APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1983, p. 1659–1669
0099-2240/83/051659-11$02.00/0

Vol. 45, No. 5

Light and Electron Microscopic Studies of Microorganisms Growing in Rotating Biological Contactor Biofilms

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Received 10 November 1982/Accepted 31 January 1983

The biofilms growing in the first compartments of two rotating biological contactors used to treat municipal wastewater were examined by light and electron microscopy. The biofilms were found to contain a complex and varied microbial community that included filamentous and unicellular bacteria, protozoa, metazoa, and (possibly) bacteriophage. The predominant microorganism among these appeared to be a filamentous bacterium that was identical to *Sphaerotilus* in both morphological and ultrastructural characteristics. It was possible to isolate a *Sphaerotilus*-like bacterium from each contactor. Both the *Sphaerotilus* filaments and the wide variety of unicellular bacteria present tended to contain poly-β-hydroxybutyrate inclusions, a probable indication that these organisms were removing carbon from the wastewater and storing it. The microbial population of the biofilms appeared to be metabolically active, as evidenced by the presence of microcolonies and dividing cells.

The treatment of municipal and industrial wastewater generated by modern society is rapidly becoming an intractable problem. The continuing demand for a pollutant-free environment is exceeding the ability of traditional waste treatment processes to produce high-quality effluents at reasonable costs. Consequently, there is an urgent need for more efficient and economical wastewater treatment techniques. The rotating biological contactor, a relatively new approach to wastewater treatment, offers a cost-effective solution to this problem.

The rotating biological contactor consists of several circular plastic disks mounted centrally on a horizontal shaft (Fig. 1). This assembly is 40% submerged in a tank containing wastewater, and the shaft is rotated by either a mechanical or a compressed-air drive. To increase the efficiency of the treatment process, the tank is usually divided into four equally spaced compartments, each containing a series of disks. The wastewater to be treated flows through the contactor by simple displacement and gravity. Microorganisms from the wastewater initially adhere to the disk surfaces and, within 1 to 4 weeks, form a biofilm ranging from 1 to 4 mm in thickness. The film sloughs off the disks after reaching a critical thickness, which is determined by factors which are not yet completely understood. Since this sloughing process occurs randomly, the biofilm thickness over a disk surface at any given time is nonuniform. Sloughed biofilm and suspended solids are washed out of the contactor as the wastewater flows through the unit. They are later removed from the effluent during secondary clarification.

In theory, the contactor combines the characteristics of the two classical methods of wastewater treatment, activated sludge and trickling filters. The biofilm growing on the disks corresponds to the trickling filter slime, and the sloughed and suspended solids in the liquid phase correspond to the activated sludge flocs. The contactor, however, adds a new dimension to traditional designs because it exposes the biofilm to both wastewater and air during rotation. Consequently, up to 97% of the carbonaceous biochemical oxygen demand of an influent municipal wastewater may be effectively removed from the waste stream.

Most of the research conducted on rotating biological contactors to date has been based on traditional engineering methods which attempted to determine the overall organic removal efficiency and other design parameters during treatment of various kinds of wastes (5, 9, 27, 30, 31, 37, 39, 62). With the increasing demand for more cost-effective designs, however, optimization of contactor performance has become an important goal. To achieve this goal, one must understand the interactions between contactor microorganisms and their physiochemical environment. Specifically, there is a need for

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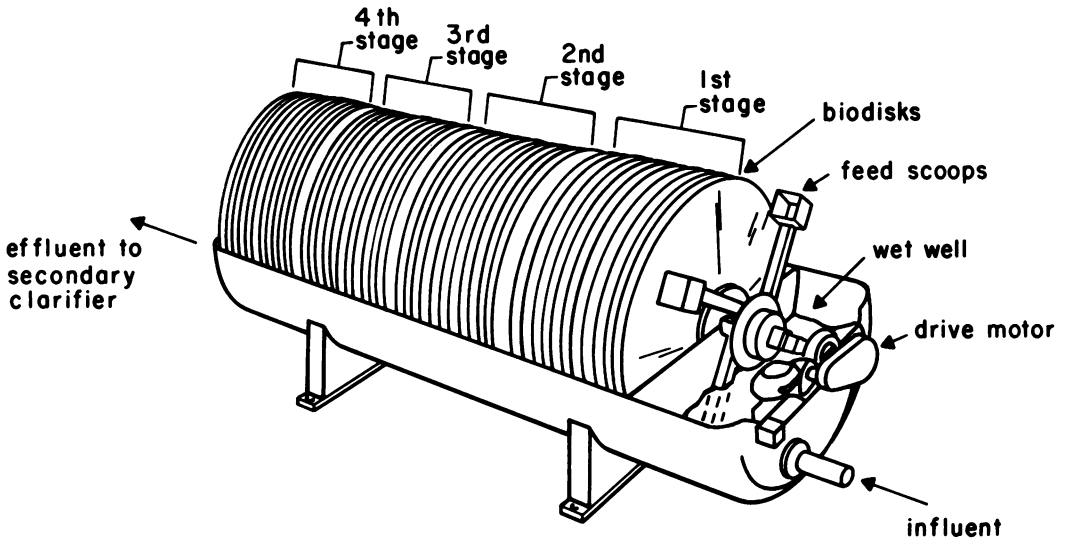

FIG. 1. Schematic diagram of a rotating biological contactor.

