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THE MECHANISM OF WASTE TREATMENT AT LOW TEMPERATURE

PART A: MICROBIOLOGY

By

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George D. Boone, and Kirke L. Martin

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THE MECHANISM OF WASTE TREATMENT AT LOW TEMPERATURE

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ABSTRACT

THE MECHANISM OF WASTE TREATMENT

AT LOW TEMPERATURE

PART A. MICROBIOLOGY

Low temperatures adversely affect wastewater treatment efficiency, partly by decreasing rates of biological oxidation. Because of the importance of adequate treatment efficiency, the feasibility of inoculating psychrophiles into a wastewater treatment system to increase biological oxidation rates at low temperatures was investigated.

Three psychrophiles capable of relatively rapid growth or oxygen uptake in sterilized sewage were selected from water and soil isolates collected in the Fort Collins vicinity. Of 97 Alaskan psychrophiles screened for maximum activity as indicated by growth rate and rate of reduction of methylene blue thiocyanate, three were selected for use in the waste stabilization experiments.

In studies with heat sterilized sewage, the effects of number of cells inoculated and culture age of the three psychrophiles (local) on percent BOD removals at different temperatures were analyzed. The effects of temperature and number of cells inoculated were the most significant. Percent BOD removals decreased as temperature decreased, and the large inoculum (10^{10} cells/200 ml) removed more BOD than the small inoculum (10^{8} cells/200 ml).

The effects of organisms used as inocula and of culture age of inocula were also significant. At 15 and 20 C, the psychrophile inocula removed less BOD than the inocula of mesophiles, but at 5 and 10 C, the psychrophiles were more efficient than the mesophiles. The 1-day old cells generally removed more percent BOD than 7-day cells, except when 10^{8} cells were inoculated at 5 C. When the small inoculum was used at 5 C, the 7-day cultures were more efficient than the 1-day cultures. It was thought that this phenomenon was the result of cold shock that affects physiologically young cells, but not stationary growth phase cells.

Using raw settled sewage as substrate at 5 C, the Alaskan psychrophiles removed substantially more BOD within a 72 hr detention period

than untreated controls; however, within a 120 hr period, BOD removals never averaged greater than 31.3%. In raw sewage studies at 2 C, it was found that the sewage inoculated with locally isolated psychrophiles had 19% BOD removals within 5 hr, but the mesophile inoculated and non-inoculated raw sewage had 15% and 14% removals, respectively. Although this difference was statistically significant, it was observed that a bacterial inoculum appreciably added to the initial BOD of the raw sewage, resulting in a higher final BOD concentration after 5 hr despite the psychrophiles ability to more rapidly oxidize wastewater nutrients.

These observations add to our knowledge concerning biological treatment as a method of wastewater stabilization; however, the system investigated appears to be too inefficient for practical use, even though it showed a statistically significant advantage over untreated or mesophilic inoculated controls.

INTRODUCTION

Modern sanitary engineering technology is largely responsible for the development of biological wastewater treatment processes capable of efficient operation in temperate regions. Although these treatment facilities have been adequately designed for efficient waste removal in temperate regions, their efficiency at low temperatures is usually not sufficient to meet current demands of pollution control agencies. In regions where very low temperatures are encountered for extended periods of time, such as the high mountain communities of Colorado or the far north, wastewater treatment presents a series of unique problems which must be resolved before waste removal criteria can be met.

Unequal loading presents a problem in communities and resort areas where weekends and holidays can introduce large increases in population and resulting waste to be treated. The principal problem encountered in low temperature wastewater treatment is decreased biological activity in the system. The secondary process of most conventional waste treatment systems is largely based on physiocochemical floculation and settling and bio-oxidation of the organic materials in the waste. The organisms responsible for the biochemical activity are usually mesophilic, having temperature optima between 20 and 40 C and, by definition, exhibiting very little activity below 10 C.

Methods of improving low temperature waste water stabilization may be approached by modifying the engineering design or improving biological activity. The engineer might design new facilities which minimize the adverse effect of low temperatures or find inexpensive methods of modifying present facilities to work more efficiently at low temperatures. However, since sewage degradation is primarily a biological phenomenon, the biologist may be able to alter the biota and enhance the biological rate of waste stabilization at low temperatures.

The approach of the study undertaken at Colorado State University was to isolate psychrophilic bacteria and evaluate their potential as inocula into sewage to increase the efficiency of waste stabilization at low temperatures. This approach could be used in already existing facilities or incorporated into the operation of newly designed facilities.

The specific objectives of this study were:

1) Isolation, identification and selection of psychrophilic bacteria capable of relatively high activity at low temperatures.
2) To determine if mutant strains with increased growth rates at low temperatures could be developed.
3) To analyze the effect of combined and pure culture inocula of different size and age on BOD and COD removals in sterile sewage at temperatures between 5 and 20 C.
4) A determination of BOD removal in raw settled sewage by the treatment organisms at low temperatures.
5) To compare the exponential growth rates of the psychrophiles with oxygen uptake rates at 5, 10, 15, and 20 C.
6) To investigate other factors which could conceivably increase the efficiency of BOD removals at low temperatures.

LITERATURE REVIEW

The prolonged persistence of organic pollution and of disease producing organisms in cold regions (1) necessitates adequate treatment of domestic wastewaters. Present treatment facilities, designed for use in temperate regions, are inefficient at low temperatures and must be improved.

The adverse effect of cold temperature on plant efficiency differs among treatment processes. Decreased temperature exerts a more adverse effect on anaerobic processes than on aerobic ones (1): thus, aerobic treatment seems better suited to cold wastewaters. Of the aerobic processes, activated sludge is less seriously affected by low temperature than is the trickling filter (39, 23, 1). Waste stabilization ponds are also adversely affected by low temperature, as winter BOD removals from ponds in Canada range from 0 to 55% compared to 65 to 90% in the summer (27). Aerated lagoons also cannot be depended upon to produce high quality effluents during winter unless provisions for process modifications which enhance efficiency are taken (7). Although aerated lagoon efficiency can be improved by having a number of lagoons in series to minimize short circuiting (99), the effects of temperature on the biological activity of the system remain. Although it has been suggested that it is possible to isolate and culture psychrophiles capable of degrading waste if inoculated into the wastewater, there is little information concerned with the design of such systems and no literature identifying capable species (1). Therefore, only literature indirectly related to the problem can be reviewed. The main topics of this review, then, are: 1) the effect of temperature on biological degradation of wastewater, 2) the properties of psychrophilic microorganisms, and 3) the feasibility of using selected strains of psychrophilic microorganisms to improve cold temperature treatment efficiency.

I. The effect of temperature on biological degradation of wastewater.

A. Biochemical reaction rates

Degradation of sewage is primarily biological, thus, inherently biochemical. Because rates of biochemical reactions vary with temperature according to the van't Hoff-Arrhenius relationship, rates of waste degradation would be expected to follow the equation

$$d(\ln K)/dT = E_a/RT^2 \qquad \text{equation 1}$$

in which E_a is the activation energy for the reaction (cal/mole), K is the reaction rate constant, R is the universal gas constant (1.99 cal/mole · ^oK), and T is the temperature (^oK). Integrating this equation yields a more useful form,

$$\ln K = -E_a/RT + C \qquad \text{equation 2}$$

or

$$\ln \frac{K_2}{K_1} = \frac{-E_a}{R}(1/T_2 - 1/T_1) \qquad \text{equation 3}$$

which shows that a decrease in temperature decreases reaction rate.

