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Title: ECOLOGICAL STUDY OF AQUATIC MYXOBACTERIA IN A WOODLAND STREAM

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Most studies of bacteria in water have concerned public health; little attention has been given to organisms which are indigenous to the aquatic environment. Myxobacteria are known to occur in soil, in the marine environment, and several have been studied in relation to diseases of salmonid fishes. However, little information is available regarding the role of myxobacteria in the freshwater environment. The major purpose of this research was to obtain data on the occurrence, distribution, and activities of aquatic myxobacteria in a woodland stream.

As a preliminary step for studying the ecology of myxobacteria in the freshwater stream, a culture medium and procedure were developed to provide a means for isolation and enumeration of these organisms. Enumeration of myxobacteria was based on the morphology of the colonies growing on the dilute nutrient medium, cytophaga

peptonized milk agar (CPM). The low nutrient concentration of this medium favored spreading of the myxobacterial colony and the production of rhizoid edge patterns which are typical of myxobacteria and distinguishable from eubacterial colonies.

The enumeration method was used to obtain data on the occurrence and distribution of myxobacteria as compared to the total bacteria population in Berry Creek. Results of the ecological study conducted over a two and a half year period indicate that myxobacteria are present in this aquatic environment all during the year with highest levels obtained in the fall (October and November) and lowest levels during the summer (July and August). Seasonal variations also occurred in the types of myxobacteria comprising the population of the stream.

It is interesting to note that peaks in the myxobacterial and the total bacterial populations occurred in advance of the peak in flow rate. Water temperature and flow rate did not seem to influence the population levels as might be expected if the myxobacteria were transient members of the microbial flora.

Additional surveys have shown that the myxobacteria are widespread in fresh water; they have been found in abundance in bottom sediments and surface films as well as in the flowing waters. Myxobacteria also appear to be well adapted to the aquatic environment. It was shown that they are able to utilize the dilute nutrients present

in water for their growth.

In an attempt to determine the possible role myxobacteria play in the aquatic environment, the predominant myxobacterial types were isolated and studied morphologically and biochemically. All the organisms studied corresponded to the classical definition of myxobacterial cells: gram negative, slender, weakly refractile rod-shaped bacteria which exhibit gliding motility. Colony morphology of these myxobacteria plated on CPM has been studied in detail and found to be a constant characteristic of the particular type of myxobacteria isolate.

Photographs of several of the predominant forms present in the stream samples illustrate the distinguishable morphology of the myxobacterial colonies. Six morphological groups have been arbitrarily designated on the basis of colony morphology; these morphological groups also show similarities in their biochemical capabilities. Biochemical studies on the myxobacterial isolates indicated that most of the strains were able to utilize simple carbohydrates. All of the isolates were capable of degrading various macromolecules, such as chitin, starch, aesculin, caesin, gelatin, and carboxy methyl cellulose. One of the isolates obtained was strictly proteolytic. The ability to hydrolyze macromolecules appears to be characteristic of aquatic isolates as well as of other myxobacteria.

One myxobacterium isolated was believed to be intimately

associated with the sheathed bacterium, Sphaerotilus natans. The latter organism was abundant in the sucrose and urea enriched section of Berry Creek. This myxobacterium could not utilize sucrose or urea and occurred only in the enriched section of the stream when Sphaerotilus was present.

The fish pathogen, Chondrococcus columnaris was also isolated from Berry Creek water. This myxobacterium can be distinguished from the other aquatic myxobacteria on the basis of its unique colony morphology. This strain of C. columnaris proved to be one of the common serological types found in the Pacific Northwest.

Based on the results obtained thus far, it is possible to speculate on the role of myxobacteria in the freshwater environment. All of the myxobacteria isolated in this study are capable of decomposing complex materials, it seems likely therefore, that these organisms may be active in the decomposition of such complex organic compounds, including the remains of other bacterial cells, which are present in the aquatic habitat. Since the isolates studied are also able to utilize the nutrients present at low levels in the stream water, these myxobacteria are not dependent on macromolecular substrates. Myxobacteria with these abilities are apparently well adapted to the aquatic environment.

Ecological Study of Aquatic Myxobacteria in a Woodland Stream

by

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--Lao-tse

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INTRODUCTION

In the field of freshwater bacteriology a disproportionate amount of work has been concerned with the enteric bacteria and with various indicators of fecal pollution. There are a great number of other types of microorganisms to which only scant attention has been paid; much work remains to be done on the bacteria indigenous to water. With increased knowledge of the occurrence, nutrition, and physiology of these indigenous bacteria, it is possible that some of these organisms could be used as indicators of the biochemical state of the environment. One of the groups of bacteria on which very little work has been done is the aquatic myxobacteria. Many myxobacteria are capable of degrading macromolecules. It seems reasonable that if the myxobacteria indigenous to aquatic habitats are able to attack a variety of complex substrates and other microbial cells, as are the terrestrial forms, they might contribute appreciably to the stabilization of organic matter introduced into a body of water.

The purpose of this study was to obtain information on the occurrence, distribution, and activities of aquatic myxobacteria in the freshwater environment. This thesis presents the results of

studies on myxobacteria carried out between April, 1966 and June, 1969. A method for isolation and enumeration of myxobacteria was developed for the collection of data in the ecological study. Comparisons were made between the occurrence of myxobacteria and the occurrence of total bacteria under controlled flow conditions in a small unpolluted, woodland stream (Berry Creek). Preliminary studies indicated that three or four myxobacteria were dominant in the stream and these were to be followed in relation to changes in the total bacterial population of the stream. However, after the enumeration method was developed, it was noted that 30 to 40 different myxobacteria occurred in the stream. The ecological study was then modified to compare the total myxobacterial population with that of bacteria other than myxobacteria. The predominant myxobacterial types were isolated, and studied further in an attempt to determine the possible role they play in the aquatic environment.

LITERATURE REVIEW

The myxobacteria, or slime bacteria, which were first described by Thaxter (60), comprise a little known group of bacteria which differ fundamentally from the eubacteria. Vegetative cells of myxobacteria closely resemble the cells of ordinary bacteria; however, they can be distinguished by their low refractility, the marked flexibility of cells which lack rigid cell walls, and their gliding type of motility. Without flagella, these organisms are able to move over solid surfaces in a slow and characteristic way; the cells arrange themselves in small groups which lie parallel to one another. These groups move as a unit on a thin layer of slime secreted by the cells. The direction of movement is away from the center of the colony toward the edge. The myxobacteria thus produce colonies which are characteristic of this movement: thin, irregularly shaped and spreading colonies (16, 53, 54, 60). A number of suggestions have been made by various workers to explain myxobacterial motility, but no adequate explanation concerning their mode of locomotion (16, 29, 54) exists at present.

Many myxobacteria are further characterized by the production of resting cells, known as microcysts. These highly refractile resting cells are produced by the shortening and thickening of single vegetative rods. In most cases the microcysts are borne in fruiting

bodies; the latter are complex structures formed by the aggregation of the vegetative cells. While not all myxobacteria produce fruiting bodies, it is because of them that the myxobacteria are best known (16, 34, 35).

Myxobacteria are saprophytes and are widely distributed in nature. They have been found in soil, water, and on a variety of decaying plant and animal materials. The most extensive studies have been carried out on myxobacteria isolated from the soil. Many of these organisms appear to live in association with true bacteria; in fact some have been shown to kill and digest other bacteria, fungi and algae. Because of the lytic activity of certain myxobacteria, these microorganisms have been considered to play an important role in the biological equilibrium of the soil (5, 20, 21, 25, 31, 41, 42, 51, 58, 59, 60).

Another characteristic property of myxobacteria is their ability to decompose such complex polysaccharides as chitin, cellulose, starch and agar (16, 53, 55, 56, 61). Cellulose decomposing myxobacteria have been reported to be among some of the soil organisms most active in the biological breakdown of cellulose. The work done on the best known members of this group, the non-fruiting myxobacteria: Cytophaga and Sporocytophaga, has been well monographed by Stanier (53, 56).

Although most of the myxobacteria which have been described

come from soil, these organisms have also been found to commonly occur in aqueous habitats. Among the aquatic myxobacteria, those which have been most extensively studied are the fish pathogens. The first myxobacterium described as pathogenic for fish was Chondrococcus columnaris. This organism was originally isolated in pure culture in 1942 by Ordal and Rucker, during an outbreak of columnaris disease in young sockeye salmon (Oncorhynchus nerka). This was the first report of myxobacteria pathogenic for animals. Bacteriological studies carried out on C. columnaris showed that the organism had a complex life cycle, producing both fruiting bodies and microcysts.

Another aquatic myxobacterium is of considerable importance as an agent of disease in young salmon. Cytophaga psychropila (Borg) is the etiological agent of bacterial cold-water disease in salmonid fish. It is a unique infectious agent in fish in that it is most virulent at low water temperatures (50F)(6, 7, 45).

A number of other aquatic myxobacteria have been found to be pathogenic to salmon. In ordinary gill disease, myxobacteria are regularly isolated from infected fish. The specific characteristics of the myxobacteria isolated vary in different outbreaks and it appears that a number of different myxobacteria might be responsible for this disease.

In their review of infectious diseases of Pacific salmon,

Rucker, Earp and Ordal (49) noted that large numbers of saprophytic, as well as parasitic, myxobacteria occur in fresh water and on the surface of fish. In a taxonomic study of myxobacteria isolated from the surface of fish, Pacha and Porter (46) attempted to differentiate between the saprophytic forms and the pathogenic species.

Studies on freshwater myxobacteria other than the fish pathogens have been mainly concerned with descriptions of particular organisms isolated from the freshwater environment. A non-fruiting myxobacterium, Polyangium parasiticum has been found to be parasitic for Cladophora, an aquatic alga (24). Grăf (26) described an aquatic myxobacterium, Sporocytophaga cauliformis, which was able to form microcysts but not fruiting bodies. Bauer described another aquatic myxobacterium which produces "spheroids" and has designated a new genus, Sphaeromyxa (5).

In a study of non-pathogenic cytophagas from water, Anderson and Ordal (3) demonstrated the presence of a fermentative myxobacterium. This myxobacterium, Cytophaga succinicans, fermented glucose with the net uptake of carbon dioxide, which was reportedly required for the fermentation. Other fermentative myxobacteria from the freshwater environment had been reported earlier by Borg (6, 7), but the nature of the fermentation was not determined and the organisms were not named.

Except for the work by Anderson and Ordal (3) very little work

had been done on freshwater myxobacteria prior to 1964 when Jeffers (30) published the results of several test media for the isolation of myxobacteria from a freshwater lake.

From the above discussion, it is apparent that while myxobacteria are known to occur in the freshwater habitat, apart from those pathogenic to fish, little is known about the role of these organisms in the aquatic environment. The recently completed studies by Burnison (10) on the interrelationships of the antibiotic substances produced by pseudomonads and myxobacteria in the aquatic environment; the studies published by Pacha and Porter (46) on the nonpathogenic myxobacteria from the surface of freshwater fish; the taxonomic studies by Nitsos (40) on freshwater cytophagas, and the results of the studies reported in this thesis have all contributed information toward explanation of the roles played by myxobacteria in the aquatic environment. Before it will be possible to adequately ascertain the precise role of these organisms in the freshwater environment, a great deal more information is needed, than is available, as to the occurrence, distribution, activities, and identity of the aquatic myxobacteria.

MATERIAL AND METHODS

Field Studies on Berry Creek

General Description of Stream

Berry Creek is a small woodland stream in the Willamette River Basin of western Oregon which has been used for experimental studies since 1959. Mason (38) and Warren, et al. (63) described the Berry Creek research area in detail. Information relating to the present study has been summarized and is presented below.

Berry Creek, one of several small streams which drain the northern slopes of McDonald State Forest, is located ten air-miles north of Corvallis in the lower foothills of the Oregon Coast Range. The south fork, on which the experimental section is located, drains 1.77 square miles of university-owned coniferous forest lands. This fork of Berry Creek then joins the north fork and flows into Soap Creek, which in turn empties into the Luckiamute River, a tributary of the Willamette River.

The flow pattern of the stream is stable from year to year; typical winter and spring flows range between 10 and 20 cubic feet per second (cfs) with occasional brief flood peaks exceeding 50 cfs. Flows as low as 0.1 cfs occur in late summer and early fall. The continuous flow of the stream is probably due to the thick vegetative

cover on the water shed and to the northward dip of the basin rocks toward the mouth of the stream. These drainage basin rocks belong to the Siletz River volcanic series and are overlaid by a sequence of sandstones.[1]

The stream bed is covered by water-worn basaltic rock ranging in size from small gravel, to 2-6 inch diameter rocks, and infrequent boulders to 12 inches in diameter. Silt and organic debris are deposited on the bottom of the deeper pools to depths of several inches; moderate to very small deposits occur in the riffles. Roots of large trees lining the stream stabilize the banks which have become undercut by high winter and spring flows.

Water quality data for 1959-1963 indicate that the mineral content of Berry Creek water is of a rather constant nature with a pH near neutrality (7.4-7.8).[2] Since the annual variation for most minerals was small and only minor differences have been noted over this five-year period, Warren, et al. (63) suggested that the neutral character of the water indicated origin in volcanic rocks and transport to the stream through rock of an insoluble nature. In the winter the stream water has a slightly turbid colloidal appearance and

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[1] See Berry Creek Progress Report, 1960, for further details on the geology of the region. Department of Fisheries and Wildlife, Oregon State University, Corvallis.

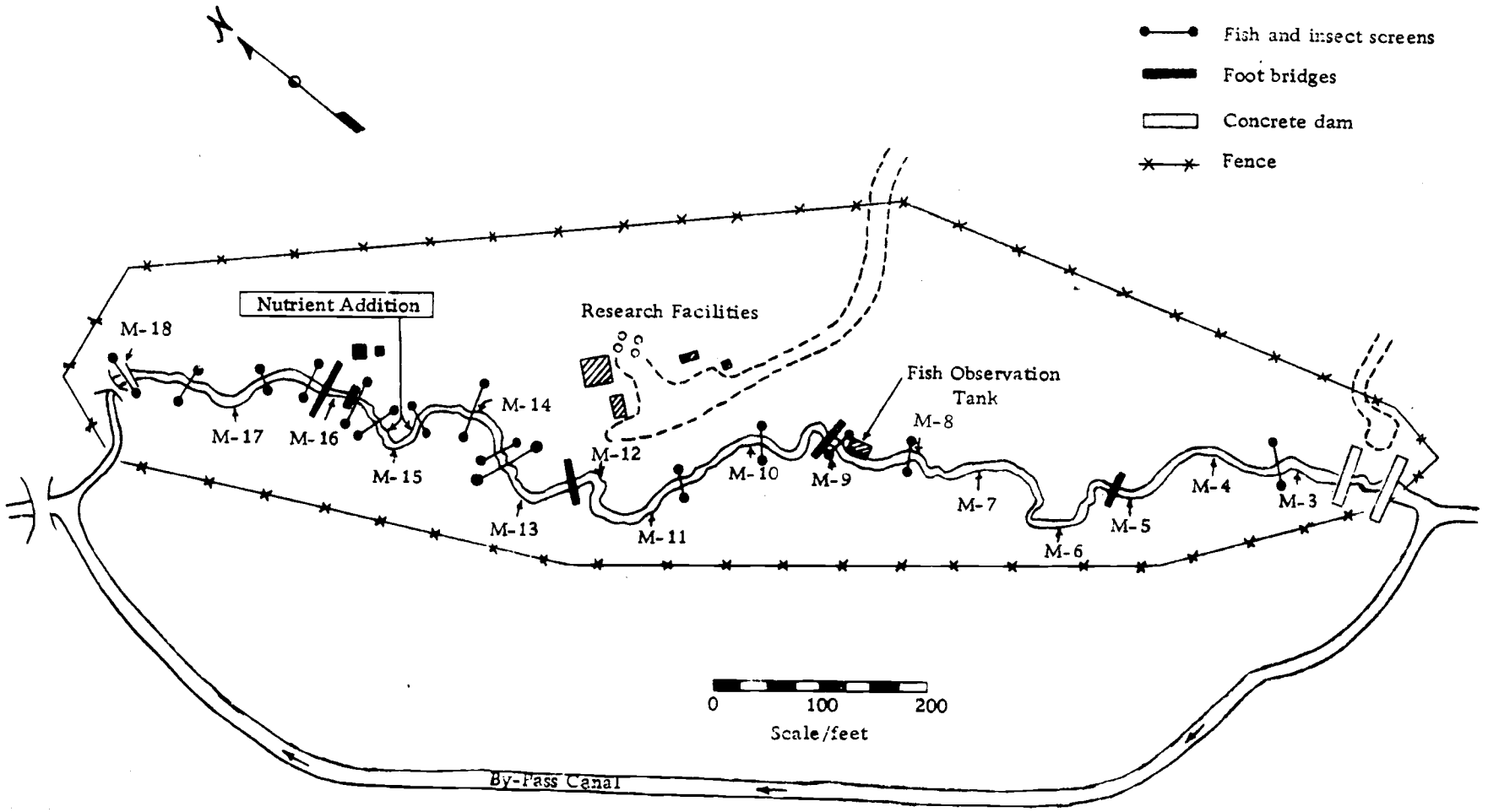
[2] Analyses were made by the U.S. Geological Survey, Portland, Oregon.

grey-brown color which results from weathering and erosion with runoff into the stream.

Comparison of temperature data reported from 1959 through the present study (36, 37, 38, 48, 63) indicated that seasonal temperature ranges for the stream are notably constant from year to year. During the winter months (December, January, February) water temperatures range from near zero to eight degrees Centigrade (C), from 8-14 C in the spring (March, April, May), from 12-22 C in the summer (June, July, August) with a rare high of 25 C, and from 20-10 C during the fall (September, October, November). The cold springs feeding the stream and the heavy vegetative cover of the watershed help to modify daily and seasonal fluctuations in the water temperature.

Experimental Section of Berry Creek

The experimental section of Berry Creek is a 1,500-foot portion of the original stream bed in which the flow can be fully controlled. A map of this section is presented in Figure 1. Flow is controlled by a concrete diversion dam and a bypass canal capable of carrying all the flow not wanted in the experimental section, up to the estimated maximum flow rate of 100 cfs. Water enters the experimental section through a 12-inch control valve just below the dam; the flow is continuously recorded at two V-notch weirs, one



Dense canopy of Maple, Ash, Fir, Oak, and Alder border the stream

Figure 1. Berry Creek Experiment Stream

immediately below the dam and the other at the exit of the experimental section (site M-18). A recording thermometer installed at M-18 also provides daily water temperature data. From late October through June, when rainfall provided adequate water supply, the flow rate could be maintained at 0.25, 0.5 or 1.0 cfs; however, in the late summer and early fall, when all available water is allowed to pass through the controlled section, the flow rate is often less than 0.1 cfs.

In the experimental section, Berry Creek has a moderate gradient of one foot in 75 feet with regularly alternating riffles and pools in each 100 foot interval. At the flow rate usually maintained (0.5 cfs) the average width of the stream is eight feet with an average depth in the riffles of one foot. At varying intervals, sections of the stream were separated by screens in order to prevent the migration of fish or the drift of aquatic insects from section to section during various experimental studies; these screens did not impede the flow rate to any extent.

A dense canopy composed mainly of red alders and bigleaf maples was present over the stream during the spring, summer and the early fall months. This covering greatly reduced the light intensity at the stream surface. Consequently, there was very little growth of autotrophic forms on the rocks and the natural periphyton of the stream was predominantly diatomaceous (14, 35, 48).

During the fall, abundant leaf material enters the stream and persists on the bottom throughout most of the winter. The control of flow rate apparently results in this accumulation of plant debris. Some of this debris is reportedly consumed by the abundant snail, Oxytrema silicula (18), and by the crayfish, Pacifastacus leniusculus trowbridgi, which feeds principally on plant material (38). Berry Creek also has a rich aquatic insect fauna of some 200 species (32); many of these species are also reported to consume plant materials.

The reticulate sculpin, Cottus perplexus, and the coastal cutthroat trout, Salmo clarki clarki, are abundant in Berry Creek. Davis (13) studied sculpin production in laboratory streams (a simplified community) while extensive studies have been conducted on the cutthroat trout in their natural environment of Berry Creek (63). Additional studies on trout growth, food habits, and food consumption have been continued at Berry Creek by McIntyre (36, 37).

As part of the cutthroat trout production study, the lower section of Berry Creek was enriched with sucrose (as a carbon source, 1960-1967) and urea (as a nitrogen source, 1963-1967) to promote the growth of Sphaerotilus natans, a sheathed, slime producing bacterium frequently associated with organic pollution. Large quantities of the plume-like growth of Sphaerotilus were attached to material along the stream edges and on the riffle bottoms. At certain times growth was extensive and covered the bottom, while at other times

only slight or no growth was visible; the amount of growth was dependent upon the continuance of enrichment. This bacterial growth provided food and habitat for insect larvae (tendipedids) which are important food organisms of trout. Enrichment of the stream in the trout production study was continuing when the present study (of aquatic myxobacteria) was begun.

Initially the stream was surveyed at 50 foot intervals, corresponding to markers along the bank designated as M-4, M-4+50, M-5, etc. Thus, the samples from these sites were also designated M-4, M-4+50, M-5, etc. Three sites were ultimately selected for study: one site in the unenriched section (M-9) and two sites in the enriched section of the stream, one immediately below the point of sucrose and urea enrichment (M-15) and the other 300 feet downstream (M-18). Water samples from these three sites were collected at regular intervals from April, 1966 through December, 1968; the myxobacteria, total bacteria and algae per milliliter were enumerated.

Later it was desirable to obtain a lateral view of the experimental section of Berry Creek. For this study, water samples were collected upstream from sites M-18 to M-9 at 100-foot intervals (i.e., M-18, M-17, M-16, etc.) every week to ten days throughout 1967.

Sample Collection

The study undertaken was designed to make comparisons between the myxobacterial population and the total bacterial population in the flowing water of Berry Creek. Several different myxobacterial types were predominant in the water samples at various times during the year. In order to determine the possible sources of these myxobacteria, samples of the stream bed (sediment and decaying leaves), surface film, filamentous algae, and flocs of the sheathed bacterium, Sphaerotilus natans, were collected in addition to the water samples. Since it was of interest to determine only the relative numbers of myxobacteria and total bacteria contributed by these sources, precise quantitative sampling was not employed.

All samples taken from the stream were collected in sterile, cold containers and kept on ice (0-2 C) during transport to the laboratory and until the plating for myxobacteria and total bacteria was completed. The samples were serially diluted in cold sterile distilled water or plated directly within two hours after sample collection. Storage in ice slowed down cell activities and prevented changes in the sample prior to plating.

Water Samples

Water samples were regularly obtained at 7-10 day intervals

from April, 1966 through December, 1968. A minimum of 200 ml of water was collected at each site by submerging a sterile 250 ml dilution bottle below the water surface and then allowing it to fill by displacement of the air. All samples were subsequently plated to determine the number of myxobacteria and total bacteria per milliliter.

Stream Bottom Samples

1) Decaying leaves: As noted in the stream description, fallen leaves accumulate in the stream and remain through the winter. Leaf material was collected with sterile forceps and transferred into sterile containers for transport to the laboratory. Known quantities were subsequently washed or blended in sterile distilled water and plated for myxobacteria and total bacteria. 2) Sediment samples: A sterile glass tube (15 mm diameter) was used to obtain a mud core sample from the stream bed. Care was taken to prevent stream water from entering the tube and diluting the sample. Known portions of the core sample were diluted in cold, sterile distilled water and plated for myxobacteria and total bacteria.

Surface Film Samples

Reduced flow rate in the spring and summer resulted in formation of a thin film on the surfaces of a few small still pools. Surface film samples were initially collected by skimming the surface

(approximately ten square inches) and suspending the film in less than 50 ml of the surface water. To standardize the sample size, surface film was later collected by placing a sterile glass slide beneath the surface film and lifting straight up. In this way the surface film adhered to the slide. These slides were then transported to the lab in sterile containers where the film was resuspended in a given volume of sterile distilled water. The results obtained are qualitative, however, since the thickness of the film layer varied with the flow rate through the sampling area.

Sphaerotilus Samples

Sphaerotilus natans grew abundantly in the sections of the stream which were enriched with sucrose and urea. This bacterium produces large slimy plumes which may provide an "enriched" environment satisfactory for the growth of other organisms. Samples of Sphaerotilus were collected by cutting the outer plume edges free from the floc mass. These portions were collected in a sterile beaker and were added to a sterile sample bottle on ice. In the laboratory, samples were shaken or homogenized in sterile distilled water in a Waring Blendor and plated for total bacteria and myxobacteria.

Algae Samples

Each spring a freshwater red alga, Batrachospermum sp., appears in the stream (from late March or early April to mid June or mid July). Samples of this organism were obtained in a manner similar to that for Sphaerotilus and the filaments were examined for the presence of myxobacteria and total bacteria.

Enumeration Methods for Berry Creek Samples

Myxobacteria

As a preliminary step for studying the ecology of myxobacteria in the freshwater stream, it was necessary to devise a satisfactory procedure for the isolation and enumeration of these organisms. The culture medium and procedure for this purpose are described in the Results and Discussion, Part I (note pp. 48-51).

Total Bacteria

The routine pour plate method recommended for the examination of water samples (1) was used to determine the total bacteria count per milliliter. Two dilutions prepared in cold, sterile distilled water were plated in triplicate and incubated at 18 C for at least one week; the recommended incubation time was extended to permit the development of colonies of those bacteria which grew more slowly at

this temperature. Comparisons were made between the spread plate and pour plate methods with the former giving slightly higher count values; however, the pour plate method was used throughout the study in order to maintain consistency in comparison of the data.

