STUDIES OF FRESHWATER BACTERIA

I. A DIRECT MICROSCOPIC TECHNIQUE

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It has long been known that the standard methods of bacteriology—pure culture isolation and observation upon artificial media—often yield only an incomplete knowledge of a particular microbic flora. Both in numbers of individuals and numbers of species, plate cultures from mouth, intestines, or vagina fall far below expectations based upon direct microscopic examination, and the same is often true of saprophytic habitats as well. This fact has been explained in part by the assumption that many of the organisms observed in smears are dead, more satisfactorily on the ground that many of them are incapable of growing upon the artificial media so far devised. From time to time we succeed in cultivating organisms which were previously known only in their natural habitat, but such accomplishments become progressively rarer. It is the author's opinion that only a minor portion of the species of bacteria existing have been cultivated and described.

Soil microbiologists have succeeded perhaps better than any other group in bringing a wide variety of organisms under artificial cultivation. The number of bacterial forms which may be isolated from soil seems to be limited only by the imagination and ingenuity of the investigators in concocting special media having a selective action. But here also one finds that after summarizing all of the information obtained by such pure culture studies, one is left without any adequate conception of the soil microflora as a whole. It is not possible, from the bacteriological analysis, to synthesize a picture of the various organisms living

and working together in their natural habitat. In recognition of this fact one finds in the recent publications of Conn, Winogradsky, and Cholodny, the beginnings, perhaps, of a movement away from the time-honored techniques of bacteriology toward a more rational study of the microbe in its natural environment. A similar development may shortly be expected in other fields of bacteriology.

The movement may in a sense be considered an extension of a movement begun some years ago in the field of general biology which has developed into a new branch of science, ecology. Ecology is essentially a revolt from the analytic and experimental methods of the laboratory, on the ground that these give no adequate conception of the organism as a whole in relation to its environment, and a return to the methods and aims of the old-fashioned field naturalist, freed however from teleologic bias and with the addition, as far as possible, of the precision of the laboratory.

The vast amount of labor that has been spent on water bacteria has been carried on almost exclusively from the standpoint of sanitation and is almost worthless from the standpoint of ecology. We have some information regarding variations in number (as revealed by plate counts) with the seasons, and with the organic content of the water. A certain number of saprophytic species have been isolated on agar or gelatin, described and named, mostly long ago. A few characteristic aquatic bacteria of striking morphology or peculiar physiology, such as *Spirillum volutans*, and the iron and sulphur bacteria, are well known. But these isolated bits of information only serve to emphasize our almost complete lack of knowledge of the types of bacteria characteristically living in fresh water, and the nature of their activities there. One is particularly struck by the brevity and meagerness of the accounts of bacteria in works on freshwater biology, as compared with the rich material available on other groups of organisms. Jordan, writing on bacteria in Ward and Whipple's "Freshwater Biology," states: "There is no special and characteristic class of 'water bacteria,' but germs from the air, from the soil, from decomposing animal and plant substances

and from the healthy and diseased tissues of animals and plants may at times find their way into water."

The present status of the problem has been well summarized by Ruttner in discussing bacteriological investigations at the biological station at Lunz:

> The investigation of the bacterial flora populating our waters has not kept pace with that of other groups of organisms. The reason is doubtless to be sought in the great difficulty which is opposed to a scientific understanding of a very large part of the bacterial flora of the water and the mud. It is true that by means of bacteriological culture methods we have succeeded in obtaining a knowledge of quite a variety of bacterial species that are present in our waters. In the majority of cases these are, however, ubiquitous, readily-growing putrefactive forms that are present everywhere. But many of the typical water bacteria, and especially those groups which are of physiological interest, which play so large a rôle in the chemical transformations of a lake, will not grow on the usual nutritive media which are used in the bacteriological plating procedure. Microscopic observation without the aid of cultural methods is of value only with regard to a few of the larger forms because of the slight morphologic differentiation of the group, and so it happens that our knowledge of the composition as well as the density of the bacterial population of the waters is still extraordinarily fragmentary.