information on the identities and in situ physiological states of the microorganisms present, yet the bacteria inhabiting contactors during secondary wastewater treatment have not been examined or characterized thoroughly. Several authors have attempted to characterize indigenous biofilm populations by light microscopic observations of wet mounts. These studies have shown that, in the first compartments of the contactor, the most commonly observed filamentous form is the bacterium *Sphaerotilus* (28, 35, 42, 47, 59, 63). Other filamentous forms, such as *Beggiatoa* (8, 42, 47), *Fusarium* (35), *Nocardia* (28), *Cladothrix* (42), and *Oscillatoria* (35), have been seen less frequently. Nonfilamentous forms observed in the first compartments have included *Zoogloea*, film-forming bacteria (42, 63), unicellular algae (35), unicellular rods, spirilla, and spirochetes (8). The final compartments have contained most of the forms seen in the first compartments, as well as *Streptomyces* (8) and *Athrobotrys* (47). There has been only one attempt to isolate all of the bacterial forms growing in contactors (4), but the specific methods used were not described in that study. Recently, scanning electron microscopy was used to observe the composition of a rotating biological contactor biofilm (1). The community was believed to comprise a stratified layering of *Beggiatoa* and *Desulfovibrio*. To the best of our knowledge, there have been no attempts to examine the ultrastructural features of biofilm microorganisms with transmission electron microscopy.

The purpose of the present study was to examine rotating biological contactor biofilms by isolation studies, light microscopy, and transmission electron microscopy to gain more detailed information about the microflora present in this environment. The first compartments in two different contactor pilot plants were studied. Particular attention was directed to determining (i) the identity of the predominant filaments, (ii) the morphological types of unicellular bacteria present, and (iii) the ultrastructural characteristics of contactor microorganisms which may serve as indicators of their physiological and ecological conditions. We describe here an active and varied contactor microbial population in which the predominant filamentous organism present was a *Sphaerotilus* species.

MATERIALS AND METHODS

Rotating biological contactor pilot plants. Two different contactor pilot plants were examined in this study. The first was operated in a fume hood in an environmental engineering laboratory. It had only one compartment constructed from an acrylic half cylinder 30 cm long and 20 cm in diameter. A horizontal stainless steel shaft supported 16 disks, each 18 cm in diameter, giving a total wetted surface area of 0.79 m^2. The equally spaced disks were made of fiberboard sealed with polyurethane. A rotational speed of 0.31 m/s was maintained by a mechanical drive. The unit was exposed to low-level fluorescent light of less than 100 lm/m^2 for up to 12 h per day. Ambient air and wastewater temperatures were 20°C. All disks were approximately 40% submerged in wastewater at any given time. The influent for this 18-cm unit consisted of raw sewage obtained in 20-liter carboys from the Durham, N.H., sewage pumping station. The carboys were stored at 4°C until used (maximum holding time, 3 days), thereby allowing solids to settle. The settled sewage was transported from the carboys to the contactor by a peristaltic pump set to deliver 67 liters per day. This

flow was sufficient to operate the unit at a hydraulic loading rate of 0.04 m^3 of wastewater per m^2 of disk surface area per day and an organic loading rate averaging 3.2 g of total organic carbon (TOC) per m^2 per day.

A second contactor pilot plant was housed in a laboratory trailer located at the Durham, N.H., wastewater treatment plant. It was a four-compartment mechanical drive Bio-Surf unit (Autotrol Corp., Milwaukee, Wis.) with corrugated polyethylene disks (0.5 m in diameter). The rotational speed was 0.31 m/s and the submergence level was 40%. The unit was exposed to no more than 10 h of natural light per day. The ambient air temperature was maintained at 20°C, and the wastewater temperature was no less than 17°C. This 0.5-m unit received 0.95 m^3 of fresh primary effluent from the Durham treatment plant each day. The effluent was pumped continuously to achieve an overall hydraulic loading rate of 0.04 m^3 per m^2 per day and an organic loading rate averaging 3.2 g of TOC per m^2 per day.

Both contactors achieved chemical steady-state operation after a 3-week start-up period, as determined by obtaining similar effluent TOC concentrations on 3 consecutive days. The TOC measurements were performed on influent and effluent samples by the ampule method outlined for the model 526 analyzer (Oceanography International Corp., College Station, Tex.) after filtration through Whatman no. 40 paper. The contactor influent wastewater (settled raw sewage or fresh primary effluent) had an average TOC of 80 mg per liter. The mean effluent concentration from the 18-cm- and the 0.5-m-diameter contactors were 17.5 and 23.0 mg of TOC per liter, respectively. Microbial samples of the 18-cm contactor biofilm for both light and electron microscopy were randomly scraped from the surface of the first disk at least 1 month after chemical steady state was achieved. Concurrently, biofilm from the 0.5-m unit was randomly scraped from the front, middle, and end surfaces of the disks in the first compartment.

Light microscopy. The biofilm samples removed from the disks of the contactors were too dense to examine directly by light microscopy. Therefore, they were rinsed in several petri dishes containing distilled, deionized water (DDW) and then drawn up repeatedly in a capillary pipette to separate the densely tangled mass. Several wet mounts of each washed sample were examined with a Nikon Biophot microscope equipped with Nomarski interference optics to assess the microflora present. Photomicrographs of each sample were made with Kodak Panatomic-X (ASA 32) film.