Algae

A modification of the method described in Millipore Technical Brochure ADM 40 was used (39). Fifty ml of water sample was filtered through a membrane filter (pore size, 0.45μ); the filter was placed on a drop of immersion oil on a glass slide and incubated in a petri dish until the membrane filter became transparent (37 C for 2-3 hours, or overnight at room temperature). Diatoms per field were counted with a calibrated microscope at 450X magnification, and the number per milliliter of water calculated using the formula below:

$$N = \frac{C \times 255}{V \times F}$$

N = number diatoms per milliliter

C = total count in F number of fields of _____ mm^2 (microscope field at 450X)

V = volume of sample filtered

F = number fields counted (at least 70)

255 = filtering area (mm^2)

Laboratory Studies on Myxobacteria

Source of the Isolates

The myxobacteria studied were isolated over a three year period from samples of the stream sediment, surface films, decomposing leaves and flowing water of Berry Creek. Those isolates selected for colony morphology and the biochemical studies represent the predominant types of myxobacteria which occurred in the stream throughout a given year.

Isolates of the fish pathogen, Chondrococcus columnaris, were obtained from Berry Creek water samples in August, 1968. This myxobacterium was distinguished from all other aquatic isolates on the basis of the colony morphology on CPM. Juvenile coho salmon (Onchorynchus nerka) from the Alsea River system were introduced into Berry Creek as food competitors with the coastal cutthroat trout (Salmo clarki clarki). Several coho salmon mortalities showed symptoms of columnaris disease (37); these infected fish are thought to be the source of C. columnaris isolated from Berry Creek since the organism had not been observed during the three prior years of this study.

Isolation of Myxobacteria and Maintenance of Cultures

Isolation Procedure

Isolates of myxobacteria occurring in Berry Creek were obtained by picking colonies of different morphological types from the CPM (cytophaga-peptonized milk agar) enumeration plates after 50 to 72 hours incubation at 18 C. Under 15X magnification, a small agar square from the colony edge was removed and transferred to cytophaga broth (2), which was incubated at 18 C for 24-48 hours. The broth culture was then diluted (approximately 10^6) in sterile broth or distilled water and plated on CPM for re-isolation. (The CPM agar plates were dried for 24 hours at 37 C before use.) The desired colony type was isolated after 50-65 hours at 18 C and transferred to fresh cytophaga broth. Cytophaga broth and caesin hydrolysate broth were used for further routine transfers in liquid culture.

Streaking for isolation often resulted in mixed myxobacterial isolates since the gliding myxobacterial cells from adjacent colonies had migrated near the desired colony's edge. The dilution and spread plate re-isolation procedure outlined above assured isolation of a single myxobacterial type. These isolates were subsequently maintained in pure culture in cytophaga agar deeps (cytophaga broth containing 0.4% agar).

Culture Maintenance

At monthly intervals, the stock cultures were transferred to fresh cytophaga agar deeps, incubated at 18 C for 2-5 days, and then stored at 5 C. Pure cultures were lyophilized in skim milk after two weeks incubation at 18 C in cytophaga agar deeps; the lyophilized cultures were stored at -10 C.

Morphological Characteristics of Myxobacteria

Cell Morphology

The isolates were stained by Gram's method after 24 hours incubation at 18 C in cytophaga broth. Cell morphology and gliding motility typical of myxobacteria were confirmed in these broth cultures by examination of a monolayer of cells in wet mount preparations with phase contrast microscope.

Colony Morphology

Colony morphology was observed on cytophaga-peptonized milk agar (CPM) after 65 hours incubation at 18 C. The low nutrient concentration of the medium favored the production of colonies which are typical of myxobacteria. Broad, flat, thin colonies spread over the solid medium and produce a periphery with an irregular rhizoidal edge. Gliding motility of cells in the outer edges of the colonies was

observed directly on solid medium by use of a phase contrast microscope with either high dry or oil immersion lens (under oil immersion, a small piece of sterile cover glass was placed on the colony edge and then the drop of oil added).

Photography: A Colony Morphology Study Technique. Many different colony types of myxobacteria were observed on the CPM enumeration field plates. These different colony patterns were either (1) artifacts of the plating technique and/or the medium used, or (2) they indicated that the myxobacterial population was comprised of several different types of myxobacteria. Since the colony patterns on CPM defied written description, photographs were made to record them and thus provide an easier means for direct comparison and study of the colonies. The most successful setup for photographing the colonies involved combination of an obliquely lighted field under 15X magnification through a stereoscopic, trinocular microscope. The transparency of the agar plating medium (CPM) aided direct photography of the detail in the colony edges. Panatomic X film was used in photographing the colonies. By using an oblique type of lighting system, the angle of incidence of the light could be varied to reveal the shape, surface texture and edge detail of the colonies. This type of illumination resulted in an unevenly illuminated background; some of this unevenness could be reduced by dodging the prints when making the enlargements.

Photographs of the myxobacterial colonies were made prior to their isolation from the CPM primary isolation plates and later, of the respective colony from pure cultures plated on CPM. In this way, single morphological types were selected for further biochemical and colony morphology studies.

Incubation conditions and plating medium used were the same for all colonies photographed. For the comparison of colony morphological types, photographs were taken after 70 hours incubation on CPM at 18 C. Entire colonies were photographed at 15X magnifications through the stereoscopic microscope; 20X magnifications were used for colony edge comparisons; and, occasionally, 25X was used in order to observe detail on very small colonies.

Biochemical Characteristics of Myxobacteria

Carbohydrate Utilization

Porter's modification (47) of the Hugh-Leifson procedure (28) was used to test for the production of acid from carbohydrates: D-glucose, maltose, D-galactose, lactose, sucrose, mannitol, and cellobiose. All carbohydrates were filter sterilized and added aseptically to the basal medium at a final concentration of 0.5%. The pH was adjusted to 7.0. Tubes of medium were inoculated with 48 hour cytophaga broth cultures; those used to detect fermentative

utilization of these carbohydrates were overlayed with sterile Ion agar (2%) and sterile Vaspar. Results were recorded at 24, 48 and 72 hours and at intervals up to two weeks.

Utilization of Macromolecules

Tests on the ability of the isolates to utilize macromolecules included hydrolysis of starch, esculin, chitin, carboxy methyl cellulose, cellulose, caesin, and gelatin. Degradation of tyrosine and tributyrin were also determined.

The methods recommended by Porter (47) were used in these tests. Two procedures were modified slightly: (1) cytophaga agar base plates were used instead of mineral salts medium in chitin decomposition method modified by Porter, and (2) the concentration of 0.2% (v/v) tributyrin used by Porter inhibited the growth of many of the aquatic myxobacterial isolates, therefore concentrations of 0.1% and 0.05% were used in the medium.

Several methods were used in an attempt to demonstrate cellulose utilization. The classical filter paper method for cytophagas and the suspension of cellulose powder in a mineral salts medium recommended by Stanier (53, 56) proved unsuccessful in demonstrating the presence of cellulose decomposers among the Berry Creek isolates. Carboxy methyl cellulose (CMC) utilization (47) could not be detected in myxobacterial isolates from fish (46) nor with

the isolates from the aquatic environment of Berry Creek.

Burnison (11) modified the Emerson and Weiser medium (19) by decreasing the agar concentration and increasing the CMC concentration. By use of this medium he was able to demonstrate CMC utilization with some of his myxobacterial isolates. A very similar medium was developed for use in this investigation. CMC concentration was increased to 1.5-2% with the agar concentration at 1.0%; the peptone concentration was reduced to 0.1%. The combination of low agar and high CMC concentrations allowed easy detection of the depressions which resulted when CMC was utilized in the medium surrounding the inoculated area. Nitsos (40) used a Waring blender to facilitate an even dispersion of CMC into solution at these concentrations. Depressions in the CMC medium were noted within 24 hours in the more active cultures; observations were made at 24, 48, 72 hours and in one week.

The method of carboxy methyl cellulose provides presumptive evidence for cellulose utilization. A sensitive method has not yet been developed for detecting the direct utilization of cellulose by the freshwater myxobacteria.

Nitrate Reduction

The ability to reduce nitrates was detected using the procedure described in the Manual of Microbiological Methods (52).

Formation of Catalase and Cytochrome Oxidase

The presence of catalase was determined by the addition of 2% hydrogen peroxide solution to colonies growing on CPM agar plates and observing for evolution of gas. Cytochrome oxidase was detected by the method of Gaby and Hadley (22).

Serological Characteristics

The rapid slide method for agglutination, using antisera provided by Dr. R. E. Pacha, was used in the identification of Chondrococcus columnaris isolates from Berry Creek.

RESULTS AND DISCUSSION

This section of the thesis is divided into three major parts: (I) the development of the method for isolation and enumeration of myxobacteria, (II) the field studies of myxobacteria occurring in the experimental stream, Berry Creek, and (III), the laboratory studies carried out on the myxobacterial isolates.

I. Development of Method for Enumeration of Myxobacteria

Introduction

A preliminary step to studying the ecology of myxobacteria in freshwater streams involved the development of a method for the isolation and enumeration of myxobacteria from an aquatic habitat. In past years ecological studies on myxobacteria have been hampered by the lack of a culture medium suitable for both differentiation and isolation of myxobacteria. Most workers have experienced difficulties in this regard because: (1) the nutrient concentration of their medium was very high resulting in production of eubacterial-type colonies by the myxobacteria, i.e., smooth, convex colonies with an entire edge, (2) on moist agar surfaces, pseudomonads and other flagellated forms overgrew the slower-growing myxobacteria, (3) the presence of myxobacteria was overlooked when samples containing high levels of other bacteria were diluted for plating, and (4)

enrichment techniques isolated a limited number of "specialized myxobacteria", e.g., chitin and cellulose decomposers.

In 1945 Soriano (as cited by Stanier, 1947) stressed the importance of using dilute media for culturing nonfruiting myxobacteria. Stanier (56) noted changes in the colony appearance of chitin decomposers when they were cultured on solid media of different nutrient concentrations, e.g., nutrient agar, yeast extract agar or mineral salts agar, all supplemented with chitin. The extent of spreading of the colony edge on peptone medium also changed when the concentration of either agar or the peptone was varied (1-2% and 0.25-1.0%, respectively). Low nutrient concentration reportedly favors spreading and production of colonies which are described as "typical" of myxobacteria. On a comparatively rich medium, the myxobacterial colonies oftentimes fail to spread and, in many cases, are indistinguishable from eubacterial colonies. Porter (47) confirmed the observations by Stanier (56) of the myxobacterial colony development as influenced by agar and nutrient concentration.

Among the media used to culture freshwater myxobacterial fish pathogens were fish infusion and a dilute tryptone medium; the nutrient concentration of the latter was reduced since higher concentrations reportedly inhibited growth (43). In studies of the myxobacterium causing columnaris disease in salmonid fishes, cytophaga agar as described by Anacker and Ordal (2) was used for culturing

Chondrococcus columnaris. Cytophaga agar contains only 0.05% tryptone (also 0.05% yeast extract, 0.02% beef extract, 0.02% sodium acetate, and 0.9% agar).

The next major study on culturing myxobacteria was reported by Jeffers (30). From several media tested, Jeffers recommended a peptonized milk agar medium for the isolation and characterization of myxobacteria from a freshwater lake.

Initial Studies

In the present investigation, preliminary studies of water samples from the experimental stream were initiated using the low-nutrient media recommended above: cytophaga agar and peptonized milk agar (PMA). For these studies a known volume of water sample was spread over the agar surface with a bent glass rod and plates were incubated at 18 C for two to three days. The resulting colonies were observed through a stereoscopic binocular microscope at 15X magnifications.

Colonies which were characteristic of myxobacteria grew on both cytophaga agar and peptonized milk agar. These "typical" colonies were generally light-yellow in color, thin, and spreading with irregular, rhizoid edges. Phase-contrast observations of gliding motility in wet mount preparations confirmed the above colonies as myxobacteria.

Both media supported the growth of numerous non-myxobacteria colonies as well; spreading colonies similar to myxobacteria were produced by a few flagellated bacteria. In addition, clear differentiation of non-spreading myxobacterial colonies (which lacked typical rhizoid edge patterns) from the eubacterial colonies was not possible on these media. This latter separation was generally easier on peptonized milk agar, however.

Control of Spreading Microorganisms

In addition to the above difficulties in colony differentiation, flagellated microorganisms (particularly pseudomonads) spread rapidly over the solid medium and interfered with the integrity of the myxobacterial colonies. The latter problem became more severe when volumes of sample greater than 0.1 ml were plated; however, plating of smaller volumes of water sample resulted in growth of only 3-15 myxobacterial colonies per plate, much less than the amount desired for statistical accuracy in enumeration of myxobacteria via a plate count method.

To reduce spreading of the pseudomonads and flagellated eubacteria, plates of culture media were first dried overnight at room temperature and at 37 C. On culture plates dried at room temperature (25 C), pseudomonad-type colonies still overgrew the myxobacteria. Plates dried for 48 hours at 37 C were able to

accomodate a larger volume of water sample with accompanying reduced pseudomonad spreading and no apparent effect on the myxobacteria colony morphology. By use of pre-dried plates, it was possible to obtain up to 200 well-isolated myxobacteria colonies per plate.

Experiments were designed to determine how long media could be dried before limiting myxobacterial growth or affecting the colony morphology. In the first of these experiments, a series of plates were dried one to eight days at room temperature (after two days at 37 C) and were then inoculated with Berry Creek water samples of a mixed myxobacterial population. The results of myxobacteria counts on these dried media are presented in Table 1.

Table 1. Effect of Dried Media on Recovery of Myxobacteria.

Drying time (days) (after 2 days @ 37 C) @ 25 C	Average No. of Myxobacteria/ml	
	Sample 1	Sample 2
0	186	131
1	182	136
2	155	109
3	168	122
4	134	111
5	127	123
6	137	102
7	120	83
8	93	82

The total number of myxobacteria per ml of water sample plated began to decrease on plates dried two days at 25 C, and a continued decrease was noted with additional drying. Small myxobacteria colony types, which spread very little on media dried two days at 37 C, could no longer be identified by their colony morphology on plates which were dried longer than an additional five days at room temperature. Colonies of this type accounted for a greater portion of the decline in total numbers of myxobacteria per sample. The "disappearance" of other myxobacterial colony types was not observed. Pseudomonad-type colonies were also notably fewer on media dried five to eight days.

The effect of reduced moisture content on the development of colony morphology is illustrated by the photographs in Figures 2 and 3. Photograph A of Figure 2 shows a spreading colony (3218-06) with abundant circle formation and a very thin edge; phase contrast observations at 1000X show layering of cells two to three deep in this edge. Note that contrary to what might be expected of gliding motility-type organisms which are dependent on a solid and moist surface for motility, colonies of culture 3218-06 spread over a larger surface area producing a very thin colony on media dried 4-5 days (Figure 2B). Comparative studies of a second predominant morphological type from Berry Creek, 3309-12 (Figure 3A and B) show no morphological changes in colony morphology when plated on media dried up

Figure 2. Effect of Dried Media on Colony Morphology of Culture 3218-06.

A. 72 hour colony on CPM dried 48 hours at 37 C. Primary isolation plate. 5X.

B. 72 hour colony on CPM dried two days at 37 C following five days at 25 C. 4X.

C. 50 hour colony on CPM dried two days at 37 C and five days at 25 C. The agar surface was moistened with distilled water prior to plating. 5X.

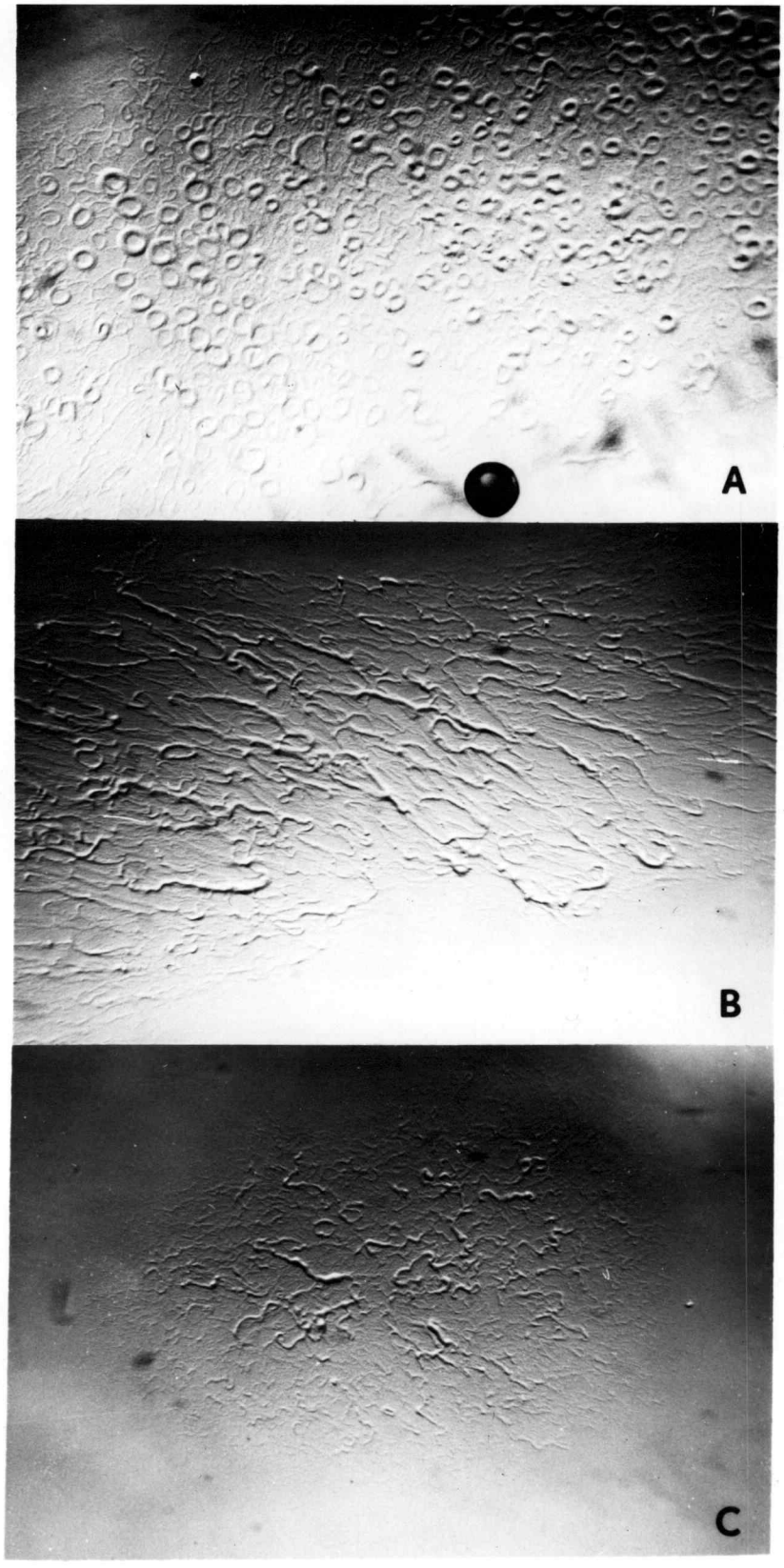
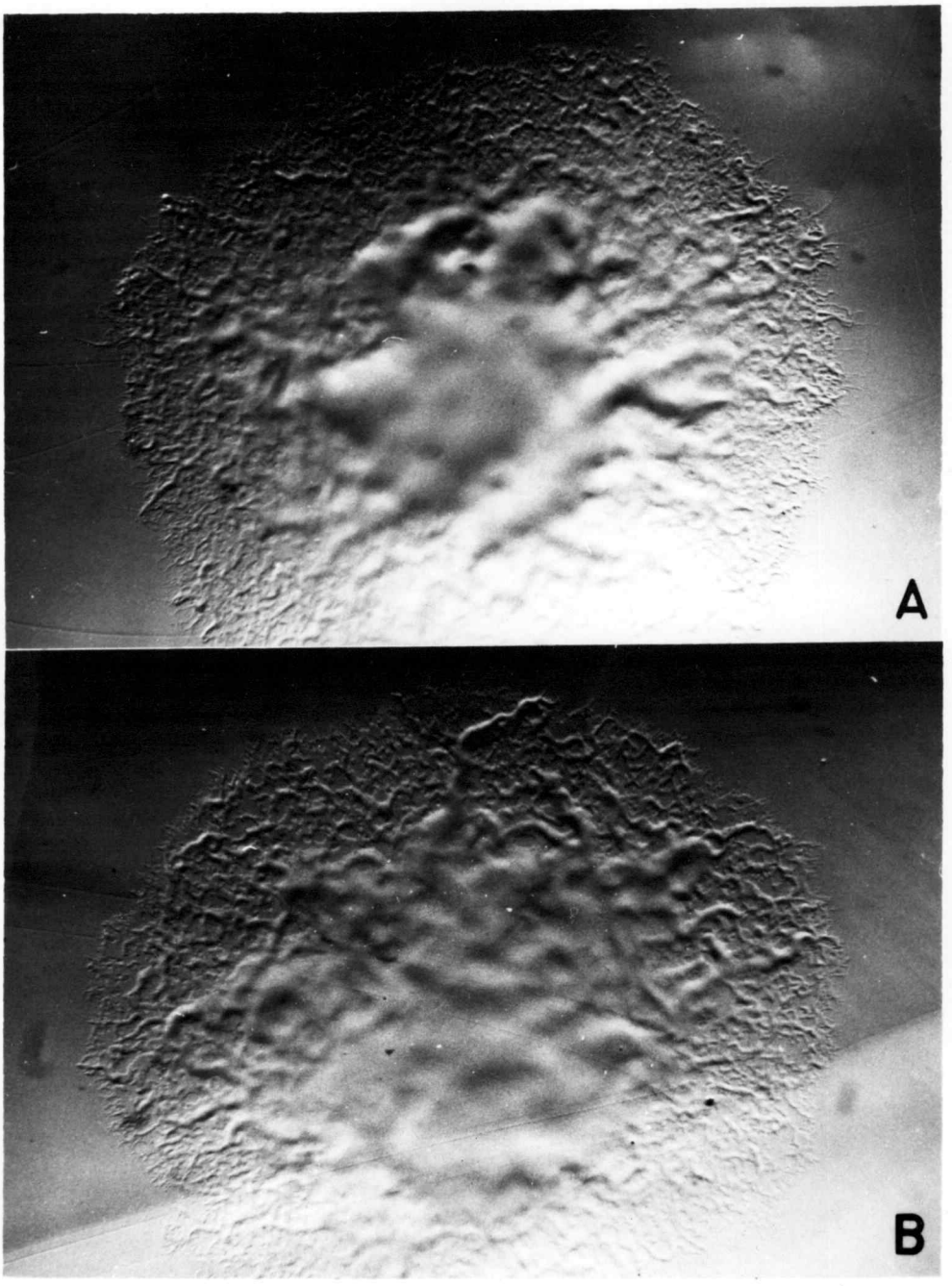

A
B
C

Figure 3. Effect of Dried Media on Colony Morphology of Culture 3309-12.

A. 72 hour colony on CPM dried two days at 37 C. 5X.

B. 72 hour colony on CPM dried two days at 37 C followed by five days at 25 C. 5X.


A
B

to five days. The effect of reducing the moisture content of the plating medium depended on the myxobacterial species involved.

Another experiment was designed to demonstrate the effect of moisture on colony morphology. A standard inoculum of strain 3218-06 was plated with varying volumes of sterile distilled water onto media dried for five days. The young colony of 3218-06 shown in Figure 1C has spread over a limited area on the artificially moistened medium rather than spreading extensively as noted on media dried five days (Figure 1B). After 72 hours incubation, no differences in colony morphology were observed between this colony and comparable colonies which had been plated on media dried for two days at 37 C.

On the basis of all the above information, all media used in subsequent studies was routinely dried at 37 C for two days prior to use; no changes in morphology of any myxobacterial types were observed under these conditions.

Selection of Enumeration Medium

For studies involving the population density of myxobacteria, it would be desirable to have a medium which supports the growth of most of the myxobacteria in the water sample and at the same time, facilitates differentiation of myxobacterial colonies from other microbial colonies. The ease in differentiation directly effects the resulting count values.

From the values presented in Table 2 note that larger numbers of myxobacteria from a given sample grew on the cytophaga medium as compared to the peptonized milk agar medium (PMA). However, many myxobacterial colonies were easier to differentiate on PMA. Because of these observations, various combinations of these two nutrient media were compared.

Table 2. Comparison of Media for Enumeration of Myxobacteria.

Sample Number	Myxobacteria Per Ml Sample				
	PMA	Cytophaga	1/10 Cytophaga	1/2 PMA	1/10 Cytophaga + 1/2 PMA
1	140	413			
2	120	392	360		
3	--	--	93	80	97
4	--	--	86	66	93
5	56	--	86	--	92
6	--	--	180	--	180
7	--	--	125	--	133

The medium consisting of one tenth strength cytophaga agar and one half strength peptonized milk agar gave the highest myxobacteria count values. This was either due to growth of more of the different myxobacterial types in the sample, or to the ease in differentiating the myxobacteria colonies from other microbial colonies, or to both. For these reasons cytophaga peptonized milk agar (CPM) was selected

as the medium of choice for use in the ecological studies.