To study the bacterial flora of a lake or pond properly by the usual methods would require an outlay of time and labor and expensive equipment for which sufficient financial support cannot be readily obtained, since the project promises to yield no results of economic importance. This paper is a preliminary communication describing a procedure which requires a minimum of equipment and labor, which will reveal both quantitatively and qualitatively the activity of at least a portion of the aquatic microflora, yielding results that may be readily correlated with the considerable data already available bearing on freshwater ecology, and which may well serve both as a control and a guide for more elaborate studies using pure culture isolation methods.

Some time ago an aquarium in my laboratory developed on its walls a growth of algae. Some microscope slides were placed in the aquarium in the expectation that the algae would also

grow on these and that permanent preparations might be made showing the organisms *in situ*. On removing and staining these slides after a week's immersion, I was surprised to find, in addition to the algae, a thin and uniform coating of bacteria of various forms, some of unusual morphology. This experiment was repeated with other aquaria, with the lily pond in the University greenhouse, and finally with the waters of Lake Alexander, Minnesota. In every case the results were the same.

The deposit of bacteria becomes apparent in a few days and increases progressively, eventually becoming so thick that individual cells may be distinguished with difficulty. That the cells are actually growing upon the glass is indicated by their occurrence in microcolonies of steadily increasing size. They are fairly firmly adherent to the glass, not removed by washing under a tap. In the case of some filamentous forms one may readily see on the slide a "holdfast" by which the organism is cemented at the base. In other cases the groups of cells are evidently surrounded by a sheath of gum which also serves to fasten the colony to the glass. While other organisms, various algae and protozoa, also grow upon the slide, there is no debris of amorphous organic or inorganic matter such as obscures the picture to such a distressing extent in slides prepared from soil by the Conn or Winogradsky techniques. The picture is beautifully clear, and any of the bacterial stains may be used.

These observations have led the author to a conception of the bacterial flora of water different from that held previously. A shallow wayside pool may occasionally develop a pellicle or turbidity due to bacteria, similar to that found in a hay infusion in the laboratory, and one may find abundant bacteria by direct microscopic examination of the surface scum immediately after a lake has "bloomed," and the excess population of blue-green algae is undergoing decomposition. But ordinarily the waters of an unpolluted lake or stream are clear, and few or no bacteria may be demonstrated by direct microscopic examination.[1] On

[1] Snow and Fred found from 740 to 29,300 bacteria per cubic centimeter in microscopic counts of centrifuged water from Lake Mendota, plate counts running from one-seventh to one-fourteenth of the microscopic counts. Microscopic counts of bottom mud will run well into the millions per cubic centimeter.

the other hand, a bit of bottom mud, even from considerable depths, stained by the Conn technique, shows an abundance of organisms comparable with the richest soil. The slime from rocks, the mucous sheaths of colonial algae, scrapings from the leaves of submerged plants, all show a microbic flora rich in numbers and in diversity of forms. It is quite evident that for the most part the water bacteria are not free floating organisms, but grow upon submerged surfaces; they are of the *benthos* rather than the *plancton*.

Microscope slides may be placed in a given aquatic environment for a definite period of time, removed, fixed, stained, and examined. It is proposed by this method to follow variations in the numbers and kinds of bacteria that develop in different sorts of freshwater habitats with the expectation that such studies will provide a clue as to the nature of the activities of the water bacteria and the part they play in the economy of lake or stream.

As far as I can determine, such a procedure has not been used before in the study of water bacteria. Naumann has pointed out that beautiful microscopic preparations of iron bacteria may be obtained by immersing slides for a time in iron-bearing waters, but apparently made no further use of the method. Hentschel has followed the development of various sessile types of aquatic organisms upon slates immersed in streams for varying periods, and clipped slides to these slates for observation of the more minute forms, but makes no mention of bacteria. Thomasson has used a technique apparently identical with that of the author, for following the distribution of diatoms in various habitats, but also makes no mention of bacteria. The precedure is identical in principle with that recently described by Cholodny for the study of soil bacteria.