For specific staining procedures, pieces of rinsed biofilm samples were washed four additional times in DDW and further separated by both the capillary pipette technique and by direct micromanipulation. Most of the constituents of the biofilm were removed from the samples by these techniques, except for the bacterial filaments and the film-forming bacteria. The presence of poly-β-hydroxybutyrate (PHB) granules in rinsed biofilm samples was tested by staining with Sudan black B by the procedure of Burdon (41). The presence of ferric iron on bacterial filaments was tested by reaction with 0.1% aqueous potassium ferrocyanide under acidic conditions to produce the Prussian blue reaction (55). Special care was taken to ensure that any soluble ferric iron in the biofilm was removed by repeatedly rinsing these samples in DDW.

Isolation experiments. The techniques used to isolate *Sphaerotilus* were similar to those described by Dondero et al. (20). Biofilm washed in four rinses of DDW and teased apart with the capillary pipette technique was mixed with 50 ml of DDW for 30 s in a blender. The homogenate was streaked for isolation on plates of Casitone-glycerol-yeast and glycerol-glutamic acid agar (20) and incubated for 48 h at 20°C. The resulting filamentous growth was examined by light microscopy and tested for PHB granules as described above.

Electron microscopy. All biofilm specimens were prepared for electron microscopy by the thin-sectioning technique. Two fixation procedures were used to prepare each sample for thin sectioning. For the Kellenberger fixation, pieces of biofilm material were suspended in Kellenberger buffer (34) and sufficient 1% OsO_4 (in Kellenberger buffer) was added to bring the final concentration of OsO_4 to 0.1%. The samples were prefixed in this suspension for 30 min at room temperature, after which they were concentrated and washed by centrifugation in fresh Kellenberger buffer. The resulting pellet was resuspended in 2 to 3 drops of tryptone-salt solution (1% tryptone [Difco Laboratories], 0.5% NaCl) and mixed with approximately 0.5 ml of molten Noble agar (Difco) at 50°C. The agar-specimen mixture was transferred to a glass slide, allowed to solidify, and cut into small blocks (less than 1 mm on a side) with a razor blade. These blocks were postfixed for 12 to 18 h at room temperature in 1% OsO_4 (in Kellenberger buffer) and prestained for 2 h at room temperature in 0.5% uranyl acetate (in Kellenberger buffer). For the glutaraldehyde-osmium tetroxide fixation, pieces of biofilm material were suspended in 0.1 M sodium cacodylate buffer (pH 7.5), and sufficient 12.5% glutaraldehyde (in 0.1 M sodium cacodylate buffer) was added to bring the final concentration of glutaraldehyde to 3%. After prefixation in this suspension for 2 h at room temperature, the specimens were concentrated and washed twice by centrifugation in 0.1 M sodium cacodylate buffer. The final pellet was resuspended in tryptone-salt solution and embedded in agar as described above. The resulting blocks of agar were then postfixed for 12 to 18 h at room temperature in 1% OsO_4 (in 0.1 M sodium cacodylate buffer).

Samples from both fixations were dehydrated through a graded ethanol series and then embedded in Spurr's low-viscosity epoxy resin (56). Thin sections were cut with glass knives or with a Diatome diamond knife on an Ultratome III ultramicrotome (LKB Produkter, Bromma, Sweden) at a cutting speed of 1 mm/s. The sections were retrieved on uncoated, 400-mesh, copper specimen grids, after which they were poststained for 15 min with 0.5% uranyl acetate (in 50% methanol) and for 2 min with 0.4% lead citrate (49).

Thin sections were viewed with a JEM-100S transmission electron microscope (JEOL USA, Electron Optics Div., Peabody, Mass.) at an accelerating potential of 80 kV. Each specimen was examined and photographed extensively to ensure that a representative sampling of microbial cells was obtained. Comparisons were also made with light microscopic observations (see above) for this purpose. Size measurements and other quantitative data were obtained from photographic enlargements of the electron micro-

