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Technical Progress Report

Microbiology of Thermally Polluted Environments

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I. Introduction

This report provides details of research carried out on this contract through the middle of the second year of its existence. Several papers supported in part or fully by this contract have been published or submitted and reprints and preprints are attached. Other progress not yet at a stage for publication is presented in the detailed narrative.

Studies using thermal environments in Yellowstone National Park as models have proceeded apace and it is now clear that these habitats are ideal for answering a number of important questions concerning the microbiology of thermal environments. Further, these habitats are very suitable for developing methodology which can then be applied to other areas. Our studies on the thermally polluted Jordan River in southern Indiana have shown us how sensitive and fragile a small stream is to the influence of a large urban complex. We have shown that even without intending to, and indeed even after taking presumably adequate precautions to avoid it, Indiana University is thermally polluting the Jordan River. One of our stations has shown a temperature, summer and winter, as high as 56°C! The studies on the Wabash River have been fraught with difficulties. Despite the presence of a very large power plant, the temperature of this large river is not increased very much, thus making difficult a clear-cut demonstration of the effects of temperature on the flora. Even more important, the rapid flow and wide fluctuation in flow rate and water level have made it very difficult to establish and maintain permanent stations. Since an important aspect of our work involves the study of the in situ development of the microflora, the inability to establish permanent stations has thwarted this work. The studies on the effect of temperature on the viability of Escherichia coli in Wabash River water have been possible and have been carried to completion. These studies have shown that not only does E. coli not replicate in warm river water but that it dies faster than in cool river water. Thus thermal pollution might actually speed up rather than inhibit self-purification. Although perhaps only a curiosity, a recent discovery of a new type of aquatic thermal habitat deserves mention. In the coal mining region of southwestern Indiana we have discovered several large refuse piles which are warm and burning slowly. A small acid stream draining one of these piles is also warm, having a temperature of 31-37°C and a pH of 2.3. The microbiota of this stream is very similar to those in Yellowstone Park with the same temperature and pH conditions. This is a further example of how a man-influenced and a natural habitat can be similar. In the case of the natural habitat, the situation would be called interesting or curious, perhaps even attractive. In the case of the man-influenced habitat, the situation would be called ugly and bad. We contend that both evaluations are valued judgements which need reexamining in more objective terms.

The Principal Investigator has spent about 10% of his time on this project, and the Co-Investigator about 25%. Other personnel involved in these studies have been Dr. Thomas Bott, a Post-doctoral Fellow of the

U.S. Public Health Service, Dr. Gary Darland, a Post-doctoral Fellow of the Federal Water Pollution Control Administration, Research Assistants Ilse Yoder, M.Sc. and Mary Louise Sullenger, B.Sc., who have spent 100% of their time on this project, and Mary Swarthout, Secretary, who has spent about 25% of her time on this project. Some of the Yellowstone work has been carried out by M.L. Brock, M.Sc., Research Associate, and W. Doemel, graduate student, of Indiana University; neither these latter two or the post-doctorates have received support from the present contract, but have made direct contributions to the work.

II. Brief Summary of Current Results

A. Studies in the Jordan River

1. Thermal Pollution of the Jordan River

As described in earlier reports, the Jordan River, which flows through the Indiana University campus, arises on the campus as a series of small cold-water springs. Most of these springs are now being influenced in one way or another by the university complex, and our temperature measurements provide an excellent picture of how such a large building complex, in no significant way different from any other urban development, has increased the water temperature of the river. This has happened even though the university does not intentionally affect the river. The thermal additions arise either from unobserved leaks in steam lines, from condensate of air conditioning units, or from cooling units of distilled water systems. In our last progress report we provided a map of the stations we have established and at which we follow temperatures on a monthly basis. Figure 1 provides temperature data at selected stations for about 1 year and illustrates the nature of the problem. Temperatures at several control areas are also given. Ellettsville Spring is a typical cold-water spring about 10 miles from the campus and remains constant in temperature at about 12°C year around. Jackson's Creek is a small creek arising in the same geological formation as the Jordan River but flowing in a different direction. Its temperature varies throughout the year in parallel with variations in air temperature. Station 15 on the Jordan River is sufficiently away from sources of heat that its temperature also varies with air temperature. The other stations on the Jordan River show heating effects of various magnitude. Station 30b is the most dramatic, its temperature ranging around 48-56°C throughout the year. This station is a small side branch immediately adjacent to one of the large steam pipes. Station 10 is the effluent from the air conditioner for the large university auditorium. Although it only flows in the summer, when flowing it averages 36°C. Station 16 is the branch arising as five springs beneath Jordan Hall. The exact source of the heat for this station is not known but we do know that the springs themselves are warm as they come out of the ground beneath the building. In addition to the general interest of these data, they have provided us with a picture of the temperature variation of habitats we have used in our isotope studies.

