Heterotrophic Bacterial Populations Associated With Leaf-Litter in an Indiana Stream

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Introduction

Temperate region woodland streams have been shown to be heterotrophic in nature and rely primarily upon allochthonous matter as a primary source of energy (8) (9) (17) (19) (20) (24). A large portion of this allochthonous material arrives in the form of autumn-shed leaves from the surrounding watershed (9) (18) (25). In addition, estimtes of leaf-litter production in different woodlands ranges from 2.5 to 6.8 metric tons/ha/yr (3), suggesting that the leaf-litter entering streams is substantial, especially in the autumn of the year.

The fate of leaves after they have entered a stream has received some attention of late. Initially, they float on the water surface and move in response to wind and water currents. Gradually, they become waterlogged and sink to the bottom accumulating in shallow margins and the downstream edges of pools. They also tend to aggregate or clump against obstacles that are located in the stream and in this respect a riffle is an excellent obstruction with which to trap leaves.

It has been documented that leaves are leached of many of their soluble products after they enter the stream, usually within the first twenty-four hours (14) (16). After leaching, a period of nitrogen enrichment occurs which has been attributed to the microbial colonization of the leaf-litter (13) (14) (16) (18). The colonization of leaf-litter by bacteria and fungi has been called "conditioning" and is important in preparing the leaf material as a palatable food source for macroinvertebrates (2) (5) (21).

The role of many macroinvertebrates in leaf-litter degradation has been identified and categorized (5). However, little attention has been paid to the bacterial species that are present in the leaf masses. The large influx of leaves in the fall of the year can lead to large accumulations of leaf-litter all of which are subject to bacterial colonization. These aggregations are in effect unique entities in terms of resources and environments and their size influences the conditions which exist within each leaf mass (2). Therefore, physical factors such as dissolved oxygen and water movements are effected. The latter parameter may be important in terms of the physical disruption of the leaf material since sediments in transport may act as abrasives. The former condition will greatly influence the microbial population and activity and is therefore of major interest in terms of the bacterial influence on leaf-litter decomposition.

Considering the fact that continual packing of leaf material occurs during

the autumn it seems reasonable to assume that reduced oxygen conditions, and possibly anaerobic conditions, are generated through the continued accumulation of new material. These conditions would, by their nature, limit the types of organisms present to those that could function and survive in microaerophilic of anaerobic environments. This can be observed in terrestial biomes in waterlogged soils where aerobic fungi and bacteria are unable to develop due to prevailing anaerobic conditions (27). Under these circumstances, the anaerobic bacteria capable of degrading non-soluble products such as cellulose and lignin would seem to be an important factor in leaf-litter decomposition. It has been suggested that the spore-forming *Clostridium* are the dominant cellulolytic bacteria in anaerobic aquatic environments (12).

Thus, the autumn leaf packs appear to sustain a dynamic mosaic community of various microbial and invertebrate species colonizing the leaf-litter. This study was undertaken to: 1) examine the dominant heterotrophic bacterial species associated with the leaf-litter and 2) to determine if anaerobic cellulolytic bacteria were present and possibly functioning in leaf-litter decomposition.

Materials and Methods

Leaves of the sugar maple (*Acer saccharum*) were collected and placed in 8 x 12 inch nylon mesh bags (mesh size: 2300 μ bs 312 μ). In September, 1976 the bags were placed above a riffle in Bell Creek, Delaware County, Indiana. Duplicate bags were attached to stakes by nylon fishing line and allowed to float downstream to become a part of the developing leaf mass. The leaf bags were sampled approximately monthly from December, 1976 to March, 1977 and analyzed for aerobic heterotrophic bacteria, total anaerobic heterotrophic bacteria, and anaerobic bacteria capable of cellulose decomposition. The analysis was performed at Ball State University, Muncie, Indiana.

When the leaf bags were sampled they were brought back to the laboratory aseptically and under both aerobic and reduced oxygen conditions. In the laboratory, samples were prepared by homogenizing the leaf material in sterile phosphate buffer (pH 7.2) at a 1:10 dilution (V/V). Homogenization was performed in a Waring Blender set at high speed for two minutes. Leaves were homogenized aerobically and anaerobically and cultured under both oxygen conditions.

Aerobic isolation consisited of spread plating decimal dilutions of prepared sample to extinction on triplicate plates of Tryptone Glucose Yeast Extract Agar (TGYEA) and incubation at ambient temperatures (20-24OC.). Typical bacterial colonies were purified through further transfers on TGYEA and subjected to biochemical examination. All incubation temperatures were at ambient temperature except those tests which required special incubation procedures.