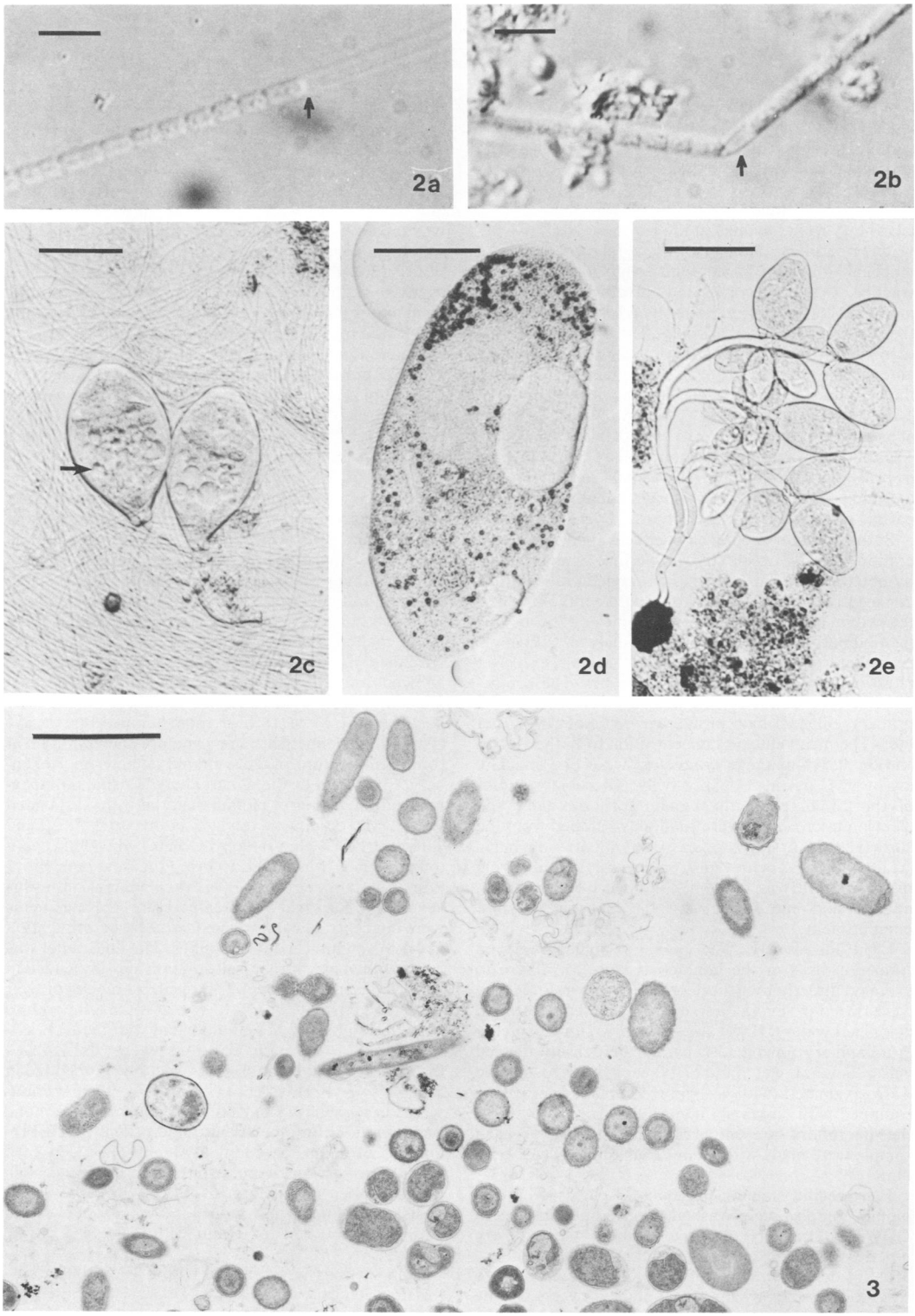

FIG. 2 AND 3. Nomarski interference light micrographs of organisms in the contactor biofilms. (a) Predominant filamentous form believed to be a *Sphaerotilius* sp. The sheath is most apparent at the section of the filament that does not contain cells (arrow). Bar, 3.0 μm. (b) The flexibility of the sheath of the predominant filamentous form is indicated by its remaining intact when filaments are bent (arrow). Bar, 3.0 μm. (c) Peritrich zooids containing numerous food vacuoles (arrow). Bar, 25 μm. (d) *Paramecium* sp. Bar, 25 μm. (e) *Opercularia* sp. Bar, 50 μm. (Fig. 3) Low-magnification electron micrograph of thin-sectioned biofilm material illustrating morphological variety of the nonfilamentous population. Bar, 2.0 μm.

graphs. Both fixation procedures gave equivalent results; micrographs made with the glutaraldehyde-osmium tetroxide technique were chosen for purposes of illustration in this report.

RESULTS

General biofilm characteristics. The biofilms on the first disk of the 18-cm contactor and on the disks in the first compartment of the 0.5-m contactor were gray-brown and filamentous, with a black subsurface layer. Growth was fairly uniform; maximum film thickness was 1 mm. Sloughing occurred randomly and recolonization appeared to be almost immediate. On the terminal disks of the 18-cm contactor and in the last compartments of the 0.5-m contactor, the biofilm was dark brown and somewhat thinner. It appeared mottled because recolonization occurred more slowly. These contactor biofilm characteristics were similar to those observed previously during domestic wastewater treatment (3, 23, 24, 27).

Light microscopy. Biofilms from the first compartments of both contactor pilot plants were extremely dense, forming interwoven mats. These mats were composed of two major constituents: filamentous bacteria, which appeared to be the predominant form, and single-celled bacteria grouped together in amorphous clumps. The latter appeared to be similar to the amorphous film-forming bacteria described by Unz and Dondero (64). Other frequently observed microorganisms included spirochetes, zooflagellates, nematodes, and free-swimming and stalked ciliates (see Fig. 2c–e).

Closer examination was afforded to the morphology of the predominant filaments. They consisted of a series of rod-shaped cells, approximately 1 to 2 μm in diameter and 2 to 5 μm long, which were tightly encased in an outer sheath (Fig. 2a). Under certain lighting conditions, individual cells appeared to contain refractile inclusion bodies. The sheath was most visible at the ends of the filaments, where the cell chain terminated and left only the empty casing. Flagellated cells were not observed exiting from the ends of the broken filaments. Concurrently, no holdfasts were seen, though these may have been lost when the samples were scraped from the contactors. The sheaths were quite flexible and were often bent to severe angles without rupturing (Fig. 2b). It was impossible to determine the overall length of the filaments because they were too intertwined with one another. False branching was rarely observed, and the filaments did not move or oscillate during examination.

Most of the cells within the filaments contained blue-black inclusion bodies after staining with Sudan black B (41), indicating that PHB was present. A small portion of the filaments did not contain PHB or contained it only in a localized region. Cells with PHB usually contained at least three of the blue-black inclusion bodies. In some filaments, the PHB granules appeared to occupy as much as 75% of the cell volume. Some of the cells in the film-forming bacterial masses also contained these inclusion bodies.

The sheaths of the filaments stained a dark Prussian blue after being exposed to potassium ferrocyanide under acidic conditions (55). As great care was exercised to ensure that no soluble ferric iron was present before staining, this apparently meant that iron precipitated out onto the sheaths of the filaments during wastewater treatment.