The integrated form also shows that a plot of the logarithm of the reaction rate versus the inverse of absolute temperature yields a curve with a slope of $-E_a/R$. If the activation energy is constant, the curve would be linear; but the curve would not be linear if the activation energy is also a function of temperature. Inorganic chemical reactions generally have activation energies which are changed only slightly by changes in temperature, and their Arrhenius plots are essentially linear. The effect of temperature on activation energies for biochemical reactions are more complex. Ware and Jex (99) found that the activation energy for the reduction of manure's BOD by a mixed microbial population was a linear function of temperature (^oC); thus, an Arrhenius plot would not be linear. Sawyer and Rohlich, however, cited numerous investigators who found that the temperature coefficient of reaction rates did not remain constant, but showed breaks at transition temperatures. Between transition temperatures,however, the coeficients were constant. Arrhenius plots of growth rates of a psychrophilic Vibrio grown in a mineral salts-glucose medium showed a temperature range where the curve was clearly linear, but at temperatures outside this linear range, the rate of growth decreased drastically (37).

The apparent discrepancy between the findings of Ward and Jex (99) and the findings reported by Sawyer and Rohlich (80) and Hanus and Morita (37) might be explained on the basis of an inherent

difference in their investigations. Ward and Jex investigated the effect of temperature on a mixed microbial population metabolizing a heterogeneous source of nutrients; whereas, the other investigators studied only single species. It is possible that the gradually changing slope (E_a not constant) of an Arrhenius plot of the rate of a complex biological reaction brought about by a heterogenous microbial population is due to 1) a change in the E_a of specific reactions as the relative abundances of the various species of the mixed population shift as temperature changes, and 2) a change in nutrient substrates being utilized as the population shifts with changes in temperature.

Although a biologist would expect an Arrhenius plot to have a temperature range where the curve is linear, a non-linear van't Hoff-Arrhenius relationship probably depicts the expected effect of temperature on biological removal of BOD from wastewater more accurately. Research has variously shown that process efficiency is: 1) independent of temperature, 2) dependent on temperature, or 3) relatively independent of temperature with temperature changes resulting in a shift of the relative importance of biological and physical processes (51). The discrepancies reported could be because the effect of temperature on treatment efficiency varies among treatment processes.

Hawkes (39) stated that seasonal temperature fluctuations have little effect on activated sludge efficiency. Keefer (48) compiled data over a twenty year period from an activated sludge plant in Baltimore and compared percent BOD and suspended solids removal to temperature. When the average flow was between 18.0 and 22.0 mgd, the average BOD removal was 86.5% at 54 F (11.7 C) and 91.4% at 79 F (26 C), but, when the flow was between 12.0 and 14.0 mgd, the average BOD removal was 89.5% at 54 F (12.2 C) and 91.5% at 76 F (24.3 C). The effect of temperature on suspended solids removal when the flow was between 18 and 22 mgd was similar to the temperature effect on BOD removal. At 53 F (11.7 C) the suspended solids removed was 80.0% and at 79 F (26 C) the removal was 92.5%.

Hunter, Genetelli, and Gilwood (40) studied the effects of temperature on BOD and suspended matter removals in a batch operated

activated sludge system with a 16 hour retention time. The results of their research are tabulated as follows:

Temperature (^{o}C)	%BOD removed	% suspended matter removed
4	79.1	81.5
20	92.3	91.8
28	90.1	83.6
35	91.1	86.6
45	93.7	93.3
55	83.6	81.3

Sawyer (78) studied the effect of temperature and sludge concentration on BOD removal in a batch operated laboratory activated sludge. He used wide mouth two-gallon jars filled with sewage and 1,000 mg/l activated sludge. The jars were placed in constant temperature baths ranging from 10 to 25 C, aerated with diffused air for ten hours and settled for one hour. The supernatant was siphoned off (the effluent) and replaced with fine screened sewage. The average percent BOD removed was 90.5, 94.3, 94.5, and 94.6 at 10, 15, 20, and 25 C, respectively. The percent BOD removal varied with sludge concentration. At 800, 1600, 2400, and 3200 ppm sludge, the BOD removals at 10 C were 84.0, 95.9, 97.2, and 96.8%, respectively.

It appears that the effect of temperature on activated sludge efficiency does not always truly follow the van't Hoff-Arrhenius relationship, because BOD removals often showed only a slight or no relation to temperature. The data of Keefer (48) showed only a 5% increase in BOD removal by activated sludge when the temperature increased from 12 to 26 C, and when the flow was relatively low, there was only 2% increase in BOD removal when the temperature increased from 12 to 24 C. The BOD and suspended solids removal data for activated sludge of Hunter et al. (40) showed no relation to temperature in the range of 20 to 45 C, although outside this range there was a definite temperature effect. Sawyer's (78) data also indicated no temperature effect on BOD removals by activated sludge between 15 and 25 C.

B. Temperature effects

Low temperatures affect equilibrium constants and saturation values of various solids and gases which may be dissolved in sewage (1) and cause a general reduction in the rates of almost all biological, chemical, and physical reactions (1, 16). Different parameters are affected by low temperatures to different degrees in each treatment system, but the overall effect is uniformly undesirable.

Conventional methods of waste disposal in the soil, including the use of leaching pits, septic tank disposal fields, the pit privy, etc., are useless in very cold regions, since decomposition and assimilation do not occur, and accumulation rather than disposal results. This problem is particularly acute in far north permafrost regions where the soil remains frozen year round (20).

When waste disposal in the soil is ineffective, one alternative is disposal into rivers and streams. However, before this can be considered feasible, adequate secondary treatment of the wastewater will be necessary. The secondary treatment process of most conventional wastewater treatment systems is a biological treatment in which organic material present in the primary effluent is microbially oxidized and removed (69) after being adsorbed on a biological floc or slime. According to Hawkes, the organisms actively responsible for this oxidation are mesophilic, having optima between 20 C and 40 C depending upon the species (39), and this is the primary reason that many conventional wastewater treatment processes are unable to do an adequate job of waste stabilization at low temperatures.

Secondary treatment in the activated sludge system is composed of physical adsorption onto the biological floc followed by aerobic degradation of the adsorbed organic materials and anaerobic digestion of the excess sludge formed by the settled floc and removed in the secondary sedimentation basin. The aerobic portion of the activated sludge process has been described as being slightly, but significantly, affected by low temperatures (11, 39, 48, 51, 101). Actual oxygen demand removals drop from the ninety percentile range

at 25 C to 30 C into the eighty percentile range at 5 C to 10 C, a difference in efficiency of 5 to 10 percent (11, 51, 52). Although little mention is usually made of detention times in activated sludge systems, a decrease in operating temperature is almost certain to require extended detention.

Pipes (72) pointed out that there are three essential biological requirements for the activated sludge process:

1) A mixed population of aerobic microorganisms must be capable of degrading the noxious components of the waste.
2) The required population must be able to grow in the aeration tank.
3) The organisms must grow in a form that will settle out in the secondary clarifier.

In describing these microorganisms as mesophiles, Hawkes indirectly provides a reason for one to suspect that Pipes' first two requirements would not be met at low temperatures. Ludzack's findings that low temperatures generally resulted in an inferior floc formation (51) indicates that the last of Pipes' biological requirements may be inadequate at low temperatures. Part of the effects of the depressed biological activity in the activated sludge process are offset by extending the detention time in the aerator to increase oxygen demand removals (11).