It was necessary to experiment a little before a satisfactory technique for placing the slides in the water was developed. The slides are attached at intervals to a line suspended from a float and fastened to an anchor at the bottom. Anchors must be of sufficient weight to hold fast in heavy wave action. I have found that a weight less than 2 pounds is insufficient. Satisfactory anchors may be made by fastening a screw-eye in the narrow end

of a small aluminum funnel, which is then filled with melted lead. Floats must be easily visible so that they may be readily found again and will not be struck or snagged by passing boats. I am using one gallon glass jugs. The screw caps are cemented on. Within the jug is a printed sign requesting the curious to leave it undisturbed.

It was necessary to give some thought to the material used for the lines. Close proximity to materials of various sorts would undoubtedly influence the type of organisms that would grow upon the slides. Thus, if one suspended the slides from cotton, linen, or silk lines, these would undoubtedly undergo decomposition, products from which would diffuse through the water and influence the growth of organisms on the glass. The use of iron wire would undoubtedly favor the development of iron bacteria, while copper or other metals would probably exhibit a certain degree of inhibiting effect from the antiseptic action of the metal dissolved in the water. The substance finally decided upon was a rubber covered copper wire. The type of wire used is known as "radio hook-up" wire. It is a flexible, stranded wire of small gauge with a thin soft rubber coating, about 2 mm. in diameter over all.

The slides are fastened to the wire in the following manner: One-inch surgeon's adhesive tape is cut into 2½-inch lengths. After cleaning the slides, one end is heated in a Bunsen flame and applied to the adhesive side of the tape, overlapping the tape for ¼-inch. The tape is then doubled on itself and fastened to the other side of the slide for ¼-inch. The slide is thus held between two layers of adhesive tape, and there extends a tab of this tape (double layer) approximately 1 inch square. A hole is punched through this tab and a number is stamped on the part of the tape which is fastened to the slide, with a rubber stamp. The tab is then dipped in melted paraffin. Slides are fastened to the line with short lengths of the rubber covered wire passed through the hole punched in the tab.

It will be seen from the above that the slides do not come in contact with any elements foreign to the environment except the relatively inert substances, paraffin and rubber. These

undoubtedly will also undergo some decomposition, but it is probable that the rate is so slow that there will be no appreciable effect on the flora developing on the glass. The slides have been fastened to the line at one-meter intervals. Thomasson states that in his diatom studies he found an appreciable difference in the flora developing, with variations of depth as little as 5 cm., and insists that slides should be suspended horizontally if they are to represent the true flora at a given depth. In preliminary observations, no such variations have been encountered in the bacterial flora. If such were the case, one should see an appreciable difference between the two ends of a single slide suspended vertically. This has not been observed.

The slides may be stained by any of the usual bacteriological methods. The clearest preparations have been obtained by simple staining with the ammonium-oxalate crystal-violet solution used in Hucker's modification of Gram's stain. Slides which rest in the bottom mud may have a certain amount of adherent debris, and in some cases Conn's rose bengal method or Winogradsky's erythrosin should be used. Sessile algae and protozoa also develop upon these slides, and the results of the investigation will undoubtedly prove more valuable if some account is taken of these other organisms in relation to the bacteria. They cannot, often, be readily identified from air dried, flame fixed, slides. Slides are therefore placed on the line in duplicates, One is stained with crystal violet for bacteria, the other is fixed in Flemming's solution and stained with Heidenhain's iron haemotoxyline for observation of the algae and protozoa.

