The biodegradation of organic substrates under arctic and subarctic conditions
Ann P. Murray, R. Sage Murphy

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by

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INTRODUCTION

The objective of this research was to obtain data on the metabolic reaction rates of the microorganisms indigenous to the cold environments of the arctic and sub-arctic in order to evaluate the natural abilities of the freshwater streams and lakes of Alaska to assimilate the wastes discharged into them. Microorganisms capable of growth even at subzero temperatures have long been known; however, most have consistently fared better at higher temperatures, usually above 20° C. Much of the work done with the biological oxidation of wastes at low temperatures has been with organisms of this type; mesophilic organisms which are able to survive at low temperatures but which are metabolically much more active in the temperature range from 20 to 45° C. Such organisms might be labeled "cold-tolerant," but they are probably biochemically quite different from the truly "cold-loving," or psychrophilic, microorganisms which are able not only to survive but also to thrive at temperatures below 20° C and which, in fact, find temperatures much higher than 25° C intolerable.

A strict definition of a psychrophile is an organism which grows rapidly enough at 0° C on solid media as to be visibly detectable within a week and whose optimum growth temperature is 20° C or lower (MORITA, 1966b). Psychrophiles differ from mesophiles in being able to grow at much lower temperatures and in being capable of greater physiological activity than the mesophile in the region where the temperature ranges for growth of the two groups overlap.

The psychrophile is an especially interesting organism when one realizes that microorganisms have no mechanism for retarding the loss of metabolic heat to their aqueous surroundings. How then are they able to maintain levels of activity at low temperatures which are comparable to those of mesophiles at higher temperatures? Their adaptation to cold environments must involve some decidedly different biochemical arrangements within the cell. Determining just what these different arrangements are has been the goal of present-day investigators, for these mechansims are the key to the distinguishing character of the psychrophiles.

Most of the research concerning these microorganisms has been aimed at discovering the biochemical bases for their ability to grow at very low temperatures and to do so at rates comparable to those of mesophiles at higher temperatures. Quite a variety of approaches has been taken. One of the first questions asked was how low temperatures affected biochemical reaction rates. The answer was not what one would expect from simple thermochemical reaction rate theory. With mesophiles and psychrophiles alike, the Q_{10} values and energies of activation are greatly increased in the lower temperature ranges for their growth. But the temperature at which this increase takes place is lower for the psychrophile. In several instances the psychrophile's energy of activation for a given process is lower than that of the mesophile. SULTZER (1961) found this to be true for saturated fatty acid oxidation and INGRAHAM (1962) for glucose oxidation.

A greater permeability at low temperatures for the cell membrane of the psychrophile has also been suggested, and it is thought that the difference in permeabilities is due to different lipids in the cell membranes, those of the psychrophile being more highly unsaturated, and hence more "liquid," than those of the mesophile at low temperatures (KATES and BAXTER, 1962; KATES and HAGEN, 1964).

Evidence has been presented supporting the thesis that mesophiles are unable to grow at as low temperatures as psychrophiles because of their inability to transport sugars into the cell at such temperatures (BAXTER and GIBBONS, 1962; ROSE and EVISON, 1965).

Research aimed at explaining why psychrophilic microorganisms have relatively low maximum temperatures for growth has led to the discovery of numerous enzymes and enzyme-forming systems in psychrophiles which are exceptionally heat-sensitive so that they are inactivated at relatively low temperatures (HAGEN and ROSE, 1962; BURTON and MORITA, 1963; UPADHYAY and STOKES, 1963; EVISON and ROSE, 1965; GRANT et al., 1968; NASH et al., 1969; MALCOLM, 1969a,b,c,; QUIST and STOKES, 1969).

Little has been done with the heat-sensitivity of the nucleic acids *in vivo*; consequently, their importance in limiting psychrophilic growth cannot yet be evaluated. However, their denaturation temperatures are such that it does not seem likely that they are involved in determining the maximum growth temperatures for psychrophiles (MARMUR and DOTY, 1959; MARMUR et al., 1963).

The same high degree of unsaturation of the lipids of the cell membrane which was assumed to permit the psychrophile to function at very low temperatures has also been implicated in causing cell death at relatively low temperatures by contributing to the disorganization of the cell membrane. There is evidence that the psychrophile loses control over permeability at low temperatures and that the subsequent leakage of cell materials leads to death (HAGEN et al., 1964; MORITA, 1965, 1966a; KENIS and MORITA, 1968).

Since truly psychrophilic microorganisms do exist and are distinguished by their ability to grow at very low temperatures and to do so at rates comparable to those of mesophiles at higher temperatures, it is reasonable to expect that the kinetic rate constants for the bio-oxidation of organics by psychrophiles under arctic conditions would not be radically different from those found for mesophiles under more temperate conditions. Consequently, it would not be valid to extrapolate from data obtained with mesophiles subjected to extreme cold to a situation where psychrophilic microorganisms are involved. Such were our thoughts when we began to investigate the metabolic reaction rates of microorganisms found in the soils and freshwaters of Alaska.

SUBSTRATE REMOVAL AND CELL GROWTH IN BATCH SYSTEMS

Theory

The rate of disappearance of an organic material from an aqueous, biologically active, system depends on many factors, including the mass of active cells and their metabolic capabilities, temperature, the concentration of the organic substrate, and the presence of the nitrogen, growth factors, and mineral elements necessary for cell metabolism. Of primary interest in the operation of biological treatment plants are the active cell mass, its age, and the concentration of the organic material.

When the active cell mass first comes into contact with a suitable substrate, there may be an initial rapid removal of the substrate from the liquid medium due to an absorption/storage mechanism. After an initial lag period, the cells enter into a logarithmic growth phase which is described mathematically by the equation:

$$\frac{dX}{dt} = \mu X \qquad (1)$$

where X is the cell population, t is the time, and μ is the specific growth rate constant for the cells under the environmental conditions with which the measurements were made. This equation can be re-written as:

$$\ln \frac{X}{X_o} = \mu(t - t_o) \qquad (2)$$

where X_o is the cell population at some initial time t_o. When $X = 2X_o$, that is, when the population has doubled, the time interval $(t - t_o)$ equals the generation time, Ω. Thus, $\Omega = \ln 2/\mu$. In theory a constant value should be obtained for both μ and Ω; however, in practice these values are continuously changing because not all of the organisms in a batch system are in the same phase of growth at the same time.

During logarithmic cell growth, the amount of substrate metabolized is directly proportional to the amount of cell growth. Eq. 2 can be re-written as:

$$\ln \frac{X_o + \Delta X}{X_o} = \mu(t - t_o) \qquad (3)$$

and, since ΔX is related to the amount of substrate removed, L_r, by the cell yield constant, a, as:

$$\ln \left[1 + \frac{aL_r}{X_o}\right] = \mu(t - t_o) \qquad (4)$$

Eventually the substrate concentration, L, declines to the level where it affects cell growth, the rate of which then diminishes. In this phase the substrate removal rate will be dependent on the substrate concentration as well as the cell mass. This dependence is frequently expressed as a first-order reaction:

$$-\frac{dL}{dt} = mX_aL \quad (5)$$

where m is a constant and X_a is the average cell mass for the period under consideration. Upon integration Eq. 5 becomes:

$$\ln\frac{L_t}{L_o} = -mX_a(t - t_o) \quad (6)$$

where L_t is the concentration of substrate remaining at time t, and L_o is the initial substrate concentration.

Following the exhaustion of the substrate, the cell population decreases as the result of auto-oxidation (endogenous respiration) and cell death.

The following first-order reaction commonly is used to describ. the over-all mechanism of substrate removal in batch systems, instead of Eq. 5:

$$-\frac{dL}{dt} = k_eL \quad (7)$$

which integrated becomes:

$$\ln\frac{L_t}{L_o} = -k_e(t - t_o) \quad (8)$$

or

$$\log_{10}\frac{L_t}{L_o} = -k_{10}(t - t_o) \quad (9)$$

where k_e is the reaction rate coefficient to the base e, and k_{10} is the reaction rate coefficient to the base 10; k_e equals $2.303k_{10}$. Since $L_r = L_o - L_t$, Eq. 9 can be rearranged to take the familiar form of the BOD equation:

$$L_r = L_o\left[1 - 10^{-k_{10}(t - t_o)}\right] \quad (10)$$

The reaction rate coefficients k_e and k_{10} vary with the temperature at which the reaction occurs. The value of k_e (or k_{10}) at a given temperature T, in degrees Centigrade, may be related to that at 20° C by the equation:

$$k_T = k_{20} \cdot \theta^{(T - 20)} \quad 11$$

The temperature coefficient, θ, has values from 1.00 to 1.18 for various aerobic biological waste treatment processes. Variations in θ are due to variations in the type of microorganisms present, the loading level, the mixing intensity and the type of treatment process (ECKENFELDER and ENGLANDE, 1970).

Experimental Methods and Results

A New Brunswick MicroFerm unit was used in all experiments which follow. This apparatus has two reaction vessels which are 2-liter cylindrical glass jars fitted with stainless steel lids and central cores through which coolant is passed to keep the medium at the desired temperature. Air can be passed through a sterile cotton filter into the medium through an orifice in the central core. It enters the liquid near the bottom of the jar and is dispersed by means of a variable-drive assembly which also mixes the medium. Sampling the medium is done by ejecting the desired volume of liquid under pressure. Additions to the medium are made aseptically through an opening in the lid (Fig. 1). The vessels and lids are autoclavable.

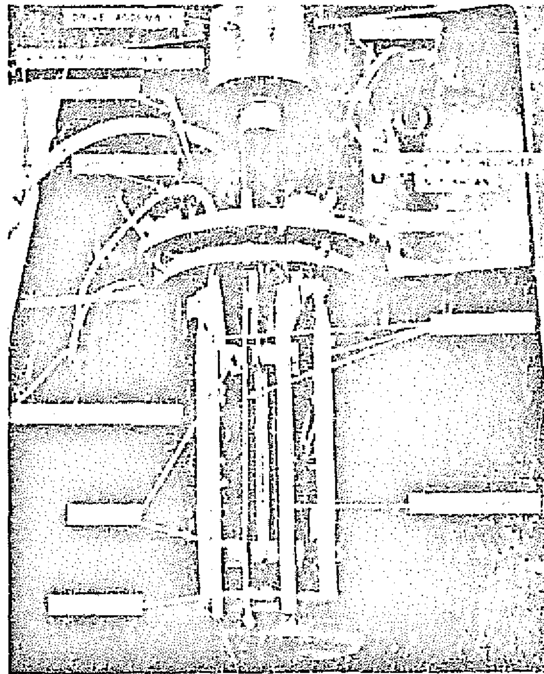

Fig. 1. Schematic of Microferm Apparatus.

The details of the analytical procedures and media used are given in Appendices A and B.

Experiment 1. Activated Sludge Microorganisms

Substrate:	Nutrient Broth – Yeast Extract
Medium:	Phosphate-buffered dilution water; pH 7.
Temperatures:	1° C and 20° C.
Organisms:	A mixed culture of microorganisms from the oxidation ditch of a brush aeration activated sludge treatment plant at College, Alaska. The temperature of the mixed liquor in the oxidation ditch was 1.3° at time of collection.
Rotor Speed:	300 rpm
Air Flow:	1.33 liter/min/liter of mixed liquor

Set-up: 500 mls of activated sludge was mixed with one liter of medium in each of 2 reaction vessels and equilibrated to the desired operation temperatures. One vessel was maintained at 1° C; the other at 20° C. The temperatures of the mixed liquors were recorded continuously. The substrate was added to each system daily for the first 15 days of operation at an average rate of 655 mg COD per day (Phase I); between days 15 and 23 substrate was added to each system on alternate days at an average rate of 346 mg COD per day (Phase II); and from day 24 to day 40 no substrate was added (Phase III). A sample of each mixed liquor was taken for analysis daily for the first week of operation and every other day thereafter. The mixed liquors were analyzed for the amount of organic substrate material remaining by the 15-minute Chemical Oxygen Demand test (COD) and for the level of Mixed Liquor Suspended Solids (MLSS) by filtration through glass fiber filters.

Results: The results of these analyses were used to calculate the rates of production of MLSS and the rates of removal of organic substrate as COD for each system. In Figs. 2 and 3 it can be seen that the rate of suspended solids production was at first greater at 1° C than at 20° C, although ultimately both systems produced the same amount of MLSS. This observation is not surprising, for these organisms were accustomed to an environment where the temperature was near 0° C and organic concentrations were high. The values of the specific growth rate, μ, are 0.50 day^{-1} at 1° C and 0.23 day^{-1} at 20° C; the corresponding generation times are 1.40 days and 2.97 days. What might be considered surprising is the fact that the cell yield constant, a, at 1° C is approximately twice that at 20° C during the logarithmic phase of solids synthesis. For every mg of MLSS produced at 1° C, 2.78 mg COD was metabolized; at 20° C, every 5.10 mg COD metabolized produced [illegible] mg MLSS. At the lower temperature 33% of the COD went for new cell material; 67% went for energy metabolism. At 20° C only 20% of the COD used went for new cell material; 80% went for energy. Thus, it appears that this particular culture of microorganisms can be more efficient at 1° C than at 20° C, at least in terms of cell growth, indicating that they may indeed be psychrophilic. However, from the standpoint of efficiency of organic substrate removal,

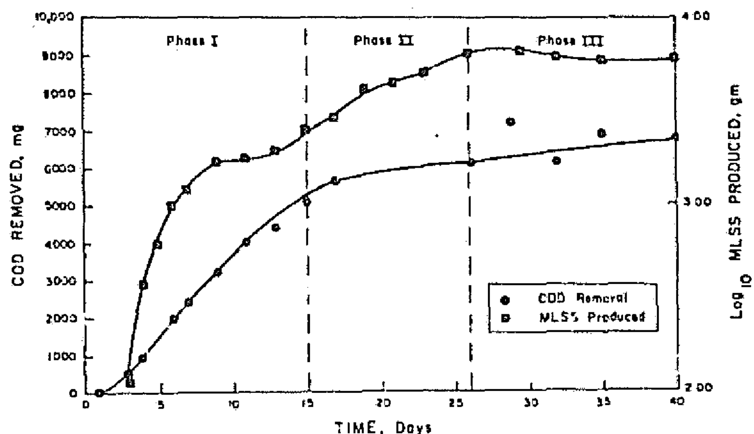

Fig. 2. MLSS Production and COD Removal by Activated Sludge at 1°C.

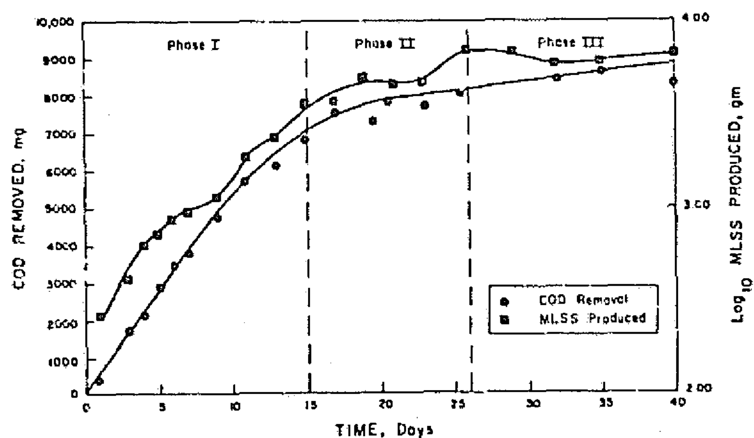

Fig. 3. MLSS Production and COD Removal by Activated Sludge at 20°C.

maintaining this microbial system at 20° C would effect a more rapid removal of COD by converting a greater proportion of it to CO_2 during logarithmic growth. During the logarithmic growth phase, 63% of the COD added to the system was removed at 20° C, in comparison, 44% of the COD added was removed at 1° C.

