EVALUATION OF NATURAL AND ARTIFICIAL SUBSTRATE COLONIZATION BY SCANNING ELECTRON MICROSCOPY[1]

ROBERT W. PAUL, JR.,[2] DAVID L. KUHN, JAMES L. PLAFKIN, JOHN CAIRNS, JR., and JUDITH G. CROXDALE
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PAUL, R. W., JR., KUHN, D. L., PLAFKIN, J. L., CAIRNS, J., JR. & CROXDALE, J. G. 1977. Evaluation of natural and artificial substate colonization by scanning electron microscopy. *Trans. Amer. Micros. Soc.*, 96: 506–519. Progressive microbial colonization of natural (sycamore leaf) and artificial (polyurethane foam) substrate material in lotic and lentic systems was studied by scanning electron microscopy. The organisms which participated in colonization showed substrate preferences and aquatic system variation. The rate of colonization was dependent not only on the aquatic system, but also on the type of substrate. Protozoa were not found on either substrate, indicating that they are lost during specimen preparation. Fungi and bacteria were principal colonizers on leaf substrates, while algae were predominate polyurethane foam invaders. In each case, colonization served to structurally modify the substrate, rendering it a more suitable habitat which was nutritionally capable of supporting other organisms.

Colonization studies of both natural and artificial substrates are becoming increasingly common for academic reasons and as pollution assessment techniques. Techniques involving substrate introduction into aquatic systems have been refined and adapted for studying decomposition, colonization, energy flow, and organism collection. Cooke (1956), Sladeckova (1962), and Wetzel (1964) have contributed extensive reviews of artificial substrate use; Cairns et al. (1969) have shown polyurethane foam to be an effective sampling device for protozoa. Kaushik & Hynes (1968, 1971), Petersen & Cummins (1974), and others have demonstrated the value of natural substrate placement in energetic evaluations. However, the visual aspects of the colonization process are not customarily included in these studies.

Early investigations on microbial colonization and succession on glass slides were conducted by Henrici (1933), Karsinkin (1934), and Gause (1936); later, on glass and plastic surfaces by Spoon & Burbanck (1967); and on plexiglass by Allen (1971) and Wetzel & Allen (1971). Although organisms collected by polyurethane foam substrates have received considerable attention, colonization and the means by which foam substrates support colonization have not been studied. Leaf material substrates in aquatic environments have also been of interest, due to the wide variety of microorganisms and invertebrates which they nutritionally support. Few authors, however, have presented direct visual evidence of leaf material colonization.

Among those who have documented colonization of natural substrate material in fresh-water environments by scanning electron microscopy (SEM) are Suberkropp & Klug (1974), who reported seasonal changes in leaf surface microorganisms of a small stream, and Paerl, who showed structural modification of detritus and commented on the nutrition of organisms colonizing organic particles in Lake Tahoe (Paerl, 1973, 1974; Paerl et al., 1975). Due to the

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increased difficulties of studying large river systems, leaf decomposition and colonization in these systems remains largely uninvestigated. However, Mathews & Kowalczewski (1969) reported that, unlike small headwater streams, leaf degradation in the River Thames was due to microbial action alone, despite the fact that invertebrates were associated with the decomposing plant material. The presence of microbes was assessed by weight, caloric, and nitrogen changes in exposed leaves, but was not documented photographically.

Willer (1923), Godward (1934), Allen (1971), and Wetzel & Allen (1971) studied the attachment of algae to aquatic macrophytes; Hutchinson (1975) provided an excellent review of this subject; and Allanson (1973) used SEM to document fresh-water microbial colonization of macrophytes. Marine studies dealing with microbial colonization of inert substrates include those of Zobell & Allen (1935) and Skerman (1956); and of organic substrates, that of Zobell (1946). Of particular interest are three SEM reports which address colonization of various natural substrates. Gessner et al. (1972) and Sieburth & Thomas (1973) studied the colonization of marine grasses; and Brooks et al. (1972), the colonization of driftwood.

Because scanning electron microscopy provides superior resolution and depth of field, this technique was chosen to monitor progressive colonization of artificial substrates in a lentic (standing water) system as well as artificial and natural substrates in a lotic (flowing water) environment. This method also facilitates comparison of relative material accumulation and differential colonization of substrate areas, in addition to indicating associated decomposition or changes in the substrate itself.

Materials and Methods

Sycamore (*Platanus occidentalis* L.) leaves from a single tree were collected immediately prior to abscission during fall 1975. They were air dried and stored in polyethylene bags until used. Two cm disks were punched from distilled water-softened sycamore leaf blades, dried at 50 C, and sewn into 10×10 cm nylon bags with a mesh opening of 3 mm. Two cm disks of polyurethane foam with a 1 cm thickness were enclosed in identical bags and fastened with stainless steel straight pins to artificial substrates, as previously described by Cairns & Yongue (1974).

Leaf disk and foam disk bags were anchored to concrete blocks and placed in the New River near Glen Lyn, Virginia, on 21 March 1976. On the same date, the foam disks were suspended at the surface of Pandapas Pond (Montgomery County, Virginia). New River leaf material was retrieved after exposures of 10 days, 3, 4, 6, 8, and 14 weeks. Foam disks from the New River were collected at 10 days, 3, 4, and 6 weeks. Pandapas Pond foam disks were collected 1, 6, 15, and 21 days following placement.