Isolation experiments. Tangled, curled, filamentous growth appeared on all of the initial isolation plates of Casitone-glycerol-yeast and glycerol-glutamic acid media. Light microscopic examination of the filaments that grew after restreaking showed that they were similar to those seen in the original biofilm samples. They consisted of rod-shaped cells within a sheath and exhibited the same morphological characteristics mentioned above. Staining with Sudan black B showed that most of the individual cells contained PHB.

Transmission electron microscopy. (i) Ultrastructure of the nonfilamentous population. Transmission electron microscopy of thin-sectioned specimens confirmed the presence of the nonfilamentous bacterial cells seen by light microscopy (see above). From low-magnification micrographs, it was evident that both a large number and a considerable variety of these organisms were present (Fig. 3). Biofilms from both contactors appeared to have similar nonfilamentous populations; no obvious differences in morphological types or ultrastructural characteristics were observed. Virtually all of the bacteria in both cases possessed typical gram-negative cell envelopes (10) ranging in morphology from short coccobacilli to long, thin rods. Spirilla were present in some samples but were seen only infrequently. Cell diameters varied considerably, ranging from 0.25 to 1.5 μm.

The ultrastructural characteristics of several representative types of the nonfilamentous bacteria are shown in Fig. 4. The majority of these organisms regularly contained one or more inclusion bodies, and some of them possessed rather prominent mesosome-like structures. In several instances, cells that appeared to be infected with bacteriophage were seen.

The nonfilamentous bacteria often appeared as groups of cells possessing identical morphological and ultrastructural characteristics. These groups, which included as many as 25 cells,

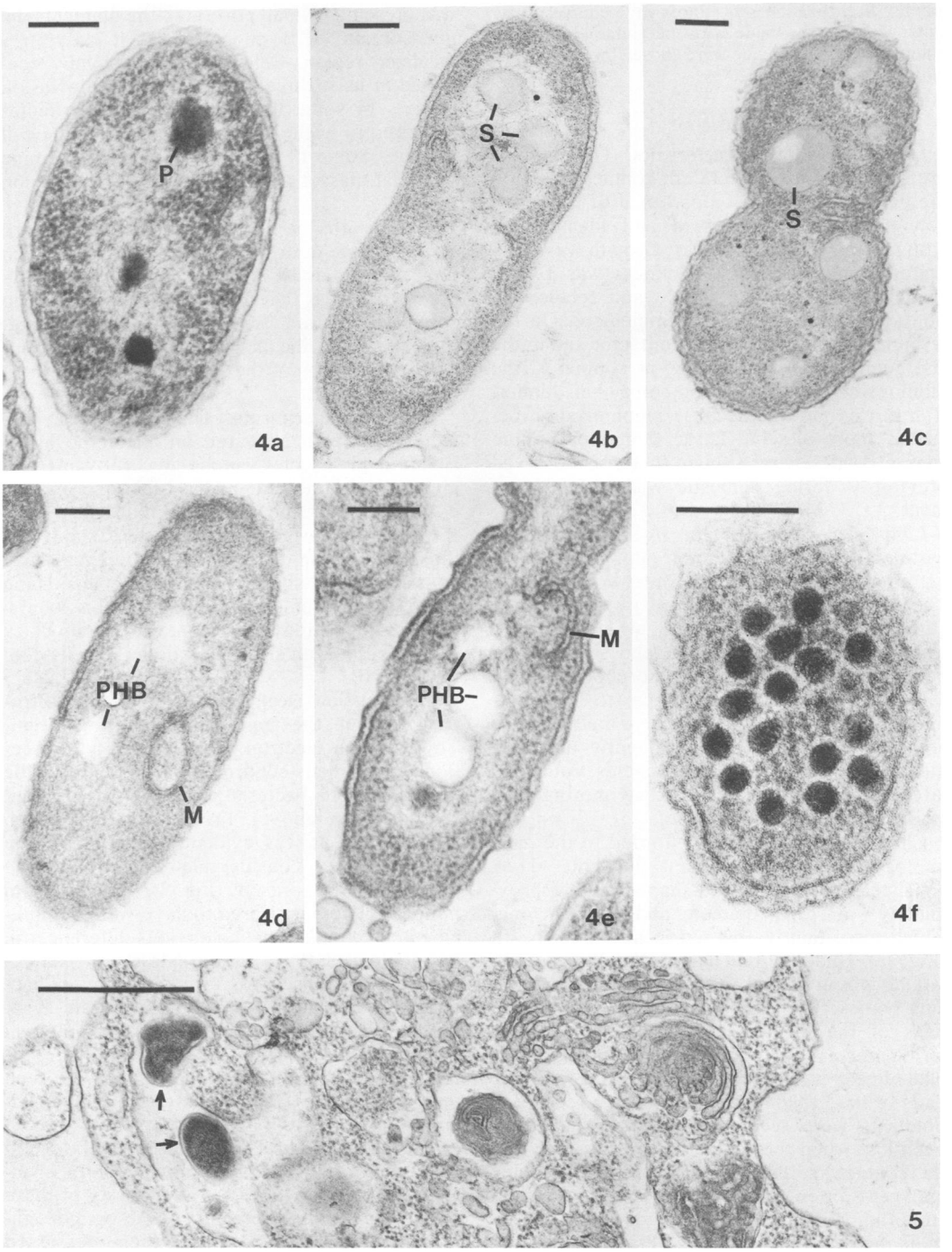

FIG. 4 AND 5. Electron micrographs of thin-sectioned biofilm material, illustrating ultrastructural features of the nonfilamentous population. M, Mesosome-like structure; P, apparent polyphosphate granules; PHB, apparent PHB granules; S, unidentified storage material. Bars, 0.2 μm. (a) Cell with apparent polyphosphate granules; (b) rod-shaped cell with unidentified storage materials, possibly PHB; (c) dividing cell with unidentified storage materials; (d) cell with apparent PHB granules and mesosome-like structure; (e) cell with apparent PHB granules and convoluted cell envelope; (f) cell apparently infected with bacteriophage. (Fig. 5) Electron micrograph of thin-sectioned biofilm material, illustrating a portion of an amoeboid cell containing bacterial cells (arrows). Bar, 1.0 μm.