Myxobacterial colonies spread extensively on this dilute nutrient medium, and produced patterns which greatly aided in their recognition as myxobacteria. This study revealed numerous variations in the patterns of the characteristic colony edges on CPM. Examples of colony edge patterns of the predominant Berry Creek myxobacteria are presented in Figures 4A and 4B below. The final magnification has been standardized to bring out details which are apparent at 20X magnifications through the binocular, stereoscopic microscope. All the colonies compared were photographed after 70 hours incubation at 18 C on CPM medium.

Colony Edge Patterns

The colony edge may be sharply defined with (1) well formed circles as noted in Edge 1 of Figure 4A, or (2) with hair-like projections as in Edges 5 and 7 of Figure 4B. Edge 8 of this latter figure further illustrates the intricate detail possible in these delicate, sheer edges. Other types of colonies do not remain discreet within a circular area, but produce elongated colonies which gradually disperse into ultra thin (monolayer) edges. Examples of these edges are shown in Edges 2, 3, 4 and 6 (Figures 4A and 4B, respectively). Some of the latter type of colony spread out in veins, often from a central point which may be raised or depressed (Figure 4A, Edges

Figure 4A. Edge Patterns of Myxobacterial Colonies on CPM. Photographs taken after 70 hours incubation on CPM.

Edge 1. strain 7 6X
circles well formed within colony.

Edge 2. strain 3 5.5X
veins superimposed over sheer layers of cells.

Edge 3. strain 42 5.5X
spreads out from center to form circles in colony edge.

Edge 4. strain BC 830 5.5X
Chondrococcus columnaris
note knots formed within the veins.

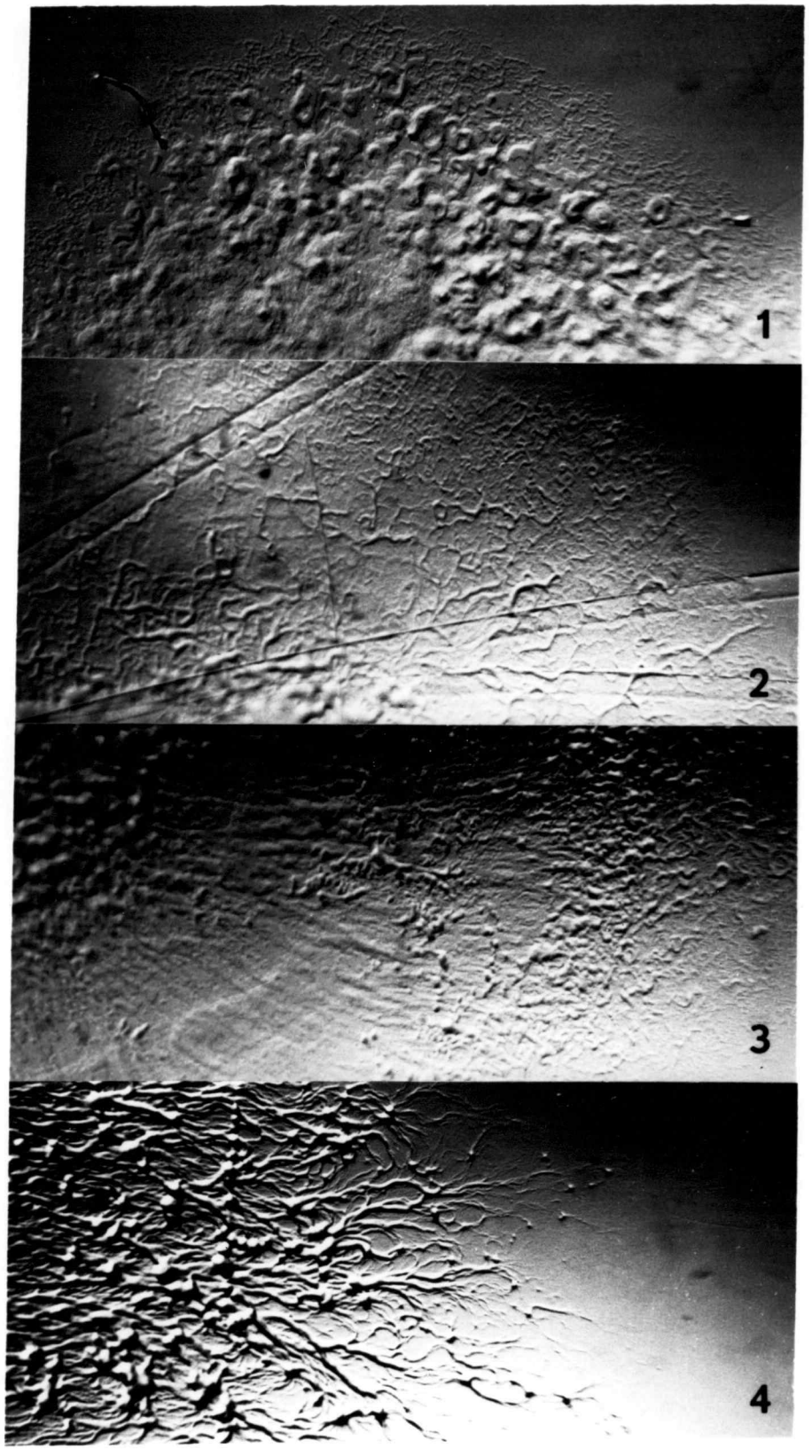

1
2
3
4

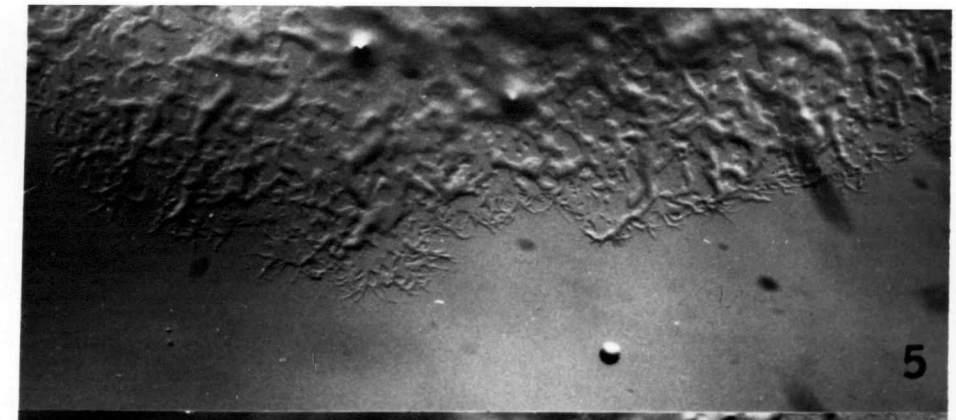
Figure 4B. Colony Edge Patterns on CPM (continued).

Edge 5. strain 34-3 8.5X
note detail of root-like projections of an edge which terminates abruptly.

Edge 6. strain 18 8.5X
note "islands" separated from major colony portion and the extremely thin colony edge.

Edge 7. strain 19 8X
similar in edge to strain 34-3, but note plateau near edge and regularity in margin.

Edge 8. strain 16 9X
intricately detailed edge of colony forming large circular structures within.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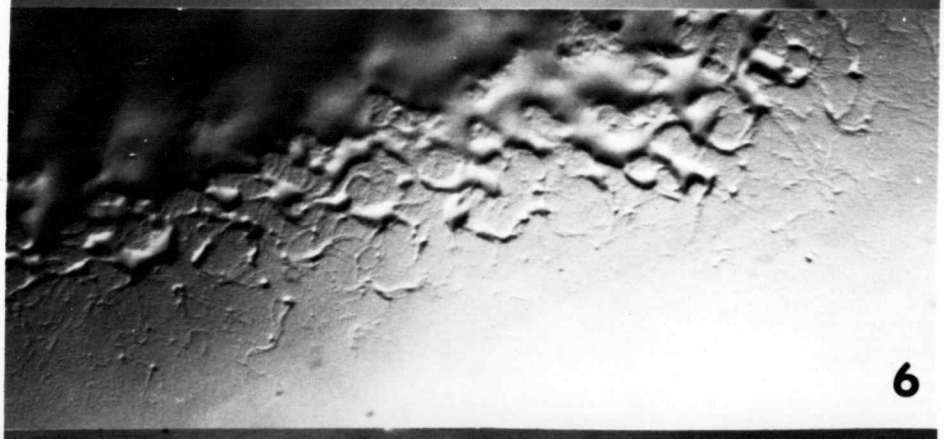
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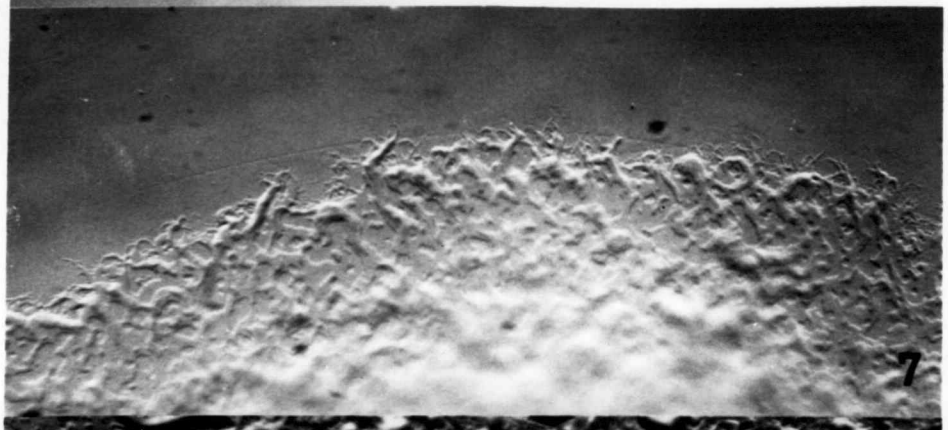
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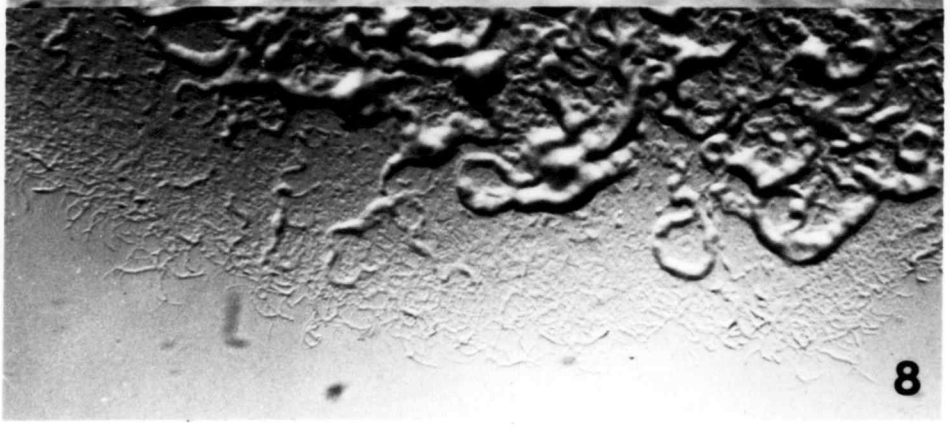
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2, 3 and 4).

Incubation Temperature and Colony Development

The rate of growth of myxobacterial colonies on any medium is effected by the incubation temperature and the medium used (as is well known for other types of bacteria). Since the myxobacteria studied in the present investigation inhabit an environment with fairly constant temperatures (yearly range, 5-25 C), the temperature of 18 C was chosen for incubation. This temperature is also recommended in freshwater myxobacterial fish pathogen studies since many of these isolates do not grow at temperatures above 20 C (6, 7).

The rate of colony growth on CPM at 18 C depended on the type of myxobacteria involved. Many myxobacterial colonies were microscopically visible 48 hours after plating; others developed more slowly at this temperature and required a longer incubation period before their colonies were visible. Because of observed differences in growth rate, counts were initially made after 50 hours incubation at 18 C. Final values per plate were in closer agreement, however, when counts were done again after 70-72 hours, thereby including those slower-growing myxobacterial colonies. By 72 hours, many colonies had spread extensively and overlapped one another; such crowded conditions did not provide for easy colony differentiation.

Further study of the myxobacterial populations indicated that a single counting could be done after 65 hours of incubation at 18 C. Values for the 65 hour counts were in agreement with those compiled by two separate tallies (Table 3).

Table 3. Comparison of Count Values after 65 and 72 Hours Incubation.

Incubation Time @ 18 C	Average Number of Myxobacteria/Ml/Site Sample*								
	Expt. 1			Expt. 2			Expt. 3		
	M-9	M-15	M-18	M-9	M-15	M-18	M-9	M-15	M-18
65 hours	134	158	204	135	136	175	269	283	296
72 hours	139	165	203	114	125	188	265	280	281

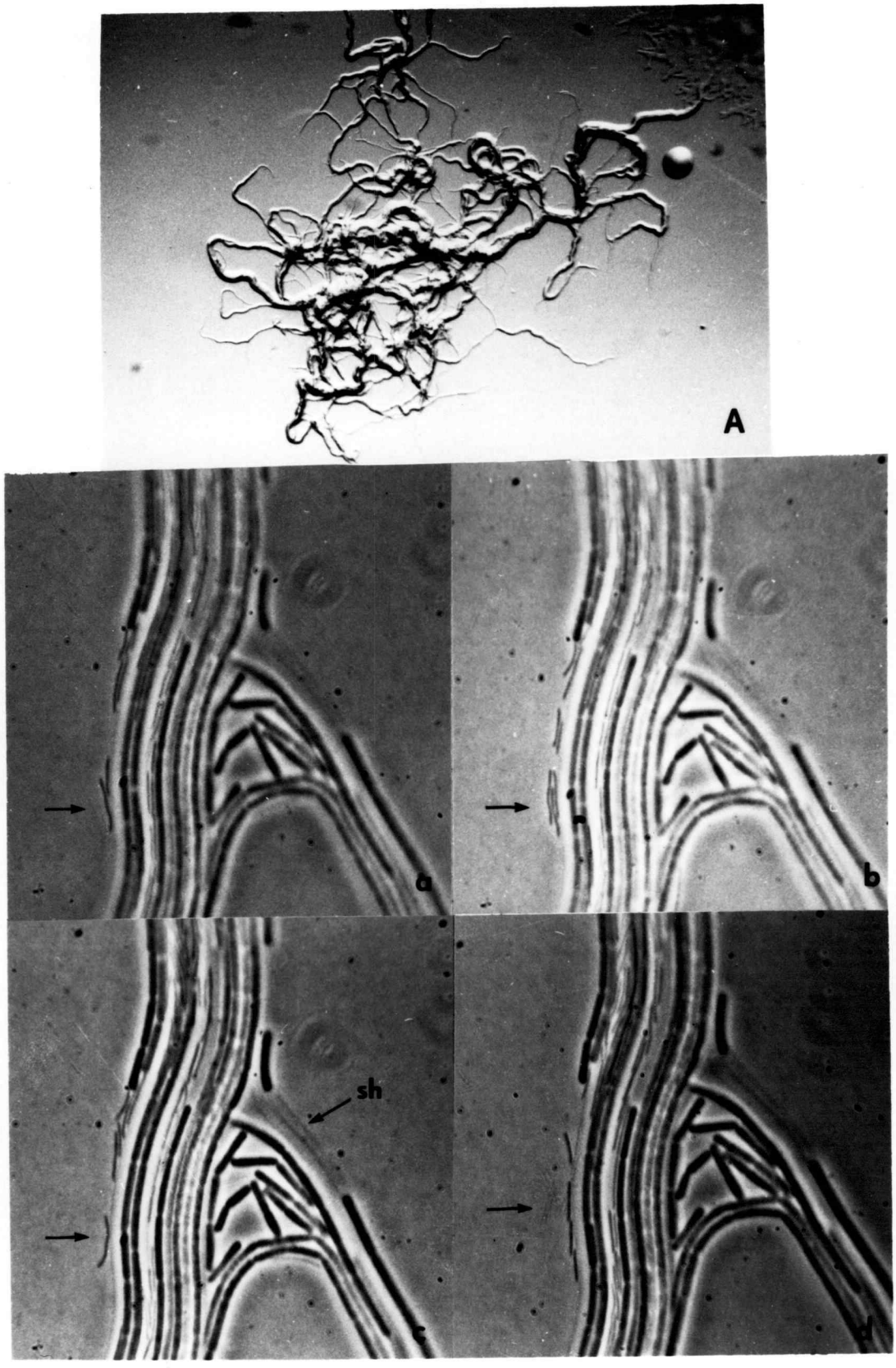
*Stream sites sampled designated as M-9, M-15 and M-18.

Sphaerotilus Growth on Media

There were further problems involved in attempting quantitative studies of the aquatic myxobacteria that deserve consideration here. During the first year of the ecological study, sucrose and urea were added to the stream as part of an enrichment program for the sheathed bacterium, Sphaerotilus natans. When large amounts of flocculent growth of Sphaerotilus were macroscopically apparent in the stream, water samples from the enriched section contained filaments of this bacterium. These filaments grew on the CPM primary isolation plates. After 60 hours incubation at 18 C, the sprawling Sphaerotilus "colonies" (Figure 5A) intermingled with the

Figure 5A. Colony of Sheated Bacterium Sphaerotilus natans on CPM Isolation Plate at 60 Hours. 6X.

Figures 5a, b, c, d. Phase-Contrast Micrographs of Filament of Sphaerotilus Colony (Figure 5A) at 15 Second Intervals. Arrow at left side indicates changing position of myxobacterial cells. Note large Sphaerotilus cells within the sheath (sh, Figure 5c) 1000X.


A
a
b
sh

myxobacterial colonies and obscured the integrity of the latter. Under phase microscopy, myxobacterial cells were noted gliding among the filament sheaths of Sphaerotilus colony. Note the changes in position of myxobacterial cells during a single minute (Figure 5a, b, c, d). Accurate myxobacterial colony counts were difficult to make in the presence of this nuisance organism. Because of this, efforts were made to devise a culture medium which would inhibit or retard the growth of Sphaerotilus without interfering with the growth of myxobacteria.

The amount of Sphaerotilus present on the plating medium was not effected by the nutrient concentration. Jeffer's PMA supported fewer Sphaerotilus colonies, but this medium also supported the growth of a smaller number of myxobacteria as well. When several nutrient media were prepared with tap water, the colonies of Sphaerotilus were greatly reduced in size. Residual chlorine of 2 ppm was detected in tap water after autoclaving and was probably responsible for the retardation of Sphaerotilus growth.

Heavy metal ions, such as zinc and copper, are reported (15) to inhibit Sphaerotilus and may also have contributed to this effect on Sphaerotilus growth. Since the quantities of these components are variable in tap water, it is generally undesirable for media preparation. A stronger objection to its use in the present study was the reduction in number of myxobacterial colonies per plate as shown in

Table 4.

Table 4. Effect of Tap Water Medium on Enumeration of Myxobacteria.

Sample Sites Enriched Section	Average No. Myxobacteria per ml on CPM	
	Distilled Water	Tap Water
M-15	191	163
M-16	220	178
M-17	218	185
M-18	290	231

Due to the reduction in numbers of myxobacteria on tap water medium, it was desirable to control the growth of Sphaerotilus in a more predictable manner. Jeffers (30) tried to use penicillin and streptomycin to retard eubacterial background on his myxobacteria isolation plates. In studies of myxobacteria isolated from fish Porter (47) found that 40% and 95% of these isolates, respectively, were sensitive to these antibiotics. In further antibiotic sensitivity disc assays, Porter found only a few myxobacterial isolates which were sensitive to neomycin and polymyxin B while all of these were sensitive to penicillin and streptomycin. Nitsos (40) reported that half of the freshwater isolates she studied were sensitive to the latter antibiotics and least sensitive to neomycin and polymyxin B. (The latter results were compiled in a recently completed study.)

Antimicrobial Agents

Neomycin was tested for its ability to control the growth of Sphaerotilus. At a final concentration of 5 μg/ml culture medium, neomycin (Neomycin Sulfate, Nutritional Biochemicals Corp., Ohio) retarded the growth of Sphaerotilus without interfering with the growth of myxobacteria or effecting their colony morphology. Molds of aquatic origin also caused interference with enumeration of myxobacteria at various times. Growth of these organisms could be inhibited by the incorporation of 10 μg/ml of cyclohexamide (Upjohn Co., Michigan) to the culture medium; neomycin does not inhibit fungi (62).

Comparative studies were carried out to determine the levels of these antimicrobial agents which would inhibit or retard myxobacterial growth and thereby effect the total myxobacterial counts. It was necessary to increase the concentration of neomycin and cyclohexamide to 25μg/ml and 100μg/ml, respectively, before a significant reduction in the number of myxobacteria appearing on the primary isolation plates occurred (Tables 5 and 6). The concentration of these agents employed routinely in the culture medium are well below these levels.

Although there was a noticeable reduction in the background bacteria on the CPM medium with neomycin added, the major

Table 5. Effect of Neomycin on Recovery of Myxobacteria.

	Neomycin (ug/ml media)										
Sample	0	5	10	15	20	25	30	40	50	75	100
1		163[a]	177	173	153	137	110	130	150	127	073
2	365	450	330	257	300	300					

a = average number of myxobacteria per ml.

Table 6. Effect of Cyclohexamide on Recovery of Myxobacteria.

	Cyclohexamide (μg/ml media)				
Sample	0	10	20	50	100
1	219[a]	--	202	208	143
2	238	239	247	--	218

a = average number of myxobacteria per ml.

objective of this portion of the study was to retard the growth of Sphaerotilus without effecting the myxobacteria totals. It is likely that other antibiotics could be used to reduce the growth of other contaminating bacteria on the isolation medium.

Summary

A culture medium and a procedure for isolation and enumeration of myxobacteria from the freshwater habitat had been developed. This medium and procedure were then used in field studies on Berry Creek to obtain ecological data on the occurrence of myxobacteria. The isolation medium was also used in subsequent laboratory studies of the colony morphology of the isolates obtained. The plating procedure and the medium which were developed are outlined below.

Enumeration and Isolation Medium

Cytophaga peptonized milk agar (CPM) contains 0.005% tryptone, 0.005% yeast extract, 0.002% beef extract, 0.002% sodium acetate, 0.05% peptonized milk, and 1.5% agar per liter of distilled water. For convenience in media preparation, a 10X concentrate of the first four ingredients was prepared and sterilized (20 minutes, 15 psi, 121 C). This concentrate was then added in required amounts to the peptonized milk agar and distilled water prior to autoclaving

at 15 psi (121 C) for 20 minutes. The pH was adjusted to 6.8 before autoclaving.

When sterile and cooled to 45 C, neomycin and cyclohexamide were added to make final concentrations of 5 μg/ml and 10 μg/ml, respectively. The medium was then poured into sterile, unscratched petri dishes (preferably glass) to solidify, and placed at 37 C to dry for 24-72 hours prior to use. The length of this drying period was adjusted so that volumes of water varying from 0.01 ml to 0.5 ml could be plated effectively.

Plating Procedure

In order to discourage further cell division, each sample was kept on ice from the time of collection until plating was completed. The samples were serially diluted in sterile, cold distilled water or plated directly onto the cytophaga peptonized milk agar field plates, which had been dried 24-72 hours at 37 C. A separate, sterile glass rod was used to spread the plating volume of 0.01 to 0.50 ml over the agar surface. Each dilution was plated in quadruplicate and incubated at 18 C for 50-72 hours.

On this dilute and transparent medium, myxobacteria produced a thin, spreading colony with a characteristic rhizoid edge. These detailed patterns of different myxobacterial colonies served to distinguish the myxobacteria from other microorganisms. Plates

containing 30-200 myxobacterial colonies were selected for counting; on more densely populated plates, the colonies tended to overgrow one another.

By use of the above procedure, it was possible to obtain reproducible results. Table 7 shows results obtained with several Berry Creek water samples plated in quadruplicate. It will be noted that each of the plates from the individual samples agrees closely with regard to the numbers of myxobacterial colonies present.

The transparency of the cytophaga peptonized milk agar medium allowed observation of the stages in colony development. Myxobacterial colonies are microscopically visible at 15X 48 hours after plating and their typical spreading development was followed by periodic observations at magnifications of 15, 20 and 25X through the stereoscopic microscope, until the colony matures at 72 hours. Results of these colony morphology studies are presented in the laboratory studies on myxobacterial isolates section.

Gliding motility at the edge of the colony was also studied directly on this medium with observations through the phase contrast microscope at 100, 450 or 1000X; observations with oil were easily accomplished when a small piece of sterile cover slip was placed directly on the colony edge. These observations confirmed the isolates as myxobacteria, i.e., long, slender, low refractile cells which exhibited gliding motility.

Table 7. Results of Quadruplicate Plating of Samples on CPM.

Sample	Number of Myxobacteria Per Plate			
	Plate Number			
	1	2	3	4
1	52	58	55	57
2	44	47	45	47
3	66	60	59	60
4	69	73	71	65
5	72	76	75	68
6	91	80	91	86
7	174	179	187	173
8	119	114	127	112
9	95	97	86	84
10	250	226	242	231
11	134	119	127	131
12	133	137	123	122

II. Field Studies on Berry Creek

Ecological Study Data

Field studies were conducted at Berry Creek from April 1966 through December 1968, with sample collection at least three times per month during this period. This investigation was subject to demands made upon the stream by other research projects which were carried out concurrently and co-operatively. Among these projects were studies on algae, aquatic insects, and trout production.

Data for two consecutive years of the ecological study on Berry Creek have been selected for presentation: Year I (July, 1966 through June, 1967) during which the stream was enriched with sucrose and urea (4 ppm and 1 ppm, respectively), and Year II (July, 1967 through June, 1968) without enrichment.

The populations of myxobacteria and total bacteria of Berry Creek were enumerated by use of two different plate count methods: the myxobacterial plate count method developed for this purpose, and the standard plate count method recommended for the examination of water (1).