Fair, Geyer, and Okun describe anaerobic digestion as a process occurring from mesophilic and thermophilic activity, with the greatest efficiency occurring at approximately 55 C by the thermophilic microorganisms and very slow digestion occurring below 20 C (23). According to Warren (100), thermophilic digestion at temperatures of 43 to 54 C can digest volumes within days that would normally require months at temperatures below 15 C. At low temperatures little digestion is going to be accomplished. After a study of wastewater disposal facilities at DEW line radar stations in Greenland, Reed and Tobiason concluded that without some form of artificial stimulation, low temperature anaerobic systems appear to serve as little more than storage devices (73).

The trickling filter is much more adversely affected by temperature than other aerobic biological wastewater treatment

systems. This is probably due to the fact that the trickling filter relies upon a microbial film for its activity, and this layer's efficiency of organic removal generally increases with an increase in temperature and decreases with a decrease in temperature (10, 58). This same effect is reflected in the statements that trickling filters mature in a few weeks in the summer, but only in several months in the winter (23) and that winter recirculation of wastes (which results in cooling) may cause an efficiency drop up to 20 percent compared to summer (10).

The trickling filter process requires the exposure of the wastewater to a large surface area rendering the system more susceptible to freezing. In severely cold areas, both the rotating distributors and the beds of trickling filters have been known to freeze solid, reducing the effectiveness of the plant to that of a primary settling basin (10).

The viscosity of the influent also results in a lengthening of the necessary detention time in a trickling filter. The detention time has been described mathematically by the formula

$$t_d = ch \frac{\nu}{g}^{\frac{1}{3}} \frac{A/V}{Q}^{\frac{2}{3}} \qquad \text{equation 4}$$

where t_d = detention time, seconds
h = filter depth, feet
ν = kinematic viscosity of the wastewater, ft^2/sec
g = acceleration of gravity, 32.2 ft/sec^2
Q = volume/(unit area) (unit time), ft/sec
A/V = surface area/unit volume of bed, ft^{-1}
c = a dimensionless constant that reflects film buildup (23).

According to Lof, Ward and Hao (50) it can be shown that

$$c = Kk^{\frac{1}{3}} \qquad \text{equation 5}$$

where K = the fraction of the pore volume occupied by the liquid, dimensionless
k = dimensionless coefficient = 5

Furthermore, equation 4 strictly applies at an air flow rate of zero. Air flowing countercurrent to the downward liquid flow would increase the detention time in the filter. Designating this increase in detention time as Δt, the total detention time, t, could be represented by the formula

$$t = t_d + \Delta t \qquad \text{equation 6}$$

They also showed that, for a G/L ratio of less than about 0.4,

$$\Delta t = 400\ (G/L) \qquad \text{equation 7}$$

where G/L = weight ratio of air-water.
Accordingly, the total detention time in a trickling may be expressed by the semi-empirical formula

$$t = Kk^{\frac{1}{3}} h \frac{\nu}{g}^{\frac{1}{3}} \frac{A/V}{Q}^{\frac{2}{3}} + 400\ (G/L) \qquad \text{equation 8}$$

If all other parameters in equation 8 remain constant, an increase in viscosity would result in an increase in detention time. This is important because water viscosity increases as temperature decreases so that water is twice as viscous at 0 C than at 25 C (77). If increased viscosity due to low temperatures results in a sufficiently extended detention time, problems could conceivably arise in loading, especially in high-rate trickling filters which recirculate secondary effluents.

Although several different types of sewage lagoons exist, the aerobic lagoon is undoubtedly the most common type found in cold regions. These systems have a relatively high dissolved oxygen content throughout their entire volume, due usually to mechanical aeration. The oxidation of organics is done biochemically by the microbial flora present in the lagoon.

According to Dawson and Grainge, short retention lagoons with detention times of two to four days characteristically produce 70 percent BOD removals in the summer, but only remove 30 percent of the BOD in the winter (17), consistent with removals obtained by primary settling (57, 69). Halvorson described a similar lagoon located in Winnipeg, Canada, which yielded an 87 percent BOD removal during the summer, but removed only 24 percent during the winter (35).

If contact times are extended for sufficiently long periods, BOD removals of 68 to 90 percent can be obtained (33, 59, 74). The time necessary to obtain any given BOD removal varies with the temperature of the wastewater being treated. Reduction of BOD as high as 88 percent at a temperature of 3 C, with a 9.3 day detention, have been reported in an aerated lagoon by McKinney and Edde (59). In contrast, an aerated lagoon operating in a high altitude Colorado ski area has been described as requiring a 32 day detention at 0.25 C to obtain a BOD removal of only 65.6 percent (see report Section B). Apparently, contact times in excess of 20 days for low temperature aerated lagoons are not unusual (17, 75).

In spite of the long detentions inherent in the low temperature sewage lagoon, it is considered to be a promising system for wastewater treatment in cold climates, and some Alaskan areas as well as mountain communities are presently making use of it (13, report Section B). The sewage lagoon is an attractive treatment system because it is inexpensive (4) and because fairly high BOD removals can be obtained as long as loading rates are low enough or enough lagoons are utilized in series to allow sufficient detention.

II. The properties of psychrophilic microorganisms

The word "psychrophile" is the result of a combination of the two Greek roots "psychros", meaning cold, and "philos", meaning to love. Taken together the word is somewhat of a misnomer, as pointed out by Ingraham and Stokes (45), since the bulk of psychrophilic organisms do not "love" cold as much as they endure or adapt to it.

Biological activity at near 0 C temperatures, characteristic of high altitude or polar region wastewater, is restricted to psychrophilic organisms. Although there is no precise definition of "psychrophile", it usually refers to a microorganism capable of forming macroscopically visible colonies within one week on solid media at 0 C (87). However, other definitions are in the literature (45, 61, 25, 26).

According to Ingraham and Stokes (45), most psychrophilic bacteria belong to the genus Pseudomonas and, to a lesser extent, Achromobacter, Flavobacterium, Alcaligenes, and Micrococcus. Many other genera of psychrophiles, however, have been isolated. These include Vibrio (63),

Serratia (34), and Cytophaga (56). Most psychrophiles are aerobic, but some anaerobic strains have been isolated (83, 96).

Differences between psychrophiles and mesophiles reportedly include range of growth temperature, temperature characteristic of growth, temperature coeficient, and endogenous respiration (90).

Psychrophiles usually have an optimum growth range of 20 to 30 C with a maximum between 30 to 40 C, while mesophiles generally grow optimally at 30 to 40 C with a maximum of about 50 C and a minimum of about 10 C (88). Obligate psychrophiles have an optimum growth temperature below 20 C (87). The majority of obligate psychrophiles show rapid thermal death at temperatures greater than 20 C (38), and exposure to melted agar is sufficient to render certain obligate psychrophiles nonviable (62). Psychrophiles also have a shorter lag period over the temperature range in which growth of both groups occurs (15).

A plot of generation time versus temperature is similar for mesophiles and psychrophiles. The major difference is that the psychrophile has a generation time minimum at a temperature lower than that of the mesophiles (41). Greater differences are seen when Arrhenius plots, logarithm of growth rates vs. inverse temperature, are compared. The maximum growth rate for the psychrophile occurs at a lower temperature than the maximum for the mesophile, and the slope of the linear region is less negative for the psychrophile than for the mesophile (42). The slope of the linear region is the negative temperature characteristic (μ) for growth; thus, on the basis of an Arrhenius plot of Ingraham's data, the psychrophile can be distinguished from a mesophile because of it's characteristic of low temperature growth. This comparison between psychrophiles and mesophiles is best when the temperature characteristic is determined between 15 and 25 C (6).