apparently represented microcolonies or parts of larger colonies that remained intact throughout preparation of the samples for electron microscopy. One or more of the cells in such groups were sometimes seen to be undergoing cell division (not shown).

(ii) Eucaryotic organisms. The only eucaryotic organisms detected with any regularity in the electron microscopic studies were amoeboid-like organisms. Interestingly, these organisms always appeared to have several vacuole-like structures containing one or more intact bacterial cells (Fig. 5). Other eucaryotes seen by light microscopy (see above) were not observed in these preparations because they were either present in relatively low numbers or lost during preparation for thin sectioning.

(iii) Ultrastructure of the predominant filaments. The predominant filaments seen by light microscopy (see above) were readily detected by transmission electron microscopy and consisted of independent bacterial cells surrounded by a common sheath (Fig. 6a). The filaments ranged from 1.35 to 1.55 μm in diameter (including the sheath). Individual cells within the sheaths ranged from 1.0 to 1.2 μm in diameter and from 1.9 to 4.5 μm in length.

The sheath material on the filaments included a relatively dense layer that was situated quite close to the surface of the underlying cell walls (Fig. 6a). This layer appeared to have a fibrous construction, especially when the plane of sectioning passed through it tangentially. Most filaments also possessed a more loosely concentrated layer of sheath material immediately outside the dense layer.

The cells within a filament were independent of one another, being separated by a fairly small gap (Fig. 6b). An amorphous substance often filled these gaps, as well as the areas between the cell surfaces and the inner surface of the sheath. The cells possessed a typical gram-negative cell envelope, including a trilaminate cytoplasmic membrane and a trilaminate outer membrane. Both membranes were markedly convoluted. A thin, electron-dense layer, possibly corresponding to the peptidoglycan material, was often visible between these two membranes.

The cytoplasmic features of the cells included nuclear material, ribosomes, and some irregularly shaped, electron-dense inclusions (Fig. 6c). The latter were similar in appearance to polyglucoside granules (54). The cells in some, but not all, filaments contained as many as 15 electron-transparent inclusions surrounded by electron-dense bounding layers (Fig. 6c). These inclusions often infiltrated poorly during preparation of the cells for thin sectioning. They corresponded in size and location to the Sudan black B-stained granules seen by light microscopy and in their ultrastructural characteristics to PHB granules (21, 54).

Most cells in the filaments contained prominent mesosome-like structures (Fig. 6b) that were peripherally located and sometimes appeared to be associated with the polar walls of the cells. Many cells also contained relatively large, complex invaginations of the cytoplasmic membrane (Fig. 6a) that sometimes resembled large mesosomes but that more often lacked the internal membranes usually associated with mesosomes (26). In the latter case, they appeared to contain amorphous material similar to that observed between the cells and the inner layer of the sheath. In almost all cases, these large invaginations originated at or near the midpoint of the cell.

DISCUSSION

This study has served to document for the first time the morphological and ultrastructural characteristics of microorganisms living in the biofilms of the first compartment of rotating biological contactors. This information is significant because available data on the physiology and ecology of these microorganisms is quite limited. The study also provides information on the contactor microorganisms as they occur in their natural environment.

Eucaryotic organisms. The fixation procedure used here to prepare the biofilm for electron microscopy did not necessarily preserve eucaryotic cells, especially large protozoa and metazoa. Amoeboid-like forms, however, were observed regularly, indicating that they may play a significant role in the trophic structure of the biofilm. Most previous studies have determined that ciliates are the major protozoa present in wastewater treatment systems, but a few researchers (6, 51, 60) have found that amoebae are often overlooked or identified as detritus. Sydenham (60) concluded that they may be ecologically as important as ciliates in wastewater treatment. The amoebae in this study contained single-celled bacteria in individual vacuoles. Ciliates in activated sludge systems have been observed to prey upon single-celled bacteria, predominantly enteric species from raw sewage (12–14). The predatory activity of the ciliate results in lower concentrations of organic and suspended solids in the effluent. The amoebae may play a similar role in the contactor biofilm. It is likely that they predominantly reside on or near the surface of the biofilm, where oxygen and influent bacteria are more abundant.