In Phase II the rate of substrate addition to the systems was reduced by 50%; consequently, the rates of MLSS production diminished. At 1° C the specific growth rate dropped to 0.099 day^{-1}, and at 20° C to 0.070 day^{-1}. COD removal likewise was reduced to 28% at 1° C and 48% at 20° C. Because the MLSS levels did not increase very rapidly during this phase of the experiment, Eq. 6

could be applied to the COD removal data to determine values for the reaction rate coefficient, m. At 1° C, m was found to be 4.7×10^{-5}/mg/day; at 20° C m was 9.6×10^{-5}/mg/day. The higher value of m at 20° C is again a reflection of the more efficient removal of organic material per unit mass of MLSS at this temperature.

When one examines the amounts of MLSS produced in this phase and the amounts of COD removed, one finds that the yield constants are very high; viz., 3.73 mg MLSS produced per mg COD removed at 1° C and 2.94 mg MLSS produced per mg COD removed at 20° C. Perhaps this observation is an indication that initially some of the substrate had been absorbed and stored in the cell mass and was being metabolized at this later time when the ratio of nutrient in the environment to the cell mass was lower.

After the 23rd day of operation no additional substrate was added to either system. MLSS production reached a plateau near 4200-4300 mg for both systems. Following the cessation of substrate addition, what little COD was removed from the mixed liquor at 20° C was compensated for by a decrease in the solids COD and its contribution to the mixed liquor. Only 2% of the COD remaining in the mixed liquor on the 23rd day had been removed from the system at 20° C by the 40th day. Thus, the value for the reaction rate coefficient m at 20° C is low compared with that for the system at 1° C wherein 51% of the remaining COD was metabolized: 2.7×10^{-7}/mg/day as compared with 9.6×10^{-6}/mg/day.

Table 1 is a summary of the three feeding rates and two temperatures used in Experiment No. 1.

TABLE I

Summary of Experiment No. 1—Activated Sludge

Temperature	Growth Phase	μ day^{-1}	Ω days	COD removal efficiency %	m mg^{-1} day^{-1}	a mg MLSS / mg COD
1°C	logarithmic	0.50	1.40	44.	--	0.360
	declining	0.099	7.00	28.	4.7×10^{-5}	3.73
	stationary	--	--	51	9.6×10^{-6}	--
20°C	logarithmic	0.23	2.97	63.	--	0.196
	declining	0.070	9.90	48.	9.6×10^{-5}	2.94
	stationary	--	--	2.	2.7×10^{-7}	--

Supplemental Studies: Shake-flask experiments were performed to determine the optimum pH for the growth of the organisms in the 1° C reaction vessel. The growth rates of this mixed culture were determined at 10 different pH values at 3.5° C in the Nutrient Broth-Yeast Extract medium. Phosphate buffers were used to maintain the pH at a given value from 6.50 to 8.42. So that the substrate concentration did not limit growth, a quite high initial COD concentration of 9000 mg/liter was used. The Optical Density (O.D.) of the culture medium, measured at 600 nm with a

Bausch and Lomb Spectronic 20, was used as an indication of the cell population. This value was recorded approximately every 12 hours over a 120-hour period for each pH regime. Direct microscopic cell counts were occasionally made, and the linear portions of the semi-logarithmic plots prepared with these values coincided with those made with the O.D. values. The growth rates and generation times are given in Table 2. The values for the generation times varied from 7.30 hours to 9.02 hours, the lowest value being that for pH 6.75. All of the growth rates in this experiment were more rapid than those observed in the original reaction vessel at 1° C by a factor of 4 or more. This result is thought to be due more to the higher nutrient: cell mass ratio in the shake-flasks (1800:1 versus 8.87:1 in the Microferm unit) than to the difference in incubation temperatures. The highest cell yield was obtained at pH 7.70, where the generation time was of an intermediate value.

TABLE 2

Growth Rates and Generation Times as a Function of pH for a Mixed Culture of Activated Sludge Organisms in Logarithmic Growth. Temperature = 3.5° C.

	μ		Ω	
pH	hr^{-1}	day^{-1}	hrs	day
6.50	0.0913	2.19	7.59	0.316
6.75	0.0949	2.28	7.30	0.304
7.05	0.0899	2.16	7.71	0.321
7.20	0.0824	1.98	8.41	0.350
7.35	0.0805	1.93	8.61	0.359
7.45	0.0855	2.05	8.11	0.338
7.70	0.0868	2.08	7.99	0.333
7.80	0.0768	1.84	9.02	0.376
8.00	0.0834	2.00	8.31	0.346
8.42	0.0780	1.87	8.89	0.370

A second shake-flask experiment with these same microorganisms was performed to measure the effect of the initial substrate concentration on cell growth. The Nutrient Broth-Yeast Extract medium buffered at pH 7.70 was used in conjunction with an incubation temperature of 3.5° C. Cell population levels were measured by the Optical Density at 600 nm of the suspensions. The specific growth rate was determined at initial substrate concentrations from 37 to 800 mg COD/liter. The results are shown in Fig. 4. A general trend of increasing growth rate to a maximum of about 0.09 to 0.10 hr^{-1} with increasing substrate concentration is noted. The substrate concentration at which the growth rate is one-half maximum is around 50 mg COD/liter.

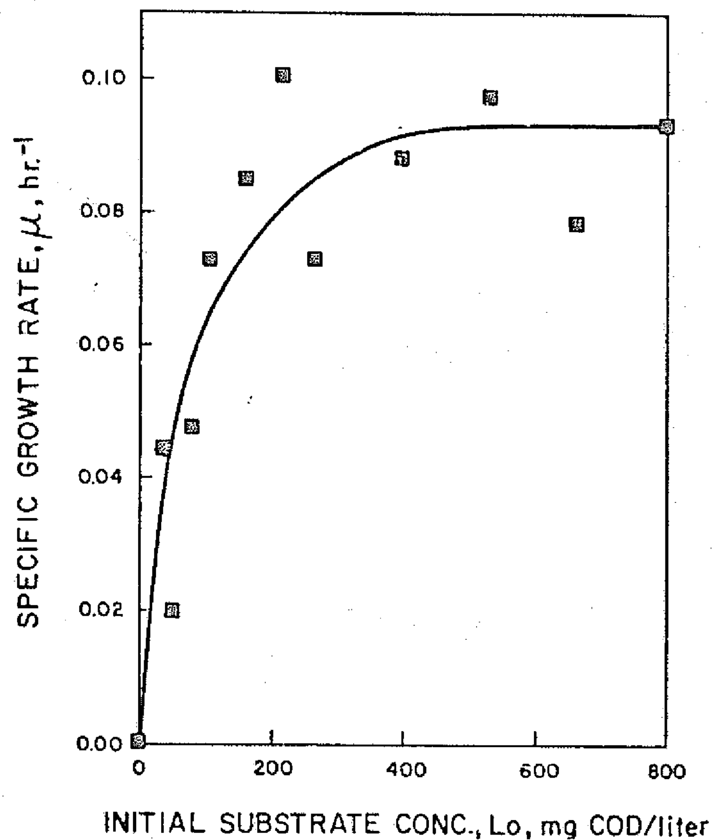

Fig. 4. Growth Rate at 3.5°C and pH 7.70 as a Function of Initial Substrate Concentration.

Experiment 2. Sub-arctic Streambed Microorganisms

Substrate: Nutrient Broth – Yeast Extract

Medium: Natural stream water

Temperatures: 2.5° C and 17.5° C

Organisms: Mud from the bed of Riley Creek in Mt. McKinley National Park was used as a source of cold-acclimated microorganisms. The samples were collected at the end of May, at which time the stream temperature was 2° C; its pH was 5.5.

Rotor speed: 200 rpm

Air flow: 1.33 liters/min/liter of mixed liquor

Set-up: In this experiment an aliquot of the Nutrient Broth-Yeast Extract substrate equivalent to 2000 mg COD was added to each of the two reaction vessels on the first day only. Instead of suspending the organisms in phosphate-buffered dilution water, the natural stream water itself was used. The disappearance of substrate was followed daily as the disappearance of COD in the mixed liquor. The 2-hour COD method was used. Mixed liquor suspended solids were measured daily.

Results: Fig. 5 shows the rates at which suspended solids were produced at 2.5° C and 17.5° C. While growth was begun within 24 hours at 17.5° C, there was a lag period of 3 days at 2.5° C. The growth rate in the logarithmic phase was much more rapid at 17.5° C: 1.69 day^{-1} compared to 0.28 day^{-1} at 2.5° C. MLSS reached a maximum of 444 mg/liter in the 17.5° C reaction vessel

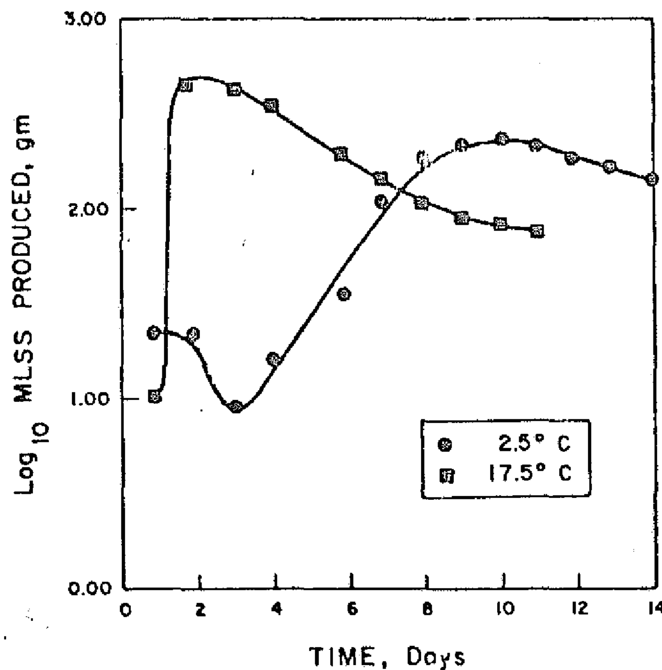

Fig. 5. MLSS Production at 2.5 and 17.5°C.

on the second day and thereafter declined. A maximum of 236 mg/liter MLSS was reached at 2.5° C on the tenth day, and thereafter the MLSS level declined. The cell yield constants during logarithmic growth were 0.169 mg MLSS/mg COD at 2.5° C and 0.804 mg MLSS/mg COD at 17.5° C. During this growth phase 70% of the COD removed from the mixed liquor in the 2.5° C reaction vessel was used for energy metabolism; only 30% went for new cell material. At 17.5° C, however, only 37% of the COD removed was used for energy; the remaining 63% went into cell material.

The COD data were used to calculate the reaction rate coefficients, k_e and k_{10}, of Eqs. 8 and 9 for the overall removal process. The values of k_e at 2.5° C and 17.5° C are 0.087 day^{-1} and 0.185 day^{-1}, respectively. COD removal was more rapid at the higher temperature (Fig. 6). In eleven days 87% of the initial added COD had been removed at 17.5° C; in comparison 72% of the initial added COD was removed at 2.5° C in 16 days.

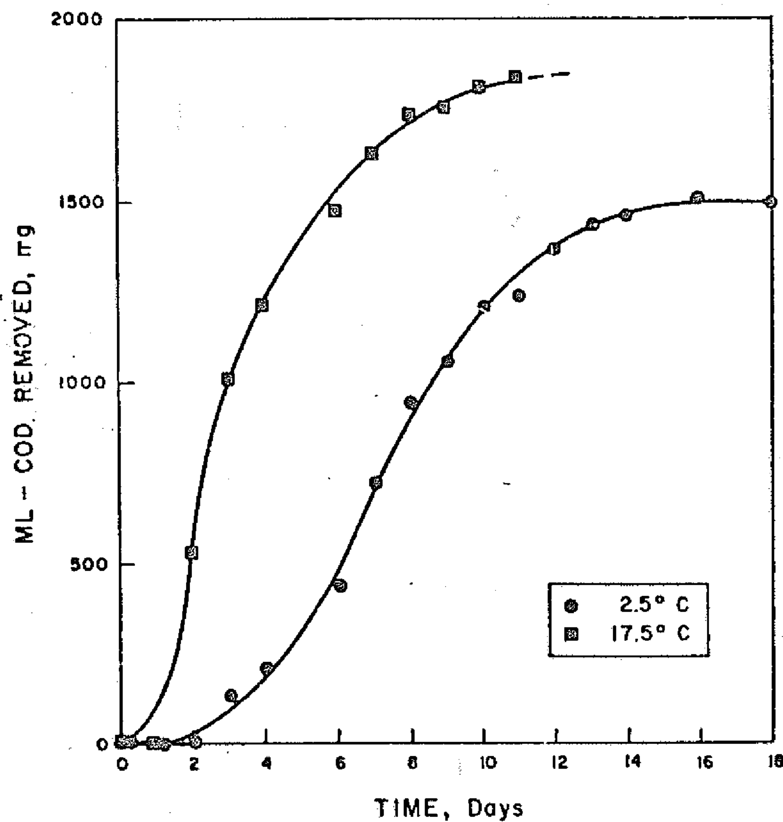

Fig. 6. Mixed Liquor COD Removal at 2.5° C and 17.5°C.

Table 3 is a summary of the experimental work done with the streambed organisms found in Riley Creek, Mt. McKinley National Park.

TABLE 3

Summary of Experiment No. 2—Sub-arctic Streambed Microorganisms

Temperature	Growth Phase	μ day^{-1}	Ω days	a $\frac{\text{mg MLSS}}{\text{mg COD}}$	k_e day^{-1}	k_{10} day^{-1}	COD removal efficiency %
2.5°C	logarithmic	0.28	2.48	0.169	--	--	--
	overall	--	--	--	0.087	0.038	72.
17.5°C	logarithmic	1.69	0.41	0.804	--	--	--
	overall	--	--	--	0.185	0.080	87.

Experiment 3. Subarctic Soil Microorganisms I

Substrate: Glucose

Medium: Mineral Salts; pH: 7.07

Temperature: 4.5 ~ 1.5° C

Organisms: A mixed culture of microorganisms from soil near Lake Chandalar, Alaska (Lat. 67½° N)

Rotor speed: 350 rpm

Air flow: 1.33 liter/min/liter of mixed liquor

Set-up: 1500 mls of the glucose-mineral salts medium was added to a sterile reaction vessel and equilibrated to temperature, at which time 5 mls of a suspension of soil in medium was added to the vessel. After the suspension was mixed with the medium, a sample was taken for analysis. The Mixed Liquor Suspended Solids (MLSS), the Chemical Oxygen Demand of the mixed liquor, (ML-COD), the Chemical Oxygen Demand of the filtrate from the MLSS determination, (F-COD), and the pH of the mixed liquor were monitored for 25 days. In order to account for wasting, a record was kept of the amounts of sample taken also. No additional substrate was added to the system until the 20th day, after which time substrate was added to the system daily. Initially the ML-COD in the system was 18,580 mg; the initial MLSS level was 294 mg.