Hanus and Morita (37), however, reported that psychrophiles could not be distinguished from mesophiles on the basis of the temperature characteristic as they found that reported values of μ showed no consistant differences between mesophiles and psychrophiles. These discrepancies reported by Hanus and Morita may by due to species variation rather than to basic differences between psychrophiles and mesophiles, since μ for a mesophilic mutant of a psychrophile Micrococcus cryophilus, was higher than μ for the parent psychrophile (91).

Psychrophiles reportedly can also be distinguished from mesophiles on the basis of their temperature coefficient of glucose oxidation (temperature coefficient = $Q_{10} = (K_2/K_1)^{10/dT}$ where K is the rate of reaction and dT is the change in temperature, C). Psychrophiles exhibit a lower temperature coefficient of glucose oxidation, with rates being based on oxygen uptake in a glucose substrate (15,44). The temperature coefficient for acetate and formate oxidation was also less for psychrophiles than for mesophiles (44). Temperature coefficients, however, differed when reference temperatures are changed, and the groups were best distinguished from one another only when reference temperatures were set at 10 and 30 C (91).

Baxter and Gibbons (8) reported that a psychrophilic yeast, Candida, had a greater endogenous respiration rate than a mesophile up to 30 C. Endogenous respiration comparisons of a psychrophilic bacterium, Micrococcus cryophilus and its mesophilic mutant gave greater rates for the mesophile (91), indicating that differences in endogenous respiration were not attributable to the ability to grow at low temperatures.

Comparisons between psychrophiles and mesophiles on the basis of temperature characteristic, temperature coefficient (Q_{10}), and rates of endogenous respiration are unreliable in distinguishing the two groups. By definition the groups can be distinguished on the basis of temperature range of growth; psychrophiles are able to function at low temperature, but mesophiles cannot. Rose (76) believed that low temperatures have two effects on the physiology of microorganisms: 1) on lipid content, and 2) on the regulation of metabolism. As temperature is lowered, the proportion of unsaturated fatty acids in the lipids of the cytoplasmic membrane of Escherichia coli increases (54). These changes in the fatty acids of the cytoplasmic membrane may change the architectural nature of the membrane and prevent permease action (25). Psychrophiles, presumably,resist structural changes as temperature decreases.

The fatty acid composition of a psychrophilic Candida remained nearly constant during growth, but that of a mesophile, Candida lypolytica changed during growth (47). Although this change was not conclusive evidence as to why mesophiles cease to grow at low temperature, it did show there was a difference in lipid metabolism between psychrophiles and mesophiles.

In addition to affecting lipid content and cell structure, low temperatures may alter metabolism. There are numerous reports of temperature changing the metabolic end products (76). Usually, only a proportional decrease in the end products occurred with decreased temperature, but occasionally the ratio of metabolic products changed or even new products could be formed (68). There was often an increase in polysaccharide synthesis at low temperature (76). Psychrophiles are capable of a wide range of metabolic activity at low temperatures, exhibiting proteolytic, lipolytic and saccharolytic abilities to varying extents (32, 35, 65, 70, 86). Most psychrophiles appear to be able to hydrolyze urea extremely well and at very high rates (35, 86). In testing 437 psychrophilic strains, Stefaniak found that 86.5 percent were capable of urea decomposition, 69.8 percent could ammonify peptones, 62 percent were capable of nitrate reduction, 23.1 percent were capable of degrading starch, and 19 percent were able to hydrolyze gelatin. It is interesting to note that, although psychrophilic nitrate reduction has been observed (12, 86), the process seems to stop short of actual dentrification. Also, in spite of highly aerobic environments, low temperatures appear to inhibit nitrification (14, 33, 60, 78).

Many enzymes and enzyme forming systems of psychrophiles are abnormally sensitive to heat (88), and this sensitivity could explain the adverse effect of temperatures above the optimum for growth (61). Formic hydrogenlyase of a psychrophilic, gram-negative, rod shaped bacterium was not synthesized above 20 C, and, once formed, is inactivated at 45 C. A mesophile, Escherichia coli, however, synthesized the enzyme at 45 C, and this enzyme is not denatured until 70 C (97).

The L-serine dehydrase of a psychrophilic Pseudomonas aeruginosa is less affected by temperatures below 15 C than is the same enzyme of a mesophilic Escherichia coli (85). Psychrophiles are apparently endowed with enzyme systems that are more temperature sensitive than those of mesophiles, accounting for the psychrophile's inability to function at relatively moderate temperatures. These enzymes function better at lower temperatures enabling them to grow at relatively low temperatures.

Other effects of temperature on enzymes exist: effects on feedback inhibition (43), induction and repression of enzyme synthesis (76), and changes in catalytic activity due to shifts in the conformation of the enzymes (93) caused by increased intra- and inter-molecular hydrogen bonding at lower temperatures (9, 53).

Psychrophily is the result of a cell structure and metabolism which allows the transport and breakdown of an exogenous nutrient supply to obtain energy for growth at 0 C. Although both cell structure and metabolism account for psychrophily, cell structure is the primary factor enabling growth at low temperature. Brown (9) and Ingraham and Bailey (44) showed that glucose oxidation rates differ between mesophiles and psychrophiles only when whole cell suspensions were used. With sonicated suspensions, the rates of glucose oxidation of the two groups were similar. These findings do suggest that cell structure determines the ability of an organism to grow at low temperature.

III. The feasibility of using selected strains of psychrophilic microorganisms to improve cold temperature treatment efficiency.

The inoculation of psychrophiles into a wastewater treatment system to improve efficiency at low temperature has been suggested, but there is little information found in the literature pertaining directly to such an approach (1). Although Boyd and Boyd (13) suggested that "starter" cultures could be developed and added to the sewage to increase mineralization at low temperatures, they did not attempt to isolate or test strains of bacteria capable of relatively high degradative ability at low temperature. The feasibility is interesting and open to conjecture. The biological degradation of wastes involves a myriad of ecological relationships; consequently, considerations for improving the biological removal of wastes at low temperature should follow an ecological approach. The objectives of an ecological study of waste are to determine what organisms are beneficial to the system and to determine how to design and operate the process so growth of the beneficial organisms is encouraged (72). The organisms most useful in stabilizing waste's organic matter, however, are not really known.

Dias and Bhat (19) felt that the most abundant organisms in their study, Zoogloea and Comamonas, were the most important, although they recognized that it was doubtful that a single bacterial species could utilize all the organic substances that occur in sewage and bring about its complete stabilization. Unz and Dondero (95), on the other hand, felt that the heterogeneous bacteria associated with nondescript flocs and zoogloeas were most important in waste stabilization, and the roles

played by branched zoogloeas and possibly Zoogloea spp. were relatively minor. The predominant genera of bacteria reported present in activated sludge varies among authors, but Pipes (72) assimilated the findings of different investigators and determined that Achromobacter, Alcaligenes, Flavobacterium,Pseudomonas, and Bacillus were the most common. The enteric bacteria present in domestic wastes were generally ignored by the various workers.

Any bacterial strain capable of increasing waste treatment efficiency at low temperature should be able to: 1) utilize the organic matter of the waste at low temperature and 2) for trickling filter or activated sludge, form an adsorptive slime or settleable adsorptive floc.

