrane of mature sporangia separates as platelets (Fig. 4). Crystal doublets decorate each platelet. Spores were visible through the transparent peridial platelets, an image not possible with gold-coated specimens. The peridium is thin and composed of an organic polymer of low atomic number presenting little barrier to backscattered electrons. Direct observation of *Aspergillus* sp. conidiophores revealed long chains of conidia on virtually every conidiophore (Fig. 5). No mechanical disruption was evident in these examinations.

*E. gracilis* is a single cell flagellate algae which lacks a cell wall and stores food material as paramylum, a beta-1:3 glucopyranoside. Just below the plasmalemma is a proteinaceous structure in the form of alternating ridges and grooves that spirals around the cell (Arnott and Walne, 1967). The cell membrane plus the underlying ridges and grooves make up what is termed the periplast. The periplast was easily visualized in gold-coated specimens (Fig. 7) and, although it is less pronounced, the periplast was visible with the ESD in uncoated, hydrated specimens (Fig. 6). The cytoplasm of *E. gracilis* cells is filled with numerous discoid chloroplasts approximately 3 μm in diameter. Vacuoles and large flattened paramylum grains located peripherally just under the periplast were apparent in BSE and ESD images of dehydrated cells (Figs. 8, 9). Chloroplasts within the cell contain magnesium in chlorophyll molecules, iron in electron carriers, and manganese in the photolytic reaction center. These elements generate backscatter signals capable of penetrating the organic periplast and provide information



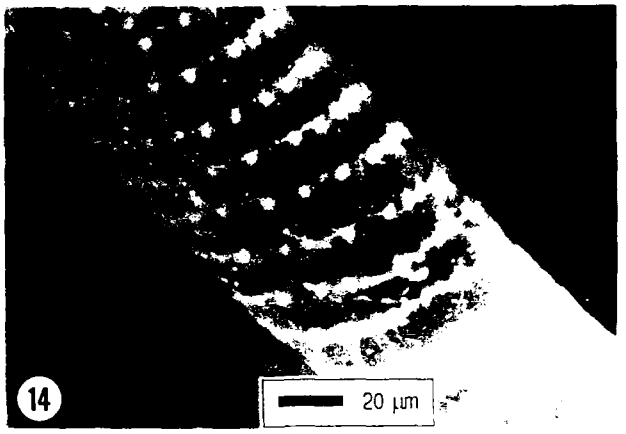




Fig. 11. Uncoated, freeze-dried, $KMnO_4$ fixed *E. gracilis* (BSE, 30 kv, 4.4 torr, 23°C, 7.2 mm).

Fig. 12. Gold-coated, freeze-dried *Spirogyra* sp. (ESD, 20 kv, 4.4 torr, 23°C, 8.2 mm).

Fig. 13. Uncoated, freeze-dried *Spirogyra* sp. (ESD, 20 kv, 4.4 torr, 23°C, 8.4 mm).

Fig. 14. Uncoated, freeze-dried *Spirogyra* sp. (BSE, 20 kv, 4.4 torr, 23°C, 9.8 mm).

on morphology (Fig. 9) and distribution of internal organelles unattainable in gold-coated specimens (Fig. 7). The distinctive spiral chloroplasts of uncoated *Spirogyra* sp. cells were displayed through the cellulose wall (Figs. 13, 14). The chloroplasts were not evident in gold-coated specimens (Fig. 12).

The backscatter electron signal in a traditional SEM is typically of lower resolution and noisier than the secondary electron signal since backscattered electrons originate from deeper in the specimen where the probe size has been broadened by multiple scattering events. Only a small number of electrons from deep within the specimen are collected by the detector. In the ESEM, specimens are usually viewed without tilting and the backscatter detector light-pipe surrounds the ESD detector directly above the specimen. This arrangement optimizes collection of high-angle backscattered electrons, increasing contrast and reducing noise.

Several staining methods have been used to impregnate biological tissue with heavy metals to increase backscatter signal and to stain specific sites within cells (Abraham and DeNee, 1973; Ulrich and McClung, 1983). Specimen preparation in such studies also usually involves deposition of a thin carbon coat to minimize charging. Carbon coating can be avoided since specimen charging is not a major concern in the ESEM.