Results: The MLSS level fluctuated for the first few days until we were able to correct a failure in the temperature regulating system. During this time ice crystals formed in the mixed liquor, incurring cell death and a drop in the MLSS. The cells apparently lysed and contributed to an increase in the ML-COD; hence, the negative COD removal shown in Fig. 7. On the fifth day the medium was re-inoculated from a flask culture of the soil organisms (black arrow). Four days later the mixed liquor, which up to this time had remained clear, was now turbid, and cell growth was in the logarithmic phase. The doubling time for the MLSS was 1.90 days in this phase; μ equaled 0.526 day^{-1}. The cell yield constant was 0.095 mg MLSS/mg COD. Shortly thereafter, the ML-COD began to be removed linearly at a rate of 414 mg per day. Between the 11th and 18th days the MLSS level did not change appreciably, so that Eq. 6 could be used to calculate a value for m; namely 2.02×10^{-4}/mg MLSS/day. After the 14th day MLSS production entered into a decline, which was reflected in a drop in COD removal 3 days later. On the 20th day, when it appeared that COD removal had reached a plateau, additional substrate (510 mg COD) was added to the system (white arrow). This addition caused a sharp increase in MLSS, accompanied by additional COD removal. Although 510 mg COD in the form of glucose was added to the mixed liquor daily after the 20th day, it was not sufficient to alter the trend in MLSS decline. By the 25th day COD removal had reached another plateau and the level of COD in the mixed liquor was increasing as a result of the daily additions of glucose.

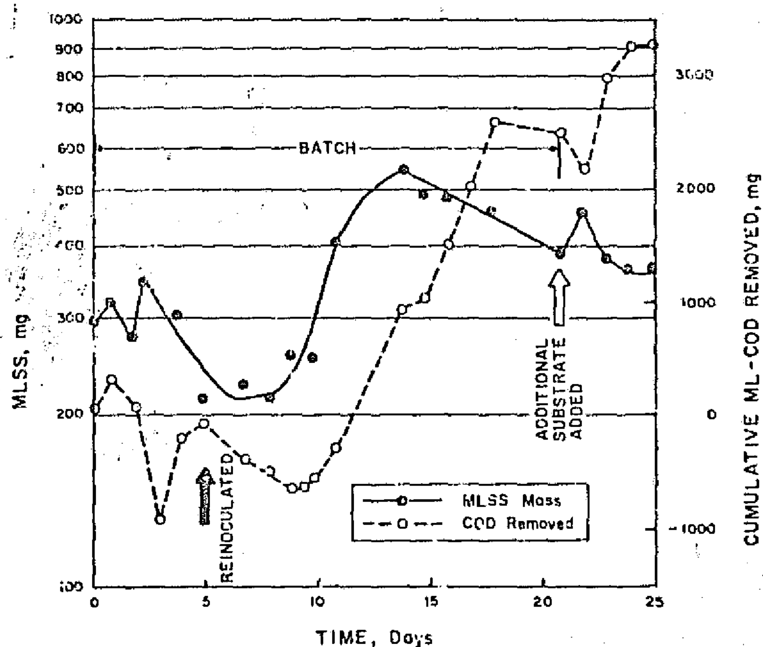

Fig. 7. MLSS Levels and COD Removals by Subarctic Soil Microorganisms Grown on Glucose Medium at 4.5°C.

The data for COD removal from the time of re-inoculation of the medium until the first plateau for COD removal was reached were used to calculate the reaction rate coefficients k_e and k_{10}. These were 0.068 day^{-1} and 0.030 day^{-1}, respectively. The COD removal efficiency for the batch operation was 76%.

Table 4 summarizes the experimental work done with soil microorganisms from a location north of the Arctic Circle.

TABLE 4

Summary of Experiment No. 3—Sub-arctic Soil Microorganisms

Growth Phase	μ day^{-1}	Ω days	a $\frac{mg\ MLSS}{mg\ COD}$	m $mg^{-1}\ day^{-1}$	k_e day^{-1}	k_{10} day^{-1}	COD removal efficiency %
logarithmic	0.526	1.90	0.095	--	--	--	18.
stationary	--	--	--	2.02×10^{-4}	--	--	49.
overall	--	–	--	–	0.068	0.030	76.

Experiment 4. Subarctic Soil Microorganisms II

Substrate: Glucose

Medium: Mineral salts; pH 7.07

Temperature: 4.5 – 1.5° C

Organisms: A mixed culture of microorganisms from soil near Lake Chandalar, Alaska (Lat. 67½° N)

Rotor speed: 350 rpm

Air flow: 1.33 liter/min/liter mixed liquor

Set-up: This experiment was a continuation of Experiment 3 with the exception that additional substrate was added to the system daily, in variable amounts, in order to determine how the COD removal efficiency varied with the loading rate. Analytical procedures were the same as in Experiment 3, with the same variables monitored. Initially the feed rate was 466 mg COD per day, or approximately 0.7 mg COD added/mg MLSS/day. Because the COD level in the system was building up too rapidly at this rate, on the tenth day the feed rate was reduced to 117 mg COD per

day, or approximately 0.15 to 0.20 mg COD added/mg MLSS/day. On the 26th day the feed rate was increased to 233 mg COD/day (about 0.6 mg COD added/mg MLSS/day), and on the 40th day it was increased to 352 mg COD/day (about 1 mg COD added/mg MLSS/day).

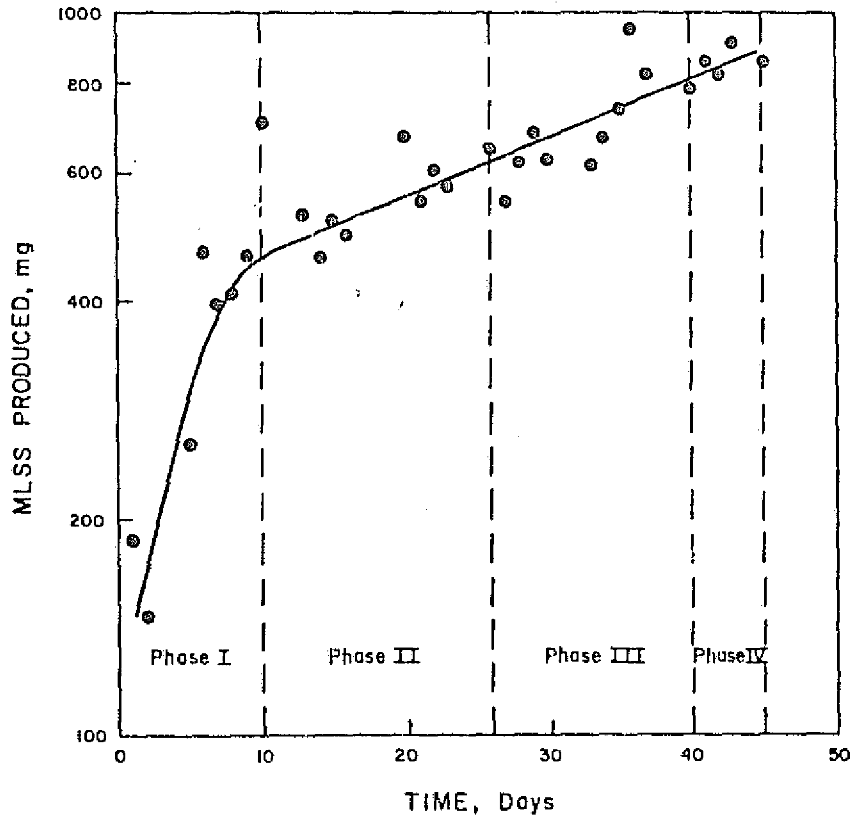

Fig. 8. MLSS Production by Subarctic Soil Microorganisms Grown on Glucose Medium at 4.5°C.

Results: Figs. 8 and 9 show the rates of MLSS production, ML-COD removal, and F-COD removal. MLSS production was most rapid in the first phase of the experiment; viz., 64 mg/day, with a doubling time of 4.8 days. ML-COD removal averaged 0.25 mg COD/mg MLSS/day; F-COD 0.30 mg COD/mg MLSS/day. (MLSS values used in these calculations were average values for the period in question). After the feed rate was reduced on the 10th day, the rate of MLSS production declined to about 12 mg/day, a doubling time of 36 days. More MLSS was wasted daily than was

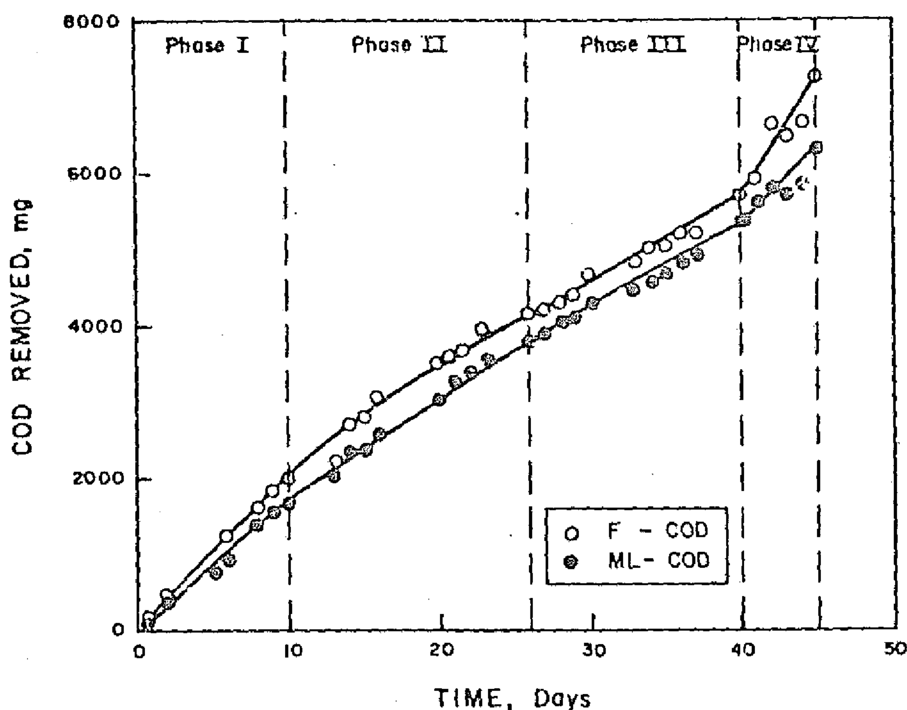

Fig. 9. ML-COD and F-COD Removal Rates by Subarctic Soil Microorganisms Grown on Glucose at 4.5°C.

produced, so that the level of MLSS in the system continually decreased (Fig. 10). Although the feed rate had been reduced in the second phase of the experiment, the rates of COD removal were just slightly lower than the rates when the feed rate was almost 5 times higher. Both F-COD and ML-COD were removed at the rate of 0.21 mg COD/mg MLSS/day. This is largely explainable by the fact that the actual loading, in terms of the average F-COD level in the system, was 1.65 mg COD/mg MLSS/day as compared to 2.55 mg COD/mg MLSS/day in the first phase. Apparently no COD was being converted into solids at this time. In the third phase of the experiment, the feed rate was increased to 0.6 mg COD added/mg MLSS/day whereas the actual loading rate was 2.06 mg COD/mg MLSS/day because of the drop in the COD level in the system due to the activity in phase II. The COD removal rates remained fairly close to what they had been: 0.24 mg F-COD removed/mg MLSS/day and 0.25 mg ML-COD removed/mg MLSS/day. In the last part of the experiment the feed rate was increased to approximately 1 mg COD added/mg MLSS/day and the actual loading rate was almost doubled, 3.89 mg COD/mg MLSS/day and F-COD removal was 0.80 mg COD/mg MLSS/day. A substantial portion of this removal of COD from the filtrate was due to incorporation into the solids. These data are summarized below in Table 5.

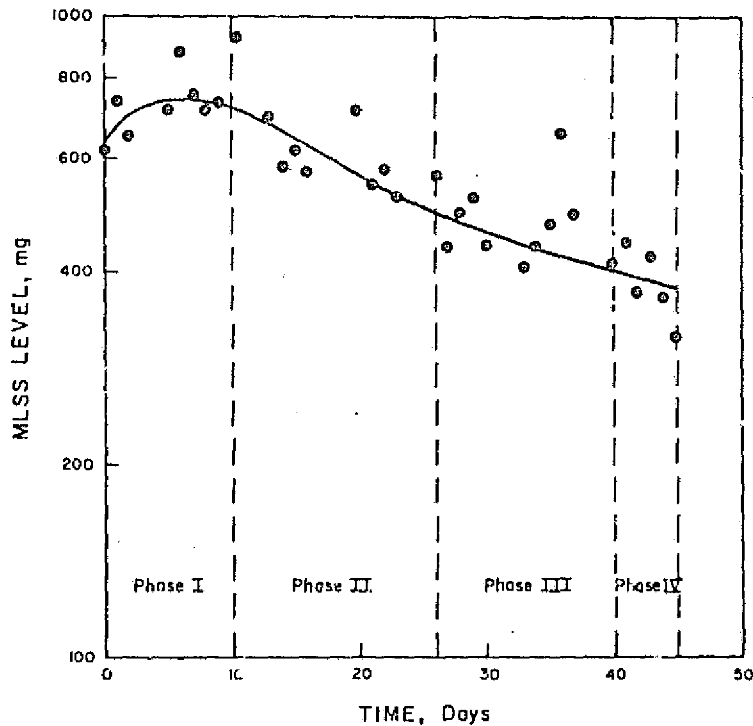

Fig. 10. MLSS Level versus Time for Subarctic Soil Microorganisms Grown at 4.5°C in a Microferm.

TABLE 5

Summary of Experiment No. 4 - Sub-arctic Soil Microorganisms

Phase	Feed Rate mg COD/mg MLSS/day	Loading Rate	COD Removal mg COD/mg MLSS/day ML-COD	F-COD	MLSS Production μ day^{-1}	Ω days
I	0.7	2.55	0.25	0.30	0.144	4.8
II	0.15 - 0.20	1.65	0.21	0.21	0.019	36.
III	0.6	2.06	0.25	0.24	0.019	36.
IV	1.	3.89	0.48	0.80	0.019	36.

The values tabulated above are averages over each phase of operation. There appears to be no relation between the feed or loading rate and the rate of COD removal. The feed rates, actual loading rates, and COD removal rates were calculated on a daily basis and compared. Again no relationship was discernible between the COD removal rates and the feed or loading rates.

Reaction rate coefficients based on Eqs. 8 and 9 were calculated from the daily F-COD removals (Table 6). The values for k_e varied erratically from a low of 0.055 day^{-1} to a high of 0.786 day^{-1} and exhibited no dependence on either the feed rate or the loading rate.

TABLE 6

Experiment No. 4 — Reaction Rate Coefficients for F-COD Removal at 4.5°C

Loading Rate mg COD/mg MLSS/day	Feed Rate mg COD/mg MLSS/day	k_e day^{-1}	k_{10} day^{-1}
0.85	0.072	0.130	0.056
0.92	0.222	0.246	0.107
0.96	0.198	0.426	0.185
1.15	0.181	0.332	0.144
1.28	0.458	0.151	0.066
1.41	0.052	0.165	0.072
1.45	0.491	0.128	0.056
1.64	0.524	0.163	0.071
1.77	0.724	0.173	0.075
1.87	0.701	0.234	0.102
1.88	0.173	0.089	0.039
2.03	0.217	0.245	0.107
2.03	0.621	0.055	0.024
2.09	0.131	0.384	0.167
2.11	0.588	0.265	0.115
2.17	0.501	0.359	0.157
2.17	0.572	0.248	0.108
2.20	0.170	0.071	0.031
2.35	0.234	0.121	0.052
2.45	0.178	0.000	0.000
2.58	0	0.074	0.032
2.61	0.152	0.369	0.160
2.75	0.956	0.184	0.080
2.85	0.724	0.125	0.055
3.07	1.23	0.000	0.000
3.55	0.178	0.149	0.065
3.65	0.927	0.786	0.342
4.58	0.903	0.099	0.043
5.74	1.18	0.490	0.213

An experiment of this type would probably have better been done with a continuous culture system in which the cell population could be maintained at some constant value while the effects of various loading rates are measured. In this experiment the fluctuations in the MLSS level and its steady decline in the last three phases of operation probably obscured any relationship between COD removal and the loading rate.