2. Temperature optima of bacteria at selected stations in the Jordan River

After we had identified a series of habitats of differing temperature characteristics we determined the temperature optima of the resident bacteria using the coverslip-isotope technique described in the methodology paper by Bott and Brock which is attached. It is assumed that the bacteria which grow on submerged cover slips are representative of those living attached to natural substrates in the water. By using colonized cover slips we can be certain that the organisms we are dealing with have developed at known temperatures. Temperature optima were then determined by measuring the rate of C^{14} acetate incorporation of replicate cover slips incubated with the isotope at different temperatures. Note that cover slips were incubated with isotope only for an hour and that

Figure 1

Annual Variation in Temperature at Selected Stations. Those stations not otherwise specified are in the Jordan River on the Indiana University campus.

Fig. 1

they were used without pretreatment and within a few hours (at most) after removal from the habitat. At no time before the isotope experiment was begun were the coverslips allowed to reach temperatures significantly different from those in the natural environment. In addition, one set of coverslips was incubated with isotope precisely at the site where colonization had occurred.

Figure 2 presents representative data. The stations studied include those given in Figure 1 which should be studied in comparison. Note that results are presented as $\log_{10}$ cpm to permit presenting several experiments on one graph and that the temperature optima are thus sharper than they appear. It is clear that the temperature optima in general parallel the temperatures of the natural habitats, the warmer habitats having higher temperature optima. These results confirm other studies carried out in Yellowstone National Park (Brock and Brock, 1968 a.) However, it should be noted that the population developing in Ellettsville Spring, where the temperature is 12°C year around, have an optima considerably higher than this temperature. This observation is in line with our studies on temperature optima of marine bacteria living in cool sea water (Kelly and Brock, 1969). It suggests that bacteria living at low temperature are not optimally adapted to their environment and will begin to metabolize faster immediately upon being warmed up. This conclusion is of obvious significance in relation to problems of thermal pollution.

However, in warmer waters the bacteria are optimally adapted to the temperatures at which they are found. The significance of this is that once a stable temperature has been maintained at a location for some time and the bacteria are optimally adapted to this temperature, if the habitat should become cooler or warmer, bacterial metabolism should slow down. Thus one cannot assume that a warming of the water will lead to an immediate increase in bacterial metabolism.

3. Thermus aquaticus as an indicator of thermal pollution

Recently we have described a new genus and species of bacteria, Thermus aquaticus, which is widespread in thermal environments, both natural and man-made (Brock and Freeze, 1969). We have described a simple means by which T. aquaticus can be cultivated and relatively specifically isolated. T. aquaticus is a non-spore-forming yellow pigmented bacterium which grows optimally at about 70°C, but grows also at 50-55°C. We originally became interested in using this organism as an indicator of thermal pollution when we isolated it from the hot tap water in our laboratory and it seemed possible that the organism might be replicating in the hot water system. Most other thermophiles studied are spore-formers whose habitats are primarily the soil and other terrestrial environments. These organisms undoubtedly wash into rivers and lakes and because they are spore-formers can remain dormant until cultured in the laboratory. T. aquaticus, on the other hand, is an aquatic bacterium and because it does not form a spore would probably not remain alive long in habitats where it does not grow. It thus seemed possible that it could be used as an indicator of thermal pollution and we set about to test this hypothesis.