On retrieval, all disks were placed in vials of water from the collection site. New River samples were subsequently fixed with formaldehyde-acetic acid-alcohol for 24 h, and Pandapas Pond disks were fixed in a mixture of 2% osmium tetroxide and saturated mercuric chloride for 15 min, as described by Párducz (1967). All material was washed for 12 h in running distilled water, dehydrated in a graded alcohol series, and transferred to 100% acetone. To minimize loss of leaf cuticle, each dehydration step was limited to 10 min. Material was then dried with a Polaron critical-point dryer using liquid CO_2, mounted on stubs with conductive silver paint, vacuum coated with gold palladium, and examined with an AMR, Model 900, scanning electron microscope. Control leaf material and polyurethane foam were processed according to the above protocol.

Results and Discussion

Microbial colonization of New River leaf material (Figs. 1–18) was rapid and organism density increased with time. Substantial colonization was achieved after 10 days (Figs. 3, 4), being particularly prominent along leaf veins. The veinal distribution of fungi on living sycamore (*Acer pseudoplatanus* L.) leaves has been previously noted by Pugh & Buckley (1971) using SEM. The differential fungal distribution may be due to aphid punctures into the phloem of the veins releasing more exudates and providing a means for mycelial entry (Pugh & Buckley, 1971), increased exudation along veins of senescing leaves (Pesante, 1963), or nutrient accumulation near the surface of veins (Wylie, 1943). Algal accumulations can also be seen at vein-blade junctions and is in general agreement with the nonrandom distribution of epiphytes about the midrib of living macrophyte leaves (Godward, 1934; Willer, 1923). Although little evidence of microbial colonization prior to New River exposure is seen in control leaves (Figs. 1, 2), vein and epidermal cells near veins may be structurally weaker as a result of parasitic invasion prior to or during senescence.

Pioneer fungi invading areas along veins trapped and retained organisms and organic debris at the junction of leaf blade and veins (Fig. 4) after 10 days of exposure. Amorphous particles can also be seen at this stage and are probably formed from dissolved organic matter (DOM) aggregation (Lush & Hynes, 1973) or calcium carbonate-DOM complexes (Allanson, 1973; Allen, 1971; Wetzel & Allen, 1971). These accumulations of detritus verify detrital aggregations reported by Allanson (1973), Paerl (1973, 1974), and Paerl et al. (1975), using SEM, and are probably of nutritional significance to microbial colonizers as they suggest. Of particular note are pennate diatom accumulations, pre-

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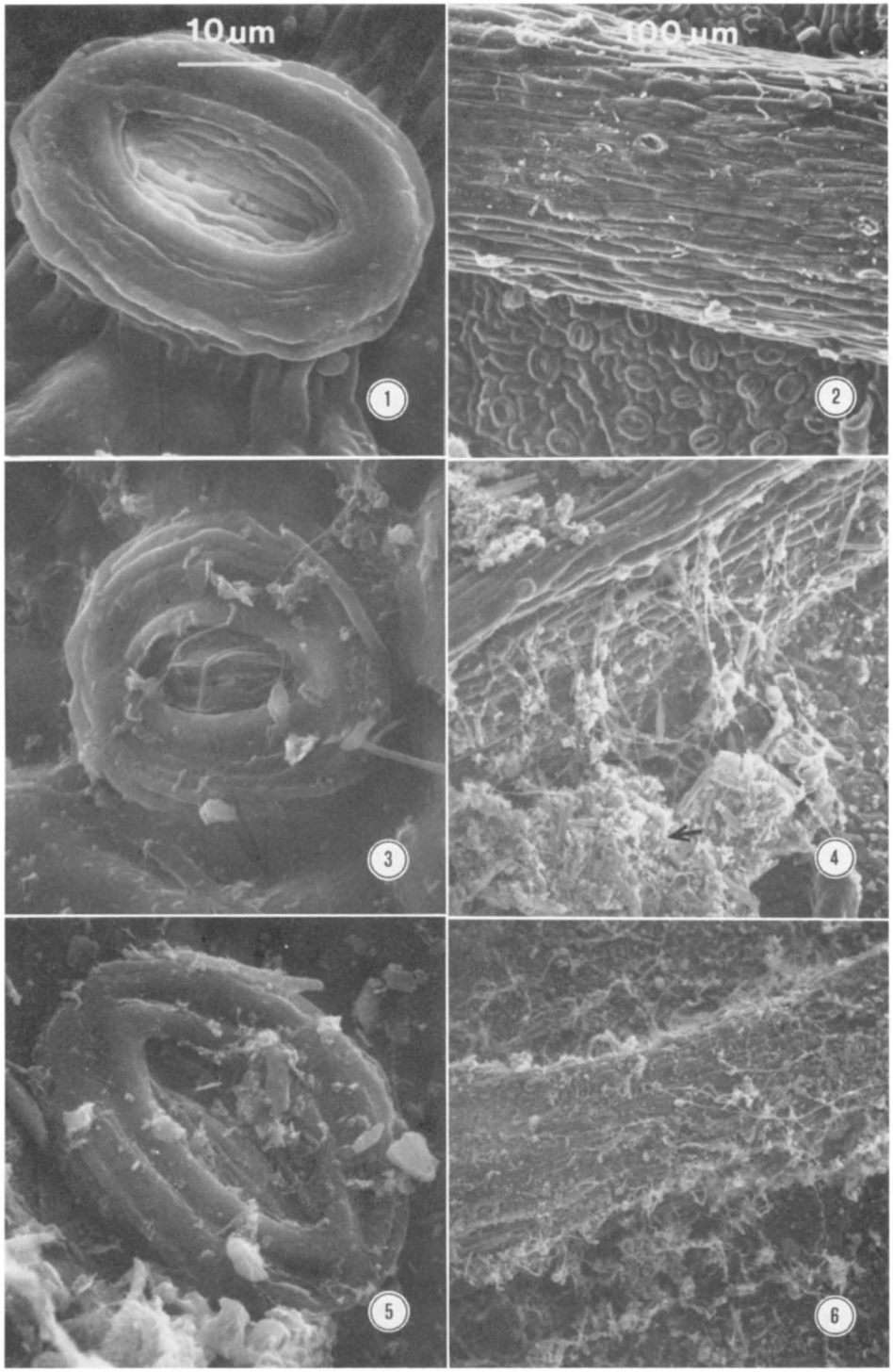
Figs. 1, 2. Sycamore leaf (abaxial surface) prior to introduction into the New River. The stomata (Fig. 1) and midrib blade region (Fig. 2) are relatively free of microbes. × 2,000, × 200. Figs. 3, 4. Leaf material exposed for 10 days in the New River. Primary colonizers are fungi, bacteria, and algae. Arrow notes the presence of accumulated organic matter (Fig. 4) which has apparently been entrapped in the fungal network or has been lodged against the midrib. × 2,000, × 200. Figs. 5, 6. Colonization at three weeks. Stomata (Fig. 5) remains normal in appearance, but shows additional accumulation of detritus. The midrib is now completely covered with fungal hyphae (cf. Fig. 6 with Fig. 4). × 2,000, × 200.