Experiment 5. A Soil Bacterium in Pure Culture I

Substrate:	Glucose
Medium:	Mineral salts and vitamins; pH 7.0
Temperature:	15° C
Organisms:	A pure culture of LC-1, a bacterium isolated from soil near Lake Chandalar, Alaska (Lat. 67° N)
Rotor speed:	250 rpm
Air flow:	1.33 liter/min/liter mixed liquor

Set-up: The purpose of this experiment was to measure the growth rate and cell yield of a pure culture of bacterium growing in a simple medium and to compare the rate of COD die-away with the rate of disappearance of glucose from the medium. A simple glucose-mineral salts+vitamins medium, from which magnesium had inadvertently been omitted, was equilibrated to 15° C and then inoculated with cells of LC-1 suspended in sterile phosphate-buffered dilution water so that the initial cell population was 5.0×10^5 cells per ml.

The die-away of glucose was followed by measuring the glucose in the filtrate from the suspended solids determination. Worthington's Glucostat Reagent, which is specific for β-D-glucose, was used. The 2-hour COD method was used to follow the die-away of oxygen-demanding substances in the filtrate from the suspended solids determination.

The increase in the cell population was measured by direct microscopic cell counts, by the Optical Density (O.D.) at 600 nm of the mixed liquor, and by the mixed liquor suspended solids. The latter measurement was made using 0.45 μ pore size membrane filters which had been washed in distilled water and dried at 103° C for one hour.

In addition, the pH of the mixed liquor, its temperature, and the volume of sample withdrawn for analysis were recorded. The experiment was of 19 days duration.

Results: The glucose was completely removed from the system within 8 days; the die-away of COD in the filtrate was less rapid (Fig. 11). As there was still considerable oxygen-demanding material in the filtrate after all the glucose had disappeared, the filtrate was analyzed for carbohydrate (CHO) by the colorimetric method of DU BOIS et al. (1956), and for volatile organic acids by a distillation and titration with Na_2CO_3. After 19 days essentially all the

carbohydrate in the filtrate was gone, but there remained an appreciable F-COD concentration of 340 mg/liter, to which the volatile organic acids contributed only 9 mg COD/liter.

The pH of the mixed liquor steadily declined from 7.02 to 6.15 as the glucose was being metabolized. Once the glucose had been removed from the system, the pH gradually returned to its initial value (Fig. 11).

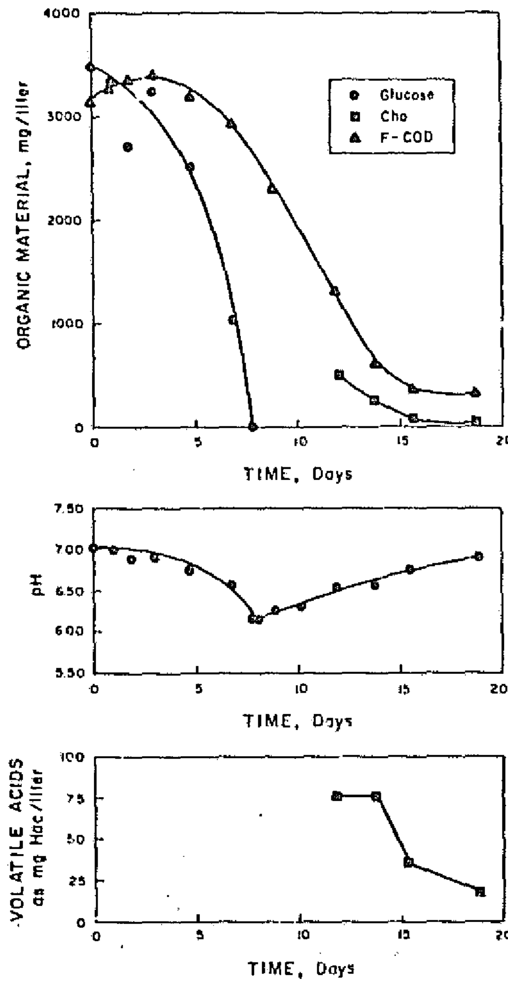

Fig. 11. Organic Matter (as glucose, carbohydrate, F-COD, and volatile acids) and pH of a Pure Culture Subarctic Organism Grown on Glucose at 15°C.

The increase in MLSS and cell counts paralleled the removal of glucose; the growth cycle was definitely in the declining phase on the eighth day when the glucose supply was depleted. The maximum growth rates, in day^{-1}, were estimated from the graphs in Fig. 12 to be 0.83, 0.62, and 2.1 as measured by O.D. @ 600 nm, MLSS, and cell counts, respectively. The corresponding generation times are 20 hrs., 27 hrs., and 8 hrs.

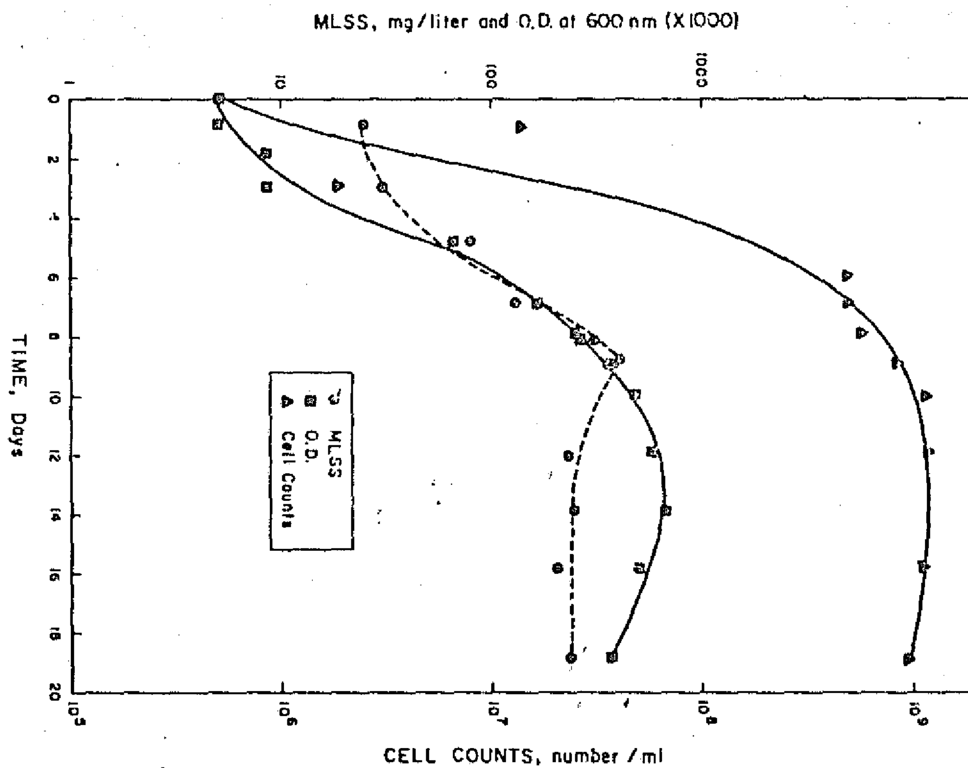

Fig. 12. Growth Curves, as MLSS Concentration, Cell Counts, and Opitical Density of a Pure Culture Subarctic Organism Grown on Glucose at 15°C.

Values for the cell yield constant were calculated on the basis of glucose removed (0.071 mg MLSS/mg glucose) and F-COD removed (0.081 mg MLSS/mg F-COD).

The reaction rate coefficient for F-COD removal when the MLSS level is stationary, m, was found to be 8.5 x 10^{-4} per mg MLSS per day. The reaction rate coefficient for the overall processes of glucose and F-COD removal k_e, was calculated to be 1.44 day^{-1} and 0.121 day^{-1}, respectively. The corresponding values for k_{10} are 0.626 day^{-1} and 0.053 day^{-1}.

The data for this experiment is summarized in Table 7

TABLE 7

Summary of Experiment No. 5 – Subarctic Soil Bacterium in Pure Culture at 15° C

Parameter	Basis	Units	GROWTH PHASE logarithmic	stationary	overall process
μ	MLSS	day^{-1}	0.62	--------	--------
Ω	MLSS	hours	27.	--------	--------
m	$MLSS^{-1}day^{-1}$ (F-COD)	$mg^{-1}day^{-1}$	--------	8.5 x 10^{-4}	--------
a	mg MLSS / mg glucose	------------	--------	--------	0.071
a	mg MLSS / mg F-COD	------------	--------	--------	0.081
k_e	glucose	day^{-1}	--------	--------	1.44
	F-COD	day^{-1}	--------	--------	0.121
k_{10}	glucose	day^{-1}	--------	--------	0.626
	F-COD	day^{-1}	--------	--------	0.053

Experiment 6. A Soil Bacterium in Pure Culture II

Substrate: Glucose

Medium: Mineral salts and vitamins; pH 7.0

Temperature: 15° C

Organisms:	A pure culture of LC-1, a bacterium isolated from soil near Lake Chandalar, Alaska (Lat 67½° N)
Rotor speed:	250 rpm
Air flow:	1.33 liter/min/liter of mixed liquor

Set-up: The same as for Experiment 5.

Results: We had intended to repeat Experiment 5 in order to obtain more data in the initial stage of the experiment; however, for some unknown reason, cell growth and glucose removal were much more rapid. (Magnesium was again omitted from the medium.) The glucose was completely removed from the system within 52 hours as compared with 8 days in Experiment 5 (Fig. 13). Carbohydrate was also absent in the filtrate at 52 hours. The COD of the filtrate decreased rapidly in the same period but thereafter diminished slowly. A residual of 160 mg COD/liter was remaining in the filtrate at 150 hours. There was very little in the way of volatile organic acids in the filtrate; only 2.1 mg/liter as acetic acid was detected at 98 hours.

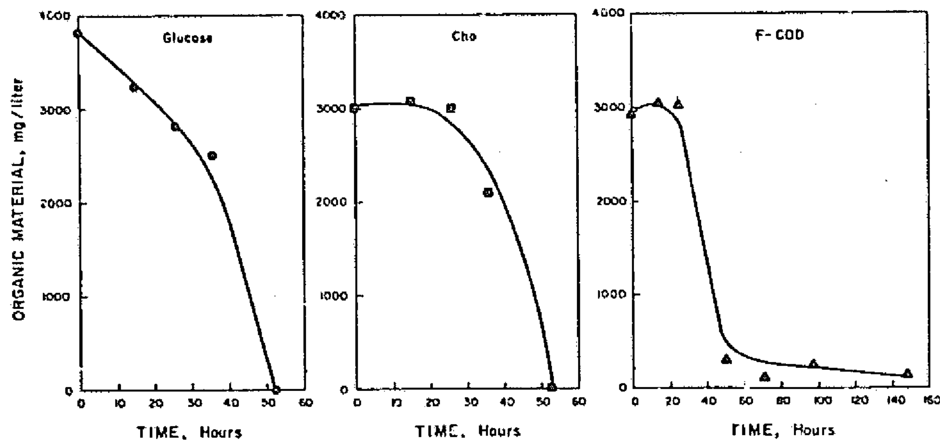

Fig. 13. Organic Matter (as Glucose, Carbohydrate, and F-COD) versus Time of a Pure Culture Subarctic Organism Grown on Glucose at 15°C.

As in Experiment 5, cell and solids growth paralleled glucose removal, reaching a maximum at the time the glucose was depleted (Fig. 14). And, in fact, the MLSS level at the maximum was twice that in the first experiment even though the initial glucose concentration and cell population had been the same. Thus, the cell yield constant was 0.245 mg MLSS/mg glucose removed and 0.335 mg MLSS/mg F-COD removed. The logarithmic growth rates were 0.32 hr^{-1}, 0.17 hr^{-1}, and 0.12 hr^{-1} as measured by direct cell counts, O.D. @ 600 nm, and MLSS, respectively.

The corresponding generation times were 2.2 hrs., 4.0 hrs., and 6.0 hrs.

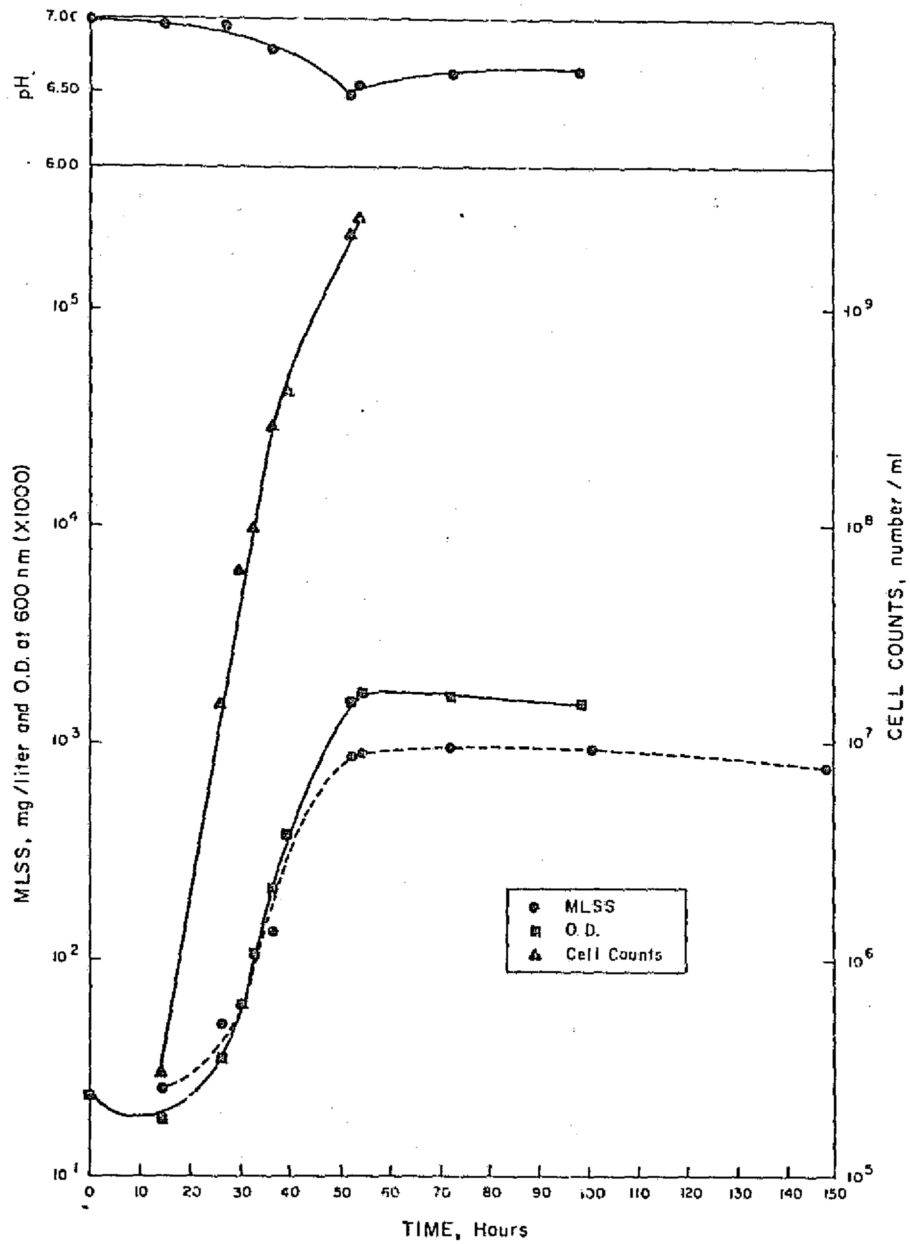

Fig. 14. Growth Curves, as MLSS Concentration, Cell Counts, and Opitical Density of a Pure Culture Subarctic Organism Grown on Glucose at 15°C.

