MASTER

Technical Progress Report

Microbiology of Thermally Polluted Environments

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I. Introduction

This is the first year of this contract and most attention has been paid to identifying appropriate study areas, hiring and training personnel, and developing necessary methodology. Several studies arising out of this preliminary work and out of the related work supported by NSF are publishable and papers have been submitted. The titles of these papers are:

- Bott, T. and T. D. Brock. Bacterial growth rates at temperatures above 90°C. in Yellowstone hot springs. Submitted to Science.
- Brock, T. D. and M. L. Brock. The fate in nature of photosynthetically assimilated ¹⁴C in a blue-green alga. Submitted to Limnology and Oceanography.
- Brock, T. D. and M. L. Brock. Effect of light intensity on photosynthesis by thermal algae adapted to natural and reduced sunlight. Submitted to Limnology and Oceanography.
- Brock, T. D. and M. L. Brock. Recovery of a hot spring community from a catastrophe. Accepted by the Journal of Phycology.
- Brock, M. L., R. Wiegert, and T. D. Brock. Feeding by Paracoenia and Ephydra (Diptera: Ephydridae) on the microorganisms of hot springs. Submitted to Ecology.
- Brock, T. D. and H. Freeze. Thermus aquaticus gen. et spec. nov., a non-sporulating extreme thermophile. Submitted to Journal of Bacteriology.

A satisfactory relationship has been developed with the Public Service Company of Indiana for studies on the Wabash River adjacent to one of their major power plants. Study areas in thermally polluted cold-water springs on the campus of Indiana University have also been identified. Methods involving the use of phase microscopy and radioactive isotopes have been developed for measuring the growth rates and temperature optima of bacteria attached to artificial substrates, and these methods are now being used in studies in thermally polluted waters. The methods were perfected first in thermal springs of Yellowstone National Park where constant temperature and flow rate, the lack of seasonality, and the simple species composition, greatly simplified the field work. The Principal investigator has spent about 10% of his time on this project, and the Co-Investigator about 25%. Personnel involved in these studies, in addition to the Principal and Co-Investigator, are Dr. Thomas Bott, a Post-doctoral Fellow of the U.S. Public Health Service, and Research Assistants Ilse Yoder and Mary Louise Sullenger who are spending 100% of their time on this project. Some hourly assistance was provided by Ronald Smith, Bryan Huber, Samuel Wylie, and William Rosser. The latter three are paid by Indiana State University from university funds. Some of the Yellowstone work has been carried out by M. L. Brock, Research Associate and W. Doemel, graduate student, both of Indiana University, neither of whom receive support from the present contract, but both of whom have made direct contributions to the work. Other people involved in the Yellowstone work have been D. Holleman, Research Assistant, and N. Doemel and T. Daley, hourly assistants. Dr. R. Lynn, Post-doctoral Research Associate, was involved in other

studies but also participated occasionally in the present work. All of these people have been supported from other funds (except M. L. Brock who cannot be paid because of nepotism regulation).

The studies so far performed, and those anticipated during the rest of the current contract year, show satisfactory progress and we anticipate no difficulty in completing the portion of the work proposed for the coming year. We anticipate that by the end of the first year we will have completed measurements of temperature optima and growth rates of bacteria both above and below sources of thermal pollution during the winter months. We will be then able to continue our studies throughout the annual cycle, and should be able to detect any significant effects which should arise during the summer when the thermal additions from the power plant are being made into naturally warmer water. Studies in Yellowstone Park on the growth rates and temperature optima of algae and bacteria at different locations along thermal gradients will be continued, with some attention to the effects of the thermal waters on the microbial populations of the Firehole River.

II. Brief Summary of Current Results

A. Wabash River Studies. Indiana State University.

It was necessary to select a proper site for experimentation prior to beginning a study of the microbial effects of thermal pollution in the Wabash River in Indiana. A large power plant of the Public Service of Indiana Corp. located in North Terre Haute was chosen since it was conveniently located near the I.S.U. laboratories and considerable data were available regarding the thermal output of the plant and its effect on the temperature at various locations along the River.