Figs. 7, 8. After four weeks exposure, stomata and vein are in initial stages of decomposition. × 2,000, × 200. Figs. 9, 10. Guard cells of stomata at six weeks exposure are no longer elevated above leaf surface (cf. with Fig. 1) and the cuticle (Fig. 10) is decomposing (arrow). Some portions of the cuticle have come loose from their associated wall. Arrow indicates triradiate hyphomycete spore. × 2,000, × 200. Figs. 11, 12. After eight weeks exposure, cuticular peeling (arrow) of the guard cells has begun. Massive detrital accumulations appear at the vein-blade junction and further decomposition of cells is apparent. × 2,000, × 200.

Figs. 13–18. Leaf material at 14 weeks exposure is in advanced stages of decomposition. Fig. 13. Stomata still recognizable, but cell beneath sub-stomatal cavity (arrow) is now protruding through aperture. × 2,000. Fig. 14. Extensive degradation of vein and heavy silt accumulation. × 200. Fig. 15. Note the degree of decomposition from leaf vein across blade. Arrow indicates area of enlargement in Fig. 17. × 50. Fig. 16. Higher magnification (× 200) of vein-blade junction in Fig. 15. Note that degradation is advanced in the same area which first showed fungal colonization (cf. Fig. 16 with Fig. 4). Epidermal blade cells completely devoid of protoplasts; only the cell walls remain. Fig. 17. Area of lamina again showing that secondary