The value for the stationary phase reaction rate coefficient for F-COD removal was 8.11×10^{-6} mg $MLSS^{-1}$ hr^{-1}.

Values for the overall reaction rate coefficient, k_e, were calculated for glucose removal, carbohydrate removal, and F-COD removal. These were 2.86 day^{-1}, 1.78 day^{-1}, and 0.47 day^{-1}, respectively. Corresponding values for k_{10} were 1.24 day^{-1}, 0.77 day^{-1}, and 0.20 day^{-1}.

The results of Experiment No. 6 are summarized in Table 8.

TABLE 8

Summary of Experiment No. 6 – Subarctic Soil Bacterium in Pure Culture at 15° C

Parameter	Basis	Units	GROWTH PHASE		
			logarithmic	stationary	overall process
μ	MLSS	hr^{-1}	0.12	--------	--------
Ω	MLSS	hours	6.0	--------	--------
a	mg MLSS / mg glucose	--------	-------	0.245	--------
a	mg MLSS / mg F-COD	--------	-------	0.335	--------
m	$mgMLSS^{-1}hr^{-1}$ (F-COD)	$mg^{-1}hr^{-1}$	------	8.11×10^{-6}	--------
m	$mgMLSS^{-1}day^{-1}$ (F-COD)	$mg^{-1}day^{-1}$	------	1.95×10^{-4}	--------
k_e	glucose	day^{-1}	------	--------	2.86
	carbohydrate		------	--------	1.78
	F-COD		------	--------	0.47
k_{10}	glucose	day^{-1}	------	--------	1.24
	carbohydrate		------	--------	0.77
	F-COD		------	--------	0.20

CONCLUSIONS

In these experiments we exposed microorganisms from different environments in sub-arctic Alaska to high concentrations of organic materials in order to determine how well they could metabolize these substrates at low temperatures. The low temperatures of incubation did not seem to hinder cell growth nor the efficiency of substrate removal; nevertheless, no direct relationship could be observed between the temperature of incubation and the rate of cell growth, the cell yield constant, or the overall reaction rate constant (Table 9). It is difficult to compare the data from different systems because of the many variables involved. In particular, it was not possible to control the population dynamics. We did not know what proportion of a mixed culture was capable of utilizing the substrates provided at the temperatures used. Nor did we know what proportion of the population was psychrophilic. However, all of the experiments were conducted within the temperature regime of the psychrophile, and there was an indication that the initial food: microorganism ratio (F/M) had more influence on the cell growth rate than did the incubation temperature within this range.

TABLE 9

Summary of Experiments 1 through 6—Cell Growth and Substrate Removal Data in Batch Systems

Source of Microorganisms	Reaction System	Substrate	Temp °C	μ day^{-1}	a $\frac{\text{mg MLSS}}{\text{mg COD}}$	k_e day^{-1}	initial F/M $\frac{\text{mg COD}}{\text{mg MLSS}}$
Activated Sludge	Microferm	NB-YE	1°	0.50	0.360	-	8.9
Activated Sludge	Microferm	NB-YE	20°	0.23	0.196	-	1.8
Activated Sludge	Shake-flask	NB-YE	3.5°	1.07	-	-	7.4
"	"	"	"	0.48	-	-	11.
"	"	"	"	1.14	-	-	16.
"	"	"	"	1.75	-	-	21.
"	"	"	"	2.05	-	-	32.
"	"	"	"	2.48	-	-	43.
"	"	"	"	1.75	-	-	53.
"	"	"	"	2.12	-	-	80.
"	"	"	"	2.34	-	-	107.
"	"	"	"	1.87	-	-	133.
"	"	"	"	2.24	-	-	160.
"	"	"	"	2.28	-	-	1800.
Streambed	Microferm	NB-YE	2.5°	0.28	0.169	0.087	44.
Streambed	Microferm	NB-YE	17.5°	1.09	0.804	0.185	22.
Soil-mixed	Microferm	Glucose	4.5°	0.53	0.095	0.068	63.
Soil-pure	Microferm	Glucose	15°	0.62	0.081	0.121	126.
Soil-pure	Microferm	Glucose	15°	2.88	0.335	0.47	126.

STREAM ASSIMILATION STUDIES

These studies were conducted in order to compare the reaction rate coefficients for oxygen consumption in sub-arctic waters alone and when sewage is added to them. Water samples were collected from the Chena River above and below the sources of pollution from Ft. Wainwright and the City of Fairbanks (1, 2); from Moose Creek upstream from Fairbanks but downstream from Eielson Air Force Base (3); and from Goldstream Creek (4). (See Fig. 15.) Samples were taken in early October, 1968, before freeze-up, when the water temperatures were 0-1° C, and again in February and March, 1969, when the waters were covered with ice.

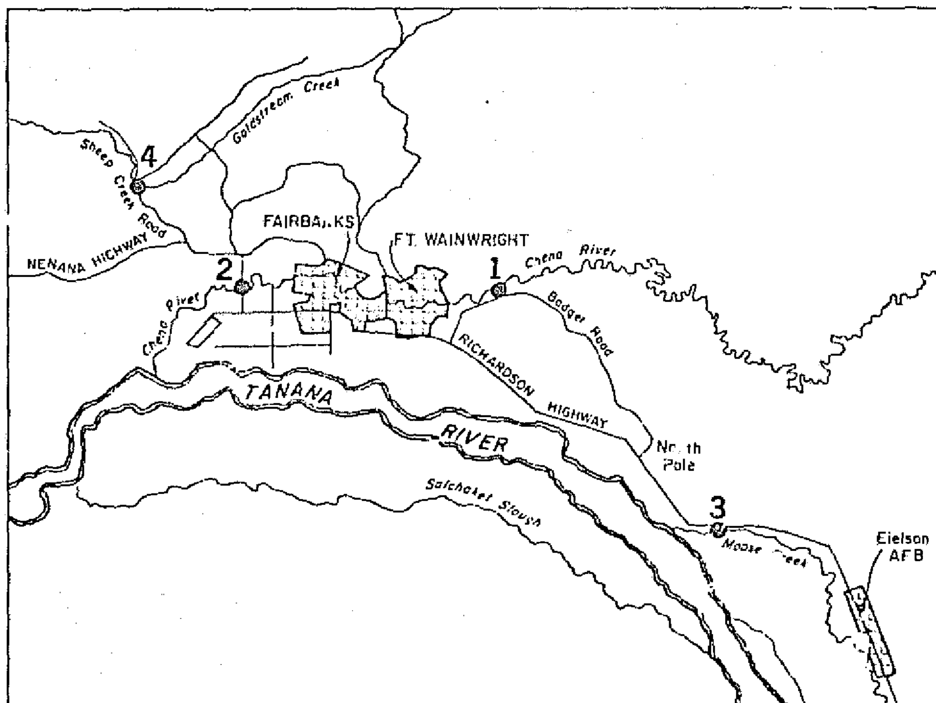

Fig. 15. Fairbanks Area Showing Sampling Sites Used in Stream Assimilation Studies.

The natural waters were siphoned into 300-ml BOD bottles and held at 4° C ± 1° C for 30-36 days, during which time the decrease in dissolved oxygen content was followed. Duplicate samples of each water were artificially "polluted" with raw municipal sewage from the City of Fairbanks treatment plant which had been filtered through Whatman GF/C glass fiber filter paper. The ratio of sewage to water was 3:100. These samples were also incubated at 4° C ± 1° C in 300-ml BOD bottles and the disappearance of dissolved oxygen followed. The dissolved oxygen concentrations

were measured according to the azide modification of the Winkler method, STANDARD METHODS, 12th Ed. The initial dissolved oxygen levels in the natural waters are given in Table 10.

The Thomas graphical method (THOMAS, 1950) for determining kinetic constants for the process in which a biochemical oxygen demand is exerted over a period of time was applied to the dissolved oxygen consumption data. This method is based on the equation:

$$Y = L_o(1 - e^{-kt}) \qquad 12$$

where Y is the Biochemical Oxygen Demand (BOD) exerted, in mg/liter, at some time, t, in days, and L_o is the ultimate BOD, in mg/liter. The Thomas method uses the approximation:

$$1 - e^{-kt} \cong kt(1 + kt/6)^{-3} \qquad 13$$

so that Equation 1 may be re-arranged to give:

$$(t/Y)^{1/3} = \frac{1}{(kL_o)^{1/3}} + \frac{k^{2/3}t}{6L_o^{1/3}} \qquad 14$$

Thus, a graph of $(t/Y)^{1/3}$ versus t will be a straight line, the slope and intercept of which will yield values for the ultimate BOD, L_o, and the reaction rate coefficient, k_e. These values are shown in Tables 11 and 12 The data for Goldstream Creek under ice-cover were too erratic to allow calculation to be made. In each case the reaction rate coefficient for the natural waters alone was lower in the sample taken under ice-cover than that taken before freeze-up. Perhaps this is attributable to the somewhat lower initial dissolved oxygen concentrations in the waters under ice-cover. According to Eckenfelder and O'Connor (1961), k_e for municipal sewage BOD at 20° C ranges from 0.10 to 0.30 day^{-1}. From their equation:

$$k_T = k_{20} \cdot 1.047^{(T - 20)} \qquad 15$$

relating the reaction rate coefficient at some temperature, T, in degrees Centigrade, to that at 20° C, k_e at 4° C should be between 0.05 and 0.15 day^{-1}. All of the values calculated for k_e for municipal sewage in these natural waters at 4° C do conform to this relationship. Thus, it would seem that there is no special oxygen requirement at this temperature for the decomposition of municipal sewage in these waters. The rate of oxygen consumption is no different from that which would be expected in temperate-climate waters held at this temperature. The rates at which oxygen demand was exerted were fairly comparable whether sewage was added to a water or not.

Our results differ from those of GORDON, 1970, who performed experiments similar to ours. He measured dissolved oxygen depletion in Chena River water, (collected December, 1968, above all sources of pollution), to which a primary effluent from the Fairbanks sewage treatment plant had been added in a dilution 1:3 (effluent to river water). A duplicate experiment was done with a

TABLE 10

Initial Dissolved Oxygen Levels in Natural Waters

Sample	Dissolved Oxygen mg/liter
before freeze-up:	
Chena River - above Fairbanks	13.2
Chena River - below Fairbanks	13.2
Moose Creek	12.1
Goldstream Creek	12.2
under ice-cover:	
Chena River - above Fairbanks	10.5
Chena River - below Fairbanks	8.83
Moose Creek	11.0
Goldstream Creek	1.67

TABLE 11

Ultimate BOD, L_o, of Natural Waters with and without Municipal Sewage Added

Sample	L_o, mg/liter with sewage	L_o, mg/liter without sewage
before freeze-up:		
Goldstream Creek	137.	0.80
Chena River - above Fairbanks	137.	0.80
Chena River - below Fairbanks	165.	2.00
Moose Creek	334.	6.33
under ice-cover:		
Chena River - above Fairbanks	121.	1.38
Chena River - below Fairbanks	241.	5.58
Moose Creek	128.	2.90

TABLE 12

The Reaction Rate Coefficient for Biochemical Oxygen Demand in Natural Waters with and without Municipal Sewage Added at 4°C

Sample	K_e, day^{-1}	
	with sewage	without sewage
before freeze-up:		
Goldstream Creek	0.106	0.030
Chena River - above Fairbanks	0.112	0.125
Chena River - below Fairbanks	0.112	0.118
Moose Creek	0.094	0.099
under ice-cover:		
Chena River - above Fairbanks	0.056	0.067
Chena River - below Fairbanks	0.095	0.085
Moose Creek	0.104	0.026

TABLE 13

Reaction Rate Coefficients for DO Depletion in Chena River Water (Collected Above Fairbanks) When Sewage Treatment Plant Effluents Are Added (From Gordon, 1970)

Temperature °C	k_e day^{-1} primary effluent	k_e day^{-1} secondary effluent
0	0.733	0.00638
10	1.97	0.0356
20	5.26	0.196

secondary effluent from a laboratory bench-scale activated sludge system operated at 0.1° C, also in a dilution 1:3 (effluent to river water). He compared the rates of oxygen depletion at 0, 10, and 20° C.

We calculated values for the reaction rate coefficient, k_e, from his data; these are shown in Table 13. The filtered raw sewage that we used should have been similar in organic content to that used by Gordon, although a large portion of the sewage microorganisms may have been removed in the filtration process. The value for k_e calculated from Gordon's data on oxygen depletion at 0° C with the primary effluent was almost 7 times greater than that which we measured at 4° C. The dilution of effluent to river water which Gordon used was about 5 times greater than ours. The value for k_e for oxygen depletion at 0° C with the secondary effluent was about 20 times smaller than that which we measured in the river water alone at 4° C. The secondary effluent apparently contained little BOD utilizable at 0° C. It would appear that temperature does not have as great as effect on the rate of dissolved oxygen depletion as does the food:microorganism ratio.

The differences between the experiments compared above is certainly of significance and should be resolved. Treatment processes, whether they be biological or chemical-physical, must be designed relative to the receiving waters into which they flow. The effects they have upon these waters is the sole purpose of treatment. Regulatory and standards-setting agencies need accurate information in order to perform their functions in a reasonable and just manner. Further efforts to more completely describe the effects of wastes (both treated and untreated) discharged into arctic and subarctic streams will be undertaken in the future.

It may be that bacterial activity is less affected by low temperatures than that of higher forms of life. In another project of the Institute of Water Resources, in which the limnology of two sub-arctic rivers, Goldstream and the Chatanika, is being investigated, it appears that temperature is indeed an important factor in the amount of biological activity observed, as indicated by algal growth and insect populations. Even though the necessary nutrients are present, biological activity is essentially nil until the water temperatures rise in the spring (NYQUIST, 1971).

Obviously there are a number of variables involved, including pH, temperature, dissolved oxygen concentrations, substrate concentrations, food:organism ratios, the organisms involved and their metabolic capabilities, as well as the presence of mineral elements and growth factors or toxic substances in either the environment or the effluent to be disposed to the water. In order to evaluate the effect of low temperatures on stream assimilation capacities, these variables must be known and carefully controlled. In short, the ecosystem must be defined.

CELLULOSE DECOMPOSITION STUDIES

We sought to isolate psychrophilic, cellulose-decomposing bacteria from Alaskan soils in order to measure the rates at which cellulose is decomposed at low temperatures in an aqueous environment. As will be shown, this last limitation on the experimental situation, while it prevented the measurement of rates of decomposition, revealed a significant dependence of the cellulose decomposition process on the physical environment.

EXPERIMENTAL METHODS AND RESULTS

An enrichment culture technique was used to isolate cellulolytic soil bacteria. Three soil samples, collected near Lake Chandalar, Alaska, were used. Erlenmeyer flasks containing 100 mls of a sterile cellulose isolation medium* were inoculated with about 0.5 g of soil. This medium contained 2 g/liter of cellulose powder as the sole carbon source, 1 g/liter of ammonium sulfate as a nitrogen source, and mineral salts; it was buffered at pH 7.0. The flask cultures were incubated at 10° C and aerated by continuous shaking on a New Brunswick rotary shaker. After two days one ml of culture fluid from each flask was aseptically transferred to a second flask of sterile medium, and these flasks were incubated at 10° C with shaking.