Through the most excellent cooperation of Public Service of Indiana (PSI), and the N. Terre Haute plant manager, Mr. H. Thurman, a wealth of important data was obtained. Figure 1 shows the data point locations used by PSI for their monitoring of river temperature over a period of years. Some detailed data are available as far back as 1956. On the basis of these data we selected several sampling sites as shown in Figure 2. Station 1 is 0.06 miles upstream from the thermal output station, 3 is 150 yds. south of the cell structure which prevents the mixing of hot and ambient waters, and station 2 is at the end of the cell block. The heat output of the plant raises the water temperature from 2 to 15°F. and some thermal effect is evident as far as 1.06 miles downstream. Our studies have shown that the techniques developed for the Yellowstone work can be applied with minor modifications to the Wabash River studies. Data on rate of uptake of H^3 -acetate by bacterial populations growing on submerged cover glasses are shown in Figure 3. The uptake is rapid and is linear for the first 45 minutes before leveling off. Controls (formalin-treated or sterile cover glasses) showed essentially no isotope uptake. The technique can be used to measure temperature optima of epiphytic bacteria developing in situ at different temperatures. At the same time, microscopic counts can be performed on the attached organisms, so that activity can be correlated with total bacterial population. The advantage of the isotope technique is that it makes possible a determination of the temperature optimum of a population which has developed in situ at a known temperature.

Terre Haute

MAP OF DATA POINT LOCATIONS

Scale 1" = 1/2 mi.

WABASH RIVER SAMPLING STATIONS

Fig. 2

Fig. 3

Uptake of ^3H -acetate by periphytic
bacteria at in situ temperature ($\approx 15^\circ\text{C}$).

B. Indiana University Studies.

Jordan Creek on the campus of Indiana University is a spring-fed stream, collecting the effluents from many springs which arise from the limestone strata of the area. The sources of all of these springs are now covered over by buildings of the university, and many of these are receiving thermal additions probably deriving primarily from steam condensate. The attached map (Fig. 4) shows the general geography of the area and the location of stations where temperature readings are being made. Temperature readings are being taken at weekly to monthly intervals, depending on the season of the year. Once we have a good picture of the temperature variation of these stations we will be in a position to undertake studies of the seasonal changes in microbial flora. As controls, we are also studying two cold-water springs in the southern part of the county, which are in undeveloped areas and receive no thermal additions. The temperatures of these springs are about 11°C. and have only minimal annual temperature variation. One of the thermally polluted effluents rises in back of Jordan Hall, the location of our research laboratory, thus providing a readily accessible study area. The temperature of this effluent is 20-25°C. and the bottom of the channel has a heavy growth of the bacterium Sphaerotilus natans, sometimes called "sewage fungus." By use of the microscope slide technique (see below), growth rates of this organism have been determined directly in the flowing water.

A paper describing a new genus and species of thermophilic bacteria, Thermus aquaticus, has just been submitted to the Journal of Bacteriology. The procedures for isolating Thermus aquaticus are described. Two key points in the enrichment procedure are the use of incubation temperatures of 70-75°C., and the use of nutrient media relatively dilute in the organic components. Strains of T. aquaticus have been isolated from a variety of thermal springs in Yellowstone National Park, as well as from a thermal spring in California. The organism has also been isolated from man-made thermal habitats, such as hot tap water, in geographical locations quite distant from thermal springs. Isolates of T. aquaticus are gram-negative non-sporulating non-motile rods which frequently form long filaments at super optimal temperatures or in the stationary phase. All isolates form a cell-bound yellow pigment, probably a carotenoid. A characteristic structure formed by all isolates is a large sphere, considerably larger than a spheroplast. These large spheres, as well as lysozyme-induced spheroplasts, are resistant to osmotic lysis. DNA base compositions of four strains were determined by CsCl density gradient ultracentrifugation and found to be: 67.4%, 65.4%, 65.4%, and 65.4% guanine plus cytosine. The growth of all isolates tested was inhibited by fairly low concentrations of cycloserine, streptomycin, penicillin, novobiocin, tetracycline and chloramphenicol. Nutritional studies of one strain were performed. No vitamin or amino acid requirements were found, although growth was considerably faster in enriched than in synthetic medium. Several sugars and organic acids served as carbon sources, and NH_4^+ and glutamate could serve as nitrogen sources. The organism was an obligate aerobe and had a pH optimum of 7.5-7.8. The optimum temperature for growth was 70°C. and the minimum about 40°C. The generation time at the optimum was about 50 minutes. The possible relationships of this new genus to the myxobacteria, flexibacteria, and