veins (arrows) are more slowly degraded than surrounding blade cells (cf. Fig. 15, arrow). × 200. Fig. 18. Higher magnification of midrib area from Fig. 14. In some cells of the veins only the walls remain. × 1,000.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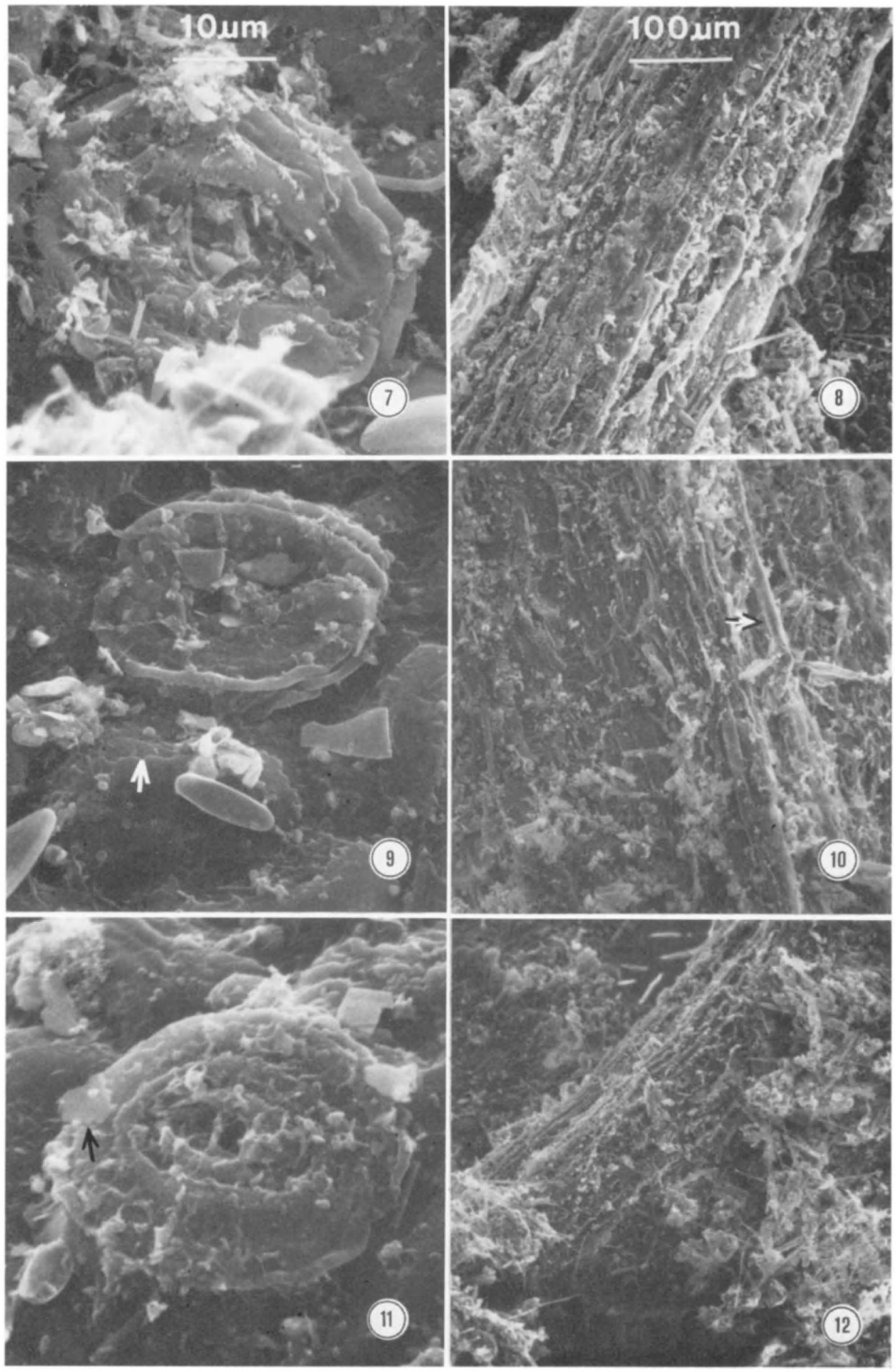
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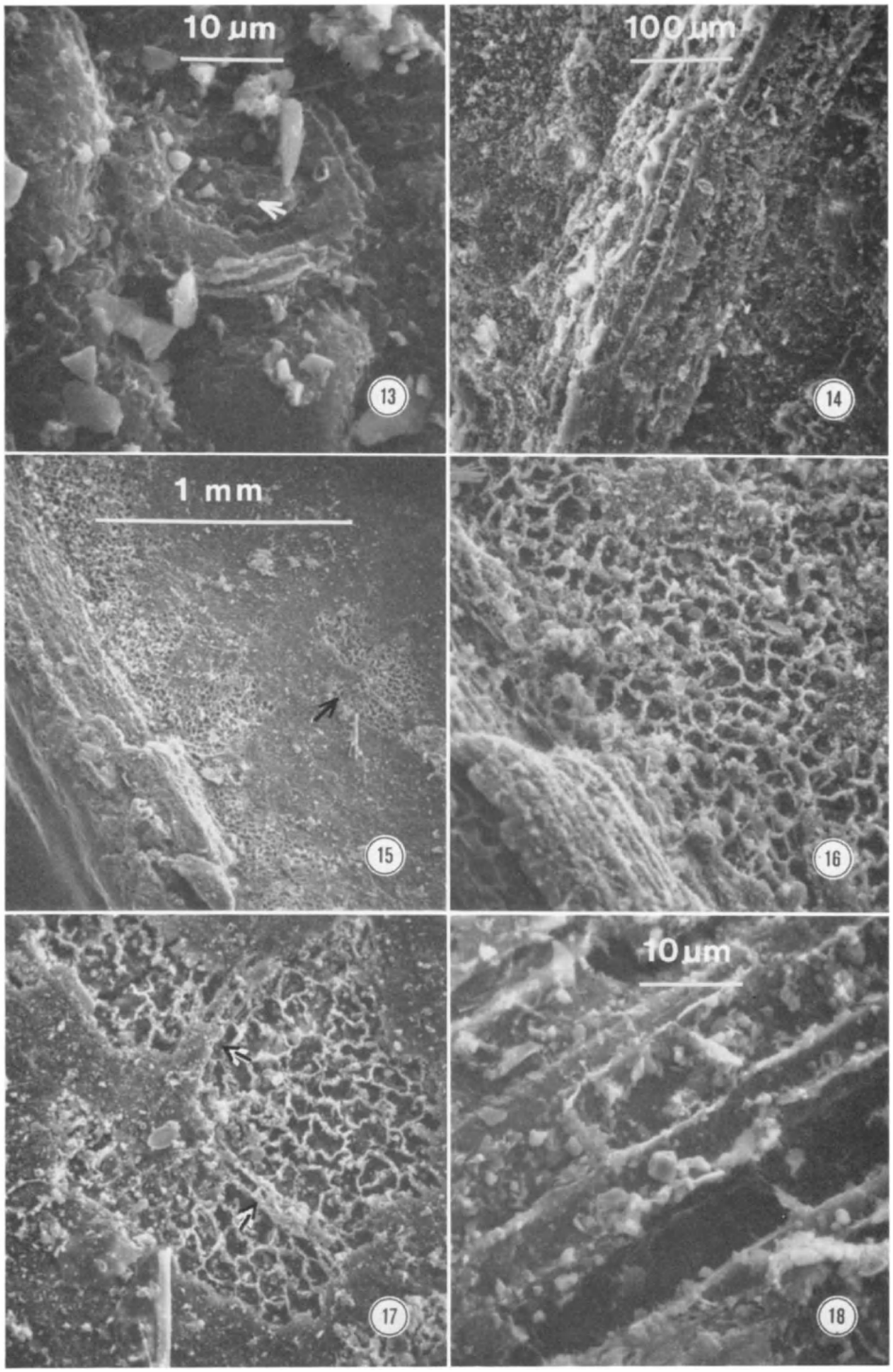
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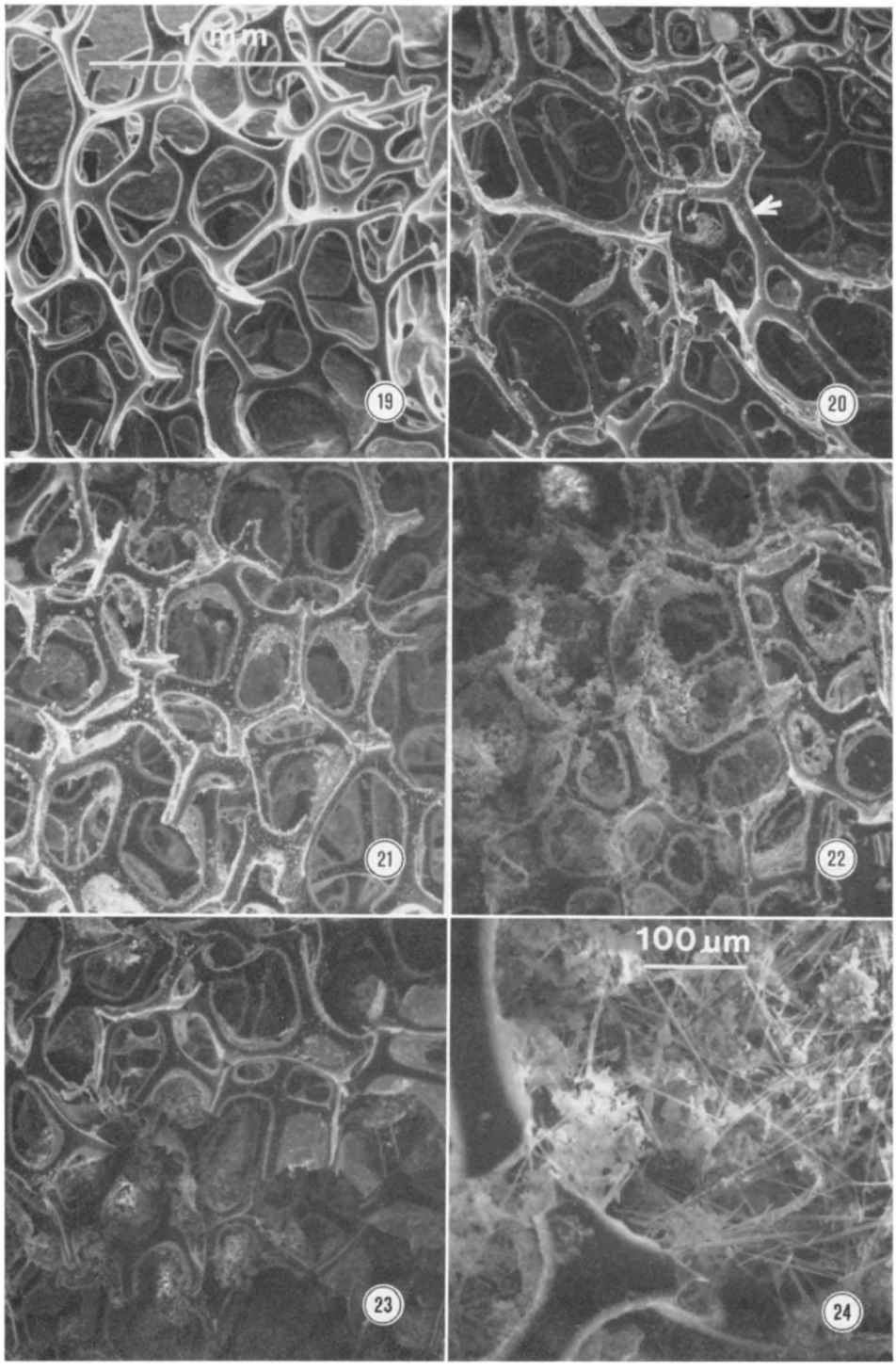
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sumably mediated by mucilagenous secretions which tend to enlarge aggregates formed by mycelial matrices, bacterial secretions, and silt buildup.