In addition to the collection of water samples for population data, limnological data on temperature, pH, dissolved oxygen, and flow rate were collected so that the effect of some environmental

factors on the microbial populations could be evaluated.

A summary of the ecological data obtained is presented graphically and discussed below. Each point on the graphs represents the monthly average of three to five samplings per site for each month. The myxobacteria and total bacteria populations are expressed as the log number of cells per milliliter of water sample. Water temperature is given in degrees Centigrade (C) and the flow rate in cubic feet per second (cfs).

Seasonal Occurrence of Myxobacteria and Total Bacteria

In order to obtain a profile view of the stream throughout the year, samples were collected at 100 foot intervals along the experimental section. Figure 6 presents the seasonal occurrence data for myxobacteria and total bacteria in Berry Creek during 1967. Table 8 summarizes the flow rate and water temperature data for these seasons. The months selected were representative of seasonal conditions observed: high flow rate and low temperature typical of winter; the opposite for summer; and intermediate flow rates and water temperatures of spring and fall. Myxobacteria and other bacterial types occurred in both the unenriched and enriched sections (M-9 through M-14, and M-15 through M-18, respectively), and were more prevalent near the end of the experimental section (site M-18) than they were in the influent water (site M-9), as noted in Figure 6.

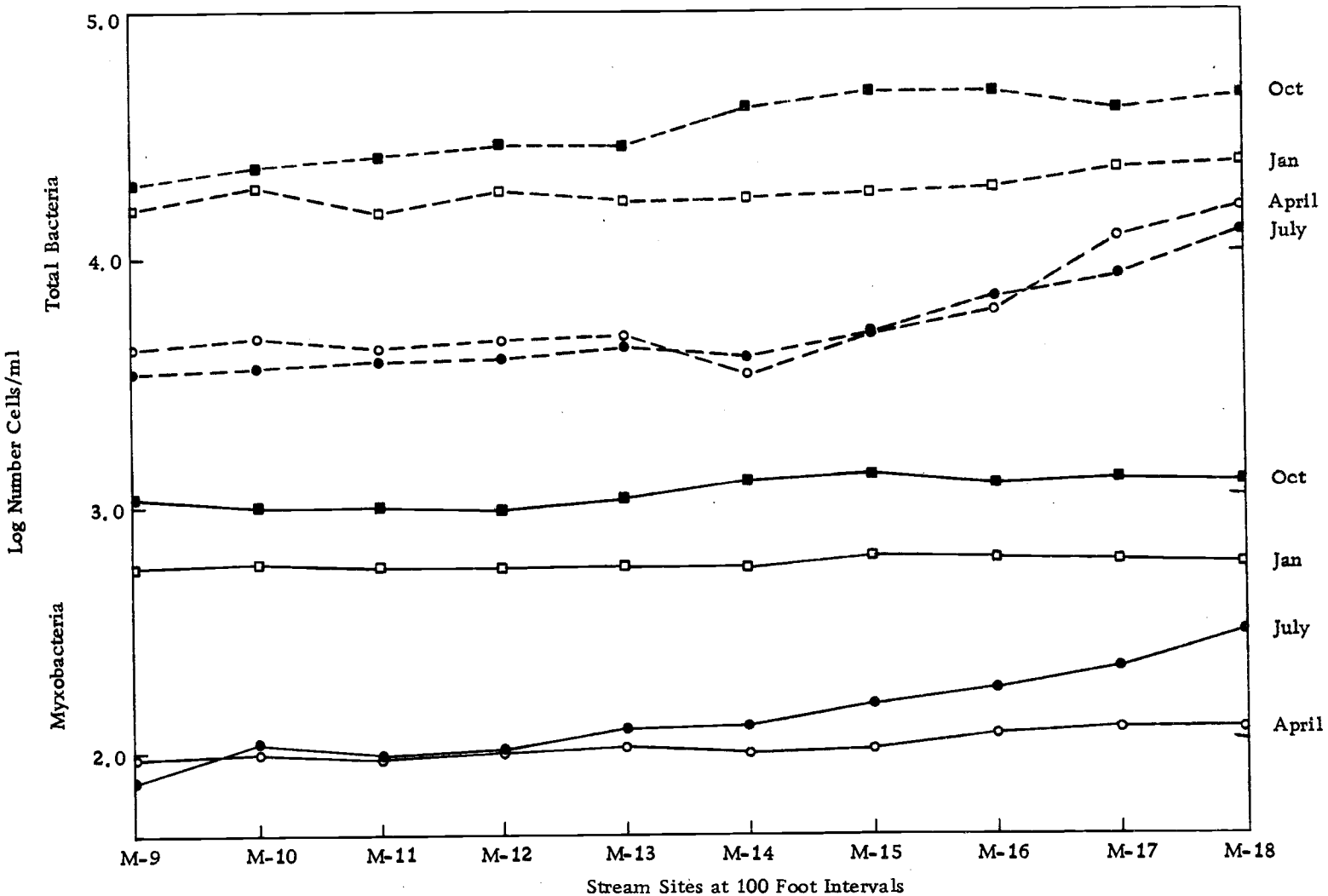


Figure 6. Seasonal Occurrence of Myxobacteria and Total Bacteria in Berry Creek during 1967. (Fall = October; Winter = January; Spring = April ; Summer = July)

Table 8. Seasonal Flow Rate and Water Temperature in Berry Creek.[a]

Season (Month)	Flow Rate (cfs)	Temperature (C)
Winter (January)	2.00	7.5
Spring (April)	0.55	8.0
Summer (July)	0.14	15.5
Fall (October)	0.17	12.5

[a]These data accompany Figure 6.

The relative level of these organisms present depended on the time of year the stream was sampled. The highest flow rate and the lowest temperatures were recorded during the winter, but the greatest number of organisms occurred in the fall when low water temperature and increasing flow rates were recorded. Reduced flow rate and warmer water temperatures occurred during the spring and summer; the lowest population values for myxobacteria and total bacteria were observed during these months (refer to Figure 6). The stream was no longer receiving enrichment during the fall of 1967; nevertheless, the myxobacterial and total bacterial populations were higher in the fall than any other time of the year.

As might be expected in a small stream with a controlled flow rate, increases in the numbers of myxobacteria and total bacteria were small over the length of the stream (M-9 through M-18) when a high flow rate was maintained (January and October). When the flow rate was regulated at a lower level, increases in the number of these

organisms in the lower section of the stream were more apparent (April and July). as noted in Figure 6.

A greater increase was noted in total bacteria in the enriched section during April and July than at any other time of the year; this might be expected since the organic content of the enriched section is greater than that of the unenriched section. The slight rise in the myxobacterial population level in the summer (July) may have reflected seasonal changes in the predominant myxobacterial type comprising the population. These changes in turn, may be associated with warmer water temperatures since a corresponding increase in myxobacteria did not occur in the spring.(April).

Water Temperature and Flow Rate Data: Year I Versus Year II

The water temperature and flow rate data (Figure 7) express similar trends for the two years under study; i.e., high temperature-low flow rate were recorded for the summer months of July, August, September; low temperature-high flow rate occurred during the late fall, winter and early spring months of December, January, February, and March of both years.

The temperature trend for the second year parallels that observed during the first year within 2 C. The lowest monthly average temperature (6.5 C) was noted in March of Year I and in December of Year II; the spring of the latter year was generally cooler than for

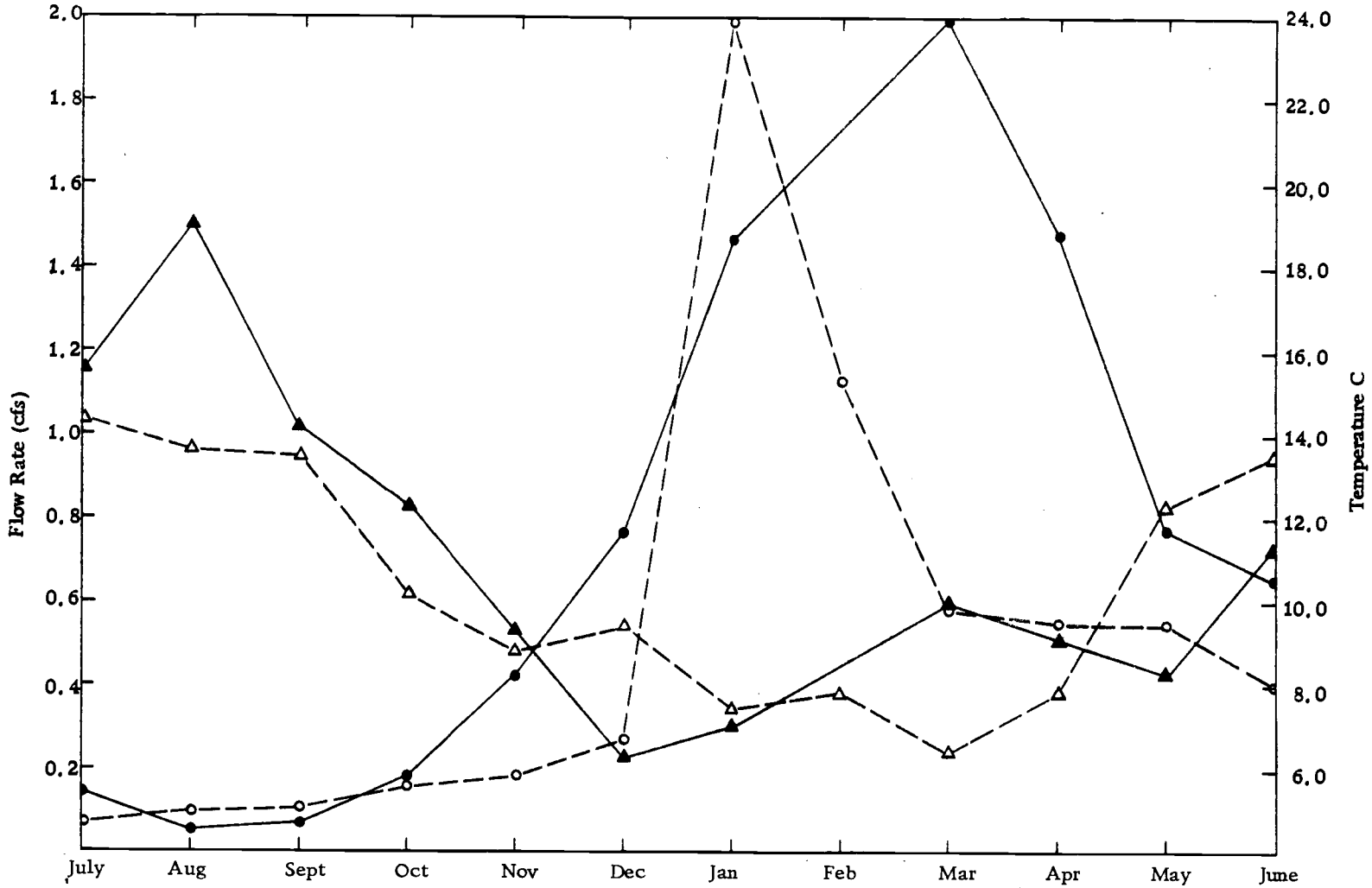


Figure 7. Berry Creek Water Temperature and Flow Rate Trends. Year I - temperature △---△, flow rate ○---○. Year II - temperature ▲——▲, flow rate ●——●.

the year preceding. A prolonged hot, dry summer resulted in the highest average temperature (20C) recorded in August (Year II).

The lowest flow rate occurred during the summer months of July, August and September, and then increased in October and November, corresponding to the onset of fall rains. The flow rate maintained through the flow-controlled section of Berry Creek, depended on the amount of water available and the regulation of flow as required for the trout production study. The flow rate peaks are of major interest since they occurred at different times during the two years. In Year I, the peak was noted in late December and through January; during Year II, the peak was observed in March, although a high rate was maintained from December through March. After the peak month each year, the flow rate continued to decrease until the low was reached in mid summer.

Occurrence of Myxobacteria and Total Bacteria in Berry Creek: Year I Versus Year II

The data obtained for myxobacterial and total bacterial counts for two years of this study are presented in Figure 8. These curves represent the level of the organisms leaving the experimental section of the stream (site M-18) during Year I and Year II. The general trend for myxobacteria and total bacteria observed during the first

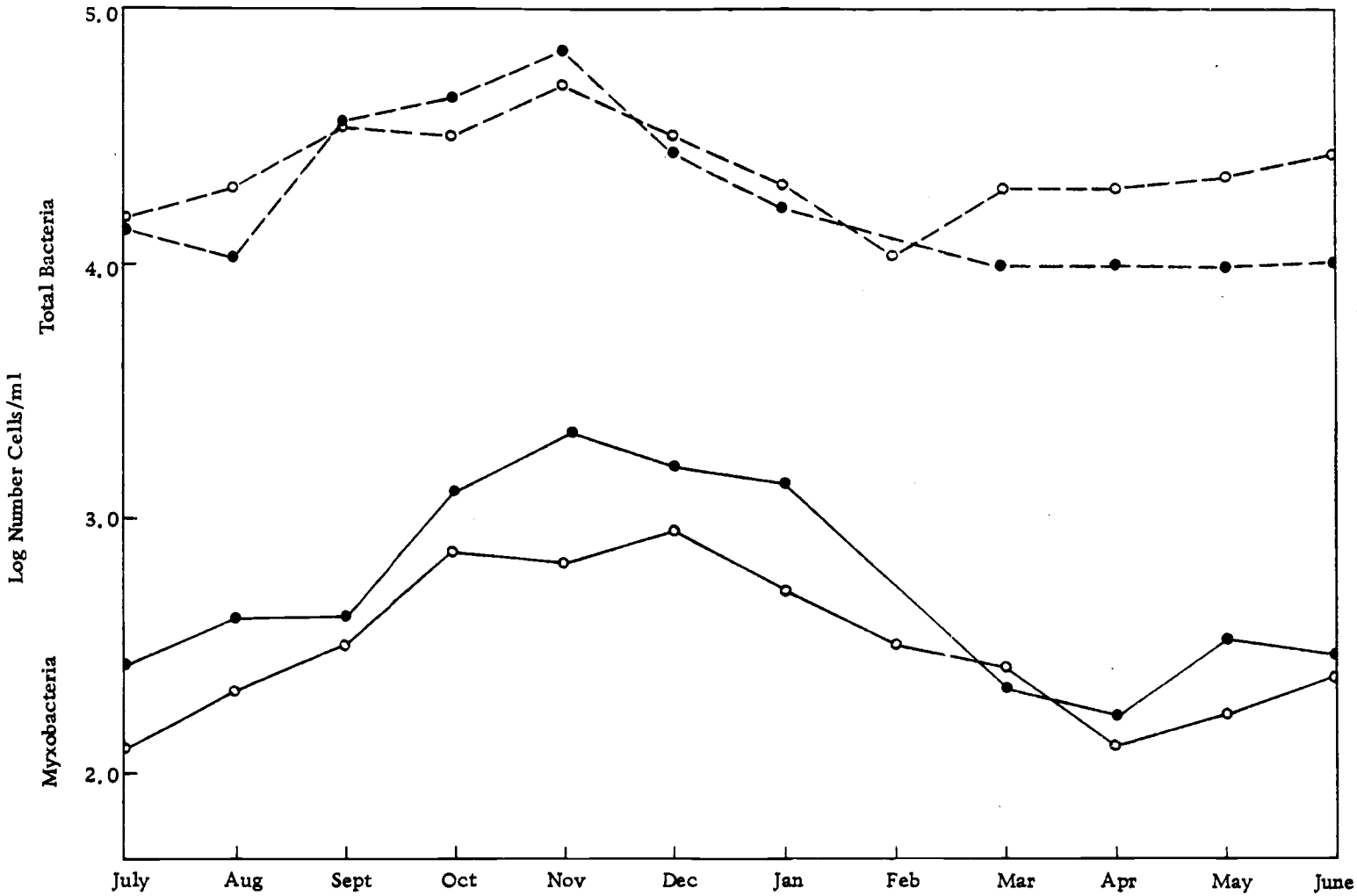


Figure 8. Myxobacteria and Total Bacteria Populations of Berry Creek during Year I (open) and Year II (solid) - site M-18.

year repeated itself during the second year. This same population trend was also noted for the first six months of the third consecutive year (data not plotted for July to December, 1968). The general trends noted for site M-18 during Year I and Year II were also recorded for site M-9 (unenriched) and site M-15 (enriched for half of Year I). Respective data for sites M-9 and M-15 are presented later in Figures 9 and 10.

Seasonal variations were observed in the populations examined. From lows obtained in mid summer (July), myxobacteria and total bacteria increased in numbers during the early fall (August, September and October), reached a maximum and remained at high levels during the winter months of November, December and January. The levels then began to decrease throughout the early spring into early summer (February through June). The number of myxobacteria was higher during the year when the stream was not enriched (Year II), while the total bacterial levels were nearly the same, except during the spring and summer of Year I when the stream received enrichment.

The peaks in the myxobacteria population occurred at the same time during both study years, i.e., in early December of Year I and in November of Year II. It is of interest to note that the total bacterial population peaked one month ahead of the myxobacterial population during Year I (Figure 8). (This early peaking was observed

at site M-15, and at site M-9 as well.) This phenomenon did not repeat during the second year, in the absence of sucrose and urea enrichment. The myxobacterial peaks are notably less sharp than that for the total bacteria during Year II.

The observations that the myxobacteria peaked later than the total bacteria (Year I) or that they maintain high levels for a longer period of time than the total bacteria (Year II), suggest that myxobacteria are actively involved in the ecology of the stream rather than being transient members of the bacterial population which are haplessly washed into the stream. If these organisms were merely washed in from soil, the two peaks (myxobacteria and total bacteria) would be expected to coincide.

Effect of Enrichment on Microbial Populations in the Stream

The relative levels of myxobacteria and total bacteria recorded for sites M-9, M-15 and M-18 are presented in Figures 9 and 10 for Year I (enriched) and Year II (not enriched). These sites were selected for study originally because they measure the number of organisms entering the stream section under investigation (site M-9), the number present immediately after enrichment (M-15), and the number leaving the enriched section, 300 feet downstream (M-18). The arrows on these graphs indicate the point at which the flow rate peak occurred during each year (flow rate data given in Figure 7).

Since Berry Creek was not enriched during the second year,

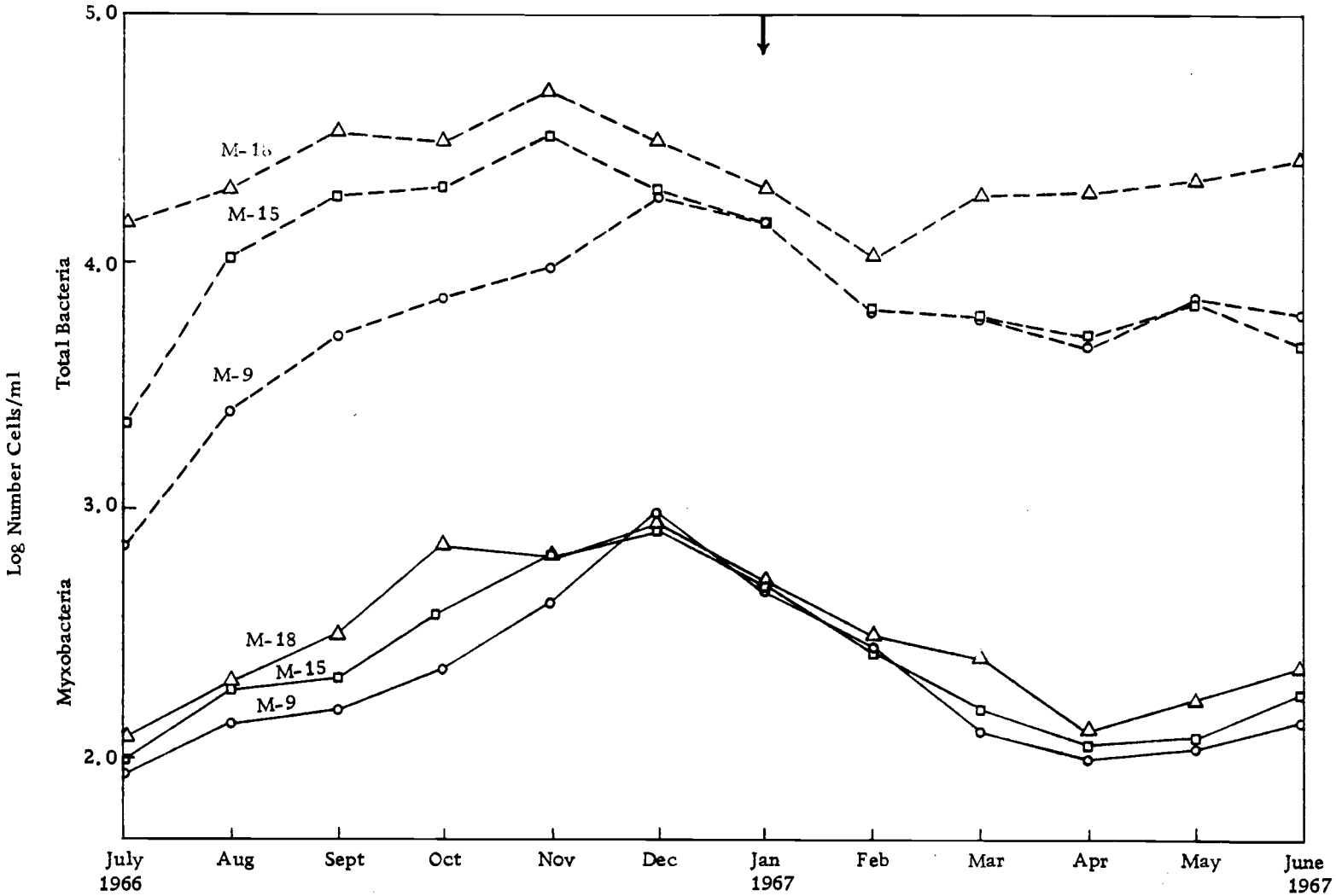


Figure 9. Myxobacteria and Total Bacteria Levels at Three Sites in Berry Creek. Year I (July 1966 through June 1967)

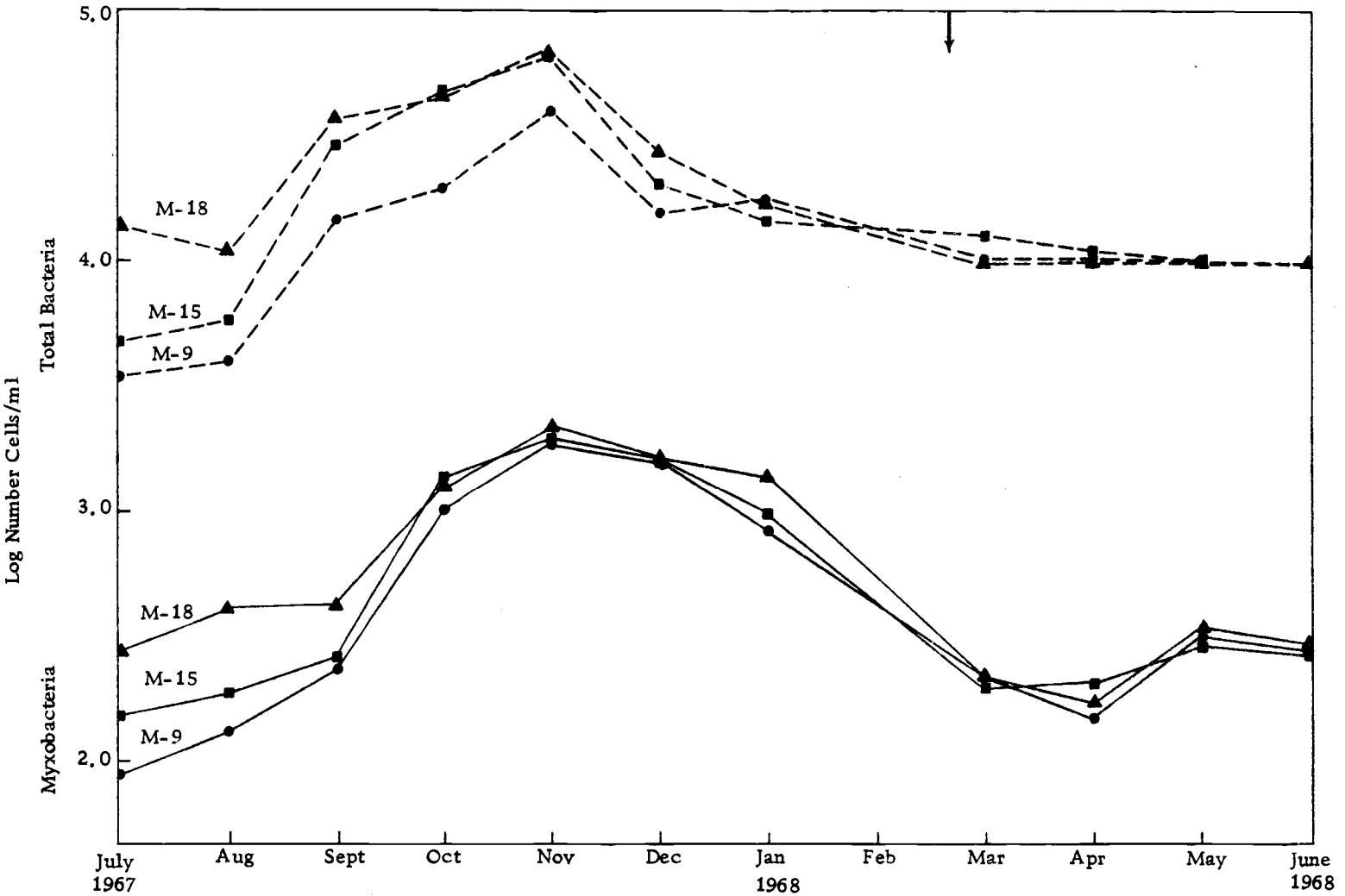


Figure 10. Myxobacteria and Total Bacteria Levels at Three Sites in Berry Creek. Year II (July 1967 through June 1968)

the data in Figure 10 indicated the "normal" levels of organisms present in the stream. Any changes in their populations due to enrichment should have been reflected when this data was compared with the data for Year I (Figure 9).