A microorganism able to utilize waste at low temperatures would likely be psychrophilic and would probably be a strain representative of the genera commonly found in sewage. Pseudomonas, Achromobacter, Alcaligenes and Flavobacterium, genera common to wastewater, have psychrophilic representatives (45). It is, thus, likely that bacteria exist that can utilize waste at low temperatures.

Because a sludge with good settling qualities is necessary for an activated sludge process to be effective, organisms capable of forming flocs are important to the treatment system. The significant bacteria in activated sludge must be able to flocculate by themselves, be flocculated by other organisms, or be able to attach themselves to larger particles which do settle out (72). Although the specific organisms most capable of forming floc are not known (30), many genera are evidently capable of flocculation. Zoogloea was once considered the most important floc former, but many other genera, including some strains of Escherichia, Alcaligenes, Bacillus, and Pseudomonas, have been isolated that readily flocculate in pure cultures (72). Unz and Dondero (95) isolated 20 strains of gram negative bacteria that were capable of forming flocs when grown in a nutrient broth. It is, thus, possible that psychrophilic bacteria are also capable of flocculation.

Because bacteria probably exist that are capable of utilizing waste at low temperatures and also capable of settling as flocs, improvement of treatment efficiency at low temperatures by using inocula of selected bacteria appears feasible. The only other alternative to biological treatment in cold environments seems to be housing and heating the entire treatment system (16).

MATERIALS AND METHODS

The methods and materials used in this study will be presented under the following headings:

I. Isolation and selection of psychrophilic organisms
   A. Local isolates
   B. Alaskan Isolates
II. Ultraviolet irradiation of sewage and selected local isolates
III. Biochemical oxygen demand determinations
IV. Psychrophilic treatment of sterile sewage with local isolates
V. Psychrophilic treatment of raw settled sewage
   A. Local isolates
   B. Alaskan isolates
VI. Psychrophilic treatment of concentrated synthetic sewage with Alaskan isolates
VII. Analysis of raw settled sewage effluent
VIII. Growth rate determinations on local isolates
IX. Oxygen uptake studies on local isolates

I. Isolation and selection of psychrophilic organisms

A. Local isolates

Water and soil samples were taken from the Fort Collins, Colorado, vicinity; the sampling locations are identified in Table 1. Either 1-ml or 0.1-ml aliquots of the water samples were spread with a glass rod on regrigerated Bacto Plate Count Agar (Difco, PCA) plates, and, for soil samples, 1 g of the soil was suspended in a 9-ml sterile buffer blank, shaken thoroughly, and either 1 or 0.1-ml aliquots were spread on regrigerated PCA plates. All plates were kep at near 4 C. Colonies which became visible within a week were picked and assigned a Roman numberal (designating the sampling location) and an Arabic number (designating the colony picked from the plate) (Table 1).

The isolates that were obtained from soil, water, irradiated sewage, and from the irradiation of previously selected isolates were inoculated into 9 ml of m-PCB contained in screw-cap tubes and placed in a test tube rack fixed at an angle of approximately

45° on a gyrotory shaker. The shaker was placed in a walk-in refrigerator (near 0 C), and the cultures were shaken at 150 rpm for 3 days. Twenty isolates, visually demonstrating the most dense growth, were transferred to and maintained on refrigerated PCA slants.

The number of isolates to be maintained was further decreased by selecting 5 isolates that grew best in heat sterilized sewage at near 0 C. Two loopfuls each, of the twenty isolates, were inoculated into 10 ml of heat sterilized sewage and incubated for 24 hr in the walk-in refrigerator with shaking. Serial dilutions were then made and spread on PCA plates which were incubated at 20 C for 48 hr. Although there were 20 cultures, only five were tested at a time, and the 1 or 2 isolates from each group yielding the highest colony counts were maintained.

Each of these five isolates was inoculated into respective 10-ml aliquots of filter sterilized sewage and incubated in a household refrigerator (4 C) for 48 hr. The density of each of the five cultures was adjusted to a percent transmittance of 80 at wavelength of 450 nm with a Spectronic 20 spectrophotometer. Because the percent transmittance of all five cultures was less than 80, adjustment with sterile deionized water to 80% was made. One ml of each adjusted suspension was inoculated into respective 100-ml aliquots of heat sterilized sewage contained in 250-ml erlenmeyer flasks, and incubated on the gyrotory shaker (150 rpm) for 24 hr in the walk-in refrigerator. Serial dilutions were plated on PCA and incubated for 48 hr at 20 C. The three isolates (XVI-4, XXII-1, XXIV-1) yielding the greatest number of colonies were maintained by monthly transer on refrigerated PCA slants. Cultures XIV-4 and XXIV-1 were selected for use in the experimental studies; the third isolate (T28B) used was from soil and was selected on the basis of its rapid oxygen uptake at 10 C.

Table 1. Sampling locations and identification of colonies which became visible within a week on PCA at refrigerator temperature.

Sample Number	Site	Number of Colonies Picked
IV	Sugar beet waste	1
V	Sugar beet waste	2
VI	Effluent-Fort Collins Trickling Filter Plant	3
VII	Effluent-Fort Collins Trickling Filter Plant	3
VIII	Effluent-Fort Collins Trickling Filter Plant	1
XVI	Poudre River-Mountain Park	4
XVII	Poudre River-Mountain Park	8
XVIII	Poudre River-Mountain Park	6
XX	Poudre River-Watson Lake	10
XXI	Watson Lake	7
XXII	Watson Lake	5
XXIII	Raw sewage-Fort Collins Trickling Filter Plant	3
XXIV	Raw sewage irradiated 5 sec	2
XXV	Raw sewage irradiated 10 sec	4

B. Alaskan isolates

A total of 97 psychrophilic isolates taken from the Chena, a subarctic Alaskan river, were obtained from Ronald C. Gordon, Ph.D., of the Environmental Protection Agency, College, Alaska. Each was subcultured into 5 ml quantities of half strength Bacto m-Plate Count Broth (½ m-PCB) in screw-cap tubes upon arrival at the laboratory and were incubated stationary at 1 C for 5 days. At the end of this five-day incubation, the culture tubes were each shaken vigorously on a Vortex mixer and 0.1 ml of the resulting cell suspension from each tube was spread plated on PCA. All plates were incubated at 1 C for 5 days and were inspected daily for signs of growth. The ten cultures exhibiting the fastest growth during this incubation period were set aside for further screening.

Gram stain indicated that the most rapidly growing organisms were gram negative rods of approximately the same size, so organism RW-25 was arbitrarily chosen as the "standard" for preparing a turbidimetric cell determination curve. A 200 ml volume of ½-m PCB was placed in a 500 ml erlenmeyer flask and autoclaved for 15 minutes at 124 C. After cooling, this medium was inoculated with organism RW-25 from the broth culture described above and incubated for 24 hours at 20 C with shaking at 113 rpm. The culture was then centrifuged in a refrigerated centrifuge at 9750 x g for 20 minutes. The supernatant was discarded and the cell pack resuspended in deionized water. Various dilutions of this cell suspension were made, each microscopically counted for total cell number per ml in an Improved Neubauer Levy Ultra Plane counting chamber and turbidity was read in a Klett Summerson Photoelectric Colorimeter using a blue No. 42 Klett filter (400-450 nm). The standard curve was a plot of the turbidity against cell numbers.