Rapid colonization at 10 days also occurred in the vicinity of stomates (Fig. 3) where mycelial penetration into internal tissues and bacterial invasion of epidermal and guard cells is evident. These observations confirm the early colonization and penetration of pignut hickory (*Carya glabra* [Mill.]) leaf stomates observed by Suberkropp & Klug (1974). The attraction of microbial populations to stomates may be further enhanced by the leaching of nutritionally valuable water soluble compounds from internal tissues through these openings. Because leaching from sycamore leaves has been shown by Kennedy & Paul (1976) to be essentially complete after one week, it is not surprising that microbial attachment is rapid and well established by 10 days.

Heavy fungal growth in close proximity to leaf veins and particulate organic matter collection near stomates characterized colonization of the lower leaf surface after three weeks of river exposure (Figs. 5, 6). The structural integrity of the tissue was retained despite fungal penetration and decompositional activity at this time. Leaf material exposed for four weeks (Figs. 7, 8) showed further colonization, particularly in the areas adjacent to stomatal openings. Vein colonization (Fig. 8) appeared to be more advanced at this stage with fungal mycelia obscured by organic debris and silt mats.

After six weeks of exposure, stomata and the surrounding epidermal cells (Fig. 9) appeared to be decomposing under colonizing bacterial attack, and cuticle peeling occurred near the vein surface (Fig. 10). Silt, organic debris, and particularly pennate diatom accumulations marked vein areas (Fig. 12) at eight weeks. In addition to these aggregations of detritus, stomata (Fig. 11) also show heavy colonization and fairly uniform coverage by bacteria.

The effect of colonizing decomposers on natural substrate integrity was noticeable after 14 weeks of exposure (Figs. 13–18). The substrates then exhibited little further fungal colonization on the surface, suggesting that fungal activity at this point in the colonization and decomposition processes was occurring in the leaf interior and that degradation at the surface was primarily bacterial. When one compares 8- and 14-week samples, a shift from large detrital accumulations to smaller, more uniform, debris coverage is seen. Stomal guard cells in 14-week samples (Fig. 13) were no longer normal in appearance and showed signs of degradation.

Vascular tissue exhibited heavy buildups of debris (Fig. 14) and the collapse of external cells (Fig. 18) after 14 weeks. Stomatal cells exposed for the same period (Fig. 13) showed that fungal mycelia were virtually absent at the surface. Degradation in the vicinity of veins was apparently the result of early colonization, penetration of fungal mycelia, and the differential colonization of these areas when compared to the balance of the leaf blade (Figs. 4, 6). Further evidence for the early colonization along veins was seen in the epidermal degradation originating near veins and progressing toward the leaf margin (Figs.

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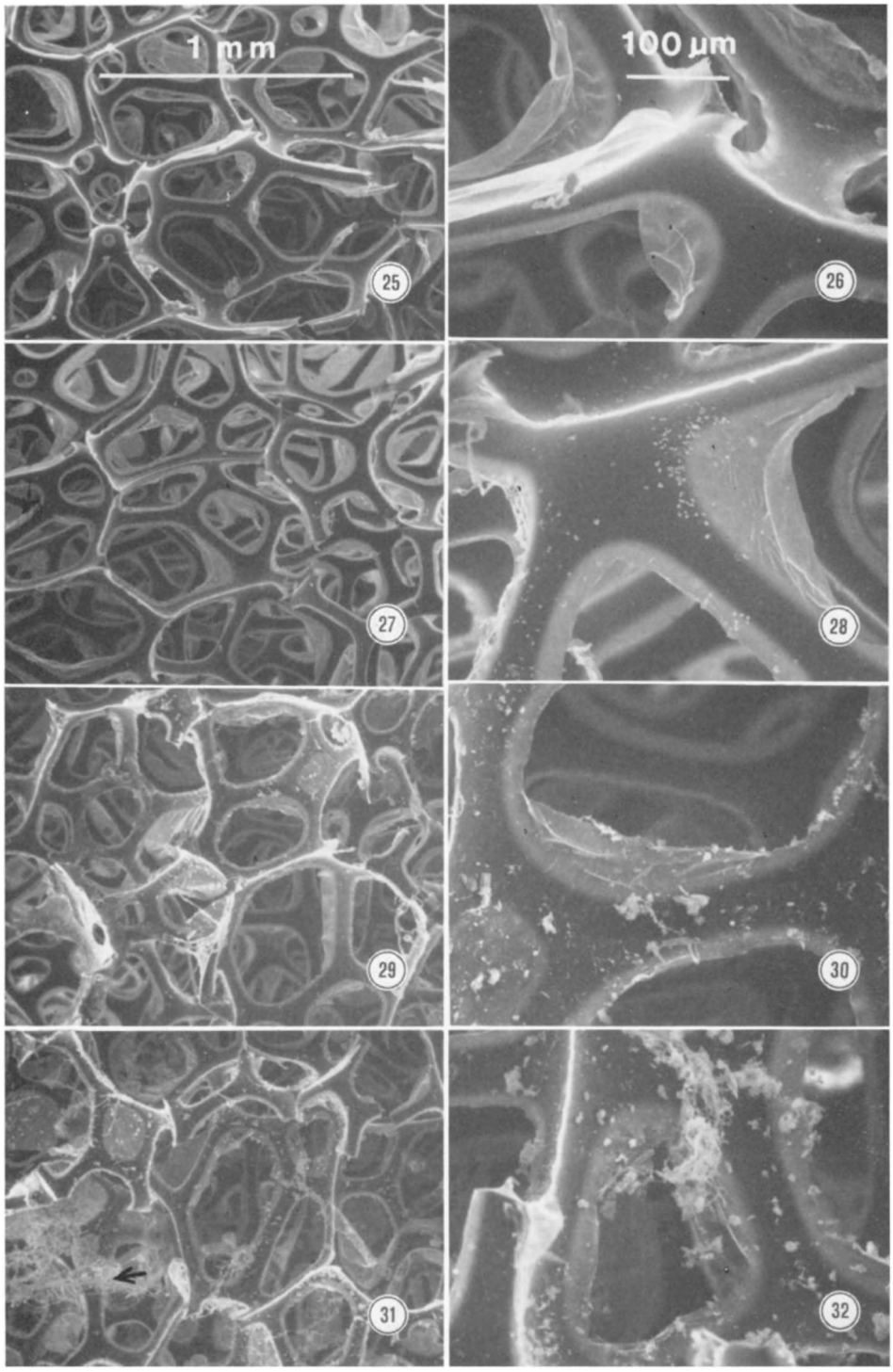
FIGS. 19–24. Polyurethane foam substrate colonized in the New River. Fig. 19. Unexposed control foam virtually devoid of microbes. × 50. Fig. 20. After 10 days exposure initial colonization by small diatoms (arrow) and small amounts of fine particulate organic matter appears on the foam pillars. × 50. Fig. 21. Colonization on pillars continues (at three weeks) and fine particulate organic matter is beginning to aggregate and occlude the interstices. Fig. 22. Greatest colonization occurs on edges of interstices with some spaces completely filled by organic matter and microorganisms. × 50. Figs. 23, 24. Foam interstices with advanced colonization. Numerous pennate diatoms are present in the detritus. × 50, × 200.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15, 16). It is interesting to note that vascular tissue appears to be more resistant to degradation than the epidermis (Fig. 17) and is presumably due to the higher fiber content in the veins than in the leaf blade as a whole. Despite earlier colonization, the rate of leaf vein decomposition seen in this study supports the findings that leaves with higher lignin contents decompose more slowly than those with lower cell wall lignin, as discussed by Wetzel (1975), and that lignin percentages recorded by Triska et al. (1975) increase with decomposition time. Although veins are colonized from the onset of exposure, longer time periods may be required to degrade the more chemically resistant cellulose and lignin of vascular tissue.