During 1966, the enriching solutions were introduced at site M-14+75; the enrichment addition point was moved to site M-15+25 from January to July, 1967, after which, enrichment was stopped. Sucrose and urea enrichment had a greater influence on the total bacterial populations (in addition to the resulting growth of Sphaerotilus) than on the myxobacterial populations (Figure 9). The numbers of total bacteria occurring below the enrichment point (M-14+75), at sites M-15 and M-18, were notably higher than upstream at site M-9. Increased organic content of the water due to leaf decomposition and increased runoff may be reflected in the separation of these three curves during the fall of Year I (Figure 9) and Year II (Figure 10) for the total bacteria populations.

The total bacterial data at site M-9 and M-15 from January through June, 1967, show nearly identical curves (Figure 9). Site M-15 no longer received enrichment during this period due to the change in enrichment addition point; this change is reflected in the decreased total bacterial level for this site. The level of total bacteria at site M-18 remained high for this same period, since it continued to receive enrichment through the rest of the year.

The Year I values (Figure 9) for site M-9 and for site M-15 (now unenriched) compare favorably with the data for these same sites during Year II (no enrichment) as seen in Figure 10. No differences were observed among the three sites for the interval from January to July, 1968. The effect of discontinued enrichment which was noted in the total bacterial population was not observed in the myxobacterial population when the enrichment point was moved below site M-15 in January, 1967 (Year I).

It is also interesting to note that the peak in maximum flow rate (refer to location of arrows) had no apparent effect on the populations of myxobacteria and total bacteria at any site. This observation is discussed further in the following section.

One myxobacterium became prevalent in the water of the enriched section when Sphaerotilus was abundant. Due to the large amounts of this single myxobacterium (as high as 1000 organisms per ml water sample) the data regarding its presence have not been included here but are presented separately in a discussion of sources of myxobacteria in the stream.

Flow Rate and Water Temperature Effects on Microbial Populations

The suggestion that myxobacteria are indigenous to the stream and not transients haplessly washed into the stream, is further

supported by the data graphed individually for each year studied (Figures 11 and 12). Data for site M-9 were selected because (1) this site did not receive enrichment at any time during this investigation, and (2) it also measured the number of organisms entering the experimental section of the stream.

During both years, the myxobacteria and total bacteria populations in the stream were highest in the late fall, at a time when the water temperature was decreasing; both populations reached their peaks a month ahead of the maximum flow rate period. The data in Figure 12 further indicate that flow rate does not directly effect the myxobacterial and total bacterial populations; here the flow rate was greatest during January, February and March while the organisms reached their peaks in late October and November, corresponding to the peak time observed for Year I (Figure 11). (No data was collected in February of Year II due to logging operations in the water shed.)

It is well known that both flow rate and water temperature play an important role in effecting the activities of micro-organisms present in the aquatic environment. The myxobacterial and total bacterial population data reported here indicate that these two parameters, while important, certainly must not be the sole influences on the population levels.

The seasonal changes observed in the populations in the stream

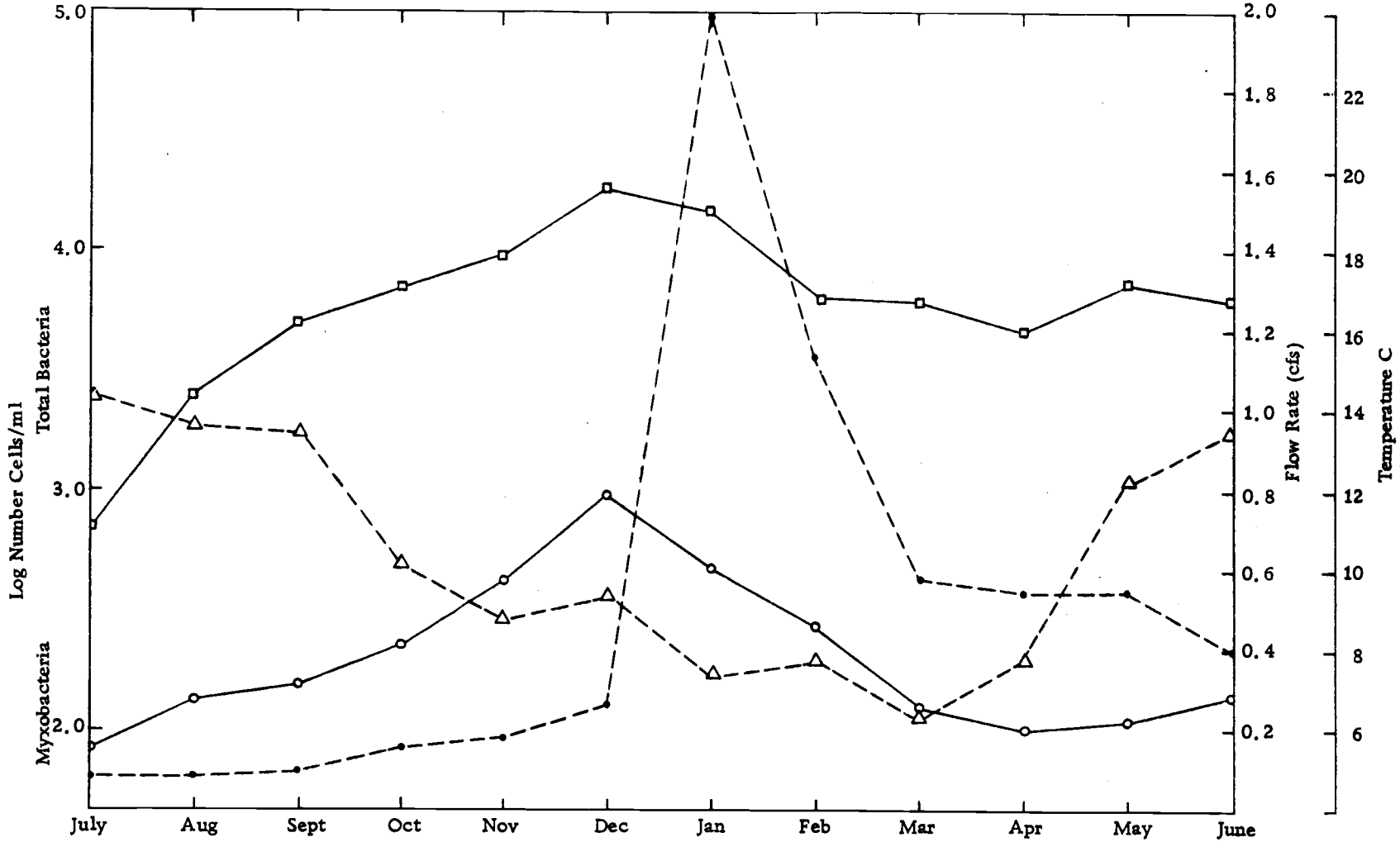


Figure 11. Effect of Flow Rate and Water Temperature on Myxobacterial and Total Bacterial Populations of Berry Creek during Year I (July 1966 through June 1967). Water temperature △---△, Flow rate •---•, Total bacteria □——□, Myxobacteria o——o.

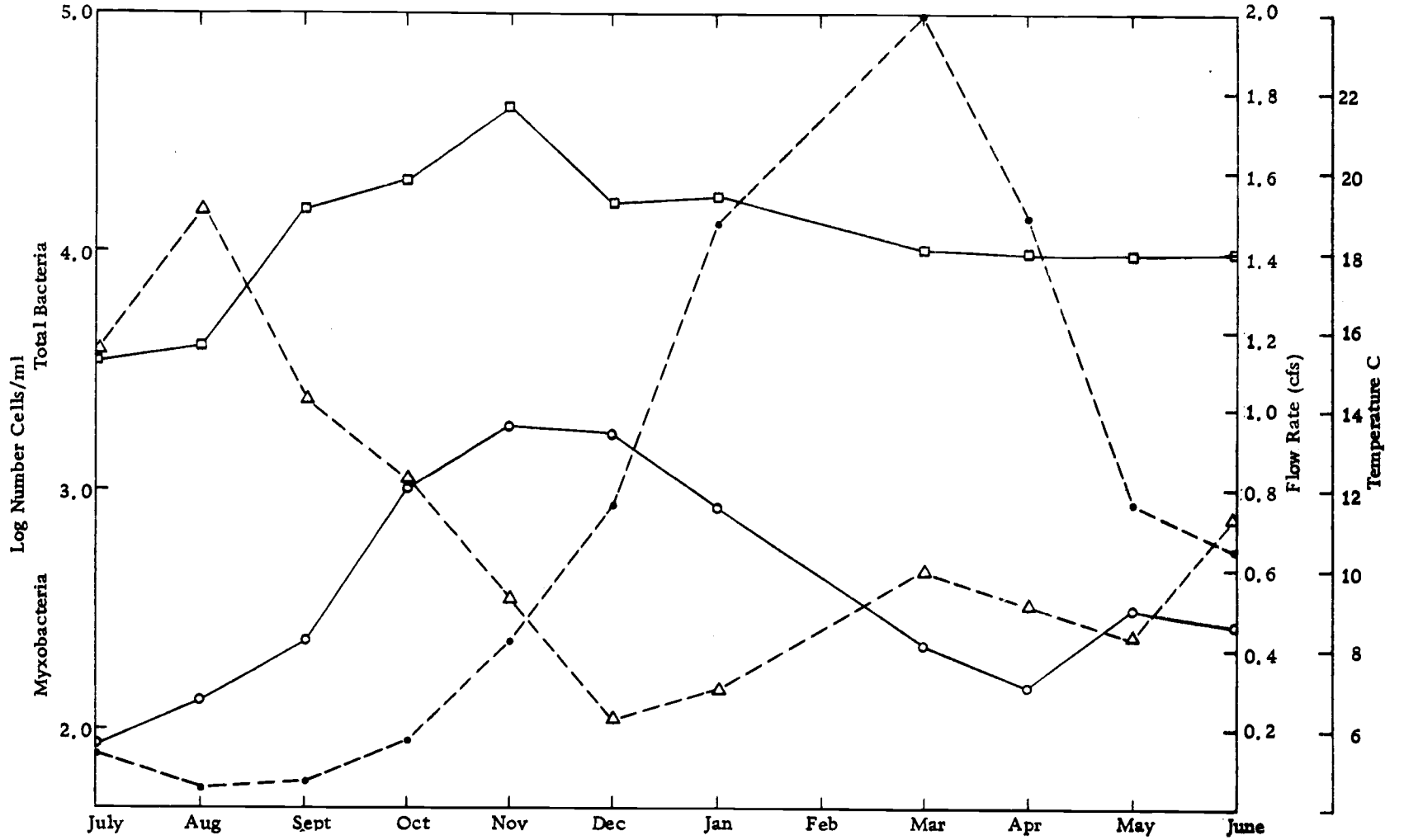


Figure 12. Effect of Flow Rate and Water Temperature on Myxobacterial and Total Bacterial Populations of Berry Creek during Year II (July 1967 through June 1968). Water temperature △---△, Flow rate •—•, Total bacteria □—□, Myxobacteria ○—○

may reflect several events: (1) the effect of washing the stream bed and resuspension of more organic material, including the myxobacteria and total bacteria present, (2) the increases due to runoff from the surrounding water shed, which increased with rainfall, (3) the natural enrichment of the stream by decomposing leaf material, etc., (4) the increased growth of the different organisms comprising the population in the stream.

Activities of Myxobacteria in the Stream Water

The pH and mineral content of the stream have been reported as notably constant over a given year (63). As will be shown, aquatic myxobacteria (and probably many other aquatic bacteria as well) are capable of growing in the stream water by utilizing the nutrients available. Since myxobacteria can grow in the stream water, the concentration of nutrients available in the microenvironment must be adequate for growth. Data supporting this conclusion are presented in the section below (p. 83-86).

To a large extent, the ecological role of a microbe will be related to its nutritional capacity. Most of these findings indicate that the myxobacteria in Berry Creek are well adapted to this habitat and are not merely transient members of the bacterial population. Even if they are, however, they may be degrading the remains of other bacterial cells, or they may be involved in the decomposition of

complex organic compounds which may be present in the stream (and which are not readily attacked by other microorganisms), while they are members of the microbial flora of a freshwater habitat.

Actinomycetes have been implicated by Silvey (50) as capable of breaking down complex organic compounds (cellulose, lignin, chitin, and others), and as capable of degrading the remains of algae and some bacterial cells. The role aquatic myxobacteria may play in the decomposition of cellulose material, for example, may be one of utilizing the initial breakdown products produced by other macro- or micro-organisms in the stream, rather than the role of a primary decomposer of cellulose. Other bacteria, such as members of the genus Bacillus and Pseudomonas, also play active roles in the ecology of the stream. Many of the interactions of the microorganisms are yet to be revealed.

Although it is likely that myxobacteria are involved in the degradation of various complex molecules in the natural environment, a great deal more work needs to be done in this area before predictions can be made for the ecological role of myxobacteria; great care must be taken in translating laboratory information correctly for the natural habitat.

Sources of Myxobacteria in the Stream

The ecological study on the occurrence of myxobacteria in

Berry Creek presented information on the myxobacterial populations of the flowing water in the stream. An attempt was made to detect the major contributors of these myxobacteria to the stream population. The stream sediment (comprised of mud and decaying leaves), the surface films, filamentous algae, insects, snails and fish populations of Berry Creek were examined as sources which possibly contributed myxobacteria to the total population in the stream water. Data in Table 9 compare the several sources with levels of myxobacteria and total bacteria found in the water.

Sediment Samples

Large numbers of myxobacteria were found in mud samples examined. The morphological types of myxobacteria represented in these samples were not different from those occurring in water samples from the same area in the stream. As noted in Table 9, mud samples were collected from two sites in the stream on different occasions. Total bacteria data for these water and mud core samples are also presented. It can be seen that about 2-20 times as many myxobacteria occur in the mud as occur in the water sample. For the total bacteria, 1-100 times as many are found in mud samples as in water samples from the same site. This finding is not surprising since a greater amount of nutrients occur in the sediments.

Table 9. Comparison of Myxobacteria and Total Bacteria from Various Stream Sources.

Sample Site and Source	Average Number per ml or gm Sample			
	Myxobacteria		Total Bacteria	
	Water[a]	Source[b]	Water[a]	Source[b]
M-13 Sediment-1	900	5000	2.5×10^4	3.5×10^6
Sediment-2	445	7500	3.6×10^4	1.8×10^5
M-16 Sediment-1	500	980	3.4×10^4	2.0×10^6
Sediment-2	435	9900	9.7×10^4	2.6×10^5
M-16 Leaves	680	8000	5.7×10^4	4.6×10^6
M-5 Surface films[a]				
Sample 1	135	1300	7.4×10^3	2.3×10^4
2	170	7325	8.0×10^3	2.6×10^4
3	170	21000	3.9×10^3	1.5×10^5
4	190	6670	4.0×10^3	1.9×10^4
5	114	13100	8.9×10^3	6.8×10^4

a = average number per ml sample.
b = average number per gm sample.

Leaf Material

The decomposing leaf debris abundant in the stream in the fall of the year was expected to yield large numbers of cellulose decomposing myxobacteria, particularly members of the genus Cytophaga. While as many as eight times more myxobacteria were recovered from leaf material in the stream than were present in water samples, no different morphological types were observed. Decomposition of cellulose could not be demonstrated for these isolates by use of Porter's method (47).

Presumptive evidence for cellulose utilization exists, however,

since the organisms had the capacity to utilize cellobiose and CMC; these results suggest that myxobacteria may be contributing to the decomposition of the abundant cellulose material. We can also speculate that decomposition may be accomplished primarily by the snail, Oxytrema silicula (18) and other cellulose utilizing organisms, such as bacteria of the genus Bacillus. The possible role of myxobacteria may be to degrade the complex molecules not utilized by these harvesters of cellulose. Further studies are necessary to accurately predict the role of aquatic myxobacteria in this particular niche of the environment.

Surface Films

Samples of the surface film which formed on the quieter pools in Berry Creek were collected and plated for myxobacteria and total bacteria content. Myxobacteria were 10-100 times more concentrated in the surface film than in water samples from the same area. From 2-7 times more total bacteria were present in these surface film samples than in corresponding water samples. Data for five samples are presented in Table 9 on the preceding page.

Algae

Batrachospermum sp. Portions of the filaments of the freshwater red alga which occurred in Berry Creek each spring, were

examined for the presence of myxobacteria and total bacteria; these samples were blended as in the procedure used for Sphaerotilus. Two yellow pigmented, motile, gram-negative rods were found associated with Batrachospermum sp. There was no evidence of myxobacteria associated with this alga.

Diatoms. Studies by Dever (14), Reese (48) and McIntire et al. (35) indicated that the algal population of Berry Creek was primarily diatomaceous. Gessner (9) has reported that algae excrete primarily amino acids. Dworkin (16) has shown that myxobacteria utilize amino acids and short peptides as sole sources of nutrient. Attempts were made to demonstrate a relationship between the occurrence of algae and myxobacteria in this aquatic environment, i.e., could the amino acids excreted by the diatoms of Berry Creek stimulate the growth of myxobacteria under natural conditions? No direct correlation was observed between the occurrence of total bacteria or the myxobacteria and the occurrence of algae (diatoms) in data collected during Year I.

During studies associated with the production of antibiotic substances by aquatic myxobacteria and pseudomonads, Burnison (10) followed the diatom and myxobacteria populations in an experimental stream. His observations suggesting that environmental conditions favored the growth of diatoms and myxobacteria lend support to the possibility of an association or interaction of the two

microorganisms in the aquatic environment. Environmental conditions which would result in enhanced growth of algae and myxobacteria may involve the production of substances by the myxobacteria capable of inhibiting various other bacteria while not affecting the algae; or, diatoms may produce substances (such as amino acids) which favor myxobacterial growth.

Sphaerotilus natans: A Source of Myxobacteria

Enrichment of the stream with sucrose and urea resulted in the growth of the sheathed bacterium, Sphaerotilus natans. The amount of Sphaerotilus visibly present in flocculent masses varied during the enrichment period. One myxobacterium also became prevalent in the water of the enriched sections when Sphaerotilus was present. For example, when the total myxobacterial count was at a level of 200/ml water sample, the level of the Sphaerotilus-associated myxobacterium alone was 600/ml. This myxobacterium, hereafter designated as BC 225, greatly decreased when flocs of Sphaerotilus were less abundant.

The myxobacterium was first noted in the stream in October of 1966. Some difficulty was experienced in identifying its colony as that of a myxobacterium. The colony was hardly visible at 48 hours on the CPM plating medium and the edge of the colony did not exhibit creeping motility after 50 hours incubation at 18 C on CPM. This

myxobacterium was not noted in samples taken from the unenriched section of Berry Creek, but was abundant in samples taken just 25 feet below the enrichment point. Due to the great abundance of this single myxobacterium in the samples, data regarding its presence was tallied separate from that of the total myxobacterial population (Table 10).

The data presented for site M-9 in Table 10 represent the number of myxobacteria entering the experimental section of the stream. Correspondingly, the data for site M-18 represent the total number of myxobacteria leaving the experimental section 1000 feet downstream. It will be noted that the myxobacterial population levels remained steady in this section from January through April (1967); slight increases in the number of myxobacteria per ml sample were noted from May into June and July. These increases represented seasonal changes in the myxobacterial population.

Myxobacteria counts which include BC 225 in the total count for this same time interval show significant increases in the number of this myxobacterium for all the sites in the enriched section (M-15^{+}, M-16^{+}, M-17^{+}and M-18^{+}). In January, with a high level of myxobacteria occurring in the stream (300 to 400/ml), an additional 500 to 1200 of the Sphaerotilus myxobacterium alone were noted per ml water sample. Late in January, the enrichment point was moved below site M-15; no colonies of BC 225 were noted again in samples

Table 10. Occurrence of Myxobacterium, BC 225.

Date	M-9	M-15	M-15+	16	16+	17	17+	18	18+
Nov. 1, 1966	178[a]	344	-	-	-	-	-	508	776
Nov. 10	354	475	813	-	-	-	-	569	1521
Jan. 7, 1967	310	408	440	378	878	380	1540	390	1560
March 28	144	158	-	166	172	178	303	152	328
April 8	119	132	-	123	143	123	148	144	246
April 25	78	82	-	92	400	84	452	93	600
May 8	84	90	-	143	258	143	934	147	1200
May 21	135	136	-	139	208	155	305	175	592
June 10	182	176	-	212	239	272	325	264	325
July 16	74	191	-	220	216	218	230	290	310

\+ = Values include BC 225.
a = Average number myxobacteria per ml water sample.

Table 11. Occurrence of Bacteria in Sphaerotilus Flocs.

Bacterial Colonies on CPM	Average Number Per Gram (Wet Weight)
Gram negative, motile rods	
pink pigment	9.0×10^5
yellow pigment	6.0×10^6
Myxobacterium BC 225	
Sample I	
initial	1.0×10^5
stirred 10 sec.	1.8×10^6
stirred 3 min.	1.6×10^7
Sample 2	
initial	2.0×10^5
blended 5 sec.	8.4×10^6
blended 10 sec.	12.8×10^6

from this formerly enriched site.

Enrichment with sucrose and urea resulted in macroscopic appearance of flocs of Sphaerotilus along the banks in the stream immediately below the enrichment point. Under these conditions, BC 225 was recovered throughout the spring of 1967 at levels of 200 to 500 myxobacteria per ml. In June the addition of nutrients was more sporatic, and by early July enrichment had ceased. During this period, a corresponding decrease in the amount of visible Sphaerotilus in the stream was noted; and, as can be seen in the data shown in Table 10, corresponding decreases in the Sphaerotilus-associated myxobacterium also occurred. By July 16, only 10 to 20 colonies of the Sphaerotilus-associated myxobacterium could be detected per ml of sample. This particular myxobacterium has not been found in any samples since enrichment with sucrose and urea has been stopped, nor since the corresponding disappearance of Sphaerotilus in early July.

To learn if BC 225 was intimately associated with Sphaerotilus flocs, samples of floc material were stirred or blended in the laboratory and plated for myxobacteria and total bacteria (refer to Table 11 on lower half of the preceding page). As many as 10^7 BC 225 colonies per gram wet weight Sphaerotilus were detected. The colony morphology of this isolated myxobacterium was the same as the myxobacterium which was noted in water samples from the

enriched section. Two other bacteria (pigmented, gram negative, motile rods) were also present in the Sphaerotilus samples, but no other myxobacteria (Figure 13A shows colony morphology of BC 225 on CPM; please refer to page 80).

Since this myxobacterium (1) did not utilize sucrose or urea, (2) was noted in such large numbers in the flocculent masses of Sphaerotilus, (3) decreased with changes in the Sphaerotilus population (completely disappearing when enrichment was stopped and Sphaerotilus was no longer abundant), the myxobacterium was believed to be intimately associated with Sphaerotilus. BC 225 may be dependent on some by-product of the sheathed bacterium, or symbiotically related to the organism; it may possibly function in the decomposition of Sphaerotilus, since it was more abundant in stream samples at times when Sphaerotilus decreased, due to discontinuous enrichment.