To isolate individual species, on the fourth and seventh days a loopful of each culture fluid was streaked onto the surface of an agar plate prepared from the isolation medium. The plates were incubated at 10° C. After two weeks incubation, pinpoint, translucent and white colonies were visible on the agar, but these did not reach an appreciable size even after several months. The low temperature was apparently not the limiting factor, for at 20° C as well only very small colonies appeared. The size of the colonies was most likely limited by the availability of the carbon and energy source, the particulate cellulose.

After twelve days the shake flasks were removed from the shaker but were left in the 10° C incubator. Because after two months the culture medium in flask No. 1 had turned orange, it was examined microscopically. The bacterial population was still viable and had even increased; rod-shaped bacteria were seen swarming around the cellulose particles. See Table 14. No change was noted in the other two flasks. Three months later the culture fluid in flask No. 1 had cleared. Microscopic examination revealed a varied, viable bacterial culture. Moreover, most of the cellulose had been dissolved.

Five isolates were obtained from this culture and another shake flask culture of soil No. 2; these were labeled LC-1 through 5. Subsequent morphological and biochemical tests indicated that these were four different species: LC-1 and LC-2 were apparently the same organism. All were rod-shaped, Gram-negative, polarly flagellated bacteria, whose sizes varied from 0.5-1 μ by 1.8 μ, depending on the age of the culture.

To enhance the growth of the organisms on agar plates and in liquid culture, the cellulose content of the medium was increased to 5 g/liter. The nitrogen and mineral salts contents were proportionately increased. Vitamins known to be beneficial or necessary to cellulose-decomposing

*The details of the media and analytical procedures used can be found in Appendices A and B.

TABLE 14

Population Levels of Mixed Cultures of Soil Bacteria in Cellulose Isolation Medium at 10°C

Cellulose: 2 g/l — Shake Flask Cultures — pH: 7.0 — Temperature: 10° C

Time (days)	Number of Bacteria per ml: Flask No. 1	Flask No. 2	Flask No. 3
7	1.0×10^7	1×10^7	7.5×10^6
12	1.2×10^7	7.8×10^6	8.1×10^6
63	5×10^8	7×10^5	8×10^6
147	1.6×10^8	--	--

rumen bacteria were also added. Unfortunately, 1 g/l of sodium citrate, to chelate heavy metals, was added at the same time. Much later it was discovered that all five isolates were able to use citrate as the sole carbon source. Thus, the data on the growth rates of these organisms in this improved medium do not reflect growth on cellulose alone. Nevertheless, the results of one experiment are reported here for comparison with the data subsequently obtained when citrate was elimiated from the medium. All five organisms grew rapidly at 4° C, as indicated by the generation times, Θ, shown in Table 15.

TABLE 15

Population Levels of Pure Cultures of Soil Bacteria in a Cellulose Plus Vitamins Medium with Citrate at 4°C

Cellulose: 5 g/l — Shake Flask Cultures — pH: 7.0 — Temperature: 4° C

Time (days)	Number of Bacteria per ml: LC-1	LC-2	LC-3	LC-4	LC-5
0.17	8×10^5	2.8×10^6	1.8×10^6	1.6×10^6	3.8×10^6
1.14	4.8×10^6	3.2×10^6	1×10^6	8×10^5	1.6×10^7
2.25	2.2×10^7	2.0×10^7	6.8×10^6	6.5×10^6	2.4×10^8
3.95	4.4×10^8	4.0×10^8	6.2×10^8	3.1×10^8	9.2×10^8
5.90	7.6×10^8	5.6×10^8	6.6×10^8	5.0×10^8	7.2×10^8
8.11	8.4×10^8	6.8×10^8	6.8×10^8	6.9×10^8	4.8×10^8
Θ, hours	9.95	7.86	6.25	9.69	6.72

When the citrate was eliminated and the shake flask experiment repeated (Table 16), there was little evidence of growth in the medium at 4° C. When the experiment was conducted at 10° C, the bacterial levels had increased only slightly over their initial levels when on the fifth day the incubator and shaker were shut off. Consequently, the cultures had been stationary and at room temperature for three days when they were examined on the eighth day of the experiment and it was found that the populations in all but one of the flasks had increased at least one order of magnitude. See Table 17.

TABLE 16

Population Levels of Pure Cultures of Soil Bacteria in a Cellulose Plus Vitamins Medium without Citrate at 4°C

Cellulose: 5 g/l — Shake Flask Cultures — pH: 7.0 — Temperature: 4° C

Time (days)	Number of Bacteria per ml				
	LC-1	LC-2	LC-3	LC-4	LC-5
0.05	1×10^6	2×10^5	1×10^6	2×10^5	1×10^6
0.96	2×10^5	1×10^6	5×10^6	2×10^5	2×10^6
2.06	1×10^6	3.7×10^6	3.8×10^6	4.2×10^6	1.4×10^7
3.75	3.8×10^6	4.6×10^6	5.6×10^6	2.6×10^6	8.4×10^6
6.78	3.0×10^6	4.0×10^6	8.8×10^6	2.8×10^6	1.1×10^7

TABLE 17

Population Levels of Pure Cultures of Soil Bacteria in a Cellulose Plus Vitamins Medium without Citrate at 10° C

Cellulose: 5 g/l — Shake Flask Cultures — pH: 7.0 — Temperature: 10° C

Time (days)	Number of Bacteria per ml				
	LC-1	LC-2	LC-3	LC-4	LC-5
0.10	7×10^5	8×10^5	1×10^6	1×10^6	1×10^6
1.01	2×10^6	3×10^6	8×10^6	5×10^5	7×10^6
2.01	3.2×10^6	1.1×10^7	1.2×10^7	1.7×10^5	6.2×10^6
2.99	6.0×10^6	1.0×10^7	1.1×10^7	2.6×10^6	1.2×10^7
4.06	8.2×10^6	1.2×10^7	4.3×10^7	2.8×10^6	1.9×10^7
8.02	1.8×10^8	1.8×10^8	7.0×10^8	2.8×10^6	2.8×10^8

Because it appeared that at least four of the isolates were capable of growth on cellulose alone, a large-scale experiment was set up to measure the rates at which cellulose was utilized and end-products were formed as well as the growth rates of the organisms in a cellulose plus vitamins medium without citrate. The reaction vessel was a 2-liter cylindrical jar fitted with a stainless steel lid and central unit through which the coolant was passed to keep the medium at 10° C. Air was passed through a sterile cotton fiber and introduced into the medium through an orifice near the bottom of the jar. The air bubbles were dispersed and the medium was mixed with a variable-speed drive assembly located just above the air outlet. The vessel and medium were autoclaved for 15 minutes at 121° C. When the desired incubation temperature had been reached and the liquid had been aerated long enough to be saturated with oxygen and carbon dioxide, the medium was inoculated with a sterile suspension of LC-1 in phosphate-buffered distilled water to give an initial cell concentration of about 4 x 10^5 per ml. The pH, number of bacteria per ml, concentration of cellulose remaining, and the concentrations of glucose and total soluble carbohydrate released into the medium were monitored for fourteen days. The bacterial population was determined microscopically using a volumetric counting chamber. Glucose was determined by the glucose oxidase medium of WORTHINGTON (1967). Soluble carbohydrate was measured colorimetrically by the phenol-sulfuric acid method of DU BOIS et al. (1956). Cellulose was determined by weight. (See Appendix.) The results of this experiment are shown in Table 18.

TABLE 18

A Pure Culture of LC-1 in a Cellulose Plus Vitamins Medium

Cellulose: 5 g/l | pH: 6.9 | Temperature: 10°C

2-liter reaction vessel

Time (days)	pH	Bacteria (+ per ml)	Cellulose (mg/ml)	Soluble Carbohydrate (µg/ml)	Glucose (µg/ml)
0	6.88	(4 x 10^5)	5.13	7.5	0.86
0.88	--	2.3 x 10^6	--	--	--
1.85	6.95	2.3 x 10^6	4.82	6.5	0.55
3.64	6.90	1.9 x 10^6	4.89	6.0	0.53
5.88	--	1.3 x 10^6	--	--	--
6.69	6.90	1.1 x 10^6	4.54	5.9	0.52
8.67	6.90	1.1 x 10^6	4.99	--	--
10.64	--	1.7 x 10^6	--	--	--
11.94	6.90	1.4 x 10^6	3.93	6.6	0.60
13.65	--	1.5 x 10^6	--	--	--

No change in any of the variables, except the cellulose concentration, was observed in 14 days. The apparent loss of cellulose was due to its adsorption onto the stainless steel parts of the reaction vessel. That the organisms used to inoculate the medium were viable and were not subsequently inhibited by heavy metals from the reaction vessel is attested to by the fact that a similar experiment with glucose as the sole carbon source instead of cellulose was performed simultaneously in an identical apparatus and the organisms grew readily.

After reconsideration of the data of Table 17, it was thought that perhaps the rise in temperature had stimulated the population increase; thereafter warmer incubation temperatures were used. Table 19 shows the results of attempting to grow isolates LC-1 and LC-5 at 20° C in shake flasks containing the cellulose plus vitamins medium without citrate. This experiment was done in triplicate. The results were all the same; namely, no change in any of the bacterial population levels for 16 days.

Similar results were obtained when flasks of sterile cellulose isolation medium with vitamins were inoculated with bacteria known to be cellulolytic and incubated at 25° C with continuous shaking. The organisms were *Cellulomonas fimi* (ATCC 484) and *Cellulomonas biazotea* (ATCC 486). As shown in Table 20, there was no change in the number of bacteria per ml in 13.5 days.

TABLE 19

Population Levels of Pure Cultures of LC-1 and LC-5 in a Cellulose Plus Vitamins Medium

Cellulose: 5 g/l — Shake Flask Cultures — pH: 7.0 — Temperature: 20° C

Time (days)	Number of Bacteria per ml: LC-1	Number of Bacteria per ml: LC-5
0.75	1.72×10^7	1.83×10^7
1.04	1.80×10^7	2.38×10^7
1.75	6.11×10^7	3.08×10^7
2.75	2.80×10^7	1.75×10^7
3.82	1.78×10^7	1.97×10^7
4.91	1.26×10^7	2.50×10^7
The flasks were re-inoculated with 24-hour old cells. The number added per ml was:		
5.04	9.2×10^5	7.2×10^5
8.20	1.91×10^7	2.75×10^7
10.02	2.92×10^7	4.48×10^7
11.30	3.00×10^7	4.73×10^7
12.32	3.43×10^7	6.15×10^7
13.55	2.60×10^7	9.3×10^7
16.20	2.80×10^7	4.75×10^7

The distinguishing feature of these experiments is the inability of a pure bacterial culture to develop in a well-mixed, aerated, liquid medium in which a particulate, cotton cellulose substrate serves as the only source of carbon, even though nitrogen, vitamins, and mineral salts, including trace elements, are provided. The fact that the same organisms are able to grow in pure culture at a rapid rate, even at the low temperature of 4° C, in the same medium to which a soluble carbon source, citrate, has been added indicates that additional growth factors are not required and that the medium itself is not inhibitory. The fact that the original cellulose isolation medium supported the mixed culture decomposition of the cellulose substrate indicates that the cellulose powder is susceptible to enzymatic hydrolysis. An important point to mention is that in both instances

where any activity was observed; that is, in the mixed culture of Table 14 and the pure cultures of Table 17, the flasks had been stationary prior to the observation. This fact suggests that a well-mixed liquid culture prevents the bacteria and the cellulose particles from having the intimate contact needed for the organism to initiate and continue cellulose decomposition.

TABLE 20

Population Levels of Pure Cultures of Known Cellulolytic Bacteria in Cellulose Isolation Medium with Vitamins

Cellulose: 2 g/l — Shake Flask Cultures — pH: 7.0 — Temperature: 25° C

Time (days)	Number of Bacteria per ml	
	Cellulomonas fimi	*Cellulomonas biazetea*
1.57	4.2×10^6	1.8×10^6
1.84	2.0×10^6	6.8×10^6
2.50	1.2×10^6	1.5×10^6
2.94	4.0×10^5	2×10^5
3.48	7.6×10^6	1.9×10^6
3.67	4.6×10^6	2×10^6
6.50	5.0×10^6	1.8×10^6
8.61	6.0×10^6	3.2×10^6
13.50	7.5×10^6	3.9×10^6

DISCUSSION

A mathematical analysis of the physical situation involved in the hydrolysis of the cellulose molecule by bacteria in a well-mixed liquid environment led to the conclusion that amont the many factors affecting cellulose decomposition, diffusion limitations were probably the most important. The degree of polymerization of the cellulose molecule, the proportion of crystalline regions, the wide variability in structures of the molecule in nature, and the extent to which it is associated with other organic and inorganic materials are all important factors in cellulose decomposition in nature. However, the extent of association of the microorganism with its substrate is probably of greater importance than any of these. The hydrolysis and assimilation of a molecule many times larger than the organism itself requires the secretion of extracellular enzymes. Direct contact of the organism with its substrate appears to be necessary to stimulate enzyme synthesis. The more intimate the association of the organism with its substrate, the more rapidly the enzymes will be able to penetrate the cellulose fiber and digest it, and the more readily will the organism be able to obtain assimiable end-products for self-maintenance. The importance of diffusion is therefore obvious: the enzymes must diffuse to and into the cellulose fiber matrix for hydrolysis to take place and the end-products of hydrolysis must diffuse back to the cell so that they can be assimilated. Thus, the amount of moisture present in the cellulose-decomposing environment directly affects decomposition by determining the extent of diffusion. In the laboratory situations studied; namely, well-mixed, aerobic, liquid shake flask cultures in which cellulose served as the sole carbon and energy source, the diffusion limitations were so great as to prevent the bacteria from sustaining themselves long enough to initiate the decomposition process and have it progress at a rate rapid enough to support a fraction of the initial cell population. Table 21 shows the expected rates of hydrolysis and glucose production for various initial cell populations under the conditions used in the laboratory, (cellulose concentration = 5 g/liter). The enzyme concentrations expected for initial cell populations from 10^3 to 10^{10} cells per ml were estimated and used to calculate the maximum rate of hydrolysis to be expected (See Appendix C). For this calculation a phenomenological reaction rate equation based on Fick's laws of diffusion was used:

$$R_h = k4\pi a(D_A + D_B)f C_{A_o} C_{B_o}$$

where k is a symmetry number, equal to 1 for collisions between unlike molecules, a is the sum of the radii of the colliding spheres, in centimeters, D_A and D_B are the diffusion constants of molecules A and B, and C_{A_o} and C_{B_o} are the initial concentrations of these molecules, in particles per ml. The factor f is a function of the electrostatic potential. For simplicity the molecules are taken to be neutral; whence f equals 1. The calculation is simplified by treating the molecules as spheres and assuming steady-state concentrations of the reactants (AMDUR and HAMMES, 1966). From the calculated rates of hydrolysis the maximum initial rates of glucose production could be calculated and compared with the rates of glucose production required solely for the energy of maintenance of the initial cell populations. It was found that the amount of glucose produced is only enough to support a population five to six orders of magnitude smaller than that originally present. Even if a glucose molceule were released with each hydrolysis, the calculated rate of release of glucose would still be well below the maintenance requirement. Obviously some part of

the original cell population must die. Those cells which lyse upon dying will release additional nutrients into the medium which will enable a larger cell population to be supported than that calculated from the rate of glucose release alone. And eventually, if some part of the population survives and cellulolysis begins the average degree of polymerization of the cellulose molecules will decrease and the probability of releasing a glucose molecule with each hydrolysis will increase. Consequently the rate of release of glucose into solution will be gradually increasing, and more and more cells will be able to survive. Table 21 also shows the times required to improve the rate of glucose release by a factor of 100. Only with very large inocula will the initial collision frequency between the enzyme and cellulose molecules be high enough to support a substantial number of cells - and then only if the cells can survive long enough on their own energy reserves to produce the postulated amount of enzyme. From inspection of the times required to increase the glucose production rates by two orders of magnitude, it would appear that at least 10^8 cells per ml are required initially for cellulolysis to begin within a time practical for laboratory observation. The collision frequency could be increased by increasing the number of cellulose particles per ml, but a more noticeable effect could be had by including small amounts of soluble nutrients in the medium to enhance the cells to survive until cellulose decomposition is well under way and they can maintain themselves. Because of the limitations which diffusion imposes on the process, it appears that cellulolysis is difficult to initiate in a well-mixed liquid environment in which no carbon and energy source other than cellulose is immediately available to sustain the cells. These findings explain why in not one of the laboratory experiments in which a pure culture of cellulose-decomposing organism was incubated in a shake flask containing a cellulose - mineral salts-vitamins medium was cellulose decomposition observed even though incubation temperatures from 4° C to 25° C and incubation times of weeks were used.