Potassium permanganate and osmium tetroxide are fixatives commonly employed in transmission electron microscopy to generate contrast by virtue of heavy metal addition. Permanganate binds readily to biological membranes and enhances backscatter signal from internal membranous organelles (Figs. 10, 11, 21, 22). *E. gracilis* fixed with permanganate showed improved preservation and definition of cytoplasmic organelles. Small discoid chloroplasts were easily distinguished from larger, flattened, peripherally located paramylum grains (Figs. 10, 11). Sufficient permanganate was bound in *Saprolegnia* sp. zoospores and oospores, both achlorophyllous, allowing them to be distinguished through the cellulose wall of the sporangium and oo-

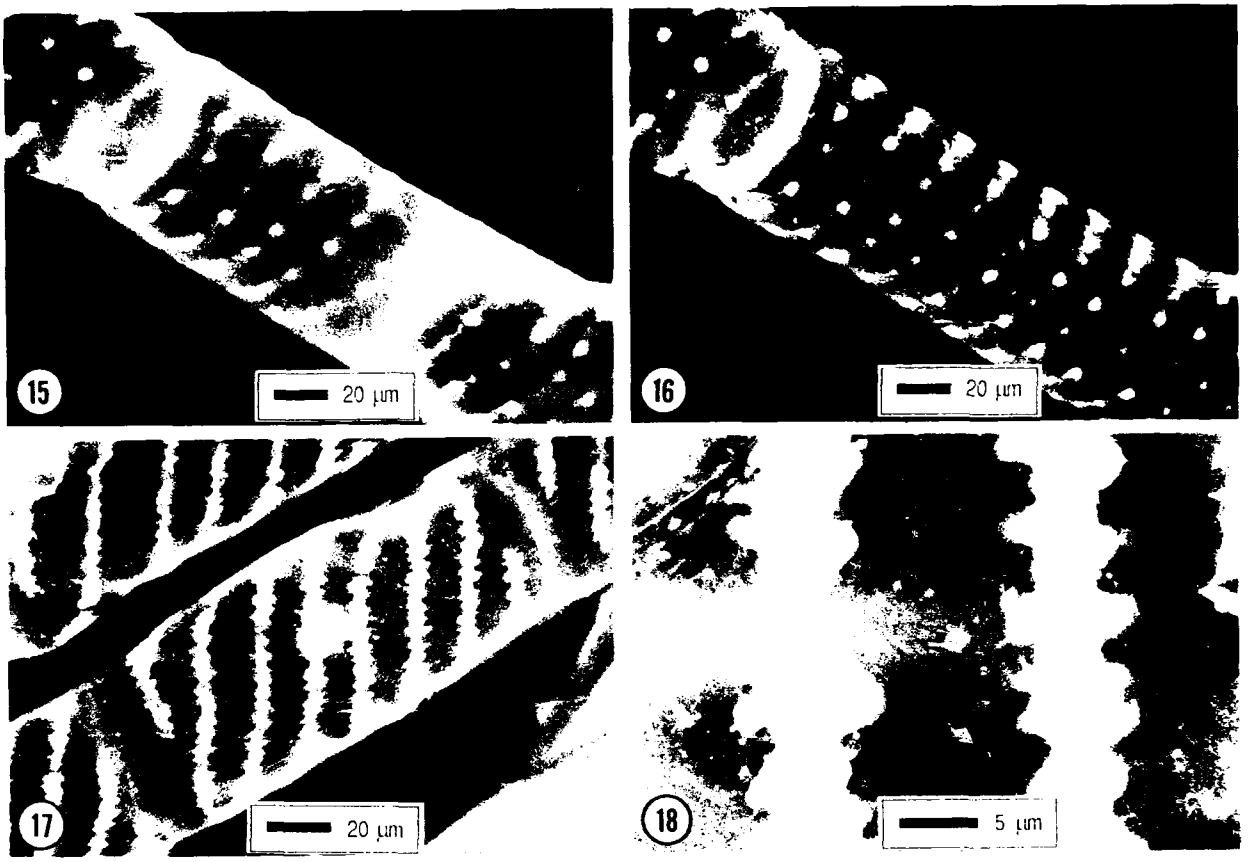



Fig. 15. Uncoated, critical point dried, iodine stained *Spirogyra* sp. (ESD, 30 kv, 4.4 torr, 23 C, 8.9 mm).

Fig. 16. Uncoated, critical point dried, iodine stained *Spirogyra* sp. (BSE, 30 kv, 4.4 torr, 23 C, 8.9 mm).

Figs. 17, 18. Uncoated, freeze-dried, iodine stained *Spirogyra* sp. (BSE, 30 kv, 4.4 torr, 23 C, 8.0 mm).

gonium, respectively (Figs. 21, 22). Osmium tetroxide reacts with cell lipids and proteins, increasing backscatter signal from cytoplasmic vesicles in actively growing hyphal tips (Fig. 23). Vesicles in some regions were in linear arrangements among what appeared to be cytoplasmic filaments. Osmium tetroxide also amplified backscatter signals from zoospore membranes (Fig. 24). Not only were individual zoospores distinguishable through the sporangial mass, but zoospore vesicles and vacuoles were evident.