Additional Sources of Myxobacteria

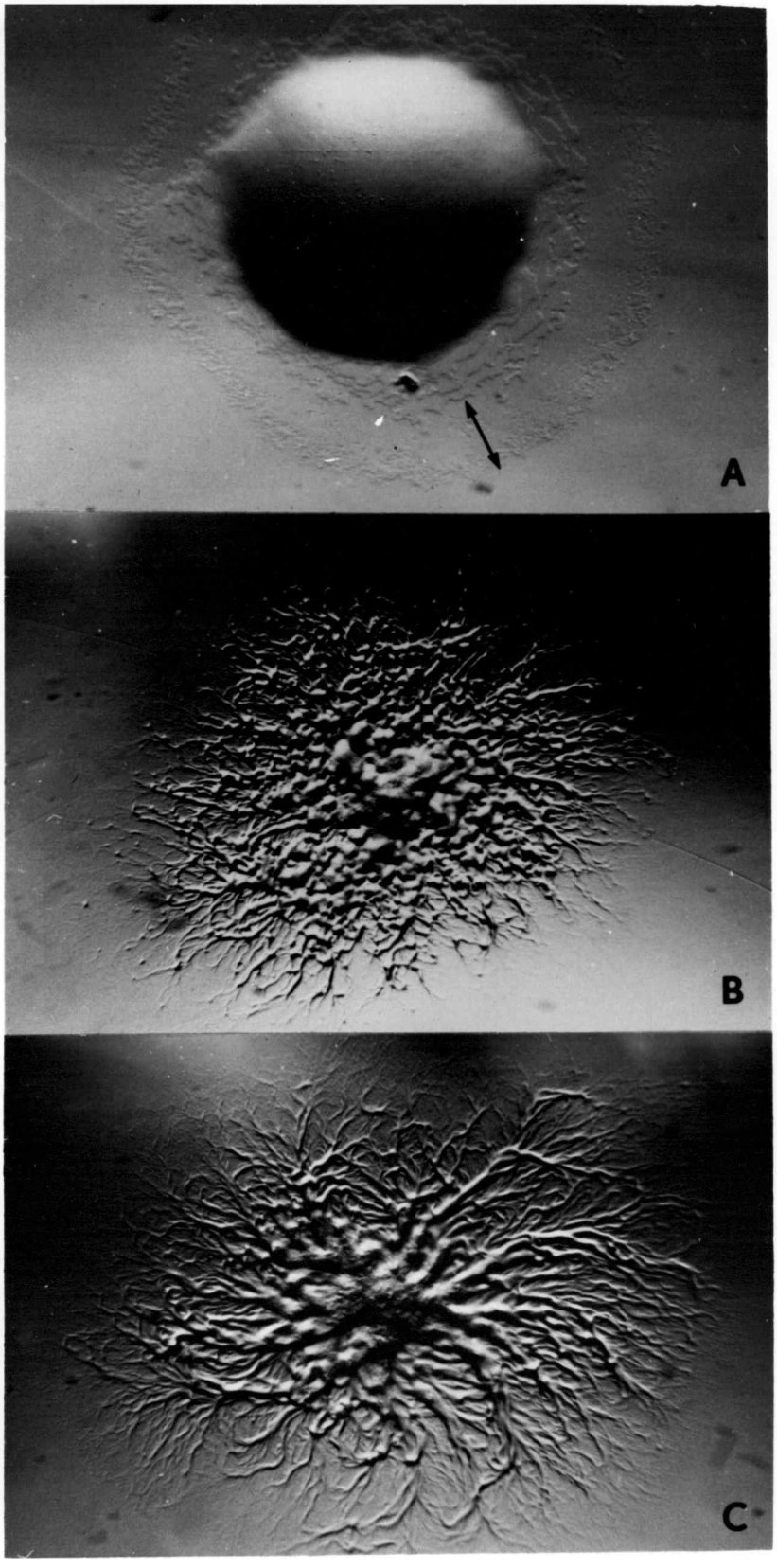
Hundreds of insects, several species of frogs, crayfish, snails, salamanders and fish are the larger members of the Berry Creek population. Myxobacteria may be normal inhabitants of these animals or associated with them in the environment. Several species of saprophytic myxobacteria have been isolated from fish (49) during studies of columnaris disease and cold-water disease caused by the

Figure 13A. Colony of Sphaerotilus-Associated Myxobacterium (Strain BC 225) on CPM. 6X.

The plateau of this colony (double-ended arrow) is the major feature in distinguishing it from pseudomonad-type colonies,as in Figure 16C; the latter form concentric layers from the colony center to the outer edge, while only a few such layers are noted in BC 225.

Figure 13B. Chondrococcus columnaris Strain BC 830. 6X. Note the formation of knots throughout this colony on CPM.

Figure 13C. Chondrococcus columnaris Strain BC 830. 6X. Note the prominent veins and the lack of knots in this colony on CPM.


A
B
C

myxobacteria Chondrococcus columnaris and Cytophaga psychrophila, respectively. Several of the myxobacteria isolated in this study may well have been normal saprophytic inhabitants of fish. No attempt was made to measure the contribution to the total population of myxobacteria from these possible sources.

Chondrococcus columnaris from Berry Creek

The fish pathogen, C. columnaris, was isolated from Berry Creek water samples for the first time on August 7, 1968. It was isolated from the stream two weeks after infected juvenile coho salmon from the Alsea River system were introduced as food chain competitors in the cutthroat trout production study. Several of the coho salmon mortalities showed symptoms of columnaris disease (37).

The colony morphology of this myxo on CPM is very similar to that described on cytophaga agar. Veins or ridges in the colonies tend to become "knotted" in places as if myxangia-production (fruiting body) is occurring there. These knots were visible in colonies first isolated on CPM; but this isolate did not always produce the knots (Figure 13B and C). The isolates of C. columnaris were serologically typed using antisera provided by Dr. R. E. Pacha and were found to possess antigens 1, 3, 8 and 9. This is a common antigenic type in the Pacific Northwest (44b).

It is of interest to note that the myxobacteria pathogenic for

fish possesses a distinctive morphology on CPM, which is unlike any freshwater myxobacterial morphological-type isolated from Berry Creek during the three year period of this study.

Surface Run-off

Another major source of myxobacteria in the small stream could be surface run-off from the surrounding water shed. During July, August and early September of 1967, a prolonged dry period resulted in minimum flow through the experimental section. If their major source was run-off, myxobacteria would be expected to disappear or to be reduced to a marginal level; this was not observed. The levels of myxobacteria occurring during the summer months were nearly the same for the three years of the study, regardless of the water temperature and flow rate during these same months. In addition, the highest levels of myxobacteria recorded in the three year period were noted in October, November and December prior to the flow rate peak of each year.

Stream Water as a Nutrient Source

Increased suspension of the organic material resulting from washing of the bed of the stream may well have contributed to the number of myxobacteria noted in Berry Creek. Increases in the myxobacterial levels may also be due, in part, to the contributions made

by the various sources already discussed. Even if the source of the microorganisms is wash from the surrounding water shed area, myxobacteria are capable of multiplying in the stream water itself. Minute fluctuations in the mineral and nutrient content during the year may be important to the microbial population.

Several simple experiments clearly demonstrated the ability of myxobacteria to utilize those nutrients present in the stream water. Filter-sterilized stream water from different sites in Berry Creek was inoculated with a quantity of stream sample, and incubated immediately in the stream in order to provide natural incubation conditions, especially temperature. Plate counts were made on the inoculum and on the bottle contents at the end of the three day incubation period. Results are given in Table 12. Both myxobacteria and pseudomonads were abundant in these stream-incubated cultures. Three to fourteen fold increase in myxobacteria were recorded; while the total bacteria increased six to ten fold. It is unlikely that an increase of this magnitude could be attributed to the utilization of reserve storage materials alone.

The observation that some myxobacteria present in Berry Creek were capable of growing in the stream by utilizing the small amount of organic nutrients and minerals present was not unexpected. Several workers have reported the ability of myxobacteria to grow on non-nutrient agar by utilizing the impurities in the agar (47, 56).

Table 12. Growth of Myxobacteria in Filter-Sterilized Stream Water.

Sample #	Site	Average Number Myxobacteria/ml	
		Initial	Three Days Incubation
1*	M-9	100	1430
2*	M-18	220	1500
3	M-9	100	750
4*	M-18	100	800
5	M-9	80	385
Culture #			
309-025		100	1000
311-020		100	1,000,000

*The total bacteria data for these samples, expressed as average number per ml; initially and after three days incubation:

Sample 1 2.75×10^3 and 3.4×10^6

Sample 2 2.5×10^4 and 1.4×10^6

Sample 4 4.5×10^4 pseudomonad-type colones/ml (at three days)

Since the number of total bacteria also increased in the filter-sterilized stream water samples, the microorganisms growing in the stream water may have been utilizing the minute quantities of substances produced by one another. It was also possible that the aquatic myxobacteria were degrading other bacterial cells and utilizing them as a nutrient source. These activities of myxobacteria are important in soil equilibrium (20, 21, 25, 51, 58, 59, 60).

Since pseudomonads and other types of bacteria could possibly enter the submerged culture bottles during the incubation period in the stream, similar experiments were conducted with bottles of sterile stream water inoculated with pure cultures of myxobacteria. Following three days of incubation, one culture had increased ten times and the other 10^4 times (Table 12).

The ability of at least some of the myxobacteria to grow in the stream must be considered as an important factor in the population increases noted for myxobacteria at different times of the year. Growth in water indicates that these organisms are well adapted to the environment and could be actively performing a function in the stream. The results of laboratory studies on the biochemical activities of several isolates are summarized in the section which follows. These data allow further speculation on the adaptation of aquatic myxobacteria to their environment.

III. Laboratory Studies on Aquatic Myxobacteria

Laboratory studies were conducted on isolates of the predominant myxobacteria types occurring in Berry Creek in an attempt to demonstrate that these isolates were different myxobacteria; and also to determine their biochemical activities from the standpoint of macromolecule attack in order to suggest their ecological role.

Morphological Characteristics

The organisms studied in this investigation were myxobacteria as indicated by their cellular morphology: individual vegetative cells were gram negative, unicellular, flexible rods with round ends. Typical myxobacterial gliding or creeping motility and low refractility was noted upon direct phase contrast microscopic examination of living, young vegetative cells. Young vegetative cells were long and slender delicate rods in size range of 0.5μ x 5-10μ. As cultures aged, the cells lost their gliding motility and many were notably shortened. All these characteristics have been reported by others as typical of myxobacteria (16, 53, 56, 60).

Cultural Characteristics

All the isolates grew at room temperature as well as at 18 C; growth at a slower rate was noted at 7 C.

A few of the isolates produced brown pigment in cytophaga broth and caesin hydrolysate broth cultures. Myxobacteria capable of producing water soluble pigments have also been reported by other workers (7, 47, 57).

Biochemical Characteristics

Biochemical studies supported the differentiation of myxobacterial types which had been separated on the basis of their colony morphology on CPM. These activities were studied in an attempt to begin to define the role of myxobacteria in the natural environment.

The biochemical tests included detection of: (1) the ability to degrade macromolecules: starch, esculin, chitin, carboxy methyl cellulose, cellulose, caesin, gelatin; (2) the ability to utilize tyrosine and tributyrin; (3) the ability to oxidatively or fermentatively attack less complex carbohydrates: glucose, maltose, galactose, lactose, sucrose, mannitol, and cellobiose; (4) the ability to reduce nitrates to nitrites or ammonia; (5) the presence of catalase or cytochrome oxidase.

The results of these biochemical tests are presented in Tables 13 and 14. Data, arranged by morphological grouping, are presented for all the isolates photographically illustrated in the thesis and for additional isolates which fall into these morphological groups (Table 15). Reference is made later in this thesis to the major biochemical

features of each of the morphological groups designated. Some general points revealed by this data will be noted below.

All the isolates produced catalase with the exception of a strictly proteolytic myxobacterium (Isolate No. 25) which produces cytochrome oxidase. Half of the isolates reduced nitrate to nitrite; several of these required four to five days to reduce nitrate. Only four isolates were noted which reduced nitrate to ammonia.

Carbohydrate Utilization and Macromolecule Degradation

Only oxidative attack on carbohydrates was detected. All but three isolates utilized glucose, maltose and cellobiose; most of these also utilized D-galactose. The ability of these isolates to attack lactose and sucrose varied; lactose fermentation was sometimes delayed or absent. Two isolates did not utilize any of these carbohydrates (Table 13).

Dworkin (16) has indicated that conflicting information is available on use of mono- and disaccharides as energy sources for groups of myxobacteria other than Myxococcus; and that, in general, glucose does not seem to be utilized to any great extent. It is apparent from the data presented that these aquatic myxobacterial isolates are capable of utilizing carbohydrates as carbon sources (Tables 13 and 14).

All the organisms investigated in this study showed some ability to degrade macromolecules. This feature has been reported

Table 13. Ability of Isolates to Oxidize Carbohydrates.[b]

Morphological Group	Strain*	Glucose	Maltose	Sucrose	Galactose	Lactose	Cellobiose	Mannitol
I	6*	A	A	-	A	-	A	-
	6-s	A	A	-	A	-	A	-
	8*	A	A	-	A	-	A	-
	24	A	A	A	-	-	A	-
	16*	A	A	A	A	A	A	-
	7*	A	A	A	A	A	A	-
II	25*	-	-	-	-	-	-	-
	32	A	A	-	A	-	A	-
	33*	A	A	-	A	-	A	-
	34*	A	A	-	A	-	A	-
	35	A	A	-	-	-	A	-
	1-17	A	A	-	A	-	A	-
III	11	A	A	A	A	-	A	-
	58	A	-	A	A	A	A	-
IV	43*	A	A	-	A	A	A	-
	9*	A	A	-	A	a	A	-
	12*	A	A	-	A	a	A	-
	37*	A	A	-	A	a	A	-
	19*	A	A	A	A	a	A	-
	20	A	A	A	A	A	A	-
	21	A	A	A	A	a	A	-
	10*	A	A	A	(±)	-	A	-
V	36	A	A	-	-	-	A	-
	39	A	A	-	a	-	A	-
	42*	A	A	-	-	-	A	-
	38	A	A	-	a	-	A	-
	30*	A	A	-	-	-	A	-
	5	A	A	-	-	-	A	-
	31*	A	A	-	A	a	A	-
VI	14*	A	A	A	A	A	A	-
	18*	a	a	a	a	a	a	-
VII	BC830*	-	-	-	-	-	-	-
	3*	A	A	-	-	-	A	-
	27	A	A	-	-	-	A	-
	28*	A	A	A	A	-	A	-
	38-p	A	A	A	A	A	A	-
	13	-	-	-	-	-	-	-
	15*	-	-	-	-	-	-	-
	45	A	A	A	-	-	A	-
	46*	A	A	A	-	-	A	-
	BC225*	A	A	-	A	-	A	-

a = delayed oxidation A = acid (-) = not utilized

b = none of isolates fermentatively attacked these carbohydrates.

* = photograph appears in thesis.

Table 14. Ability of Isolates to Degrade Macromolecules and Tyrosine.

Morphological Group	Strain*	Starch	Esculin	CMC[a]	Cellulose	Chitin	Caesin	Gelatin	Tyrosine[b]	Tributyrin
I	6 *	+	-	+	-	+	+	+	+	+
	6-s	+	-	+	+	+	+	+	+	+
	8 *	+	+	+	-	-	+	+	-	+
	24	+	+	+	-	+	+	+	-	+
	16 *	+	+	+	-	-	+	+	+	+
	7 *	+	+	+	-	-	+	+	-	+
II	25 *	-	-	-	-	-	+	+	+	-
	32	+	+	+	-	+	+	+	-	+
	33 *	+	+	+	-	+	+	+	-	+
	34 *	+	+	+	-	+	+	+	-	+
	35	+	+	-	-	+	+	+	-	+
	1-17	+	+	+	-	-	+	+	-	+
III	11	+	+	-	-	-	+	+	-	+
	58	+	+	-	-	-	+	+	-	+
IV	43*	+	+	+	-	+	+	+	+	+
	9*	+	+	+	-	+	+	+	+	+
	12*	+	+	+	-	-	+	+	+	+
	37*	+	+	+	-	-	+	+	-	+
	19*	+	+	+	-	-	+	+	-	+
	20	+	+	+	-	-	+	+	+	+
	21	+	+	+	-	-	+	+	+	+
	10*	+	+	+	-	-	+	+	(+)	+
V	36	+	+	-	-	+	-	+	+[b]	+
	39	+	+	-	-	+	-	+	+[b]	+
	42*	+	+	-	-	+	+	+	+[b]	+
	38	+	+	-	-	+	+	+	+	+
	30*	+	+	+	-	+	+	+	-	+
	5	+	+	-	-	+	+	+	+[b]	+
	31*	+	+	+	-	+	+	+	-	+
VI	14*	+	+	-	-	-	+	+	-	+
	18*	+	+	(+)	-	-	+	+	-	+
VII	BC830*	-	-	-	-	-	+	+	+	-
	3*	+	+	+	-	-	+	+	(+)	+
	27	+	+	+	-	-	+	+	-	+
	28*	+	+	-	-	-	+	+	-	+
	38-p	+	+	-	-	-	+	+	-	+
	13	+	-	-	-	-	+	-	+	-
	15*	+	+	+	-	-	+	+	-	+
	45	+	+	+	-	+	+	+	+	+
	46*	+	+	+	-	+	+	+	+[b]	+
	BC225*	+	+	+	-	+	+	+	+	+

a = carboxymethyl cellulose.

b = brown pigment also produced.

* = photograph appears in thesis.

for myxobacteria and appears to be an important characteristic of this group of bacteria (16). Most of the isolates hydrolyzed starch, esculin, caesin and gelatin. Nearly half utilized chitin while a lesser number degraded tyrosine and tributyrin. Presumptive evidence exists for utilization of cellulose by these aquatic myxobacteria. All the isolates in which carboxy methyl cellulose degradation could be demonstrated were also able to utilize the smaller components of cellulose: cellobiose and glucose.

Only one isolate from a surface film sample attached cellulose powder suspended in mineral salts medium (56). The consistency of the cellulose changed, becoming viscous after prolonged incubation at room temperature (two months); vegetative cells and germinating microcysts of a Sporocytophaga were noted in the culture. It would be desirable to devise more sensitive methods of detecting possible cellulose degradation by these myxobacteria.

All isolates from fish studied by Pacha and Porter (46) hydrolyzed gelatin and caesin; several did not utilize starch or chitin. Only one of the myxobacterial isolates from Berry Creek failed to utilize starch. One isolate was strictly proteolytic (No. 25) and did not utilize any carbohydrates, simple or complex. Myxobacteria living on the surfaces of fish might be expected to be proteolytic, while those inhabiting water may be able to attack other substrates as well. Thus, the habitat of strain 25 may have been the surface of fish.

Approximately 40 different types of Cytophaga and Sporocytophaga are believed to be present as based on the morphological, biochemical and physiological differences recorded. Myxobacterial isolates were designated as members of the genus Cytophaga when only vegetative cells were produced; and as Sporocytophaga when both vegetative cells and microcysts were noted (53, 56, 57). The fish pathogen, Chondrococcus columnaris, was the only fruiting myxobacterium isolated from Berry Creek.

To date about 30 species of Cytophaga have been described by other workers; of these 17 were isolated from the soil or wood and ten were isolated from the marine environment. Very little work has been done on the freshwater cytophagas, which are not included at present in Bergey's Manual of Determinative Bacteriology, Edition 7 (1957). A recent taxonomic study on freshwater myxobacteria by Nitsos (40) contains a classification scheme for these organisms. As indicated by several workers (40, 56, 58), the classification of freshwater myxobacteria has not been solved completely. Continued study should facilitate a workable scheme for the nonfruiting, freshwater myxobacteria including data of the type presented herein.

Colony Morphology Study

Enumeration of the myxobacteria in the ecological study was based on the recognition of myxobacterial colonies growing on the

dilute nutrient medium,CPM (cytophaga peptonized milk agar), which was developed for this purpose. The colony morphology observed on CPM was a constant characteristic of the particular myxobacterial type and distinguished it from other microbial colonies compared under the same conditions; CPM, 18C, etc.

The colony morphology on CPM was also consistent after several laboratory transfers. Separation of the different myxobacteria on the basis of their colony morphology was further supported by biochemical studies. Isolates of the same morphological type utilized the same biochemical substrates. The data tables providing this information for 40 isolates were included in the previous section (Tables 13 and 14). The colony features observed are thus not artifacts of the method used for their study, but do represent different myxobacteria.

Photographs of the dominant and interesting forms present in the stream samples have been compiled and those selected for this thesis illustrate the distinguishable morphology which is typical of myxobacteria. In some instances photographs of myxobacterial colonies were made prior to their isolation from the CPM primary isolation plates. Magnifications of 15 X were used consistently for the differentiation of the myxobacterial colonies from other colony types present in the plated sample. Unless otherwise specified, colonies were photographed after 70 hours incubation on CPM at 18 C.

Table 15. Photographs of Myxobacteria Representing Each Morphological Group. *

Morphological Group and Main Features	Strain or Isolate Number	Figure and Print Number	Thesis Page Number
I. Circles within colony:			
spreading colony	6, 8	14A, B	96
	6	2	34
confined colony	7	4A, edge 1	39
	16	4B, edge 8	40
II. Colonies with ridges or veins	33, 34	14C, D	96
	47-10	14E	96
	25	17C	108
III. Granular surfaced colonies	28	14F	96
IV. Smooth centered colonies with detailed rhizoid edges:			
a. fine projections	34-3	4B, edge 5	40
	10	15A	99
	37	15C	99
	12	3	35
	12	19	111
	19	4B, edge 7	40
b. blunt projections	43	18	110
	9	15B	99
V. Spread from central point	31	15E	99
	30	15F	99
	42	4A, edge 3	39
VI. Colonies appear "splashed" on agar surface	14	20	113
	18	21	114
	18	4, edge 6	39
VII. Miscellaneous group:			
Sphaerotilus-associated myxo	BC 225	13A	80
pink pigmented-dull	28	14F	96
-smooth	28	16A	104
"bubbly" colony	15	17B	108
sheer colony-veiny (II)	3	4A, edge 2	39
plateau colony (IVa)	46	15D	99
	46	17A	108
Chondrococcus columnaris	BC 830	4A, edge 4	39
		13B, C	80

*Biochemical data for these isolates are summarized in Tables 13 and 14.

Although great variations in edge patterns, shapes and sizes, thickness of the colonies were observed, the common characteristic of (1) a spreading colony, and (2) a rhizoid edge generally describe the colony "typical" of a myxobacterium.

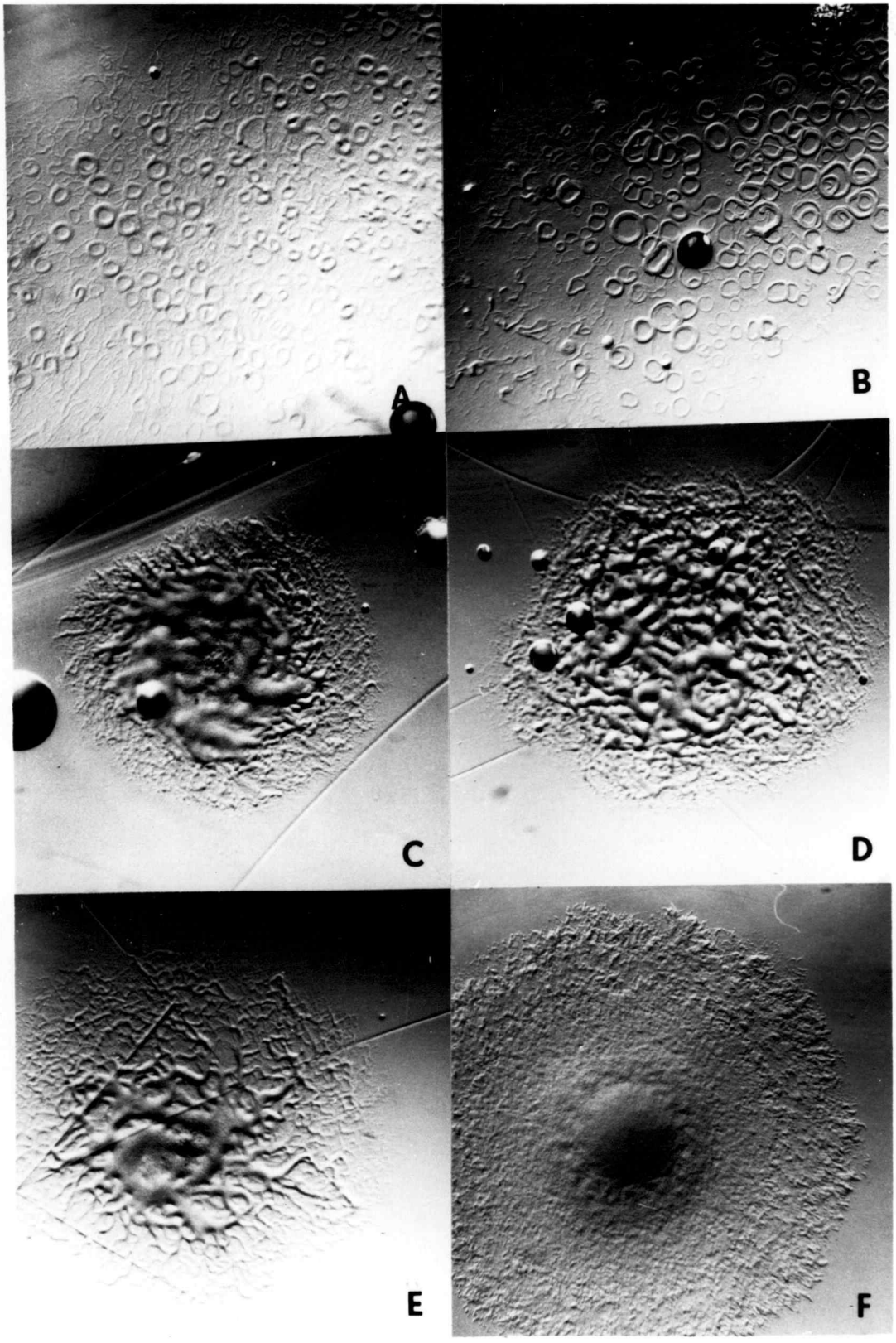
From the colony types of aquatic myxobacteria isolated, seven morphological groups were designated. A brief description of the major features of each group follows; pertinent biochemical data, and photographs of isolates representing the group are also included. These photographs clearly illustrate the distinct morphological features used in the grouping. Since examples representing these morphological groups are presented in other sections as well, Table 15 summarizes the location of these photographs in the thesis.

Morphological Groups

Type I. Colonies in the first group are thin and spread over a large area forming a mat of circles in slightly varying sizes. They often form colonies with curled ridges throughout which suggest, and later form, complete circles scattered throughout the colony (Figure 14A & B; Figure 2). A second colony type which forms circles remains circular and confined in area (rather than spreading) in both the developing and mature colonies (50 and 72 hours, respectively), Figure 4, edges 1 and 8. Colonies of this morphological type (I) are present in the stream samples all during the year; they are more

Figure 14. Representatives of the Colony Morphology Groups. 70 hour colonies on CPM.