Procaryotic organisms. The predominant organism in the biofilms examined here was a filamentous bacterium consisting of rod-shaped

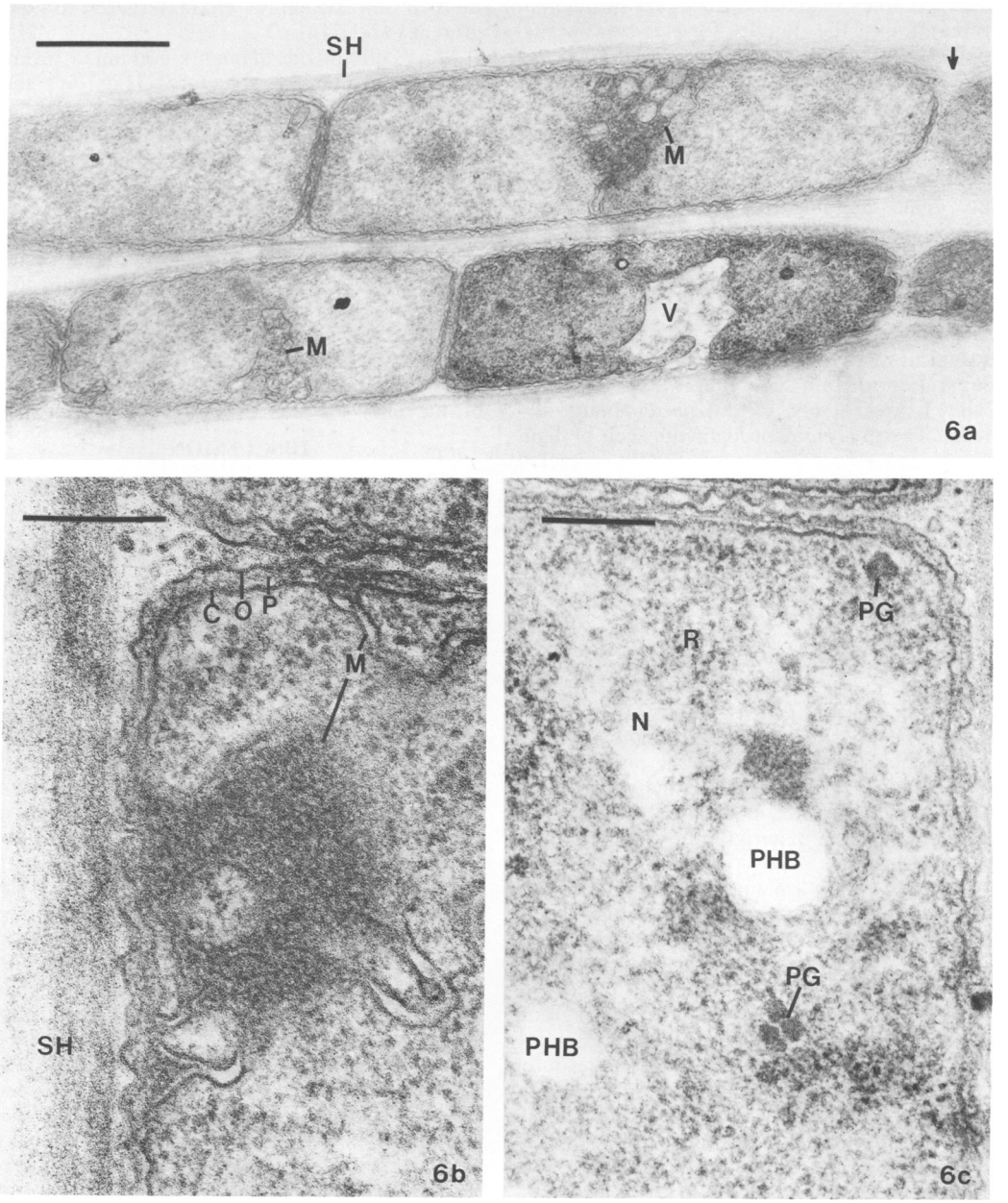

FIG. 6. Electron micrographs of thin-sectioned biofilm material, illustrating ultrastructural features of the predominant filament believed to be *Sphaerotilus* sp. C, Cytoplasmic membrane; M, mesosome-like structure; N, nuclear material; O, outer membrane of cell envelope; P, peptidoglycan layer of cell envelope; PHB, apparent PHB granule; PG, electron-dense inclusion, possibly polyglucoside granule; R, ribosomes; SH, sheath; V, large invagination of the cytoplasmic membrane. (a) Overall arrangement of cells in filaments. Note fibrous appearance of the sheath when sectioned tangentially (arrow), large mesosomes, and invagination of the cytoplasmic membrane. Bar, 1.0 μm. (b) Detail of cell structure, illustrating cell envelope and sheath construction. Sheath appears to have two layers. Two mesosome-like structures are present, one originating from the cross-wall (or division septum) and one originating from the longitudinal wall. Note small separation between cells. Bar, 0.25 μm. (c) Detail of cell structure, illustrating cytoplasmic contents. Bar, 0.25 μm.

cells enclosed by a common sheath. The data presented suggest strongly that this bacterium is a *Sphaerotilus* sp., on the basis of the taxonomic structure of the *Sphaerotilus-Leptothrix* group recently established by van Veen et al. (65). The principal criteria for identification of *Sphaerotilus* at the genus level are based on microscopic examination, isolation, and ruling out other filamentous forms (19). The light microscopic morphology of the biofilm organism was identical to that described as *Sphaerotilus* by several authors (19, 29, 38, 57, 65) in regard to both the sheath characteristics and the morphology of the cells within the sheath. Other filamentous forms were eliminated because the contactor bacterium lacked (i) the ultrastructural features of cyanobacteria, (ii) endospores, (iii) cross-walls, and (iv) active motility. In addition, the filaments isolated on both Casitone-glycerol-yeast and glycerol-glutamic acid media were similar to *Sphaerotilus* isolates described by Dondero et al. (20). The ultrastructural characteristics of the filaments in the biofilm were also similar to those of *Sphaerotilus* sp. studied in pure culture (17, 29, 57, 65), especially the strain described by Petitprez et al. (44). The principal similarities in ultrastructure included sheath morphology, wall structure, and presence of PHB granules and prominent mesosomes. Taken together, the above observations leave little doubt that the predominant filaments in the contactor biofilms were members of the genus *Sphaerotilus*. The data presented here did not permit identification of the biofilm filaments at the species level, and no further attempt was made to do so because the taxonomy of the *Sphaerotilus-Leptothrix* group has been somewhat controversial (19, 22, 48, 65).