The anaerobic isolations procedures were performed as above but were carried out in a bacteriological transfer hood (Lab. Con. Co.) which had been altered to maintain an anaerobic atmosphere. The hood was evacuated and flooded with CO_2 several times before any operations were performed. A

constant stream of CO_2 was allowed to run during the isolation procedures to insure a positive pressure inside the chamber. The TGYEA plates were incubated at ambient temperature in gas-pack jars (B.B.L.). Typical colonies were purified and transferred to two TGYEA plates. One plate was incubated aerobically and the other was incubated anaerobically. Colonies growing on aerobic plates were picked and transferred to diferential media for biochemical identification. Colonies growing only on anaerobic plates were gram stained and inoculated into chopped meat-glucose medium and peptone-yeast extract medium for identification by gas-liquid-chromatography and by the V.P.I. system for culturing and identifying anaerobes.

Anaerobic cellulolytic bacteria were investigated using a multiple tube test with Imshenetskii medium No. 1. This medium was prepared according to Rodina (23) with the notable exception that the cellulose was homogenized into a slurry (1.5% cellulose). Whatman #1 filter paper was used as a source of cellulose. Each tube in the series contained approximately ten milliliters of cellulose medium. All tubes were overlaid with sterile heavy weight mineral oil after inoculation to insure that anaerobic conditions were maintained.

After approximately two weeks inocula from each tube in the series were transferred to another set of tubes containing the same medium and again overlaid with sterile oil. The tubes that eventually showed a marked reduction in the amount of cellulose present were considered positive. Enumeration was carried out according to the 14th edition of *Standard Methods for the Examination of Water Wastewater* for the multiple tube tests (1). Inocula from each reduced tube were streaked onto TYGEA plates and incubated anaerobically. Colonies were purified and transferred to two TGYEA plates and incubated as above.

Aerobic isolation on non-selective medium

Typical bacterial colonies from the higher dilution TGYEA plates were identified biochemically. Pigmented bacteria of the genus *Flavobacterium* were the most numerous on nearly all plates and comprised between thirty-five and forty-five percent of the recovered bacterial population. Members of the genus *Pseudomonas* were also dominant and comprised between thirty and forty percent of the recovered population. Another organism suggestive of the genus *Beijerinckia* was isolated routinely on all high dilution plates and comprised between twenty and thirty percent of the bacterial population recovered by the spread plate technique.

Members of the genera *Bacillus, Achromobacter, Chromobacterium,* and various members of the family Enterobacteriaceae, especially *Klebsiella*, were isolated routinely at most lower dilutions. Enumeration of aerobic bacteria demonstrated an average maximum value of 1.5×10^8 bacteria/ml in the March sample. Low values of 9.0×10^5 bacteria/ml were recorded in January.

Anaerobic isolation on non-selective medium

A number of facultative bacteria were isolated on TGYEA under anaerobic conditions. Almost all of the organisms represented in this group were members of the genus *Pseudomonas*. Two of these were tentatively identified as *Ps. effusa* and *Ps. mira,* both of which are reported to attach cellulose according to Breed

et al. (4). Members of the genus *Klebsiella* were also isolated under these conditions and were the only other facultative organisms recovered using this technique. Plate counts from this procedure ranged from 6.0 to 10^4 bacteria/ml in January to 1.8 x 10^5 bacteria/ml in March.

Anaerobic bacteria were also isolated which were capable of growth on TGYEA after the initial inoculation but failed to grow upon subsequent transfers. Gram stains of these organisms revealed that they were gram positive rods or filaments. Gram stains of other anaerobes that grew weakly or well upon transfer showed that most were gram positive rods with terminal spores which is suggestive of the genus *Clostridium*. Purified isolates of these organisms were subjected to gas-liquid-chromotography. All such cultures showed a very large butyric acid peak and a somewhat smaller acetic acid peak. These data are again suggestive of the genus Clostridium. Biochemical tests performed on these organisms with the V.P.I. anaerobic system did not prove useful in identifying the bacteria to species.

Anaerobic isolation in cellulose medium

Several facultative organisms were isolated in the cellulose medium including *Ps. effusa* and *Ps. mira* along with various members of the family Enterobacteriaceae. However, all tubes which demonstrated a reduced cellulose content were found to contain gram positive sporogenous rods. The same characteristic acid peaks were obtained from analysis of these organisms as described above for the sporeforming bacteria isolated anaerobically on TGYEA. The number of cellulolytic anaerobes/ml ranged from a low of 93 in December and March to a high of 150 in January.

A list of the dominant and common genera of bacteria that were routinely isolated during the investigation is shown in Table I.