The organisms develop upon the slide in a fairly uniform film. If this is of the proper density, the bacteria may be counted with some degree of accuracy. The density increases with the duration of immersion, and is evidently determined by two factors: the number of organisms brought to the slide by water currents or their own motility, and the rate at which they multiply upon the slide. Quantitative studies of the rate of increase of the organisms upon the slide should prove interesting, and will have to be made eventually before more general quantitative studies can be properly evaluated. But preliminary studies at Lake

Alexander indicate that in general slides immersed for two weeks will develop enough organisms to allow a reasonably accurate count, without becoming too dense. This time period will probably have to be varied to suit the season and habitat. Preliminary observations indicate that significant quantitative variations will be noted with the season, the depth of water, and the character of the bottom. The number of bacteria developing upon a unit area of the slide in a unit period of time may serve as a measure of microbic activity in a given environment.

While it is true that bacteria vary too little in morphology to allow of their identification by the microscope alone, I do not feel as pessimistic as Ruttner about the limitations imposed by a purely morphologic study of the water bacteria. A glance at one of the slides prepared by the method described above will astonish one who is not acquainted with these organisms, by the great variety of morphologic types which are present. A few of the larger and well known forms, such as *Spirillum volutans* and *Cladothrix dichotoma*, may be recognized at a glance. But even with the more minute forms, there is a great variation in size, form, and arrangement of the cells, which, if observed systematically, must lead to some orderly understanding of the number and distribution of the species.

Some difficulty has been experienced in deciding whether certain forms encountered are large bacteria or smaller species of *Cyanophyceae*. All of the definite blue-green algae encountered so far have proved to be strongly Gram-positive while nearly all of the definite bacterial forms have been found to be Gram-negative. A few minute spherical bacteria in clusters from bottom mud are Gram-positive, and certain of the larger rod-shaped bacteria show Gram-positive granules within the cells, but in general it has been rather surprising to note the almost complete absence of Gram-positive bacteria. No spore formers have been encountered.

It is obvious that, until more completely known, these water bacteria cannot be fitted into existing classifications, but some sort of a working classification must be adopted in order to analyze the slides intelligently. The forms encountered so far may

be immediately divided into two great groups—those which occur in filaments or chains, and those which occur singly or in irregular aggregates, corresponding to the *Trichobacterinae* and *Haplobacterinae* respectively, of Alfred Fischer. The filamentous types may be further subdivided into those which are continuous and those which are articulated, the latter according to size, shape, and internal structure of the elements. Further noteworthy characters of filamentous types are the presence or absence of sheaths, of true branching, of false branching, and of holdfasts. No true branching filamentous forms have been encountered so far. The non-filamentous types are of widely varying form and size. There are minute spherical forms, singly or in clusters, larger round or oval cells with a reticulated protoplasm much like Azobacter; there are rods of varying length and thickness, some with deep-staining granules, others uniform in their staining. There are comma-shaped cells and true spirilla varying in size. While it is true that one would hardly be justified in naming these organisms and describing them as species on the basis of their morphologic appearance alone, it is quite evident that the morphology is sufficiently diversified to warrant a tentative classification of the organisms observed which will be sufficient for at least preliminary ecologic surveys.

This procedure is not offered as a substitute for pure culture studies. Undoubtedly each of the bacterial forms will, eventually, have to be isolated in pure culture before they may be understood with any degree of completeness. But the method does offer a short cut towards an ecologic survey of the water bacteria. It will indicate what sorts of organisms are to be sought in cultures, and when morphologic types have been correlated with habitats, it may indicate what sorts of media or conditions of cultivation are to be used for their isolation.

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PLATE 1

This collection of photomicrographs is presented to illustrate the wide variety of morphologic types of bacteria which have been encountered. The magnification is 800 times.

Figures 1 to 21 inclusive are filamentous types which the author would classify as trichobacteria; the remainder are haplobacteria. Figures 1 and 2 show the holdfast and the "false" branching, respectively, of *Cladothrix dichotoma*. Articulation or its absence, sheaths or their absence, are illustrated in the other filamentous forms.