Figure 2

Temperature Optima of Microflora Developing on Substrates Immersed at the Stated Locations. For temperatures of the stations through the year, see Figure 1.

Fig. 2

We used for this study the Jordan River and the other habitats near Bloomington whose temperature characteristics we had studied (see Figure 1 and Technical Report C00-1804-1).

The procedure used was based on sanitary bacteriological water analysis. A nine-tube Most Probable Number method was used, with three tubes at each dilution of the water sample. Incubation was at 70°C in the medium of Brock and Freeze (1969) and all tubes showing turbidity after 1, 2 and 3 days were noted. On the third day of incubation, all tubes showing turbidity were streaked on agar medium and after incubation at 70°C the presence of typical yellow-pigmented colonies noted. Yellow colonies were examined microscopically for the presence of absence of spores and for the typical T. aquaticus morphology.

Representative data are given in Table 1. Note that these studies were done in summer when the normal streams are fairly warm, although the springs of course remain cool. The results show that T. aquaticus was not found in the cool springs or a normal stream, but was found in the thermally polluted Jordan River. The counts at station 30b, temperature 56°C, were quite high. This technique clearly shows promise and studies will continue.

Table 1

Thermus aquaticus as an indicator of thermal pollution

<u>Cold-water Springs</u>	Date	Temperature °C	MPN Index/100 ml
Abandoned Farm	7/2/69	9.0	N.D.
Ellettsville	7/2/69	11.5	N.D.
<u>Unpolluted Stream</u>			
Bean Blossom Creek	8/19/69	24.0	N.D.
<u>Jordan River</u>			
Music branch tile (#13)	7/16/69	34.0	20
Below station 12			
Jordan Hall "pool" (#16)	7/16/69	39.0	N.D.
Steam Bridge (#30b)	8/19/69	56.0	15,000

MPN index calculated as described in text

N.D. None detected

B. Wabash River Studies. Indiana State University

As was described in the last progress report, the initial phases of work on the Wabash River concerned the establishment of sites within the thermal gradient at the power plant outflow where in situ estimations of growth rates of the microflora could be made. It was established at that time that the usual temperature difference between an upstream station and the outflow of the power plant was 10°C, and during late summer and early fall the stations were typically 26°C and 36°C respectively. During that time of the year the river stage is very low (about 1-2 ft.) and the thermal gradient is well defined, some thermal effect being noted several miles down river. At that time it appeared that the submerged coverslip method used successfully in lakes and small creeks would be applicable to the study.

Subsequent work during the current grant period has revealed formidable obstacles to the successful application of the coverslip technique to large river waters. Some difficulties are merely minor problems of modification of technique but, one, the dynamic, unpredictable nature of the river, has not been circumvented as yet.

Several experiments were terminated because a great proportion of the coverslips were broken during immersion in the swiftly flowing river waters. This problem has been obviated by the use of microscope slides which were scored 14 mm and 28 mm from the end and inserted scored end down into a plastic coated test tube rack. After immersion in the river for various time intervals, slides were removed, inserted in racks in pans containing river water and taken to the laboratory. The slides were then broken along the score and one 14 mm section was used for ³H-acetate uptake, the other 14 mm segment for protein determination, and the remaining long section for microscopic observations.

The dynamic nature of the river as well as a high concentration of suspended soil particles poses several serious problems to sample replication.

a) Rate of flow of river: The position of a weighted test tube rack containing slides relative to the water surface was not necessarily constant during an experiment. During dry periods the rate of flow of the river was sufficiently gentle so that the slide rack remains approximately verticle about 8 inches beneath the surface of the river, with the long axis of the rack along the lines of flow. During such times slides in the rack (in alternate rows) present a given side upstream at a 45° angle. When the river rises following rain the incidence of the top of the rack toward the upstream changes so that at times the top of the rack (and hence the edges of the slide) faces upstream. In addition, rapid flow tends to disarrange the slides in the racks. Therefore slides, whether assessed by ³H-acetate incorporation or microscopic counts or even microscopic observation, differ greatly among replicates.