TABLE 21

Expected Rates of Hydrolysis and Glucose Production for Various Initial Cell Populations

Initial number of cells per ml	Enzyme concentration (No./ml)	Hydrolysis rate R_h (rxs/ml/sec)	Initial rate of glucose production R_G (molecules/ml/sec)	Rate of glucose prod'n required R'_G (molecules/ml/sec)	Time required to improve R_G by 100X
1×10^3	1.8×10^7	7.92×10^4	5.29×10^1	2.59×10^7	1.74×10^4 days
1×10^4	1.8×10^8	7.92×10^5	5.29×10^2	2.59×10^8	1.74×10^3 days
1×10^5	1.8×10^9	7.92×10^6	5.29×10^3	2.59×10^9	1.74×10^2 days
1×10^6	1.8×10^{10}	7.92×10^7	5.29×10^4	2.59×10^{10}	17.4 days
1×10^7	1.8×10^{11}	7.92×10^8	5.29×10^5	2.59×10^{11}	41.7 days
1×10^8	1.8×10^{12}	7.92×10^9	5.29×10^6	2.59×10^{12}	4.2 hours
1×10^9	1.8×10^{13}	7.92×10^{10}	5.29×10^7	2.59×10^{13}	25 minutes
1×10^{10}	1.8×10^{14}	7.92×10^{11}	5.29×10^8	2.59×10^{14}	2.5 minutes

Circumstances in nature are more favorable for cellulose decomposition than those employed in the laboratory experiments. In the natural environment one finds a variety of species of microorganisms capable of utilizing the food sources such as sugars, organic acids, proteins, hemicelluloses, and lignins which are available in addition to cellulose. Through a complex symbiosis a microorganism population is established and supported. Hence, cellulose decomposition should be initiated more readily in nature.

Aerobic cellulose decomposition is probably maximum in a terrestrial environment such as the upper layer of soil, a rotting log, or the leaf litter on the forest floor. Provided the humidity is suitable, diffusion limitations should be minimal, because the organisms remain in intimate contact with the substrate. The fungi generally dominate the cellulolytic process in the soil, unless the soil is very alkaline or has been fertilized (IMSHENETSKY, 1968), for they require less nitrogen than the bacteria to metabolize an equivalent amount of carbohydrate. The fungi penetrate the cellulose fiber by releasing cellulase at the tips of their extending hyphae (REESE, 1959). Because the cellulolytic enzymes do not have to diffuse far to reach the substrate and digest it, the local concentrations of both the enzyme and the hydrolysis products are effectively greater, in relation to the substrate and the cell, than in an aquatic environment. A similar description applies to cellulose decomposition by bacteria in this situation. In addition, the protoplasmic contents of dead fungal cells help support the bacterial population, both cellulolytic and non-cellulolytic.

Although a variety of microorganisms and food sources are available in natural aquatic environments as well, the large volume of water and lower cellulose concentrations essentially decrease organism-substrate interactions, so that diffusion hinders the progress of cellulolysis here just as it does in the shake flask. One would expect the benthic regions of a body of water to be the site of cellulolytic activity rather than the water itself. Insoluble cellulosic materials settle to the bottom and sink into the upper layer of mud where close contact between the cellulose and anaerobic cellulolytic bacteria is possible. Few fungi exist in muds, so that their contribution to cellulolysis in this situation is negligible (LAURENT, 1969). Cellulose decomposition is probably initiated readily by anaerobic bacteria but may not be prolonged, for these organisms produce organic acids in the process of assimilating the cellulose. Unless the cellulolytic bacteria are accompanied by species capable of metabolizing the acids, the pH of their environment will become so low as to prevent them from functioning. In the absence of organic acid-metabolizing organisms, cellulose decomposition probably occurs sporadically. Data substantiating this picture of cellulose decomposition have been reported by LAURENT (1969), who found that cellulolytic activity in the water of a pond was very weak compared with that in the underlying mud. He observed that maximum cellulolytic activity occurred in the mud immediately below the mud-water interface and declined in the deeper mud layers. As would be expected for a process limited by diffusion, increasing the temperature from 5° C to 25° C had no influence on cellulolytic activity in the water but did have a distinct positive effect in the mud.

In waste treatment practice cellulose is treated primarily by anaerobic methods. The major portion of the cellulose found in domestic sewage is separated from the soluble waste matter in an initial sedimentation step. The settled waste, or primary sludge, as it is called, is then digested anaerobically. Cellulose decomposition is promoted by the close organism-substrate contact that is possible and by the variety of microorganisms and food sources present in the sludge. The cellulose is largely converted to organic acids by acid-forming bacteria in the first stage of digestion. The acids are subsequently metabolized to methane and carbon dioxide by another group of bacteria. In the process these bacteria raise the pH and permit further degradation of cellulose by the first group.

The extent to which the cellulose remaining in the liquid portion of the waste after primary sedimentation is subsequently decomposed will depend on the process by which the waste is treated. The activated sludge process is the conventional biological method of treating soluble wastes. "Activated sludge" is a conglomeration of bacteria, protozoa, possibly some fungi, and particulate organic matter which is in the process of being degraded or is not biologically degradable. If particles of cellulose become attached to this active mass, it is conceivable that their

decomposition would be easily initiated if cellulolytic bacteria were present. It is unlikely that the decomposition would be extensive by the time the activated sludge is settled out and the treated effluent is disposed of, for contact times between the activated sludge and the waste are typically a matter of hours. But if the cellulose remains attached to the sludge when it is settled and returned to the aeration tank to be mixed with untreated waste, it would probably be degraded eventually. However, the extent to which this attachment occurs is not known.

The cellulose which does not become attached to the active mass but remains in suspension as the waste is aerated probably passes out of the treatment plant unaffected. Although the bacterial population in the liquid portion of the activated sludge-waste mixture is higher than in most natural aquatic environments, the proportion which is cellulolytic is not known and would be a function of the composition of the waste. Also, the concentration of cellulose found in domestic sewage which had undergone primary sedimentation is about one-thousandth of that used in the laboratory experiments (HURWITZ, et al., 1961), so that the opportunity for interaction between cellulose and cellulolytic organisms is likely to be substantially less.

CONCLUSIONS

Although originally intended to measure rates of cellulose decomposition at low temperatures, these experiments have revealed the dependence of the microbial cellulose decomposition process on the physical environment. Some moisture is required to coat the cellulose fiber surface and to provide a medium for diffusion, but too much water tends to defeat the process. This is evidenced by the observation that pure cultures of cellulolytic bacteria were unable to develop in a well-mixed, aerated, aqueous medium in which a particulate, cotton cellulose served as the only source of carbon, even though nitrogen, vitamins, and minerals, including trace elements, were provided; yet they were able to grow under the same conditions when a soluble carbon source was added or when the medium was not mixed. Apparently a well-mixed liquid culture prevents the bacteria and the cellulose particles from having the intimate contact needed for the organisms to initiate and continue cellulolytic decomposition. The closer the association of the organism with the substrate, the more rapidly its enzymes will be able to penetrate the cellulose fiber and digest it, and the more readily it will be able to obtain assimilable end-products for self-maintenance.

As emphasized by the laboratory experiments and an analytical evaluation of the role of diffusion in cellulolysis, the key factor in the process of cellulose decomposition appears to be the extent to which organism-substrate interaction is possible. This requirement for intimate contact between the organism and the cellulose molecule leads to the conclusion that cellulose decomposition is minimal in aqueous environments. One would expect the benthic region of a body of water to be the site of cellulolysis rather than the water itself.

REPORT SUMMARY

In this exploratory survey of the metabolic activities of microorganisms indigenous to the cold environments of the Arctic and sub-Arctic, we measured the rates of disappearance of organic substrates in aqueous biological systems at low temperatures (1 to 20° C) using microorganisms from different ecological systems: from an activated sludge waste treatment plant operating near freezing, from the mud of a sub-arctic streambed, and from soil in an area of Alaska where the air temperature rarely exceeds 70° F. These experiments yielded values for the various parameters applicable to batch systems, including growth rates, cell yields, and reaction rate coefficients. We could observe no over-riding relationship between temperature and any of these variables, but it is difficult to make comparisons between different systems because of the many variables involved. However, we did find that micro-biological activity at low temperatures was comparable to that at higher temperatures. There was also some indication that the initial food: microorganism ratio had variations in more influence on the cell growth rate than did variations in the incubation temperature within this range.

Stream assimilation studies were conducted at 3.5° C to compare the reaction rate coefficients for oxygen consumption in sub-arctic river waters alone and when sewage is added to them. We found the reaction rate coefficients to be comparable whether sewage was added to a water or not. Furthermore, the rates of oxygen depletion were no different from what would be expected in temperate-climate waters held at the same temperature. Comparison of our results with those of GORDON, 1970, indicated that temperature may have less of an effect on the rate of oxygen depletion by microorganisms than does the amount of nutrient present.

We had set out to measure the rates at which cellulose is decomposed in sub-arctic aqueous environments and ended by discovering how dependent the cellulose decomposition process is on the physical environment, in particular on the amount of water present. It appears that in an aqueous environment, the diffusion limitations are so great as to prevent the initiation of cellulolysis unless other energy sources are available and/or there is present a large population of cellulose decomposers. We concluded that the bottom mud is a more likely site for cellulose decomposition than the water itself.

We had wanted to measure the effect of low temperatures on the metabolic reaction rates of microorganisms found in normally cold environments but found it difficult to isolate temperature effects from other effects when working with mixed populations of organisms of unknown composition. And, in fact, there are so many factors involved that in a natural situation low temperatures need not necessarily be rate-determining. For example, we have seen how the proximity of an organism to its food may have a greater effect on the rate at which that food is metabolized than does the temperature-dependence of the metabolic capacity inherent in the organism. To evaluate the natural abilities of the freshwaters of Alaska to assimilate wastes discharged to them, it would probably be of more benefit to view each situation from the systems viewpoint. An ecosystem could be carefully analyzed, with each variable examined and its relationship to the others established. Temperature would be one of these variables. Taking this approach, one would sooner arrive at a working model which would allow one to make predictions regarding the waste-assimilation capacity of the system. Thus, if the experiments which we have

reported here had been conducted in conjunction with experiments on other variables in each type of system, then the usefulness of the information obtained would have been increased many times through its relationship to them.

APPENDIX A

Analytical Procedures

Mixed Liquor Suspended Solids, MLSS, were measured by filtering a known volume of mixed liquor, usually 50.0 mls, under vacuum, through a washed, dried, and tared filter. The filter was usually a 0.45 μ membrane filter, but when the solids consistency was very thick, a Whatman GF/C glass fiber filter was used. After the sample had been filtered, the filter was dried in an oven at 103° C for one hour and re-weighed. The difference in weights gave the MLSS.

Chemical Oxygen Demand (COD) analyses were performed in two ways. The 15-minute method used a 5.00-ml sample, 10.00 mls, 0.125 N $K_2Cr_2O_7$, and 15.0 mls concentrated H_2SO_4 containing 13.33 g Ag_2SO_4 per liter, and a 15-minute reflux time. This method was subsequently replaced with the 2-hour method found in STANDARD METHODS, 12th ed., p. 510, which uses a 10.00-ml sample, 5.00 mls 0.2500 N $K_2Cr_2O_7$, 15.0 mls concentrated H_2SO_4 containing 9.81 g Ag_2SO_4 per liter, 0.2 g $HgSO_4$, and a 2-hour reflux time. After being refluxed the sample was cooled and diluted to 70 mls with distilled water. The excess dichromate was then titrated with a standardized solution of $Fe(NH_4)_2(SO_4)_2$. The difference in $K_2Cr_2O_7$ initially and that remaining after refluxing yielded the oxygen demand exerted by the sample.

Volatile Organic Acids were measured by the following procedure: 5.00 mls of sample were added to a distillation flask; 3 drops of Cresol red indicator were added and the pH was adjusted to about 2 (indicator turned from yellow-orange to pink-orange) with a few drops of 6 N H_2SO_4. Then 35 mls of distilled water were added to the distillation flask, which was connected to a West condensor and wrapped in asbestos. Thirty mls of distillate were collected in three 10-ml portions. These were transferred quantitatively to 125-ml Erlenmeyer flasks and titrated to the phenolphthalein end-point (pH 8.2-8.3) with 0.0200 N Na_2CO_3. The equivalent organic acid concentration was expressed as mg acetic acid per liter.

Soluble carbohydrate was determined by the coloremetric method of DU BOIS et al., (1956). Because this method can be used for polysaccharides as well as oligosaccharides and simple sugars, the filtrate from the cellulose or MLSS determination was used. To a 2.00 ml sample, in a ½" B & L colorimetric test tube, was added 1.0 ml of a 5% (w/w) phenol solution. Then 5.0 mls of concentrated reagent grade sulfuric acid were added rapidly, in 10-20 seconds. The acid stream was directed against the liquid surface rather than the side of the tube. After 10 minutes the sample was shaken for 10 to 20 minutes in a water bath at 25-30° C. The absorbance of the resulting orange solution was measured at 490 nm in a Bausch and Lomb Spectronic 20 against a blank prepared by treating 2.00 mls of distilled water in the same manner as the sample. Standard glucose solutions, containing between 5 and 50 μg of sugar per ml, were used to prepare a standard curve which was linear in this concentration range.

Cellulose was measured by filtering a 20.00 ml sample of culture medium through a 5μ porous-bottom crucible which had been previously dried and tared to constant weight at 103° C. Although a volumetric pipet was used for sampling, for consistency it was necessary to rinse it out with distilled water in order to flush out the cellulose particles adhering to the walls of the pipet.

(This was done after a portion of the filtrate had been collected for the glucose and soluble carbohydrate determinations.) After most of the moisture had been drawn off by suction, the crucible was again dried to constant weight at 103° C. The difference in weights gave the cellulose remaining in suspension in the medium.