flavobacteria groups are discussed and it is concluded that it differs in significant ways from members of all these groups. This work is of significance because this organism may be a useful indicator of thermal pollution. Studies along these lines are proposed for the next contract period.

C. Yellowstone studies.

These studies have lead to the development of methods being used in the above work. The Yellowstone work has been supported also by research grants from the National Science Foundation.

Technique for measuring bacterial growth rates in flowing waters. Bacterial growth rates have been measured by immersing microscope slides in the flowing water for various periods of time, removing the slides, and counting the number of bacterial cells. To distinguish between growth on the slide and passive attachment from the water, a new technique has been developed which makes use of ultraviolet radiation. One set of slides is untreated, the other set is irradiated at intervals. The principle is that cells which passively attach to the slides will be killed by irradiation and cannot form colonies, and counts of irradiated slides give a measure of the attachment rate. This technique, first worked out at Yellowstone, is now being applied to both the Wabash River and Jordan Creek studies. The results are clear-cut: most of the bacteria which appear on the slides are a result of growth rather than passive attachment. Thus, bacterial counts on the unirradiated slides give a measure of growth rates on the slides. This technique has wide applicability in flowing water systems. Using this technique, growth rates of bacteria living in boiling springs at Yellowstone National Park have been measured (see preprint). The estimated generation times range from 2 to 7 hours, values which are comparable to those of aquatic bacteria living in less extreme environments. The results suggest that the upper temperature for life has not yet been defined. Serious consideration should be given to the possibility of bacteria living and growing in high temperature boilers and hot water heaters.

Isotope methods for measuring temperature optima of attached bacteria. Cover slips are immersed in special racks in the flowing water and left for a period of time sufficient to obtain a heavy bacterial population. From the above results we know that these bacteria have actually grown on the cover slips at the ambient thermal environments. The cover slips are then transferred to vials of river water equilibrated at different temperatures and a radioactive isotope added. After incubation the bacteria are killed with formalin, the cover slips washed, and then counted in a scintillation counter. This technique provides a measure of the temperature optimum for the incorporation of the isotope into bacterial protoplasm. We have used ^{14}C -acetate, ^3H -acetate, and ^3H -thymidine, and other isotopes could be used. We expect to use this technique to compare temperature optima of the bacteria in normal and thermally polluted habitats, as well as to follow changes in temperature optima throughout an annual cycle.

Algal studies. Because both the Wabash River and Jordan Creek are unfavorable habitats for the growth of algae due to high turbidity, the algal studies have been carried out in thermal waters at Yellowstone. The goal of these studies is to determine the optimum temperature for algal growth in nature, and to determine the factors controlling this optimum. For much of this work, the ^{14}C method is used. In studies on photosynthesis of natural algal populations using carbon-14 it would be desirable to know the fate of the radioactive label after assimilation. To ascertain this it is necessary to return the labeled algal population to the natural environment and sample periodically. With planktonic algae this procedure would be difficult, but it can be done with benthic algae since the habitat of these organisms is in a fixed position. Even with benthic algae, complications can arise from the washout of labeled cells or their replacement by unlabeled cells. We have overcome this difficulty by combining macroscopic assay of radioactivity with quantitative microscopic autoradiography, since the latter procedure permits a direct assessment of radioactivity of cells irrespective of the presence of unlabeled cells. The alga which we have used is the unicellular blue-green alga Synechococcus, which forms relatively uniform and compact mats in the effluent channels of hot springs, making it possible to obtain a number of replicate samples from the same site. The conclusion from the present study is that about 50% of the ^{14}C assimilated by the alga is later lost, probably by respiration, and that a significant proportion of the remaining radioactivity is converted from one biochemical form to another. We suggest that much of the ^{14}C is originally incorporated into a storage product whose carbon is later utilized as a source of energy as well as for biosynthetic reactions. These studies have important implications for the use of the ^{14}C method for assaying primary productivity, and suggest that similar experiments should be done in other natural environments.