b) Suspended material in river: The Wabash River, typical of many midwestern rivers, is very muddy especially following rain. The soil particles rapidly coat the slide even during the first day of immersion so that microscopic observations of the various bacteria adhering to the slide is difficult if microscopy is done with transmitted

light. A technique was developed which allows one to observe bacterial cells in the presence of large amounts of detritus. A Wild MK5 microscope was equipped with reflectance optics (10x, 20x, 40x, and 100x objectives) and observations were made under dark field. Bacterial cells were clearly discernable against a background of mineral material, decaying vegetation and unidentifiable debris. c) Comparison of upstream slides with slides from power plant outfall. If one intends to compare the in situ growth in the hot and cold areas, it might be reasonable to assume that incubation for the same time interval in the two habitats would lead to populations on the slides which would reflect the types of organisms in the two habitats and their growth rates at that temperature. It was noted, however, that slides immersed upstream in the colder, more slowly flowing water quickly became coated with soil and debris (occasionally as soon as four hours after immersion) while those in the thermal area were relatively 'clearer' even after several days immersion, because hot water flowing from the power plant moves very rapidly with turbulence for a considerable distance downstream. That is, rate of flow and turbulence are variables in addition to temperature, the effect of which one hopes to estimate.

The rapidly changing nature of the river following even moderate rains upstream has caused much damage to or destruction of sampling stations above and along the thermal gradient (see report C00-1804-1 for their location). Floating debris, especially large logs or trees, has in many cases destroyed a sampling station and ruined an experiment. Typical damage includes a) tearing test tube rack from the float, b) carrying station including anchor downstream for several miles, c) leaving the station intact but moving it to a place with a different temperature.

Recent failure to maintain sampling stations even during late summer when the river is usually quiescent has led to the design of a new station buoy which should be much more stable under varying river conditions.

Despite these difficulties, a sufficient number of experiments have been carried out to show clearly that bacterial growth rate and metabolism are faster below than above the thermal source. Table 2 shows data obtained with the H^3 -acetate incorporation technique on colonized coverslips immersed above and below the thermal source. Other experiments have given essentially the same picture. These studies show clearly that rate of isotope uptake is considerably faster below than above the power plant. They also show that the rate of bacterial development on the coverslips (as indicated by the isotope uptake rate on coverslips colonized for different time periods) is also markedly faster below than above the power plant.

Table 2

H^3 -Acetate Incorporation
Counts/Min.

Station No.	T(°C)	Days Colonization in River		
		1	2	5
1 Above power plant	25-27	42.6	557	877
2 Below power plant	32-35	274	742	3500

Incubation in 0.1 μ C/ml H^3 -acetate for 1 hour at 25°C

The other major study done at the Wabash River site was one concerning the effect of temperature on viability of Escherichia coli in river water. As noted in the original contract proposal, it is of considerable practical importance to ascertain whether intestinal organisms such as E. coli, derived from or indicators of fecal pollution, might replicate in thermally polluted habitats when the temperatures reached those similar to the human body. The data presented here show that not only does E. coli not replicate, but that it actually dies faster in warmer water than in cooler. We conclude that in nutrient-poor river water E. coli dies of starvation and that death is faster at higher than at lower temperature.

To permit assay of the viability of E. coli in natural river water containing many other kinds of bacteria, a streptomycin resistant mutant of E. coli was used, and viable counts were performed on agar medium containing streptomycin. The antibiotic effectively inhibited the growth of all natural bacteria so that only E. coli was assayed. The inoculum of E. coli was grown in normal nutrient medium and diluted into river water so that a fairly small inoculum was present, around 10^6 /ml, to prevent any secondary effects.

Figure 3 presents a representative experiment (over 20 separate experiments were done). It can be seen that in natural river water death is fairly rapid at 35°C and much less so at 24°C. Other experiments showed that death was even slower at temperatures below 24°C. Death might be due either to action of a phage or parasite of E. coli such as Bdellovibrio, or to the ingestion of the bacteria by a protozoan or other animal. To eliminate the latter causes of death, the experiment was also done with filter-sterilized water. This shows that death is not due to a predator. A search for a phage or Bdellovibrio was carried out by placing samples of river water on agar plates spread with E. coli and examining the plates after incubation for plaques. No plaques were found. The finding that death was faster in filtered water suggests that the bacterium may be

Figure 3

Death Rate of Escherichia coli in Filtered and Unfiltered Wabash River Water at Various Temperatures.

partly protected from death by particulate matter such as silt present in the water. Other experiments have shown that death does occur in distilled water or phosphate buffer at rates similar to that in river water. This suggests that death is not due to some toxic agent present in river water. The protection against death by particulate material is of interest and could be investigated further although it is outside of the scope of the present study.