Glucose was determined enzymatically using Worthington Biochemical Corporation's *Glucostat Special*. This preparation permits the determination of glucose in the presence of poly- and oligosaccharides since it is essentially free of carbohydrases and is specific for glucose (WORTHINGTON, 1967). The glucose concentration in the filtrate from the cellulose determination was measured as follows: To 2.0 mls of sample, in a 16 x 150 mm test tube in a 37° C water bath, was added 2.0 mls of the *Glucostat Special* reagent. The sample and the reagent were mixed and incubated for 30 minutes, at which time the reaction was stopped with one drop of 5 N HCl. The absorbance of the resulting orange solution was measured at 400 nm in 1 cm cells in a Beckman DB Spectrophotometer against a blank consisting of 2.0 mls of distilled water treated in the same way as the sample. Standards, containing 10 to 30 μg of glucose per ml, were treated simultaneously, and the concentration of glucose in the sample was calculated from the formula:

$$\text{Concn.}_{\text{sample}} = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times \text{Concn.}_{\text{standard}}$$

Cell counts were made microscopically using a Zeiss phase-contrast microscope at 1200X magnification. A measured volume of sample was mixed with at least an equal volume of a 36% formaldehyde solution containing methylene blue. This solution kills the cells and stains them at the same time. About 0.1 ml of the mixture was transferred to a Peiroff-Hausser bacteria counting chamber. This device consists of a glass base with a grid of squares of known size and a cover slip which provides for a known depth of liquid over the grid. One can thus count the number of bacteria found in the liquid over x number of squares, determine the volume which this represents, and calculate the number of bacteria per ml. This value is then multiplied by the dilution factor to give the number of bacteria, living and dead, per ml of culture fluid.

Glassware used in the analyses and in the preparation of media was washed in detergent and hot water and rinsed at least three times in tap water and three times in distilled water. It was then treated with acid-dichromate cleaning solution and rinsed thoroughly in distilled water.

APPENDIX B

Media

Phosphate-buffered dilution water was prepared according to STANDARD METHODS, 12th ed., p. 417. It contained, per liter of distilled water, 8.5 mg KH_2PO_4, 21.75 mg K_2HPO_4, 33.4 mg $Na_2HPO_4 \cdot 7H_2O$, 1.7 mg NH_4Cl, 22.5 mg $MgSO_4 \cdot 7H_2O$, 27.5 mg $CaCl_2$, and 0.25 mg $FeCl_3 \cdot 6H_2O$. Final pH was 7.0.

Nutrient Broth - Yeast Extract Medium. Nutrient Broth is composed of Beef Extract and Peptone and is a general cultivation medium for organisms not too exacting in their nutrient requirements. The Peptone has a high amino acid content and only negligible quantities of more complex nitrogen compounds. Yeast Extract contains B-complex vitamins which stimulate growth (DIFCO, 1953).

Difco's Bacto-Nutrient Broth and Bacto-Yeast Extract were used to prepare a stock substrate solution, aliquots of which could then be diluted with distilled water to obtain working substrate solutions of the desired COD: 9.6 g of Nutrient Broth plus 6.0 g of Yeast Extract were dissolved in distilled water and diluted to 500 ml. This solution was then autoclaved at 15 p.s.i. for 15 minutes. Its COD was 36,000 mg/liter.

Glucose-Mineral Salts Medium contained, per liter of distilled water, 10.0 g glucose, 5.5 g KH_2PO_4, 10.0 g Na_2HPO_4, 3.0 g $(NH_4)_2SO_4$, 2.0 g trisodium citrate monohydrate, 0.25 mg $FeCl_3 \cdot 6H_2O$, 27.5 mg $CaCl_2$, and 0.2 mg of each of the following trace elements: $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The medium was sterilized by filtration through a sterile 0.45 μ membrane filter. Its final pH was 7.07; its theoretical COD is 13,774 mg/liter.

Glucose-Mineral Salts + Vitamins Medium contained, per liter of distilled water, 3.00 g glucose, 2.00 g $(NH_4)_2SO_4$, 2.75 g KH_2PO_4, 5.00 g Na_2HPO_4, 0.17 mg $FeCl_3 \cdot 6H_2O$, 18.4 mg $CaCl_2$, and 0.13 mg of each of the following trace elements: $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. In addition the following six vitamins were added to a final concentration each of 10^{-9} molar: biotin, pyridoxine hydrochloride, folic acid, riboflavin, paraminobenzoic acid, and cyanocobalamine (B_{12}). The medium was sterilized by filtration through a sterile 0.45 μ membrane filter. Its final pH was 7.02; its theoretical COD is 3200 mg/liter.

Cellulose powder was prepared by mild acid hydrolysis of non-absorbent cotton. 10.0 g of cotton was suspended in one liter of 3 N HCl in a 1500-ml beaker and heated to 80-90° C. The mixture was stirred continuously until the fibers broke and formed a loose, fairly homogeneous floc. The beaker was removed from the heat and allowed to cool to about 60° C. The floc was separated from the liquid by filtration through a Whatman 41H filter paper in a Büchner funnel with vacuum applied. It was then washed with distilled water until the final washing was of neutral pH. To do this rapidly the floc had to be resuspended in distilled water and filtered several times. After most of the moisture had been removed by suction, the floc was dried overnight in a 103° C oven. It was then pulverized in a mortar and pestle. Microscopic examination revealed that the powder was made up of flat, rectangular particles with an average size of 100μ x 20μ x 5μ.

Cellulose isolation medium contained, per liter of distilled water, 2.0 g cellulose powder, 1.0 g $(NH_4)_2SO_4$, 2.5 g Na_2HPO_4, 1.26 g KH_2PO_4, 0.05 g $MgSO_4 \cdot 7H_2O$, 15 mg $CaCl_2$, 0.4 mg $FeSO_4 \cdot 7H_2O$, and 0.2 mg of each of the following trace elements: $ZnSO_4 \cdot 7H_2O$, $CuSO_4 \cdot 5H_2O$, $MnSO_4 \cdot H_2O$, $CoCl_2 \cdot 6H_2O$, and $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$. The medium was adjusted to pH 7.2 with NaOH and autoclaved for 15 minutes at 121° C. Its final pH was 7.0.

Cellulose isolation medium with vitamins. Prior to sterilization, each of the following six vitamins was added to the cellulose isolation medium so that the final concentration of each was 10^{-9} molar: biotin, pyridoxine hydrochloride, folic acid, riboflavin, paraminobenzoic acid, and cyanocobalamine (B_{12}).

Cellulose plus vitamins medium contained, per liter of distilled water, 5.0 g cellulose powder, 3.0 g $(NH_4)_2SO_4$, 5.0 g Na_2HPO_4, 2.75 g KH_2PO_4, 0.10 g $MgSO_4 \cdot 7H_2O$, 7.5 mg $CaCl_2$, 0.2 mg $FeSO_4 \cdot 7H_2O$, and 0.1 mg of each of the trace elements added to the cellulose isolation medium. In addition, each of the six vitamins was added to give a final concentration of each of 10^{-9} molar.

APPENDIX C

Estimation of Enzyme Concentrations Used in Hydrolysis Rate Calculations

Before the phenomenological equation could be used to calculate a maximum rate of hydrolysis, some idea was needed of the enzyme concentrations that could be expected. Surprisingly, there is little decisive information on enzyme synthesis rates to be found in the literature. But enough data are available to enable rates to be calculated for two enzyme systems if certain assumptions are made. One of these systems involves the extracellular enzyme, α-amylase; the other; the intracellular enzyme β-galactosidase.

MARKOVITZ and KLEIN, (1955), measured rates of biosynthesis of α-amylase by resting cells of *Pseudomonas saccharophila* and found that, with maltose as the inducer, about 5 enzyme units per hour (per ml, presumably, since the assay volume equaled 1 ml) were synthesized for 3 hours, after which time the rate declined. Presumably the cells were initially grown in a medium containing 0.2% sodium lactate as the carbon and energy source. If a cell yield of 0.5 g of cells (dry weight) per g of lactic acid is assumed, the final resting cell suspension, concentrated fourfold, contained 0.32 g of cells (dry weight) per 100 mls.

MARKOVITZ and KLEIN defined one unit of α-amylase as that amount of enzyme which caused a loss in color with iodine at 660 nm equivalent to 0.5 mg starch in 30 minutes at 37° C and pH 5.5. Since the average degree of polymerization of soluble starch is about 300 glucose residues (WHISTLER and SMART, 1953), 0.5 mg starch corresponds to 5.6×10^{15} starch molecules. Starch molecules with a DP less than 20 units do not give a color with iodine that would be detected at 660 nm. To reduce a starch with a DP of 300 to one with a DP less than 20 would require breaking at least 15 bonds per molecule, and most likely more than 15 bonds would be broken in the actual process. If the turnover number and molecular weight of bacterial α-amylase are presumed to be similar to the values for pancreatic α-amylase, 25,000 (LONG, 1961) and 45,000 (DIXON and WEBB, 1964) respectively, and if only one active center per enzyme molecule is assumed, then one unit of enzyme corresponds to at least 1.11×10^{11} enzyme molecules or 8.3×10^{-6} mg enzyme. Thus, the enzyme synthesis rate observed by MARKOVITZ and KLEIN, 5 enzyme units per hour per ml, is equivalent to at least 1.74×10^{2} enzyme molecules per cell per hour or 2.16×10^{-7} mg enzyme per mg cells per minute.

When starch was used as the inducer, α-amylase was synthesized at the rate of 0.22 units per hour per ml of cell suspension for the first three hours, and then the rate declined. In this case one unit of α-amylase was defined as that amount of enzyme which released 0.021 mg of reducing sugar (as maltose) in 3 minutes at 37° C and pH 5.5. One unit thus corresponds to 4.68×10^{11} enzyme molecules or 3.50×10^{-5} mg enzyme, and the observed rate of enzyme synthesis equals 32.1 enzyme molecules per cell hour or 4.01×10^{-8} mg enzyme per mg cells per minute.

Rates of α-amylase synthesis by *P. saccharophila* were also calculated from the data of SCHIFF, EISENSTADT, and KLEIN (1959) using the following assumptions: (1) that 70% of dry cell material is protein; (SETLOW and POLLARD, 1962); (2) that the dry weight of a bacterium is about 10^{-9} mg(SISTROM, 1962); and (3) that the molecular weight of α-amylase is 45,000 (DIXON and WEBB, 1964). Resting cells synthesized the enzyme at an average rate of 1.7×10^{3}

molecules per cell per hour or 2.1 x 10^{-6} mg per mg cells per minute, whereas growing cells synthesized 6.0 x 10^{2} molecules per cell per hour or 1.0 x 10^{-6} mg enzyme per mg cells per minute. Note that α-amylase was manufactured more rapidly by resting cells than by cells growing on starch.

MAY and ELLIOTT (1968) have described a somewhat different biphasic production of α-amylase by 24-hour old cells of *Bacillus subtilis.* These cells were washed and incubated at 30° C in a medium supplemented with a mixture of 16 amino acids. Initially α-amylase was synthesized at a rate of 9.2 units per ml of culture per hour, but after 75 minutes the rate increased to 26.6 units per ml of culture per hour. As no estimate of the cell population was given by the authors, this value was taken to be 10^{9} cells, or 1 mg dry weight. per ml. In a previous paper (COLEMAN and ELLIOTT, 1961) it was stated that 1 mg of enzyme nitrogen was equivalent to 38,000 enzyme units. If the enzyme protein is 16% nitrogen, then α-amylase was initially formed at the rate of 1.83 x 10^{4} molecules per cell per hour or 2.47 x 10^{-5} mg per mg cells per minute. During the second phase it was produced at the rate of 5.32 x 10^{4} molecules per cell per hour or 7.16 x 10^{-5} mg per mg cells per minute. The fact that these values are an order of magnitude higher than those previously calculated is not unreasonable in view of the supplemental amino acids with which the cells were provided.

From previously induced cells of *Escherichia coli,* KAMEYAMA and NOVELLI (1962) isolated a cell-free system that catalyzed the synthesis of β-galactosidase. The isolated system represented 5.71% by weight of the original cell protein, if one assumes that the cells were originally 70% protein on a dry weight basis. KAMEYAMA and NOVELLI defined one unit of β-galactosidase activity as that amount of enzyme required to hydrolyze one μmole of o-nitrophenyl-β-D-galactoside in one hour at 37° C. According to JACOB and MONOD (1961) this hexameric enzyme has a molecular weight of 810,000 and a turnover number of 240,000 at 28° C and pH 7.0. If at 37° C the turnover is 1.8 times faster than at 28° C, then one KAMEYAMA and NOVELLI enzyme unit is equivalent to 2.32 x 10^{10} hexameric enzyme molecules. At 37° C it was observed that 2.51 mg of the cell-free protein formed 215 enzyme units in 60 minutes; this rate corresponds to 84 molecules per cell per hour or 1.78 x 10^{-6} mg enzyme per mg cells per minute.

In another experiment with cell-free extracts from pre-induced cells of *E. coli,* EISENSTADT, KAMEYAMA, and NOVELLI (1962) measured an enzyme synthesis rate of 150 units per hour per 2.76 mg of cell-free protein. This rate is equal to 52.5 hexameric enzyme molecules per cell per hour or 1.18 x 10^{-6} mg enzyme per mg cells per minute. When particulate protein from non-induced cells was mixed with supernatant fluid from an induced cell preparation, an enzyme synthesis rate equivalent to 16.2 molecules per cell per hour or 3.63 x 10^{-7} mg enzyme per mg cells per minute was observed. And when particulate protein from induced cells was mixed with supernatant fluid from non-induced cells, 42.9 hexameric molecules were formed per cell per hour or 9.64 x 10^{-7} mg enzyme per mg cells per minute.

MANDELSTAM (1957) measured the rate at which methyl-thiogalactoside induced β-galactosidase synthesis in leucine-starved cells of *E. coli.* His results were in terms of μmoles of o-nitrophenyl β-D-galactoside hydrolyzed per hour per mg dry weight of cells at 30° C and pH 7.0, so that the turnover number for β-galactosidase reported by JACOB and MONOD (1961) could be used to calculate the rate of enzyme synthesis. Although the rate was not constant throughout the incubation period, 1 mg of cells formed 6.27 x 10^{11} molecules in three hours. Thus, the average rate of synthesis was 2.08 x 10^{2} hexameric molecules per cell per hour or 4.68 x 10^{-6} mg enzyme per mg cells per minute.

Table C-1 summarizes the enzyme synthesis rates calculated. The fact that data from different sources yielded similar values invites confidence in the results. The most rapid rate of synthesis was that at which *Bacillus subtilis* manufactured α-amylase; namely, 7.16 x 10^{-5} mg enzyme per mg cells per minute. But because the medium in which these cells were incubated was supplemented with amino acids, and because the estimate of the number of cells per ml used in this calculation may be an order of magnitude or two in error, 5 x 10^{-6} mg enzyme per mg cells per minute is probably a more reliable estimate to use. This rate is equivalent to about 3 x 10^3 cellulase molecules per cell per hour, a liberal value but one not out of line with observed rates of enzyme synthesis by bacteria.

Supposing that a resting cell can maintain this rate for three hours, and that at this time it will have exhausted about half its synthetic capacity, then from an initial inoculum of 1 x 10^3 cells per ml, one could expect there to be an eventual cellulase concentration of 1.8 x 10^7 molecules per ml, provided the cells remain viable long enough to synthesize this amount.

TABLE C-1

Summary of Enzyme Synthesis Rates

Enzyme	Organism	Temperature °C	Enzyme molecules per cell per hr	mg enzyme per mg cells per min
	Pseudomonas saccharophila			
	resting cells	25	1.74 x 10^2	2.16 x 10^{-7}
	"	25	32.1	4.01 x 10^{-8}
α-amylase	"	30	1.7 x 10^3	2.1 x 10^{-6}
	growing cells	30	6.9 x 10^2	1.0 x 10^{-6}
	Bacillus subtilis			
	resting cells	30	1.83 x 10^4	2.47 x 10^{-5}
	"	30	5.32 x 10^4	7.16 x 10^{-5}
	Escherichia coli			
	cell-free extracts	37	84	1.78 x 10^{-6}
	"	37	52.5	1.18 x 10^{-6}
β-galactosidase	"	37	16.2	3.63 x 10^{-7}
	"	37	42.9	9.64 x 10^{-7}
	leucine-starved cells		2.08 x 10^3	4.68 x 10^{-6}

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