Fresh raw sewage was obtained from the Fort Collins #1 trickling filter plant immediately following grit clarification, was supplemented with 0.5 gram per liter Bacto Yeast Extract and autoclaved. After cooling, this sterile supplemented sewage was centrifuged for 20 minutes at 12,100 x g and the supernatant vacuum filtered simultaneously through a double thickness of fiber-glass prefilters and a 0.45 membrane filter. The filtrate was again autoclaved and refrigerated at 5 C.

A methylene blue thiocyanate solution was prepared by dissolving an 8.23 mg tablet of the dye in 50 ml boiling deionized water and brought to a volume of 200 ml by adding cool deionized water. The solution was refrigerated at 5 C in a foil covered flask.

The ten cultures obtained from the primary screening procedure were inoculated into 10 ml quantities of sterile supplemented sewage in sterile screw-cap test tubes. All tubes were incubated at 5 C for 4 days after which they were vigorously shaken on a Vortex mixer and each cell suspension adjusted to a total cell concentration of 1.5×10^8 cells per ml (Klett value of 10) by adding deionized water.

The final screening step involved the use of two incubation systems. The first was composed of eleven test tubes to each of which

had been added 8 ml of the sterile supplemented sewage, 2 ml of cell suspension and 1 ml of the methylene blue thiocyanate. The last tube (control) contained no psychrophilic cell suspension. The second system was identical to the first except that 8 ml of raw sewage which had been allowed to settle in the 1 C incubator for one hour was used as substrate instead of the sterile supplemented sewage. Both systems were incubated at 5 C for 5 days after which methylene blue reduction was determined visually from no reduction (++++) to complete methylene blue reduction (0). Both systems were compared and the three psychrophiles demonstrating greatest methylene blue reduction in both systems were selected as treatment organisms. These, designated B-6, B-39, and C, were maintained, from this point on, in sterile supplemented sewage as stock cultures.

Determination of pH effects on treatment organisms was done using an acetate buffer at pH values of 4 and 5 according to the method of Walpole (98), a tris-maleate buffer and pH values of 6, 7 and 8 according to the method of Gomori (31), and a carbonate-bicarbonate buffer at pH values of 9.2, 10, and 10.7 according to the method of Delory and King (18). One ml of buffer at each pH value was placed individually into three sterile screw-cap test tubes. Sterile supplemented sewage in 8 ml quantities was then added to all tubes. The three tubes buffered at each pH value were inoculated with 1 ml of the stock culture cell suspensions of each treatment organism, one organism to a tube. This was done so that at each pH value, three tubes would be represented, each tube containing a different treatment organism. Klett turbidity and pH measurements were made on all mixtures at the beginning and at the end of a three day 5 C incubation. Initial and final pH values were averaged together and recorded along with the difference between initial and final Klett turbidity measurements. The data presented graphically gave expression of the effect of pH on growth.

The effect of pH on substrate oxidation was experimentally determined using the same experimental design with the exception

that to each tube was added and thoroughly mixed, 1 ml of the methylene blue thiocyanate solution described above. At the end of the incubation period, all tubes were compared to a set of visual standards made by using 0.0041, 0.0033, 0.0025, 0.0016, 0.0008, 0.0004 and 0 mg per 1 of methylene blue thiocyanate in deionized water. Amount of methylene blue reduction (substrate oxidation) was recorded in mg per 1 methylene blue which compared most closely to the appropriate standard. Percent reduction was calculated and used for plotting.

II. Ultraviolet irradiation of sewage and selected local isolates

Approximately 20 ml of either raw sewage or broth cultures of the locally isolated psychrophiles were placed in clean glass petri dishes and irradiated from a distance of 37.5 cm with a 15 watt General Electric ultraviolet light source (2537 Å wavelength). The raw sewage samples were irradiated for 2, 5, or 10 seconds, and the isolates for 1 min. One millileter of the irradiated sample was then spread with a glass rod on PCA plates and incubated near 0 C in the walk-in refrigerator. Colonies which became visible within 4 days were isolated for possible use as inocula in the experimental studies.

III. Biochemical oxygen demand determinations

All standard 5-day, 20 C biochemical oxygen demands (BOD) were determined by the azide modification of the iodometric Winkler method described in Standard Methods for the Examination of Water and Wastewater (2). All dilutions were made directly in 300-ml BOD bottles by adding a known volume of sewage plus a 0.3 ml (equivalent to 0.1%) raw sewage seed into the BOD bottle and then siphoning aerated dilution water into the bottle until filled. The concentration of sewage added was determined by dividing the volume of sewage added by 300 ml. Only 5-day, 20 C BODs were determined.

IV. Psychrophilic treatment of sterile sewage with local isolates

Either 10 ml (large inoculum) or 0.1 ml (small inoculum) of the 24-hr or 7-day old culture suspension was inoculated into a 1000-ml flask containing 600 ml of sterilized sewage. A known volume (usually 3-6 ml) was aseptically withdrawn and placed into a 300-ml BOD bottle

for determination of the initial 5-day, 20 C, BOD. This inoculated sewage was decanted equally into three sterile 500-ml erlenmeyer flasks and incubated at the appropriate temperature (5, 10, 15, or 20 C) for 23 hr in an incubator shaker with aeration accomplished by shaking (60 rpm). After the 23-hr aeration period, the sewage was decanted into sterile 250-ml flasks and allowed to settle for 1 hr at the same temperature used when aerated. From this settled sewage, the final BODs were determined. The percent BOD removed was used as the parameter of treatment efficiency, and the data was analyzed by a four-factor analysis of variance. It was necessary to modify the three-factor analysis described in Snedecor and Cochran (84) to fit four treatments.

With combined culture inocula the experimental method was essentially the same as described for single cultures. Here, however, only large inocula (10 ml), each consisting of one of the four possible combinations of the three isolates, cultured and harvested as described previously, were used. These combinations were: 1) 5 ml XVI-4 + 5 ml T28B, 2) 5 ml XVI-4 + 5 ml XXIV-1, 3) 5 ml XXIV-1 + 5 ml T28B, and 4) 3.3 ml XVI-4 + 3.3 ml XXIV-1 + 3.4 ml T28B. The incubation conditions and BOD determinations were the same as described previously. The data were analyzed together with the large inocula data of the single culture experiment using a three-factor analysis of variance described by Snedecor and Cochran (84).

Two mixed, mesophilic populations were employed in this study as controls. The first control consisted of an inoculum obtained from a raw sewage culture and the method was the same as that used in single culture inocula studies. The data from this control were analyzed together with the data from the single culture inocula studies. The second control consisted of the raw sewage with no added inoculum. Approximately 200 ml of the freshly collected raw sewage was added to a clean 500-ml flask and the initial BOD was determined as described earlier, except seeding was not necessary. The sewage was incubated concurrently with the single and combined culture inoculated sewage and the final BOD was determined. The percent BOD removed was calculated, but the data was not analyzed statistically with the other data. This control was also used as the seed material for determining BODs on the heat sterilized sewage.

V. Psychrophilic treatment of raw sewage

A. Local isolates

A loopful of each of the three psychrophilic isolates, or 1 ml of raw sewage, was inoculated into a 250-ml erlenmeyer flask containing 100 ml of heat sterilized sewage supplemented with 1/5 strength m-PCB, and incubated at room temperature (20-25 C) on a gyrotory shaker at 150 rpm for 1 day (18-24 hr) or for 7 days. The cultures were centrifuged in a refrigerated centrifuge and the cell pellet was suspended in sterile deionized water so that a 1:50 dilution gave a reading of 50 Klett units as measured by a Klett-Summerson photoelectric colorimeter using the blue filter (400-450 nm). This cell suspension contained approximately 1.2×10^{10} cells/ml (Appendix 1).