Morphological Group	Print	Final Magnification
Type I.	A. Strain 6	5X
	B. Strain 8	5X
Type II.	C. Strain 33	5X
	D. Strain 34	4X
	E. Strain 47-10	4X
Type III.	F. Strain 28	4X


A
B
C
D
E
F

abundant in the spring and fall with fewer occurring in summer. Generally, they attack all macromolecules and most of the less complex molecules; all utilize glucose and maltose and cellobiose.

Type II. Colonies of group II form multiple ridges or veins which swirl at the center, often counterclockwise. This type is predominant in the summer but present in smaller numbers throughout the rest of the year. Examples of these are seen in Figure 14C, D, E and Figure 4A, edge 2. Strains 33 and 34 cannot be separated biochemically and probably represent the same type. Macromolecules utilized included chitin and CMC; no utilization of sucrose and lactose was noted by isolates of this group.

The Type II isolate in Figure 17 C1, C2 is very interesting biochemically; it is the only isolate studied which attacks proteins but does not utilize any carbohydrates. It also possesses cytochrome oxidase, while lacking catalase. Morphologically, it is also unique and perhaps a separate group should be established for myxobacteria of this type (strictly proteolytic isolates as strain 25).

Type III. These circular colonies form a granular center which is slightly thicker than the inner periphery and the edge. Colonies of this type are abundant in stream samples in the summer months. The isolate in photograph F of Figure 14 produces a pink pigment; most of the granular-type colonies produce yellow pigments, however. All sugars are utilized by isolate 28 with the exception of lactose; chitin is not attacked but the other macromolecules are utilized. Other granular isolates utilized these same substrates.

Type IV. Colonies with this general morphological pattern

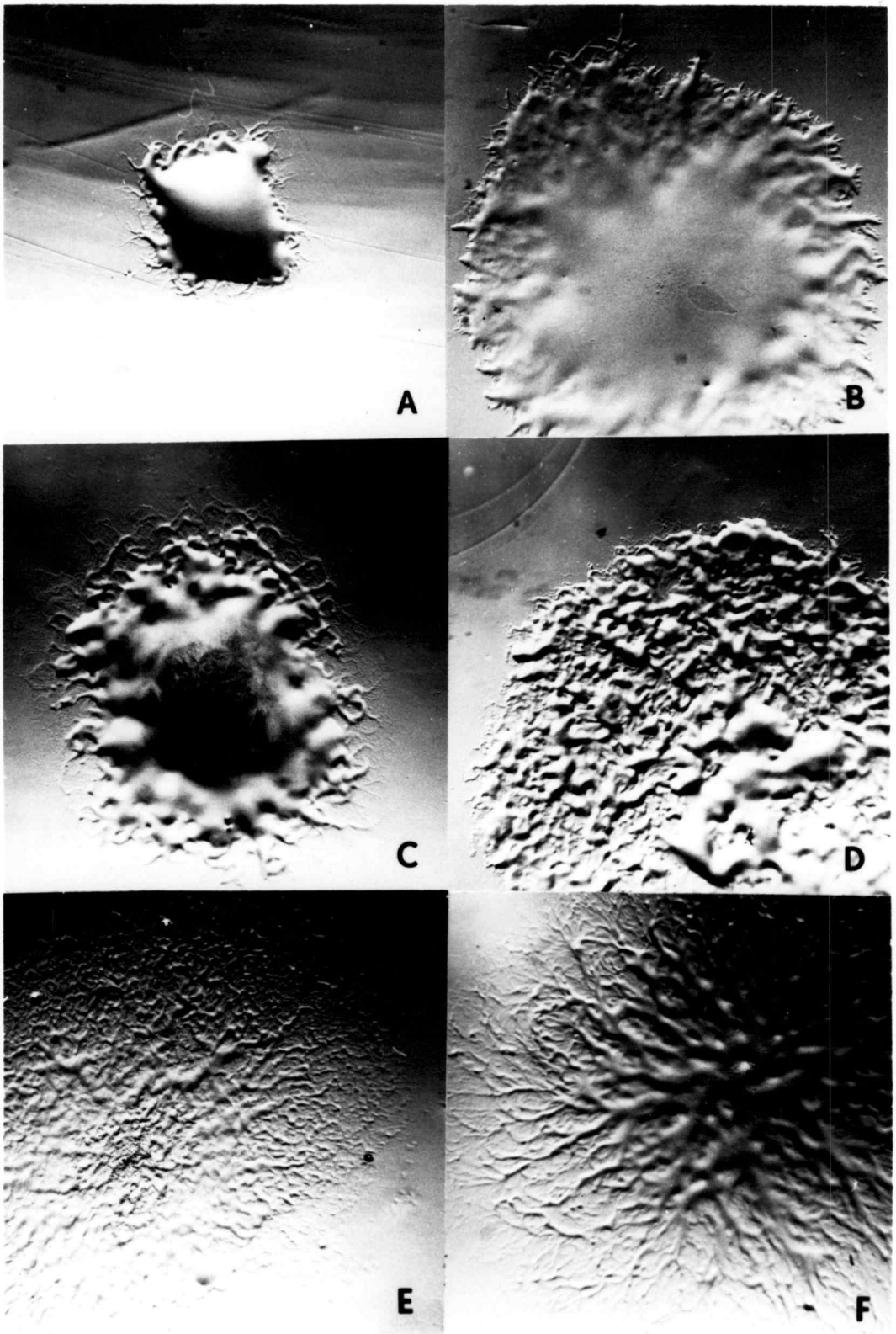
generally possess smooth centers with variously detailed rhizoid edges. They vary mainly in size, with subtle differences in their edge patterns; these differences are particularly noticeable in the young colonies. The raised colonies are circular or slightly irregular in shape with a smooth center and a slightly ridged periphery which extends into a finely detailed rhizoid edge (Figure 15A, B, strains 10 and 9).

When viewed in reflected light, strain 37 appears to be irridescent and the center reflects light in kaleidoscopic patterns (Figure 15C). Some colonies of Type IV form blunt projections in the colony edge (strain 43), others possess fine hair-like projections (strain 12). These two strains are compared further in a study of their colony development (refer to pp. 110 and 111).

Type IV colonies are abundant in Berry Creek in the fall, winter, and spring, with fewer occurring in the summer. All of these isolates utilize the macromolecules tested and tyrosine. The blunt-type of colony (Figure 18) is separated from the colony type which form fine-projections (Figure 19) on the basis of oxidative attack on lactose and chitin utilization; none of the fine-type utilized chitin while they do metabolize all the sugars tested, except sucrose. The blunt-type colony, strain 43 (Figure 18) utilizes chitin and lactose, but not sucrose.

Figure 15. Representatives of the Colony Morphology Groups. 70 hour colonies on CPM.

Morphological Group	Print	Final Magnification
Type IV.	A. Strain 10	5.5X
	B. Strain 9	7.5X
	C. Strain 37	4.5X
Type VII.	D. Strain 46	4.5X
Type V.	E. Strain 31	4X
	F. Strain 30	4.5X


A
B
C
D
E
F

More recently a Sporocytophaga with a similar morphology to a fine-Group IV colony was found; it slowly utilized cellulose powder in mineral salts broth base. The other isolates studied showed the ability to degrade CMC but not cellulose directly.

Type V. Three different isolates of colonies which radiate outward from the center in fan-like manner are presented below: (1) a colony with the center depressed resembling a crater (Figure 15E), (2) a colony with a raised center and smooth ridges similar to the morphological Type II colonies (Figure 15F) and (3) a colony with a large streaming plateau which flows into circle formation and then to the rhizoid edge. (Figure 4, edge 3). Colonies of Chondrococcus columnaris spread in a manner similar to Figure 15F and could be tentatively placed in this morphological group.

Myxobacteria of colony Group V are abundant in the fall and not very prominent during the rest of the year. All members of this group hydrolyze tyrosine producing a brown pigment; glucose and maltose are the only sugars attacked; in addition to cellobiose and CMC, chitin was also utilized by Type V isolates.

Type VI. Colonies of this group appear to be "splashed" on the agar (Figures 20 and 21). Smooth, irregularly-shaped dense areas of cells occur in the center of the colony and gradually merge as the colony matures. Some of these small, irregular areas

remain isolated near the very thin, finely detailed rhizoid edge of the mature (72 hour) colony (refer to Figures 4-6 and Figures 20D and 21C; pages 40, 113 and 114, respectively).

Myxobacteria in this morphological group occur mainly during the spring and summer in Berry Creek. All attack macromolecules, including tributyrin, as well as all the sugars tested. CMC degradation could not be demonstrated, although all were able to utilize cellobiose and glucose.

Studies on the development of these colonies with time (Figures 20 and 21) revealed two basic types: one which has several circles evident in the young colonies, and a second one in which these circles are never present. The mature colonies show very little difference morphologically and have not been separated biochemically. Strain 18 (Figure 21) exhibited a three day delay in utilization of sugars. Further studies are necessary to determine whether delayed oxidation of carbohydrate is an important characteristic in the identification of this myxobacterium.

Type VII. Colony types which could not be included in the above groups were placed into a miscellaneous group (Type VII). Photographs of a number of these unusual colonies are shown in Figures 13, 14, 16 and 17. The pink pigmented myxobacterium with the smooth and dull-surfaced colonies was included in this group (Figures 16A and 14F). The smooth colony produces acid from all

sugars tested except lactose, while the granular colony does not attack maltose.

The colony in Figure 17B is finely dispersed, thin and circular; the "bubbly" center becomes more dense as the colony matures. This type does not utilize sugars but does degrade the more complex macromolecules. Strain 15 was noted most often in the fall, but was never abundant in the stream.

The myxobacterium associated with Sphaerotilus designated as BC 225 was placed into Type VII. This organism was found only in the enriched sections of Berry Creek and in the flocs of Sphaerotilus; it diminished with a decrease in the latter and disappeared entirely following termination of the enrichment experiment. Biochemically this isolate did not attack sucrose or urea, but could utilize starch, caesin, gelatin, esculin, tyrosine, glucose and maltose. Data regarding the occurrence of this isolate was presented in an earlier section of the thesis along with a photograph of the colony on CPM (Figure 13A).

Other isolates in Group VII include strain 3 (Figure 4A-2) which forms a circular colony with a smooth, but bumpy center and a wide, sheer plateau with small veins moving toward the rhizoid edge. Like strain 46, it hydrolyzes chitin, as well as other macromolecules; the sugars utilized are glucose and maltose. Strain 46 can attack sucrose in addition to these two sugars; its colony morphology is also quite

different (Figure 15D, 17A).

Chondrococcus columnaris was isolated from Berry Creek in August 1968 after infected juvenile coho salmon were introduced into the stream. The morphology of this myxobacterium on primary isolation plates was unlike any other isolate obtained from this environment. Figure 13B and C presented with the Berry Creek Ecological Data section illustrate its morphology in pure culture.

Smooth Colony Types and Non-Spreading Myxobacteria

A few of the isolates studied produced non-spreading variants along with their usual spreading forms. Examples of two such non-spreading colonies are presented in Figure 16. Colony A shows a smooth form with an entire margin. The eubacteria-like colony seen in Figure 16B has wavy "tracks" etched on its surface (arrow) as well as an irregular margin. The "parent" strain is characterized by the formation of circles within the colony margin (Morphological Group I). These irregular-edged colonies developed in aged broth cultures did not form when the cultures were transferred weekly. However, the smooth isolate (Figure 16A) retained its morphological identity at any age. Both of these smooth types of myxobacterial colonies showed typical gliding motility and no differences in their biochemical reactions as compared with their spreading or related strains.

Both a smooth and a spreading form of colonies have been

Figures 16A and 16B. Non-Spreading Myxobacteria Colonies on CPM.

A. Smooth form strain 28.

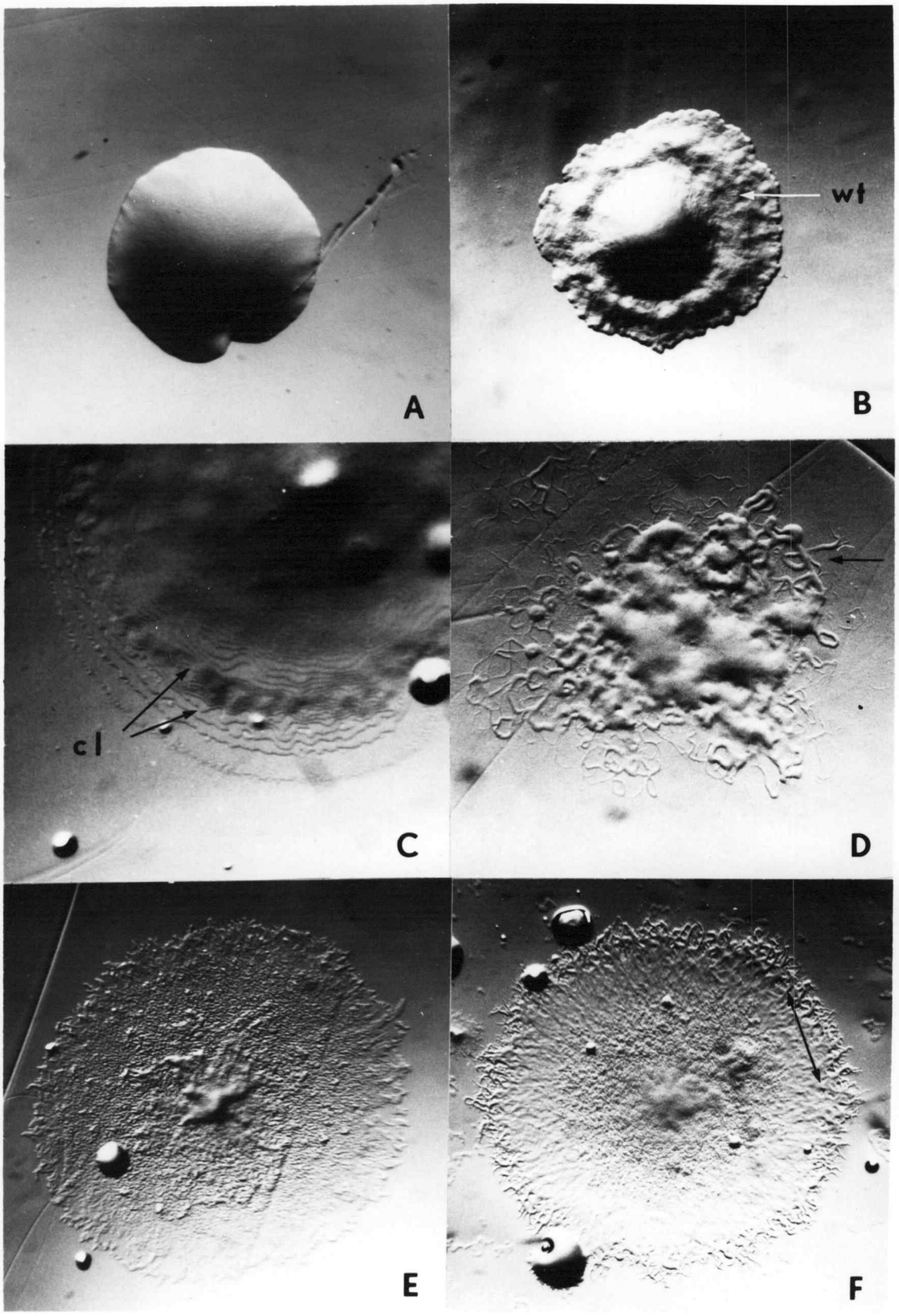
B. Eubacterial-like colony of strain 3218-06. Note the etched surface with wavy "tracks" (wt).

Figures 16C, D, E, F. Non-Myxobacteria Producing Colonies Similar to Myxobacteria on CPM Primary Isolation Plates.

C. Colony of Pseudomonas sp. 6X
Concentric layers (cl) are characteristic of colony on CPM.

D. Colony of Bacillus sp.
The curly extensions of colony terminate abruptly rather than spread in fine root-like extensions typical of myxobacteria.

E. and F. Bacillus sp. colonies.
Note thickness in colony at edge (⟷) and in granular center.


A
wt
B
cl
C
D
E
F

isolated by R. A. Holt (masters thesis in preparation) and by R. E. Pacha (44a) during studies on cold-water disease of coho salmon (Oncorhynchus nerka). Both types of colonies were formed on cytophaga agar as well as on CPM by the myxobacterial disease agent, Cytophaga psychrophila.

Non-Myxobacteria Producing Colonies Similar to Myxobacteria

Bacterial colonies with colony patterns similar to myxobacteria were noted on CPM on occasion. Some pseudomonad colonies possessed edges similar to those of the myxobacteria. At various times during the year, these colonies were abundant on the CPM enumeration plates (Figure 16C). Pseudomonads, however, possess a distinguishable morphology of their own. Note the series of concentric layers prior to the plateau near the periphery; all colonies of this type were motile by means of flagella when checked under phase-contrast microscopy. Familiarity with the pattern of their colony allowed these pseudomonad-type colonies to be more easily separated from the myxobacteria.

Some colonies of Bacillus sp. can be confused with myxobacteria until one notes their colony features more carefully. In Figure 16D, a Bacillus colony is shown; note the sheer mono-layer of cells, so typical of myxobacterial edge, is absent (arrow). No myxobacteria

colonies were observed which appeared to be as stringy as this type of Bacillus species.

Figures 16E and 16F show very thick colonies with a dense, grainy appearance in the center. This grainy appearance served to distinguish this colony from those formed by myxobacteria. None of the myxobacteria colonies examined possessed this type of center. Examination of the thick colony edge (two-way arrow in Figure 16F) under phase contrast microscopy removed all doubt regarding the identity, since the cells can be seen to possess flagellar type of motility and are also very large as compared with most myxobacterial cells observed.

Comparison of Young and Mature Colonies on CPM

For enumeration purposes in the initial stage of the ecological study, myxobacterial colonies were counted after 50 and 72 hours growth on CPM at 18 C. It was soon obvious that mature colonies of many of the myxobacteria had colonial features which were also recognizable in younger colonies. These young colonies show the very delicate differences existing in different myxobacteria; these details become somewhat obscured as the colony develops. Comparison of edge patterns and the colony center in three examples of aquatic myxobacteria will clearly point out typical features of young (50 hour) and mature (72 hour) colonies of myxobacteria. Note the

edge of the 72 hour colony in Figure 17A-1; the same detail is evident in the young colony Figure 17A-2).

Culture 15 is an interesting isolate with a very thin, finely dispersed circular colony which develops small mounds of cells in the center; the latter becomes more dense as the colony matures. The finely dispersed, nearly invisible edge never becomes very thick in the mature colony. The center mounds become larger and somewhat confluent with age, but the edge remains dispersed, as in the young colony. This is apparent when a close comparison is made between photographs B1 and B2 of Figure 17.

Another example of a colony "filling in" at the center is shown in Figure 17, C1, C2. The heavier veins, which appear superimposed on a sheerer layer of cells become "smeared" together upon aging.

These observations indicated that the different colony types could be recognized at different stages in their development. Myxobacteria types can be distinguished with ease at 65 to 72 hours.

Development of Myxobacterial Colonies with Time

Colonies representing two different morphological groups have been selected to illustrate the changes which occur in the development of myxobacterial colonies on CPM. Colonies are followed from the time they are microscopically visible (48 to 50 hours) until no further

Figure 17. Comparison of Features in Young and Mature Colonies of Myxobacteria on CPM.

A1. strain 46 72 hours 4.5X

A2. strain 46 50 hours 6X

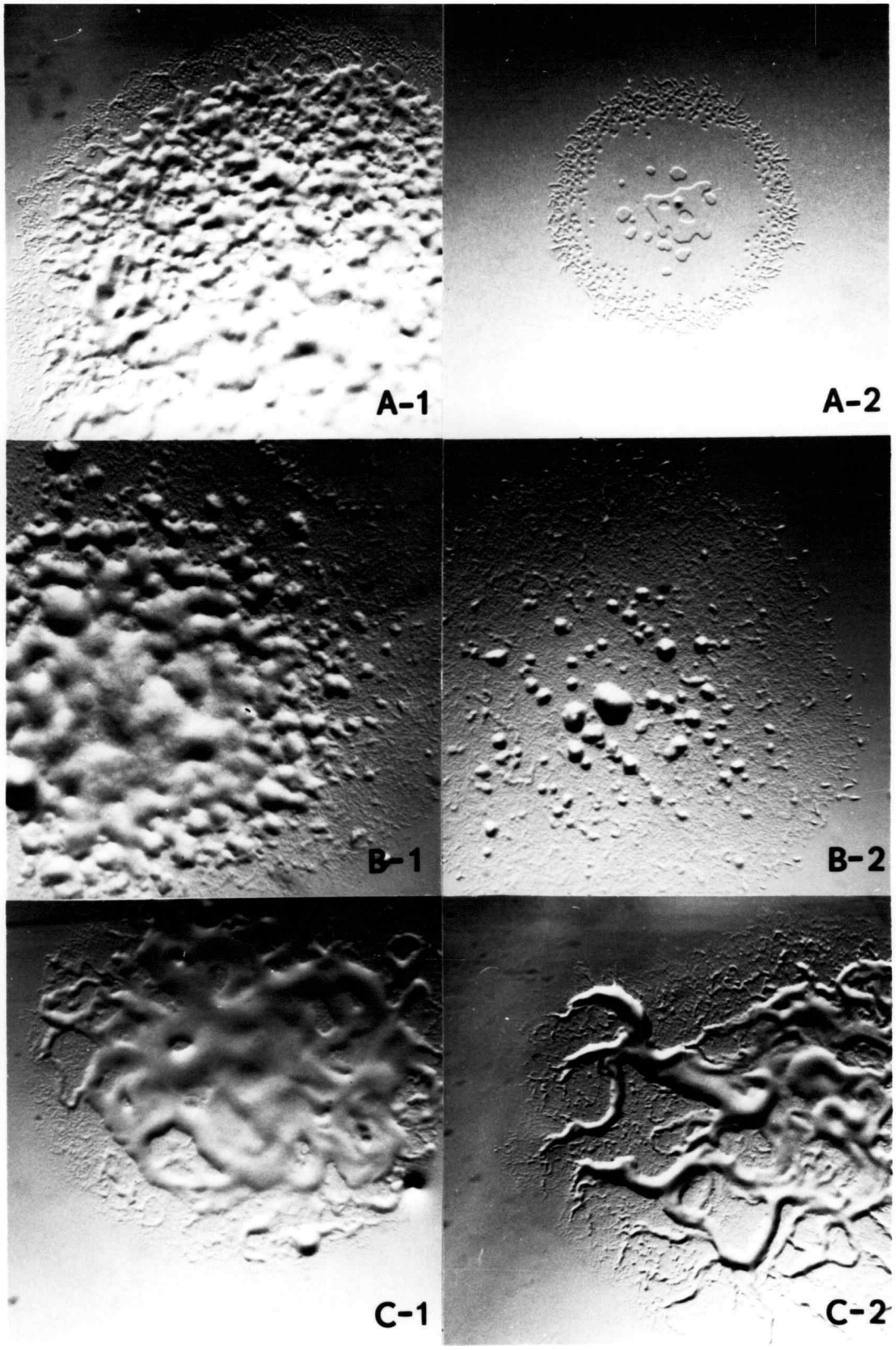
B1. strain 15 70 hours 8X
on primary isolation plate

B2. strain 15 62 hours 7X

C1. strain 25 70 hours 7.5X

C2. strain 25 56 hours 7X

This is the strictly proteolytic isolate from Berry Creek.


A-1
A-2
B-1
B-2
C-1
C-2

changes occur, usually within 72 hours incubation at 18 C. To record the complete development of the colony, photographs were taken after 50, 56, 65, and 72 hours incubation. Because of their thinness, young colonies are invisible by transmitted light but when examined in reflected light, their matte surface shows up in contrast to the surrounding agar. (For enumeration and for photography, the field of view was illuminated obliquely.) Major changes occurred during this 22 hour period but the characteristics typical of myxobacteria are recognizable in younger colonies.

The development of two Type IV colonies (strain 43 and strain 12) is compared in the first example of myxobacterial colony formation. Note the blunt projections of the edge on the young colony of strain 43 in Figure 18A. As the colony grows larger and the center becomes thicker and more smooth in appearance, the edge detail remains similar to that noted in the younger colony. The fixed, dark speck at the right in these photos marks the progress of the developing colony at the indicated ages (Figure 18B and C).

A similar time sequence is presented for the second morphological type in the group, strain 12 (Figure 19). Note the detail of a sharply defined colony edge as compared to the more blunt edge of the strain 43 colony. The edge detail is clearly visible in the younger colony (Figure 19A and B). By 65 hours, the colony is merely a smaller version of the 72 hour colony (Figure 19C and D).

Figure 18. Colony Development of Myxobacterial Strain 43 on CPM. (Morphological Type IV)

A. 50 hour colony 7.5X

B. 65 hour colony 5X

C. 72 hour colony 4X

The speck at right marks the increase in size as the colony developed.