The presence of *Sphaerotilus* as the predominant filamentous form in the rotating biological contactor biofilms examined here is significant in view of the metabolic capabilities of this organism. Initially, the iron encrustation on its sheath led investigators to believe that *Sphaerotilus* was an autotroph (67, 68); however, it is now considered to be an aerobic heterotroph (18, 43, 65). *Sphaerotilus*-based films growing in laboratories and in natural environments can remove 0.5 to 7.4 g of TOC per m^2 per day (7, 15), suggesting that this bacterium may contribute significantly to the organic uptake capacity of the contactor biofilm. Although *Sphaerotilus* requires oxygen as a terminal electron acceptor, it can function in microaerophilic conditions (16, 65). This fact is particularly significant because the filaments may continue to remove organic carbon from the waste stream under oxygen-limited conditions which may exist with depth in the biofilm. *Sphaerotilus* can exist as a filament or as a free-swimming flagellated cell (43, 45, 57). Its variable morphology may also be uniquely suited to the contactor process. Swarmers may rapidly recolonize disk surfaces after sloughing. Filaments may attach to the disks and may serve as a stabilizing structural force within the biofilm. The maximum growth of *Sphaerotilus* occurs when the fluid velocity is between 0.18 and 0.45 m/s (46), rates which coincide with the optimal rotational speeds used for rotating biological contactors (2, 24).

The ultrastructure of a bacterial cell can sometimes be indicative of the cell's physiological condition. The data in this study confirm the presence of a metabolically active population in the biofilm. It was apparent that the population was quite active from (i) the numbers of cells seen, (ii) the variations in cell size, (iii) the presence of microcolonies, and (iv) the presence of dividing cells within these microcolonies. The presence of mesosomes in both filaments and nonfilamentous cells may also be evidence of active metabolism and growth. Although mesosomes are currently somewhat controversial in terms of their true ultrastructural characteristics and their functions (if any) in the bacterial cell, they are often seen in dividing or in metabolically active cells (26).

Both the *Sphaerotilus* filaments and many of the nonfilamentous bacteria contained PHB granules. PHB is stored by bacterial cells when the carbon concentrations available in the environment are not limiting (25, 29). The large number of PHB granules found in the contactor bacteria indicates that excess carbon was present and had been metabolized. The storage of PHB by these bacteria may serve as an important intracellular sink for organic carbon in contactors. PHB can account for 11 to 22.5% of the dry weight of *Sphaerotilus* (50) and 12.0 to 50.5% of the dry weight of *Zoogloea* (11). The variation in the percentage of cell volume involved in PHB storage may in part be a function of the amount of organic matter available to the cells. Therefore, as the degradable organic concentration in the wastewater increases, the biofilm bacteria may store more carbon as PHB until some critical amount of the cell's volume is occupied by this substance. The storage of PHB, however, cannot be considered exclusively of other cellular metabolic processes, because it acts concomitantly with them in determining the fate of assimilated carbon in the biofilm. PHB also serves as a carbon and energy source for the cells during low nutrient concentrations (40, 58), and in this capacity it may help mitigate the effects of fluctuating hydraulic and organic loadings in the contactor. In addition, PHB has been shown to accumulate in some bacteria during oxygen-limiting conditions because the reductive compound formed during the synthesis of

the polymer can act as a terminal electron acceptor in place of oxygen (52, 53, 66). Therefore, the presence of PHB in the bacteria observed here may also indicate that oxygen concentrations are limiting within the biofilm.

In *Sphaerotilus*, the thickness of the sheath and the formation of an additional layer of sheath-like material has been observed in cells exposed to high organic loadings (29, 45). These external cell structures may function in a manner similar to PHB or may additionally function like the extracellular polysaccharide matrices described for film-forming bacteria (33), or both. In aerobic waste treatment systems, film-forming bacterial matrices are important as (i) a storehouse of carbon and energy, (ii) an effective adsorbent of metals and organic compounds, (iii) a floc-forming mechanism, and (iv) a buffer during high carbon and nitrogen growth conditions (33). The extracellular layers of *Sphaerotilus* may serve similar functions.

Some understanding of the ecological conditions in the biofilm may also be drawn from examining the biofilm microorganisms. Both light and transmission electron microscopy revealed the presence of many different types of bacterial cells, along with a few eucaryotic forms and (possibly) bacteriophage. This work supports the contention of other researchers who found a variety of bacteria present in wastewater treatment systems (32, 36, 61, 64). The presence of a diverse biofilm community may increase the ability of rotating biological contactors to efficiently degrade wastes and withstand environmental fluctuations. The presence of apparent phage within the microbial cells in the contactor may be indicative of deteriorating conditions in the biofilm. The bacteriophage may act as natural enemies of biofilm bacteria and may reduce their ability to assimilate organic matter from the wastewater. These and other aspects of the microbial ecology of contactor biofilms are presently the subject of continuing studies in our laboratories.

ACKNOWLEDGMENTS

We thank Sandra A. Nierzwicki-Bauer for technical assistance and Charles W. Walker for the use of his Nikon Biophot microscope.

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