The rapidity with which colonization occurred was surprising, especially when artificial substrates were considered. Artificial substrate colonization in the New River (Figs. 19–24) was characterized by the appearance of different colonizing organisms. Although Cairns et al. (1969) and Cairns & Yongue (1974) have shown that protozoa rapidly colonize polyurethane foam, they either do not survive or are washed from the foam during the preparation procedure. Presumably, the inert nature of the polyurethane foam has a profound influence on the types of colonizing organisms. Colonization rates and the types of colonizing organisms, with the exception of fungi, of both New River substrate types do, however, seem similar. These results are in agreement with the findings of Patrick et al. (1954) and Castenholz (1960).

Comparison of control (Fig. 19) and 10-day (Fig. 20) foam disks showed that, unlike leaf substrates where fungi and bacteria were early colonizers, small diatoms were the first organisms to appear on the polyurethane foam. These observations support Skerman's (1956) and Sieburth & Thomas' (1973) premise that initial bacterial colonization is not necessary for ensuing organism accumulation. Apparently, the secretion of algal slime substances during their attachment (Fritsch, 1935) also serves to influence the accumulation of organic matter and silt at three weeks (Fig. 21). The presence of these small particles was restricted to the polyurethane "pillars" and was attributed to the previous algal colonization. Further aggregation of organic matter, algae, and bacteria seen at four weeks (Fig. 22) appeared to originate from points of attachment on the foam pillars and proceeded into the interstitial spaces of the foam. Accumulations of material after four weeks of exposure closed the foam spaces, enhancing the entrapment of additional material previously washed into the foam. Easily assimilated organic compounds from decaying organic matter, CO_2 liberation by heterotrophs, and extracellular product release from previous colonizers probably make the interior of the foam attractive for further microbial accumulation. After six weeks of colonization (Figs. 23, 24) pennate diatoms, in vast numbers, were present and filled much of the foam's interior.

Pandapas Pond substrates had a similar pattern of colonization; however, colonization appeared to be slower and the succession of organisms appeared to be different from New River substrates. Polyurethane foam exposed for one

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FIGS. 25–32. Artificial foam substrates exposed in Pandapas Pond from one day to three weeks. Each pair of figures is arranged to show a low magnification view on the left (× 50) and a closer view of the colonizing organisms on the right (× 200) at increasing exposure times. Figs. 25, 26. Foam exposed for one day shows virtually no colonization compared to control (Fig. 19). Figs. 27, 28. After six days exposure invading colonizers, bacteria appear. Figs. 29, 30. Colonization and fine particulate organic matter remained confined to the foam pillars at 15 days. Figs. 31, 32. After three weeks exposure in Pandapas Pond, the degree of colonization is nearly as great as in New River samples. Filamentous algal colonizers are seen in Fig. 31 (arrow).

day (Figs. 25, 26) and six days (Figs. 27, 28) in Pandapas Pond provided evidence that bacteria were the principal early colonizers and that their attachment to the substrates was simply adhesion to a suitable surface. Initial bacterial colonization of inert (Henrici, 1933; Karsinkin, 1934; Zobell & Allen, 1935) and organic (Allanson, 1974; Zobell, 1946) substrates, subsequent slime deposition, and algal aggregation are documented by these photographs. DOM uptake by the substrate probably preceded all biological colonization, as Hutchinson (1975) suggests. Bacterial secretion may enhance organic debris retention seen after 15 days (Figs. 29, 30), but the amount of accumulation was slight. Filamentous algae were the predominant colonizers of foam exposed for three weeks (Figs. 31, 32). The collection of organic matter and organisms in Pandapas Pond samples at three weeks was less than for the New River samples during the same exposure period (Fig. 21). The differential accumulation of material was probably due to a faster water exchange rate through the New River samples.

Protozoan colonization of polyurethane foam substrates may be rapid or gradual, reaching equilibrium after ca. 1–5 weeks (Cairns et al., 1969; Yongue & Cairns, 1976). Colonization of these substrates by these organisms and others is probably enhanced by organism and organic debris resource accumulation.