Discussion

The identification and enumeration procedures used in this investigation revealed a variety of bacteria associated with the leaf-litter habitat. Aerobic bacterial plate counts reached 1.5 x 10^8 bacteria/ml which compares favorably with the counts obtained by other investigators (15) (22). Obviously the method employed enumerates only those organisms that are nutritionally and competitively able to develop on the TGYEA plates. However, the developing microorganisms, as well as those that are not culturable, utilize common substrates in the stream and should respond similarly to environmental changes. (Witkamp) (28) has reported on the validity of the serial dilution technique in relation to counts from terrestrial leaf-litter.

It should be realized that the enumeration of microorganisms cannot act as a measure of their importance in the process of decomposition. It has been suggested that there may be no relationship between the number of microorganisms present and metabolic rate (10) and that the importance of bacteria in organic decomposition is determined by the latter parameter rather than the former (28). This investigation does not attempt to equate the mere presence of bacteria in the leaf-litter with a direct function in regards to decomposition. Nevertheless, the complexity of the microbial colonization of

large leaf masses suggests a "community" concept and offers the potential for both direct and indirect interactions among the community members.

TABLE I *Dominant and common genera of bacteria isolated from leaf-litter in Bell Creek, Delaware County, Indiana, from December, 1976 to March, 1977.*

Dominant Genera	Common Genera
Flavobacterium	Enterobacter
Pseudomonas	Citrobacter
Beijerinckia-like organism of unknown taxonomy	Klebsiella
	Bacillus
	Achromobacter
	Chromobacterium
	Clostridium

Most of the bacteria isolated during this investigation were not capable of degrading the cellulose of lignins found in large quantities in leaves. The dominant genera isolated from the leaf-litter were *Flavobacterium, Pseudomonas*, and a Beijerinckia-like organism of unknown taxonomy. These data are similar to those of Suberkropp and Klug (26) who found *Flavobacterium* and *Pseudomonas* to be among the major genera associated with oak and hickory leaves in a woodland stream. *Flavobacterium* were also found to be a dominant member of the microbial population associated with organic matter in transport in streams (6) and an organism identified as *Flavobacterium* has been isolated which clearly stimulated the growth of the cellulolytic bacterium Sporocytophaga in mixed culture (11). This type of association may be a factor in the roles played by bacteria in leaf-litter decomposition.

Similar indirect associations may be hypothesized for other non-cellulolytic bacteria found to be present in the leaf-litter. the Beijerinckia-like organism is suspected of fixing atmospheric nitrogen and might possibly provide fixed nitrogen to the community. This function might also be attributed to the *Enterobacter* and *Klebsiella*, which were found routinely, since members of these genera have been implicated in nitrogen fixation (7). The various members of the genus *Pseudomonas* may also impact some indirect influence on the amount of leaf material which is degraded. Certain members of the genus *Bacillus* have also been found to degrade cellulose and the isolation of this genus from the leaf-litter should not be considered unimportant.

The scope of this study and the complexity of the microbial community within the leaf-litter precludes any detailed analysis of specific relationships. However, we do emphasize that the potential for commensal, mutual, and synergistic relationships exists.

When the leaf-litter habitat is viewed with respect to community interactions it is possible that a small number of bacteria could influence decomposition more readily than a larger number of less active species. A case in point is the relatively small number of cellulolytic bacteria found in the leaf mass. No attempt was made to directly measure the importance of these bacteria

in the decomposition process. However, the continued accumulation and packing of leaf-litter should result in microenvironments capable of sustaining anaerobic or facultative organisms. Therefore, it is assumed that if the organisms were routinely isolated from the leaf mass then the proper conditions exist in the field to allow them to be metabolically active. Since gram positive sporogenous rods (*Clostridium* spp.) were found in all tubes showing cellulose reduction, it is probable that these organisms are also active under field conditions.

These data by no means represent the total bacterial composition of the leaf-litter habitat. Only the dominant species associated with this particular leaf mass have been identified. Since only those bacteria that grew primarily on TGYEA were isolated many bacterial species were not cultivated. Cellulolytic members of the genera *Cytophaga* and *Sporocytophaga* have been isolated from leaf-litter in other investigations (22) (26) but were probably not selected for by the methods employed here. It must be emphasized that we are not suggesting that the bacteria are the most important organisms involved in leaf-litter decomposition. Other data suggest that the fungi might be more important, at least initially (16) (26). However, the value of bacteria associated with stream leaf-litter decomposition should not be overlooked, especially in the microenvironments that are more suited for their colonization. Hopefully, further studies will be performed relative to the function and relationships of bacteria in the overall energy budget of heterotrophic steams.