Labelling of specific cytoplasmic structures was achieved by staining *Spirogyra* sp. cells with iodine. The chemical reaction of iodine with starch is an accepted qualitative test (Hanker et al., 1964). The accumulation of iodine by starch in *Spirogyra* sp. cells intensified backscatter signals from circular areas spaced along flattened spiral chloroplasts (Figs. 15–18).

Backscatter signals originating within cells are impeded by cytoplasmic water when hydrated specimens are directly examined in an atmosphere of water vapor at reduced temperatures. Critical point drying increased backscatter signals from specimens. Organic solvents typically used during SEM specimen preparation solubilize chlorophylls in algal specimens (Parsons and Strickland, 1963). Freeze drying provided maximum backscatter signal in samples by removing free water without removing chlorophyll (Figs. 17, 18).

## CONCLUSIONS

The ESEM is an effective tool for viewing delicate biological samples directly with no specimen preparation. Backscatter signals from internal cellular structures were often sufficient to penetrate overlaying membranes, providing useful information about cytoplasmic morphology. Backscatter signals from specific cell structures were enhanced by the introduction of heavy metal stains, including potassium permanganate and osmium tetroxide.

## ACKNOWLEDGMENTS

This work was supported by the Office of Naval Research, Program Element 0601153N, the Defense Re-

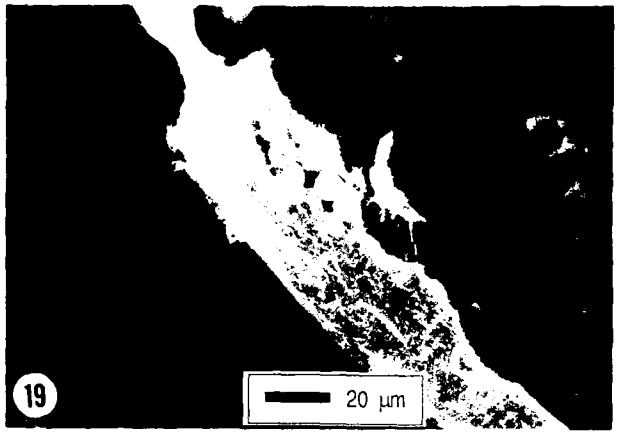



Fig. 19. Gold-coated, freeze-dried *Saprolegnia* sp. (ESD, 20 kv, 4.4 torr, 23°C, 8.6 mm).

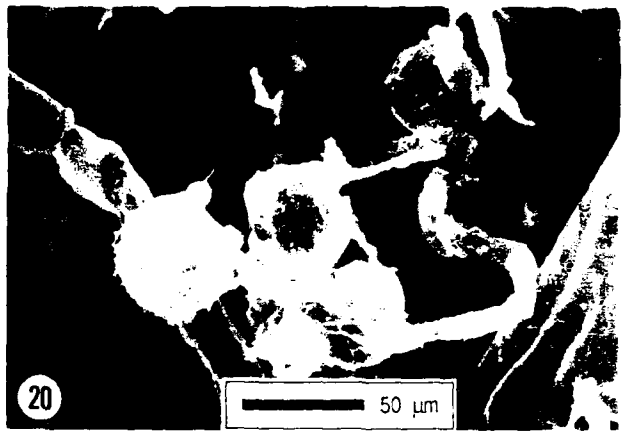
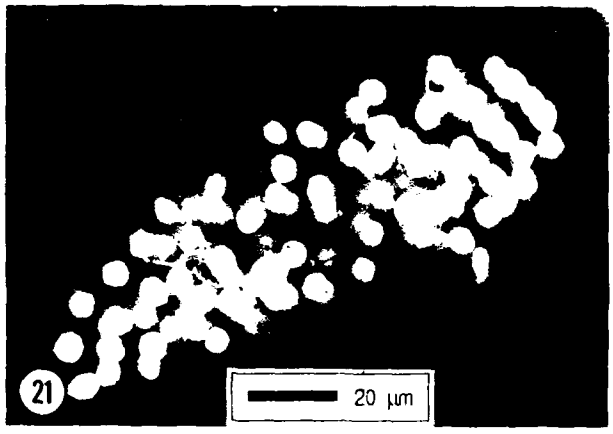
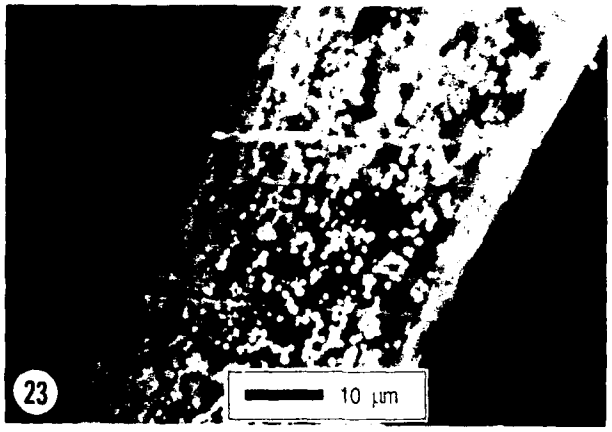