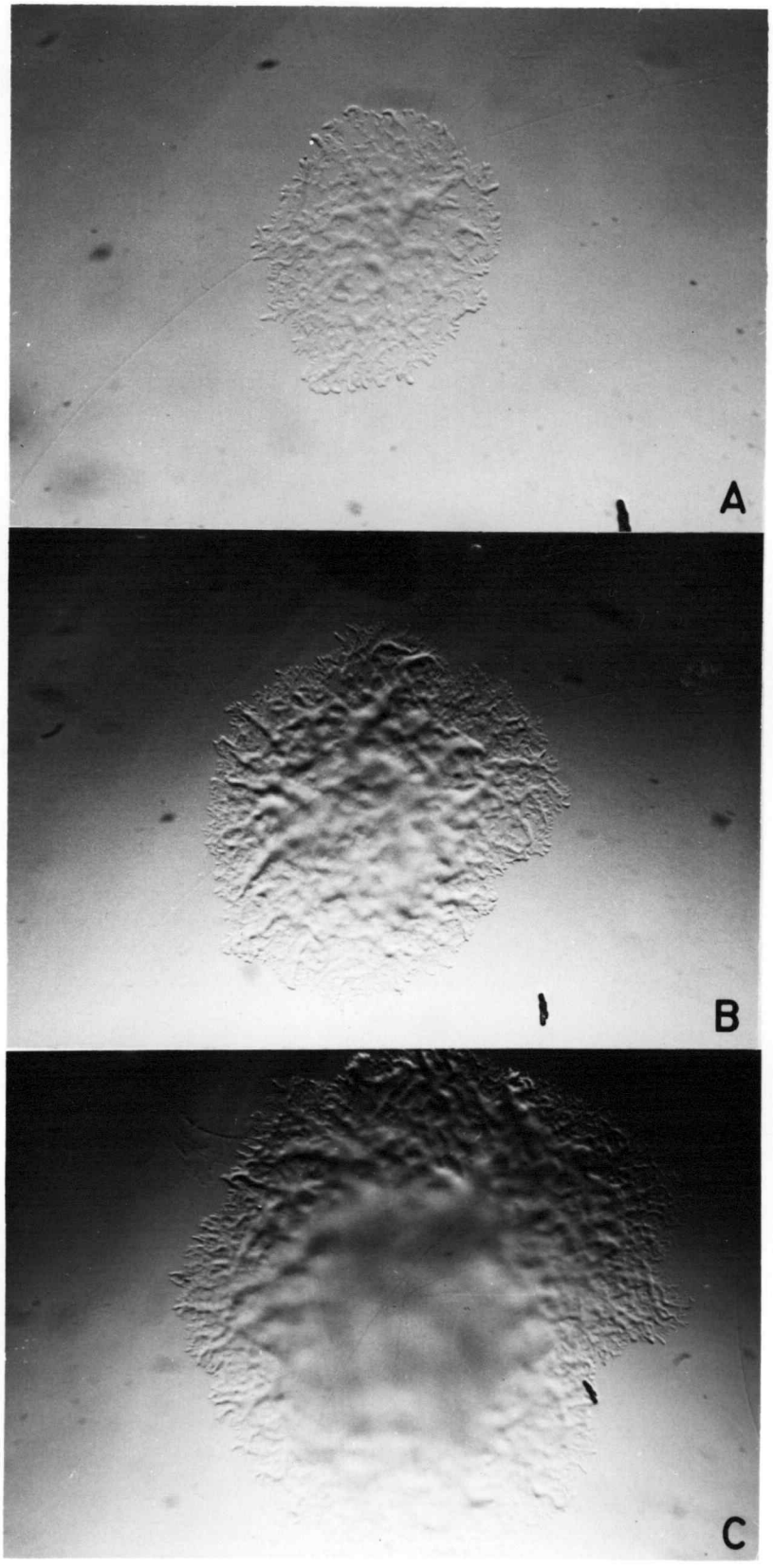

A
B
C

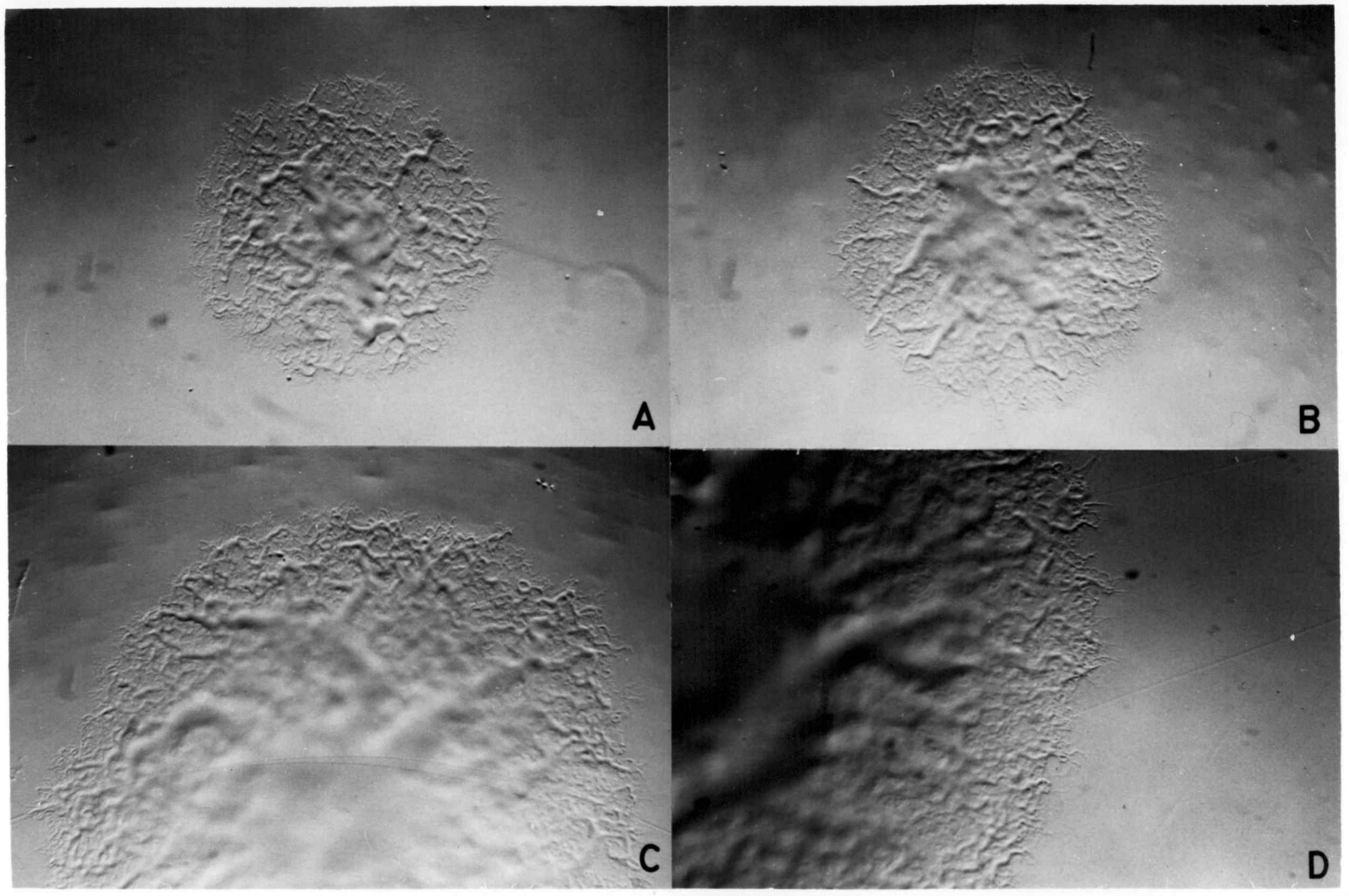
Figure 19. Development of a Colony of Strain 12 on CPM. (Morphological Type IV)

A. 50 hour colony 5X

B. 56 hour colony 5X

C. 66 hour colony 4X

D. 72 hour colony edge 7.5X


A
B
C
D

These two strains differ in their ability to oxidatively attack lactose, and particularly in their ability to utilize chitin; thus the fine morphological differences serve to help in distinguishing two different myxobacteria.

The colony development of two strains from a second morphological group (Type VI) illustrate further differences notably in younger and developing colonies. Figure 20 (A, B, C, D) shows development of strain 14; the circles which form over a thinner layer of cells become a confluent mass in the mature colony. The edge retains the same appearance and the circular structures are no longer noticeable by 72 hours.

In Figure 21, development of a similar Type IV colony was followed (strain 18). While the edges of these two mature colonies at 72 hours are nearly indistinguishable, the difference between the younger colonies of these types was the very obvious lack of circular structures at any point in the development of the colony in strain 18. The experiment was repeated several times with the same results. Recognition of these colonies as two different myxobacteria can be assured after 65 hours incubation but, it is more difficult, if not impossible at 72 hours or thereafter.

These two isolates attack the same substrates biochemically, but strain 18 required three additional days incubation in order to utilize the same sugars as strain 14. Further studies are necessary

Figure 20. Development of Colony of Strain 14 on CPM. (Morphological Type VI)

A. 56 hour colony 8X

B. 60 hour colony 8X

C. 63 hour colony 7X

D. 72 hour colony 8X

The fibre at left was used as a location marker for the colony.

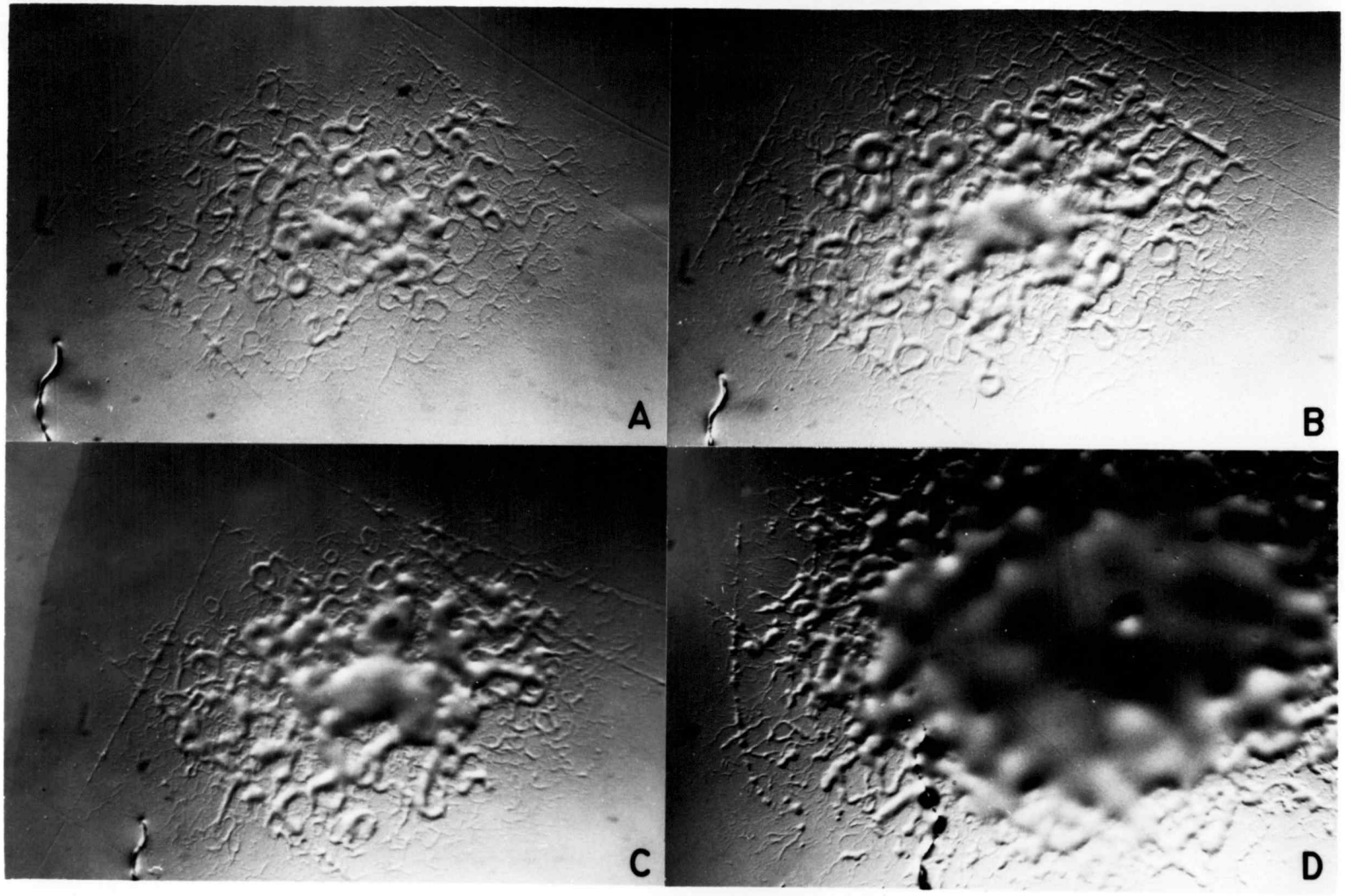

A
B
C
D

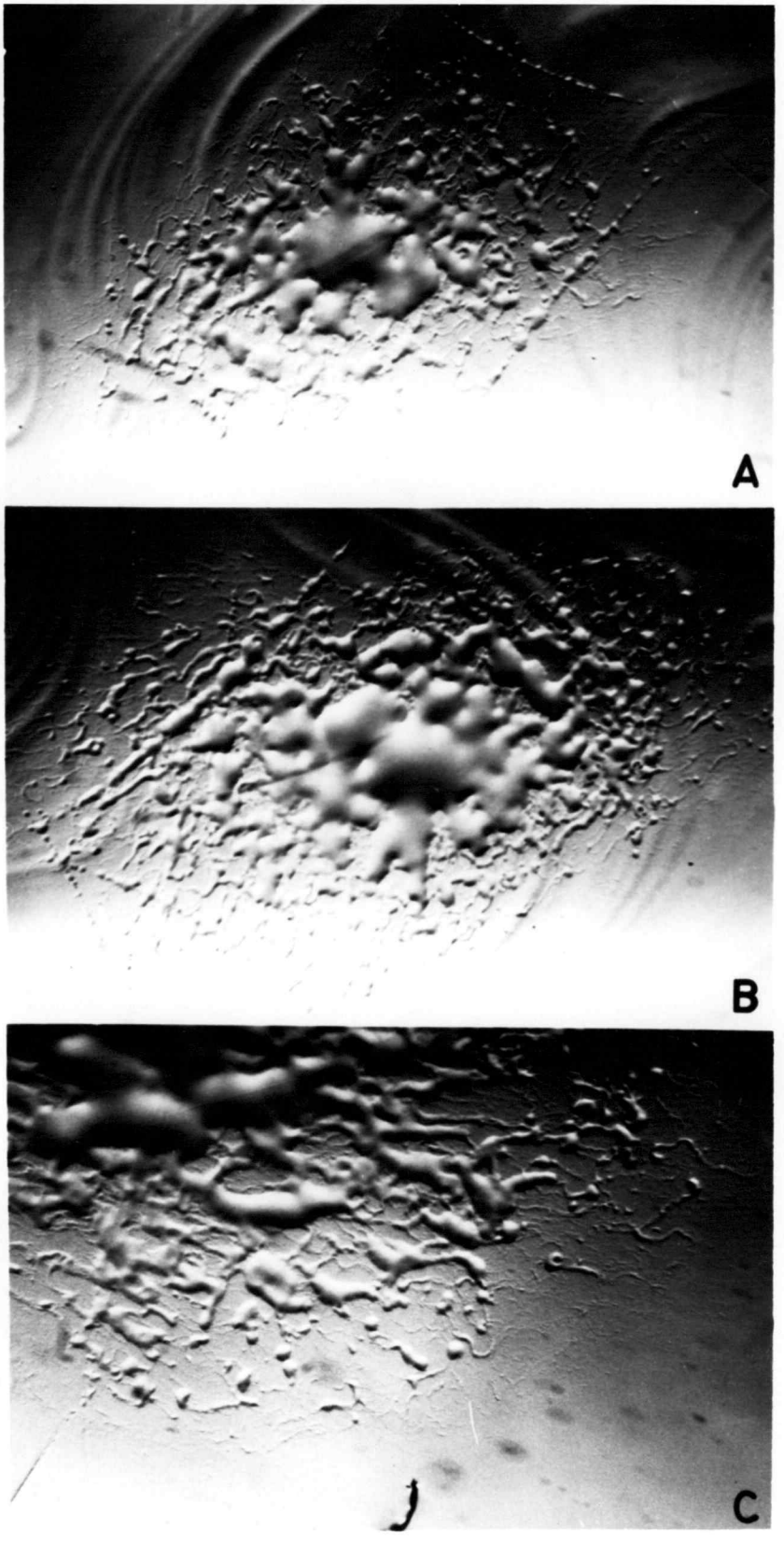
Figure 21. Development of a Colony of Strain 18 on CPM. (Morphological Type VI)

A. 65 hour colony 4X

B. 70 hour colony 4X

C. Edge of 88 hour colony 7X

Note the absence of circular structures in this strain as compared to strain 14 in preceding figure.


A
B
C

before these two myxobacterial types may be designated as individual types, or as the same myxobacterium.

The morphological features of the colonies presented in this thesis, as well as additional types studied, provide a useful laboratory tool for preliminary differentiation of myxobacteria from one another. The colony patterns on CPM have been studied in detail and are a constant characteristic of the particular myxobacterial isolate or type producing them. The youngest, and most actively gliding cells, are usually noted in the colony edge and, at this point, one can only speculate that the spreading of the colony on low nutrient medium (to provide the final pattern observed) may be the result of cells creeping to an area where the food supply is more abundant. Stanier (56) has suggested this possibility but, to date, it has not been supported by unequivocal experimental evidence. Nevertheless, the colony patterns formed are very useful in the separation of myxobacteria from other microbial colonies.

Care must be taken to clearly define the conditions under which the colony morphological features are observed. Manipulation of time and temperature of incubation, or the nutrient concentration, in particular,will greatly alter the colony appearance. Similar observations have been made by Stanier (53, 56), Anacker and Ordal (2) and Porter (47).

SUMMARY

As a preliminary step in studying the ecology of myxobacteria in a freshwater environment, a culture medium and satisfactory procedure were devised for the isolation and enumeration of these microorganisms. This technique was then used in field studies on Berry Creek to obtain data on the occurrence and distribution of the myxobacteria present in this freshwater habitat. Laboratory studies were also conducted on myxobacteria isolates in order to provide some insight into their role in the stream.

The development of the enumeration and isolation medium for the myxobacteria was based on their ability to glide over solid surfaces on the thin layer of slime secreted by the cells. Since the direction of this gliding movement is away from the colony center toward the edge, the colonies, which result are characteristic of this movement, i.e., thin, spreading, and irregularly-shaped. The nutrient concentration of the medium was adjusted to support the growth of as many myxobacteria as possible, while allowing for ease in differentiating the myxobacterial colonies from other microbial colonies able to grow on the medium.

On the cytophaga-peptonized milk agar medium (CPM) ultimately developed, the colony patterns produced by different myxobacterial isolates proved to be a constant characteristic of the

particular isolate. The low nutrient concentration of this medium favored spreading of the myxobacterial colony and the production of rhizoid edge patterns which were typical of myxobacteria and distinguished them from eubacterial colonies. Hence, the myxobacteria in a plated sample could be separated from one another, and from other microbial colonies, on the basis of their distinctive colony morphology.

The additional problems encountered in developing the isolation and enumeration medium were solved without effecting the growth rate or morphology of the myxobacteria colonies. The spreading colonies produced by flagellated members of the samples were controlled by the use of pre-dried plates and varied plating volumes. The interference caused by Sphaerotilus "colonies" was reduced by the addition of neomycin to the culture medium.

The predominant members of the stream's myxobacteria population were isolated from the flowing water samples throughout the investigation. Isolates were also obtained from other sources in the stream: surface films, sediment, decaying leaves, algae, fish, and the flocs of Sphaerotilus natans. All of the isolates were confirmed as myxobacteria on the basis of the low refractility and the marked flexibility of their cells, and their gliding type of motility, as noted under the phase contrast microscope. Forty isolates were then selected for additional study.

The colony morphology of the myxobacteria on CPM was studied extensively. The transparency of the medium allowed observation and photography of the stages in colony development. Myxobacteria colonies were microscopically visible 48 hours after plating and their typical spreading development was followed by periodic observations until the colony matured (after 72 hours at 18 C). Features present in young colonies were useful in "tagging" the isolate as a particular type of myxobacterium. The numerous variations in the type of edge pattern produced by the colonies on the CPM medium were used to differentiate the individual myxobacteria types.

This differentiation of the isolates, on the basis of their CPM colony morphology, into individual and separate myxobacterial types, was supported further by biochemical data concerning the utilization of various substrates, both simple and complex in nature. Isolates which possessed discreet, and sometimes subtle differences in their colony morphology on CPM also exhibited differences in their ability to utilize the substrates tested.

On these bases, six morphological groupings have been designated. A brief description of the major morphological features of each group, pertinent biochemical data and photographs of the isolates representing the group are included in this thesis. These photographs clearly illustrate the distinct morphological features used in the group designations.

Isolates which possessed a unique colony morphology and/or unique biochemical capabilities, or which occurred only rarely during the extensive sampling of the stream, are included in a seventh group. One example in this group is Chondrococcus columnaris, which was isolated from Berry Creek water samples following the introduction of infected juvenile coho salmon. The unique morphology of the C. columnaris colony on CPM facilitated easy preliminary identification of this myxobacterium, a pathogen of salmonid fishes. The identification was confirmed using specific antisera provided by Dr. R. E. Pacha.

The ecological study involved collection of data on the myxobacteria population of Berry Creek as influenced by the total bacteria and algae populations and the main physical parameters of flow rate and water temperature. Comparison of two years of the data obtained revealed the following information. The general trends in water temperature and flow rate were relatively constant from year to year. Water temperature data for the second year paralleled that observed during the first year within two degrees, except for an unusually hot August (20 C) during the second year. The flow rate could be regulated and was, therefore, similar during both years of the investigation, except for a period beyond the December-January peak into April of the second study year when a higher flow rate was maintained.

The general trend observed in the populations of myxobacteria and total bacteria during the first year repeated itself during the second year of the study, and into the third year as well. Myxobacteria and other bacterial types occurred in both the sucrose and urea-enriched and the unenriched sections of Berry Creek, and as might be expected, were more prevalent near the lower end of the experimental section than they were in the influent water. The relative level of these microorganisms present in the stream varied seasonally.

The lowest levels were recorded in mid summer when the water temperature was highest and the flow rate minimal. The numbers of myxobacteria and total bacteria increased during the early fall (August, September), reached a maximum (October-November) and remained at high levels during the winter months (December, January), and then decreased throughout the early spring and into early summer (February through June).

The greatest number of myxobacteria and total bacteria occurred in the fall when water temperatures of 10-15 C and increasing flow rates were recorded. Peaks in the myxobacterial and total bacterial populations occurred at least a month ahead of the flow rate peak. The respective peaks would be expected to coincide if these organisms were transient members of the stream populations. From the data obtained, neither of the physical parameters appeared

to directly influence the populations as studied.

The effect of sucrose and urea enrichment of the stream during the first year of the myxobacteria study, was reflected in the higher numbers of total bacteria recorded at sites in the enriched section and the abundance of flocs of Sphaerotilus natans, the slime producing, sheathed bacterium. The myxobacteria population was not generally influenced by this enrichment; in fact, higher levels of myxobacteria were recorded during the year without enrichment. An exception involves a single myxobacterium (BC 225) which was predominant in Berry Creek enriched sections when Sphaerotilus natans was also abundant. This myxobacterium, believed to be associated with Sphaerotilus, was never noted in the unenriched section and was absent from all samples in the enriched section after enrichment ceased.

It was shown that myxobacteria were capable of growing in filter-sterilized stream water; apparently the low concentration of nutrients available in their micro-environment is adequate to support growth of these microorganisms. The pH and mineral content of Berry Creek water have been reported as constant over a given year, however, slight changes may greatly influence the myxobacterial type which is expressed as the predominant member of the population. These noted seasonal changes may be dependent upon the available minerals or upon specific nutrients, or the presence of

products of some other microorganism's metabolism.

All the myxobacteria investigated in this study showed some ability to degrade macromolecules. This feature has been reported for other myxobacteria and appears to be an important characteristic of this group of bacteria. Of the substrates tested which would be present in the natural habitat, starch, esculin, caesin and gelatin were hydrolyzed by most of the isolates; nearly half were also able to utilize chitin. Direct attack of cellulose could not be demonstrated, however, presumptive evidence exists for the utilization of cellulose by these aquatic myxobacteria. The isolates were able to degrade carboxy methyl cellulose and the smaller components of cellulose: cellobiose and glucose. With the exception of the known isolate of Chondrococcus columnaris, all of the aquatic isolates obtained from Berry Creek are believed to be members of the genus Cytophaga. Among these isolates, cellulose decomposers would be expected to be more abundant. One strictly proteolytic isolate was obtained from Berry Creek, in addition to C. columnaris. The habitat of this isolate may also have been the surface of fish.

The results of these studies on the myxobacteria have yielded a useful technique for the initial identification of myxobacteria on the basis of their colony morphology. Field studies have yielded information on the occurrence and distribution of myxobacteria in the stream; and laboratory studies have yielded information on their

biochemical capabilities, thus allowing speculation on the role of myxobacteria in the freshwater environment.

The aquatic isolates studied possess capabilities very similar to the terrestrial myxobacteria, which have been investigated in other studies, however, these findings indicate that myxobacteria are well adapted to the freshwater habitat. They are not transient members of the stream's microbial population, since the myxobacteria population does not fluctuate directly with changes in flow of the stream. The myxobacteria indigenous to this aquatic habitat were able to attack a variety of complex substrates, and thus, may be contributing to the stabilization of organic matter naturally introduced into the water.

It seems likely that aquatic myxobacteria could be involved in the primary or secondary degradation of various complex molecules of the natural environment, such as chitin, cellulose, etc., or possibly the remains of other bacterial cells, as well. Further insight into the precise role of myxobacteria in the aquatic environment however, depends upon extended investigation.

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