Literature Cited

1. AMERICAN PUBLIC HEALTH ASSOCIATION, AMERICAN WATER WORKS ASSOCIATION, and WATER POLLUTION CONTROL FEDERATION. 1976. Standard Methods for the Examination of Water and Wastewater. 14th Edition Amer. Public Health Assoc. Inc. New York 1193 pp.
2. BOLING, R. H., E. D. DOODMAN, J. A. VANSICKLE, J. O. ZIMMER, K. W. CUMMINGS, R. C. PETERSEN, and S. R. REICE. 1975. Toward a model of detritus processing in a woodland stream. Ecology **56**:141-151.
3. BRAY, J. R. and E. GORHAM. 1964. Litter production in forests of the world. Adv. Ecol. Res. **2**:101-157.
4. BREED, R. S., E. G. D. MURRAY, and A. P. HILCHENS. 1957. Bergey's Manual of Determinative Bacteriology. Seventh Edition The Williams and Wilkens Co., Baltimore 1094 pp.
5. CUMMINS, K. W. 1974. Structure and function of stream ecosystems. Bioscience **24**:631-641.
6. CUMMINS, K. W., M. J. KLUG, R. G. WETZEL, R. C. PETERSEN, K.F. SUBERKROPP, B. A. MANNY, J. C. WUYCHECK, and F. O. NEWARD. 1972. Organic enrichment with leaf leachate in experimental lotic ecosystems. Bioscience **22**:719-722.
7. DALTON, H. 1974. Fixation of dinitrogen by free-living microorganisms. Crit. Rev. Microbiol. **3**:183-220.
8. DARNELL, R. M. 1964. Organic detritus in relation to secondary production in aquatic environments. Verh. Internat. Verein. Limnol. **15**:462-470.
9. FISHER, S. G. and G. E. LIKENS. 1972. Stream ecosystems: Organic energy budget. Bioscience **22**:33-35.
10. HARGRAVE, B. T. 1972. Aerobic decomposition of sediment and detritus as a function of particle surface area and organic content. Limnol. Oceanogr. **17**:583-596.
11. HOFSTEN, B. V., B. BERG, and S. BESKOW. 1971. Observations on bacteria occurring together with Sporocytophaga in aerobic enrichment cultures on cellulose. Arch. Microbiol. **79**:69-79.
12. HOFSTEN, B. V. and N. EDBERG. 1972. Estimating the rate of degradation of cellulose fibers in water. Oikos **23**:29-34.

13. HYNES, H. B. N. and N. K. KAUSHIK. 1969. The relationship between dissolved nutrient salts and protein production in submerged autumn leaves. Verh. Internat. Theor. Angew. Limnol. **17**:95-103.
14. IVERSEN, T. M. 1973. Decomposition of autumn-shed beech leaves in a spring brook. Arch. Hydrobiol. **72**:305-312.
15. KAUSHIK, N. K. and H. B. N. HYNES. 1968. Experimental study on the role of autumn-shed leaves in aquatic environments. Jour. Ecol. **56**:229-243.
16. KAUSHIK, N. K. and H. B. N. HYNES. 1971. The fate of the dead leaves that fall into streams. Arch. Hydrobiol. **68**:465-515.
17. KING, D. L. and R. C. BALL. 1967. Comparative energetics of a polluted stream. Limnol. Oceanogr. **12**:27-33.
18. MATHEWS, C. P. AND A. KOWALCZEWSKI. 1969. The disappearance of leaf-litter and its contribution to production in the River Thames Jour. Ecol. **57**:543-552.
19. MINSHALL, G. W. 1967. Role of allochthonous detritus in the trophic structure of a woodland spring brook community. Ecology **48**:139-149.
20. NELSON, D. J. and D. C. SCOTT. 1962. Role of detritus in the productivity of a rock-outcrop community in a Piedmont stream. Limnol. Oceanogr. **7**:396-413.
21. REICE, S. R. 1974. Environmental patchiness and the breakdown of leaf-litter in a woodland stream. Ecology **55**:1271-1282.
22. RODINA, A. G. 1963. Microbiology of detritus of lakes. Limnol. Oceanogr. **8**:388-393.
23. RODINA, A. G. 1972. Methods in Aquatic Microbiology. University Park Press Baltimore 346 pp.
24. SCOTT, D. C. 1958. Biological balance in streams. Sewage Ind. Wastes **20**:1169-1173.
25. SLACK, K. V. and H. R. FELTZ. 1968. Tree leaf control on low-flow water quality in a small Virginia stream. Environ. Sci. Techn. **2**:126-131.
26. SUBERKROPP, K. and M. J. KLUG. 1976. Fungi and bacteria associated with leaves during processing in a woodland stream. Ecology **57**:707-719.
27. WAKSMAN, S. A. and F. C. GERRETSEN. 1931. Influence of temperature and moisture upon the nature and extent of decomposition of plant residues by microorganisms. Ecology **12**:33-60.
28. WITKAMP, M. 1963. Microbial populations of leaf-litter in relation to environmental conditions and decomposition. Ecology **44**:370-376.