Freshly collected raw sewage was decanted as 1000-ml aliquots into each of three 2000-ml flasks and placed into a constant temperature bath at 2 C. After the sewages equilibrated with the temperature of the bath (approximately 2 hr), one was inoculated with 5 ml of a suspension containing equal amounts of the three psychrophiles. The second flask was inoculated with 5 ml of a mixed, mesophilic population suspension, and the third flask was not inoculated. A volume of 7.5 ml was withdrawn from each flask for determining initial BOD concentrations (no seed was required). Each of the three sewages was aerated by bubbling compressed air, first into a flask containing water equilibrated with the temperature of the bath to filter and cool the incoming air and then into each of the three sewages. After a 4-hr aeration period, three 250-ml flasks were filled with each of the three sewages and allowed to settle for 1 hr before two 9-ml volumes from each flask were withdrawn for duplicate determinations of BOD. Percent BOD removed was calculated as the parameter of treatment efficiency and the data were analyzed statistically by a two-way analysis of variance (14).

B. Alaskan isolates

One hundred ml of sterile supplemented sewage was placed in each of three sterile 250 ml erlenmeyer flasks, inoculated with

each of the treatment organisms and incubated in a shaker at 5 C for 2-3 days, shaking at 92 rpm. When relatively heavy growth became visually apparent, turbidity was determined and converted to total cell number per ml. A sufficient amount of inoculum was placed in 50 ml centrifuge tubes to yield approximately 3×10^{10} total cells. These suspensions were centrifuged at 12,000 x g for 20 minutes, the supernatant discarded and the cell packs resuspended in 10 ml deionized water by vigorous shaking. This wash cycle was repeated once and the 10 ml suspensions were ready for use as inocula.

Several liters of raw sewage were obtained immediately following grit clarification and allowed to settle for one hour at 1 C, after which the supernatant was drawn off and 300 ml quantities placed in one-liter flasks. These were inoculated with a 10 ml cell suspension described above and 5 ml of centrifuge wash water were added; the control contained 15 ml of deionized water. The inoculation of treatment organisms gave a total psychrophilic cell count of approximately 1×10^{8} cells per ml. All treatment flasks were incubated in the temperature controlled shaker at 5 C for 5 days at 92 rpm.

Treatment flasks were sampled initially and at 24 hour intervals for 5 days for BOD determinations (3). D_1 and D_2 values for the BOD were determined in duplicate for each treatment and averaged to yield a single BOD value at each sampling period.

Initially and at 24 hour intervals for 5 days appropriate dilutions of samples from each of the psychrophilic treatment systems as well as of the control system were made, using sterile buffered water blanks. Each dilution was spread plated in 0.1 ml amounts in duplicate on PCA. All plates were incubated for 2 weeks at 1 C in the walk-in cold incubator and plates showing 30 to 300 colonies were counted and duplicates averaged.

VI. Psychrophilic treatment of concentrated synthetic sewage with Alaskan isolates

Psychrophilic inoculum preparation in this experiment was identical to that described earlier for Alaskan isolates. A fecal coliform which failed to exhibit visual evidence of growth for 2 weeks at 5 C

was used as a mesophilic control. This organism was inoculated into a 100 ml volume of sterile supplemented sewage, incubated for 24 hours at 37 C without shaking, harvested and used in the same manner as the psychrophilic inocula.

The synthetic sewage of Pipes (71) with the modification that boron was not added as one of the constituents was used. This synthetic material was made in triple strength and used in the same manner as the raw settled sewage described earlier with one modification, tris-maleate was used to buffer the flasks, containing organisms B-6, B-39 and control at pH 7.0 to 7.2 and the treatment flask containing organism C to a pH of 7.4 to 7.6. Dow Corning Antifoam A was used periodically to control excessive foaming.

BOD determinations were identical to those previously described and the procedure for psychrophilic counts was the same as described earlier with the following exceptions: 1) dilutions of 10^{-2} of the fecal coliform control were made and 0.1 ml plated in duplicate and 2) psychrophilic treatment plates were incubated at 15 C for 60 hours.

VII. Analysis of the raw settled sewage effluent

Inoculum preparation was identical to that described earlier in this paper for Alaskan isolates, except that the inoculum was adjusted to yield a cell count of 6×10^8 cells for each treatment organism.

Substrate preparation was also identical to that described earlier, with the exception that 2 liter flasks were used to contain 600 ml of the raw settled sewage. Initially and at 24 hour intervals, 90 ml quantities were removed from each flask, vacuum filtered simultaneously through a double thickness of prefilters and a 0.45 μ membrane filter. Aliquots of the filtrate from each treatment were used for analysis.

A. Analysis for nitrate-nitrite nitrogen

Aliquots of 20 ml from each treatment filtrate were colorimetrically analyzed for nitrate-nitrite nitrogen by the method of Schall and Hatcher (81), with the modification that the protein precipitation was not carried out. All colorimetric tests in the effluent analyses were read for optical density in a Spectronic 20 spectrophotometer.

B. Analysis for sugar

One ml aliquots from each treatment filtrate were colorimetrically analyzed for sugar by the method of Morris (64).

C. Analysis for orthophosphate

Aliquots of 35 ml from each treatment filtrate were colorimetrically analyzed for orthophosphate by the Vanado-Molybdate methods as described in Standard Methods for the Examination of Water and Wastewater (3).

D. Analysis of viscosity

Viscosity measurements of all treatment filtrates were made using a Gilmont V-2100 falling ball viscosimeter.

VIII. Growth rate determinations

Each of the three selected local isolates were inoculated into respective 200 ml portions of heat sterilized sewage contained in 500 ml erlenmeyer flasks, incubated in a shaker at 25, 20, 15, 10, or 5 C, and shaken at 100 rpm. Standard plate count determinations were made in triplicate for each culture four times, either once hourly, when incubated at 25 C, or once every other hour, when incubated at lower temperatures, while the cultures were in exponential growth (16-24 hr after inoculation). Growth rates were determined using the equation:

$$\frac{\log_{10} N_{T_2} - \log_{10} N_{T_1}}{0.301} \times \frac{1}{T_2 - T_1} = \text{generations/unit time} \qquad \text{equation 9}$$

where:

N_{T_2} = number of colonies counted at time T_2

N_{T_1} = number of colonies counted at time T_1

$0.301 = \log_{10} 2$

IX. Oxygen uptake studies on local isolates

The determination of oxygen uptake was performed in a Precision Warburg Apparatus as developed by Unbreit, Burris, and Stauffer (94).

The main compartment of each Warburg vessel received 2 ml of heat sterilized sewage and the side arm received 0.5 ml of a cell suspension (prepared as described previously for local isolates). The center well contained 0.2 ml of a 20% KOH solution and a fluted piece of filter paper (approximately 1 cm^2) to absorb CO_2.