Figures 22 to 25 show spiral types. The large spirillum shown in 24 and 25 is probably *S. volutans*.

Figures 33, 34 and 35 show peculiar vibrio types with long attached filaments. These are probably not flagella, as no mordant was used. It is the author's opinion that they are *stalks* by which the bacteria are attached to the glass, similar to the stalks of some diatoms.

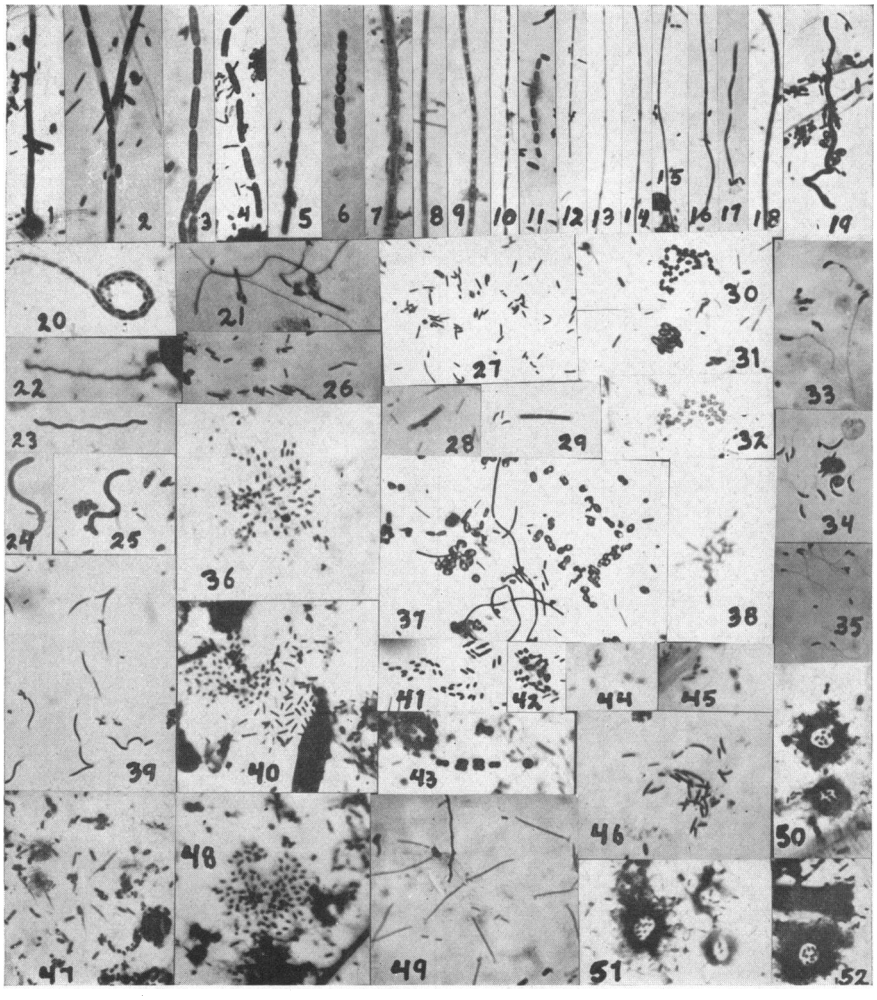
Figures 28 and 29 show rod forms with peculiar faintly stained areas at the poles, probably a plasmolytic phenomenon.

Figure 37 shows large vacuolated oval cells somewhat resembling *Azotobacter*.

Figures 44 and 45 are encapsulated forms.

Figure 47 shows, among others, spindle-shaped types which in form and staining reactions resemble fusiform bacilli of the human mouth.

Figures 50, 51, and 52 show minute colonies of *Siderocapsa major* Molisch. The bacteria are contained within a mucoid capsule, about which ferric hydroxide is deposited in a ring.



(Arthur T. Henrici: Studies of Freshwater Bacteria)