The ways in which lentic and lotic artificial substrates became colonized appeared to differ. However, in both environments bacteria or algae were the primary colonizers. These organisms apparently condition the substrate by their secretions and physically enhance the opportunity for the aggregation of organisms, organic matter, and debris as Allen (1971), Hutchinson (1975), Skerman (1956), Wetzel & Allen (1971), and Zobell & Allen (1935) suggest. Our observations indicate that initial colonization of polyurethane foam was bacterial in the lentic environment and algal in the lotic system studied. However, because the retrieval of New River samples did not occur before 10 days, bacterial colonization may have preceded algal attachment. The specific type of colonizer is probably of less importance than its role, which in both cases appears to be similar. Of major consequence, however, is that further accumulation of particulate matter and organisms appears to be abetted by algal or bacterial secretion and matrix formation by the algae themselves, thus rendering the polyurethane foam nutritionally rich and inviting to other organisms. The role of fungi in artificial substrate colonizing appears to be of minor significance. A more likely determining factor in the amount of ensuing colonization is the DOM, particulate matter, and organism load carried by the water and the movement of water into and through the artificial substrate.

Fungal and bacterial colonization of leaf material during initial exposure almost certainly has a nutritional as well as a physical basis and is perhaps similar to macrophyte colonization (Allanson, 1973; Allen, 1971; Gessner et al., 1972; Sieburth & Thomas, 1973; Wetzel & Allen, 1971). Since the first retrieval of leaf material occurred after 10 days of exposure, substantiation of either bacteria or fungi as the initial aquatic colonizer could not be determined. The type of initial colonizer, again, appears to be of little consequence with bacterial slime secretion and matrix formation by fungal mycelia (perhaps) being of greater importance. Aggregation of organically rich products suggests that the production of easily assimilated compounds accelerates degradation and attracts organisms. Clumping and cementing of particulate organics into aggregates is enhanced by bacterial, algal, and fungal secretions as well as matrices formed by fungal mycelia and pennate diatoms. These observations lend support to the macrophyte-periphyton metabolic model of Wetzel & Allen (1971) as do the observations of Allanson (1973) and suggest that this model may be applied, in a general way, to leaf colonization and particulate matter decomposition. Attachment and

subsequent substrate degradation occurred initially at vein-blade junctions and was accompanied by mycelial penetration into the leaf interior along veins and at stomatal openings. Although aquatic hyphomycetes were noted on leaf material, the New River fungal flora was not dominated by the hyphomycetes observed by Suberkropp & Klug (1974), but rather was similar to that seen by Allanson (1973), Gessner et al. (1972), and Sieburth & Thomas (1973). Furthermore, decomposition in this study appeared to be more rapid than in that of Suberkropp & Klug (1974), possibly because of seasonal differences in processing rates or a different bacterial and fungal flora. Rapid New River decomposition may also be due to heterotrophic activity stimulation by macrophyte, bacterial, or algal release of nutrients and organic products. Leaf decomposition which accompanies colonization in a large river, like the New River, indicates that decomposition is primarily initiated and completed by microorganisms and not macroinvertebrates as Mathews & Kowalczewski (1969) suggest.

Like artificial substrates which are conditioned for protozoan colonization, leaf material has been shown (Barlocher & Kendrick, 1973a, b, 1975; Cummins et al., 1973; Petersen & Cummins, 1974) to be nutritionally desirable to aquatic invertebrates only after an initial colonization or conditioning period; it also serves as an important mechanism in leaf degradation. Additionally, in marine environments the importance of microbial-organic matter complexes on leaf surfaces to grazing primary consumer nutrition has been emphasized by Brooks et al. (1972) and Gessner et al. (1972); and in lentic systems, by Allanson (1973).

Colonization of both natural and artificial substrates thus serves to structurally modify these substrates into ecologically more complex systems capable of supporting a diverse array of species.

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STUDIES ON THE PRESERVATION OF THE CILIATE *DIDINIUM NASUTUM*[1]

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McGRATH, M. S., DAGGETT, P.-M. & NERAD, T. A. 1977. Studies on the preservation of the ciliate *Didinium nasutum*. *Trans. Amer. Micros. Soc.*, 96: 519–525. Two strains of *Didinium nasutum* (Müller, 1786) Stein, 1859 were successfully frozen using 10% dimethylsulfoxide as a cryoprotectant. A suspension consisting partially or wholly of cysts frozen at a controlled rate of 1 C per min gave rise, when thawed, to active feeding trophozoites which did not appear different from the original stock. Dried cysts were similarly recovered from −150 C. An accelerated storage test designed to gauge stability of dried material proved that shredded filter paper was preferable to glass as a drying substrate, but indicated that viability would gradually decrease with storage at 4 C. The ease, reliability, and advantages of maintaining this protozoon cryogenically, especially in a dried condition, are presented to encourage more widespread acceptance and use of cryopreservation for encysting protozoa.

Cryopreservation has become of greater interest to protozoologists in the past two decades. Its alternative, continuous subculturing, is associated with the risk of morphological and/or physiological changes rendering the organism atypical. Among protozoa, the least explored group in reference to cryopreservation is the ciliates. Pioneering work was accomplished with the two nonencysting ciliate complexes *Tetrahymena pyriformis* (Hwang et al., 1964) and *Paramecium aurelia* (Simon, 1971). Since some species can frequently be stored in the lab-

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