It seems safe to conclude that E. coli, and probably other intestinal bacteria, would not replicate in warm river water, but rather would die even faster than otherwise.

C. Yellowstone studies

As noted in our last report, studies of thermal algae and certain bacterial studies can be carried out much more easily at Yellowstone than other places. Some of these studies integrate with those supported by our NSF grant.

1. Bacteria living at temperatures greater than 90°C

As reported earlier (Brock, 1967) many boiling springs at Yellowstone have bacteria living in them, and we recently reported (Bott and Brock, 1969) that growth of these organisms is surprisingly rapid. Unfortunately culture studies have not yet been successful so that in the summer of 1969 we elected to study the physiology of these organisms directly in the springs, using the coverslip-isotope technique described earlier. We have concentrated on one location, Boulder Spring, which is favorably situated geographically and in which the bacteria show rapid growth. Physiological studies have been done by measuring the rate of incorporation of radioactively labeled compounds into bacterial populations which have colonized coverslips. A number of replicate cover slips are used and incubated under different conditions. By use of this technique we have shown that the probable energy source of these bacteria is sulfide, that they are microaerophilic, and that their temperature optimum for isotope incorporation is about 87-90°C. By use of this information it may now be possible to devise culture methods.

A second aspect of this problem is the relationship between pH and temperature. Although bacteria are found at temperatures up to and above the boiling point in virtually all neutral and alkaline springs, in acidic springs such is not the case. Dr. Gary Darland, a post-doctoral associate, has surveyed a large number of hot springs of various temperature and pH characteristics for the presence of bacteria and has found that at pH values below 5 there is a progressive lowering of the upper temperature limit. It thus appears that bacteria are unable to evolve the ability to grow at both high temperature and high acidity. This conclusion is of significance in relation to thermal pollution of industrial waters in which high acidity may occur.

Algal growth rates at various temperatures along hot spring thermal gradients have been measured directly in nature by the darkening technique

described earlier (Brock and Brock, 1968b). Algal doubling times of 12 hours to 1 week have been found at different temperatures. The optimum temperature for algal growth rate is about 70°C. (No growth occurs at temperatures above 73°C.) However, standing crop is maximal at a lower temperature, 55-60°C. If productivity is calculated from the growth rate and standing crop, productivity is maximal at about 65°C, a temperature somewhat lower than that at which growth rate is optimal. The techniques used in this work may have wider application in thermal environments.

2. Physiological ecology of Cyanidium caldarium

As noted in the last report, the alga Cyanidium caldarium is of considerable interest because of its ability to colonize a niche closed to other algae, that of warm acid waters. Doemel has carried his work on the temperature and pH limits of this organism to completion and has shown that it has an upper temperature limit of 55-56°C and an upper pH limit of about 5. It grows down to pH of about 0, and its lower temperature limit in nature is about 30-35°C, being conditioned by its inability to compete with other acidophilic algae able to grow better at lower temperatures, such as Euglena and Chlamydomonas. Of considerable interest is the lack of temperature strains of C. caldarium, since strains of the organism isolated from temperatures ranging from 36-56°C have precisely the same temperature optimum. This is in contrast to the situation with the blue-green algae living in alkaline waters where temperature strains do exist. The lack of temperature strains of the acidophilic thermophile is of practical interest because it suggests that algal adaptation to changing acid thermal environments is not possible. Another interesting point of general significance is that at acid pH values blue-green algae are completely absent, even though they are the dominant thermal algae of neutral and alkaline waters. The work on C. caldarium is now being written up by Doemel and the Principal Investigator for publication.

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