It is important to know the actual growth rates of the algae in the natural environment. We took advantage of a natural catastrophe to measure the recovery rate of an algal population. The ability of ecosystems to repair damage due to outside influences is often stated, but it is rare that it is possible to follow such recovery through its various stages. Because of the relatively rapid growth rates of microorganisms, it would seem reasonable that recovery of microbial ecosystems would be relatively rapid. Recently, we have had the opportunity to observe and quantitate the recovery of a hot spring ecosystem from a catastrophic destruction of its biological components. The hot spring is Mushroom Spring, a moderately large spring in Yellowstone National Park which has served as a model for a number of previous studies from our laboratory. On August 30, 1967, there was a brief, violent localized hailstorm in the Lower Geyser Basin of Yellowstone National Park. Observations at Mushroom Spring on September 1, 1967, showed that essentially no algal-bacterial mat remained undamaged anywhere in the effluent channel from the source down to the foot except where neutral density glass plates has been established over the mat. On the eighth day after the hailstorm it was already clear that recovery from the catastrophe was occurring, as a faint, greenish color could be seen on the bottom of the effluent channel. Quantitative cores were then taken periodically and the algal density determined. The initial recovery

rate was essentially exponential and the doubling time for station II was calculated to be about 10.5 days. The data for stations I (71°C.) and III (65°C.) gave doubling times of 17 and 10 days respectively. When these doubling times are compared with the generation times calculated for the steady state from the darkening experiments, it was found that the generation times in the steady state are considerably shorter than are the recovery rates. At least one reason for this difference is that the recovery rate as measured is not the actual algal growth rate during the recovery period, but represents a balance between growth and wash-out. If we assume that the wash-out rate is constant throughout the recovery period, and is the same as that measured in the steady state, we can calculate that the algal growth rates during recovery are slightly faster than the steady-state growth rates.

Further measurements of algal growth rates have been done by use of experimental artificial channels, which provide a virgin substrate for algal colorization.

One of the important factors controlling algal standing crop is the presence of animal grazers. The main grazers are ephydrid flies which are extremely numerous when the temperature of the system drops below 50°C. We have used isotope methods to show that these flies do feed on algal and bacteria. Algae were labeled with ^{14}C from CO_2 and bacteria were labeled with ^{14}C from glucose. Transfer of label from microorganisms to flies was measured directly, and differential extraction procedures showed that much of the assimilated radioactivity was incorporated into macromolecular constituents in the flies and their larvae. It is also concluded that these insects promote mineralization of organic carbon fixed in microorganisms.

We have completed two summers of work on the physiological ecology of Cyandium caldarium, a eucaryotic alga which lives only in warm acid waters. This work has been carried out by William Doemel, a graduate student at Indiana University. C. caldarium is the only alga found at temperatures above 35-40°C. in acid hot springs, and lives only at pH values below 4. Its temperature optimum in nature, as shown by $^{14}\text{CO}_2$ fixation, is 45°C., and its upper temperature limit is 55-60°C. No algae are found at temperatures above 60°C. in acid springs and, significantly, blue-green algae are completely absent throughout the whole temperature range. Doemel has developed methods for measuring growth rate in nature and has shown that the optimum temperature for growth, as well as for photosynthesis, is 45°C.