Fig. 20. Gold-coated, freeze-dried *Saprolegnia* sp. (ESD, 20 kv, 4.4 torr, 23°C, 8.7 mm).







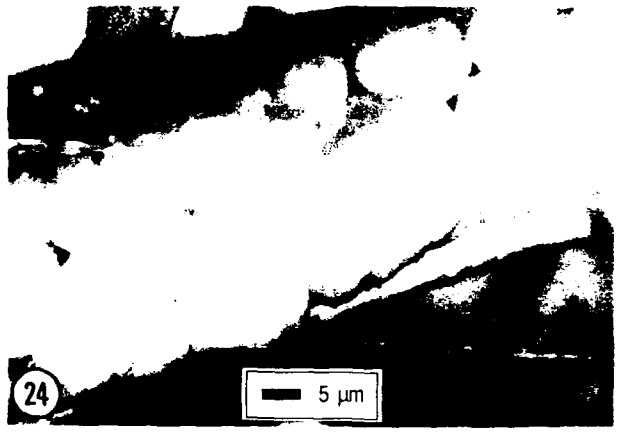




Fig. 21. Uncoated, freeze-dried, $KMnO_4$-fixed *Saprolegnia* sp. (BSE, 30 kv, 4.4 torr, 23°C, 8.7 mm).

Fig. 22. Uncoated, freeze-dried, $KMnO_4$-fixed *Saprolegnia* sp. (BSE, 30 kv, 4.4 torr, 23°C, 8.1 mm).

Fig. 23. Uncoated, freeze-dried, osmium tetroxide-fixed *Saprolegnia* sp. (BSE, 20 kv, 4.4 torr, 23°C, 7.9 mm).

Fig. 24. Uncoated, freeze-dried, osmium tetroxide-fixed *Saprolegnia* sp. (BSE, 30 kv, 4.4 torr, 23°C, 7.0 mm).

search Sciences Program, NRL contribution NRL/JA/7333—92-0004.

## REFERENCES

Abraham, J.L., and DeNee, P.B. (1973) Scanning electron microscope histochemistry using backscattered electrons and metal stains. Lancet, 1:1125.

Arnott, H.J., and Walne, P.L. (1967) Observations in the fine structure of the pellicle pores of *Euglena granulata*. Protoplasma, 64: 330–344.

Danilatos, G.D. (1983) A gaseous detector device for an environmental SEM. Micron and Microscopica Acta, 14:307–319.

Danilatos, G.D. (1988) Foundations of environmental microscopy. In: Advances in Electronics and Electron Physics. P.W. Hawkes, ed. Academic Press, Boston, pp. 109–250.

Everhart, T., and Thornley, R.F.M. (1960) Wide-band detector for micro-microampere low-energy electron currents. J. Sci. Inst., 37:246–248.

Gautier, A. (1960) Technique de coloration histologique de tissus inclus dans des polyesters. Experientia, 16:124.

Hanker, J.S., Seaman, A.R., Weiss, L.P., Ueno, H., Bergman, R.A., and Seligman, A.M. (1964) Osmiophilic reagents: New cytochemical principle for light and electron microscopy. Science, 146:1039–1043.

Little, B.J., Wagner, P.A., Ray, R.I., Pope, R., and Scheetz, R. (1991) Biofilms: An ESEM evaluation of artifacts introduced during SEM preparation. J. Industrial Microbiol. 8:213–222.

Parsons, T.R., and Strickland, J.D.H. (1963) Discussion of spectrophotometric determination of marine plant pigments, with revised equations for ascertaining chlorophylls and carotenoids. J. Mar. Res., 21:155–163.

Ulrich, R.G., and McClung, J.K. (1983) Analysis of erythrocyte-mediated microinjection by backscattered imaging. J. Ultrastruct. Res., 82:327–334.