The flasks were equilibrated to bath temperature before they were tipped and the cells mixed with the sewage. Readings were recorded at 15-min intervals for the first half-hour and then at 30 min intervals for the duration of the 6 hr incubation period. Uptakes were determined at 25, 20, 15, 10 and 5 C for each of the three isolates cultured in either ½ strength m-PCB or sterilized sewage supplemented with 1/5 strength m-PCB for less than 24 hr or for 7 days. Since all suspensions did not contain the same number of cells, all readings were corrected to a standard cell concentration (1.2×10^9 cells/ml) using the relationship of cell number to Klett reading (Appendix 1). It was assumed that the rate of oxygen utilization was proportional to the concentration of cells present in the reaction vessel.

RESULTS

Three locally isolated psychrophiles, two from aquatic environments and the other from soil, were selected as test organisms for experiments in raw and sterilized sewage. Improved growth rates at low temperatures of these and other isolates were not accomplished by ultraviolet irradiation. Of the Alaskan isolates, three were selected for use as inocula in raw sewage and concentrated synthetic sewage to determine the potential of psychrophiles used to increase wastewater stabilization at 5 C.

The results obtained in this study will be presented under the following headings:

I. Analysis of the effect that single and combined local culture inocula of different size and of different age have on BOD removals in sterilized sewage at temperatures ranging from 5 to 20 C.

II. Comparison of the BOD removal ability of psychrophiles inoculated into raw sewage to the removal ability of raw sewage's indigenous flora.
   A. With local isolates.
   B. With Alaskan isolates.

III. Comparison of the ability of Alaskan psychrophilic isolates to stabilize a concentrated synthetic sewage, measured by BOD and percent BOD reduction, with the stabilization ability of a known mesophile (fecal coliform) in the same substrate.

IV. Comparison of the effects of inoculated Alaskan psychrophiles on the chemical and physical composition of the wastewater substrate with effects exhibited by the raw sewage's indigenous flora.

V. Comparison between growth rates and oxygen uptake rates of the three local psychrophilic isolates in heat sterilized sewage.

VI. The effect of pH on the growth and oxidative ability of the Alaskan isolates in sterile sewage supplemented with 0.05 percent yeast extract.

I. Analysis of the effect that single and combined local culture inocula of different size and of different age have on BOD removals in sterilized sewage at temperatures ranging from 5 to 20 C.

The first experimental design involved determining what effect age and size of inocula of the three local psychrophilic isolates and of a mesophilic population had on percent BOD removals from sterilized sewage at temperatures ranging from 5 to 20 C. The composite results obtained with the four treatments are presented in Table 2 and the analysis of variance, shown in Table 3, revealed that the effects of all four treatments and their interactions were significant at the 0.5% level.

The most significant two-way interactions were size-age, temperature-age, and organism-temperature; the other two-way interactions were not as significant. The only three-way interactions significant at the 0.5% level were temperature-size-age and organism-temperature-age.

The results of BOD removals obtained by the single treatment variables are shown in Tables 4 through 7. The data in these tables were derived from Table 2 by averaging the percent removal values of the separate variables of each single treatment over all other identical variables. The BOD removal for the significant interactions, obtained similarly to the single treatment variables, are shown in Table 8 through 14 and Figures 1 and 2.

Table 4 reveals that the percent BOD removed varied among isolates. The difference among isolates was relatively slight although statistically significant (Table 3).

The differences in percent removals among temperatures (Table 5) were greater than differences among organisms; thus, the effect of temperature was greater than the effect of organism used as the inoculum, a fact shown by the larger F value for the temperature treatment. There was little difference between removals at 5 and 10 C, although the differences between successively higher test temperatures were large.

Table 6 shows that a large inoculum (10^{10} cells) removed more BOD than a small inoculum (10^{8} cells). As seen by the large difference between the two size variables and by the F value from Table 3 for the size treatment, this treatment had the greatest effect on BOD removals.

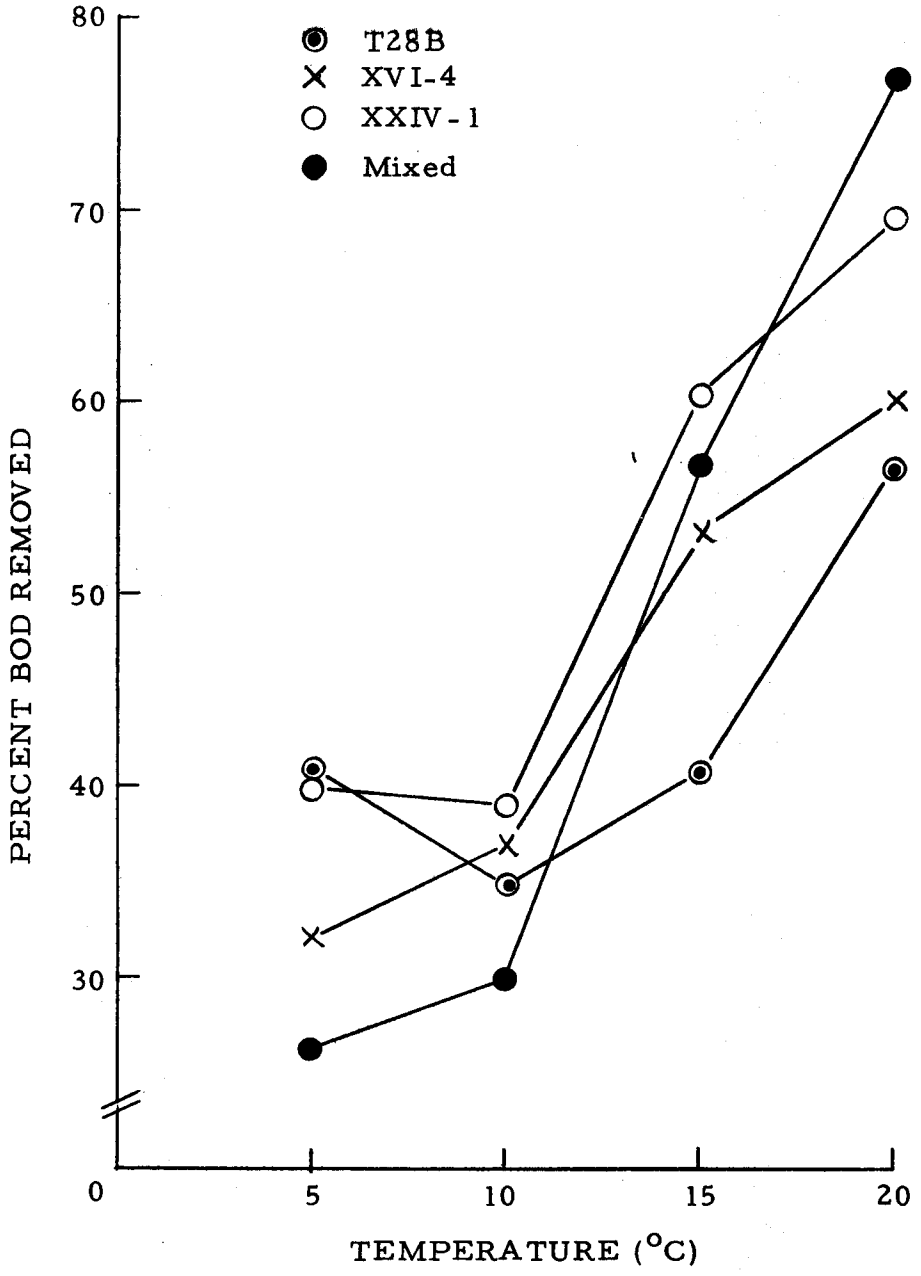


Figure 1. Average percent BOD removals by the four inocula at different temperatures.

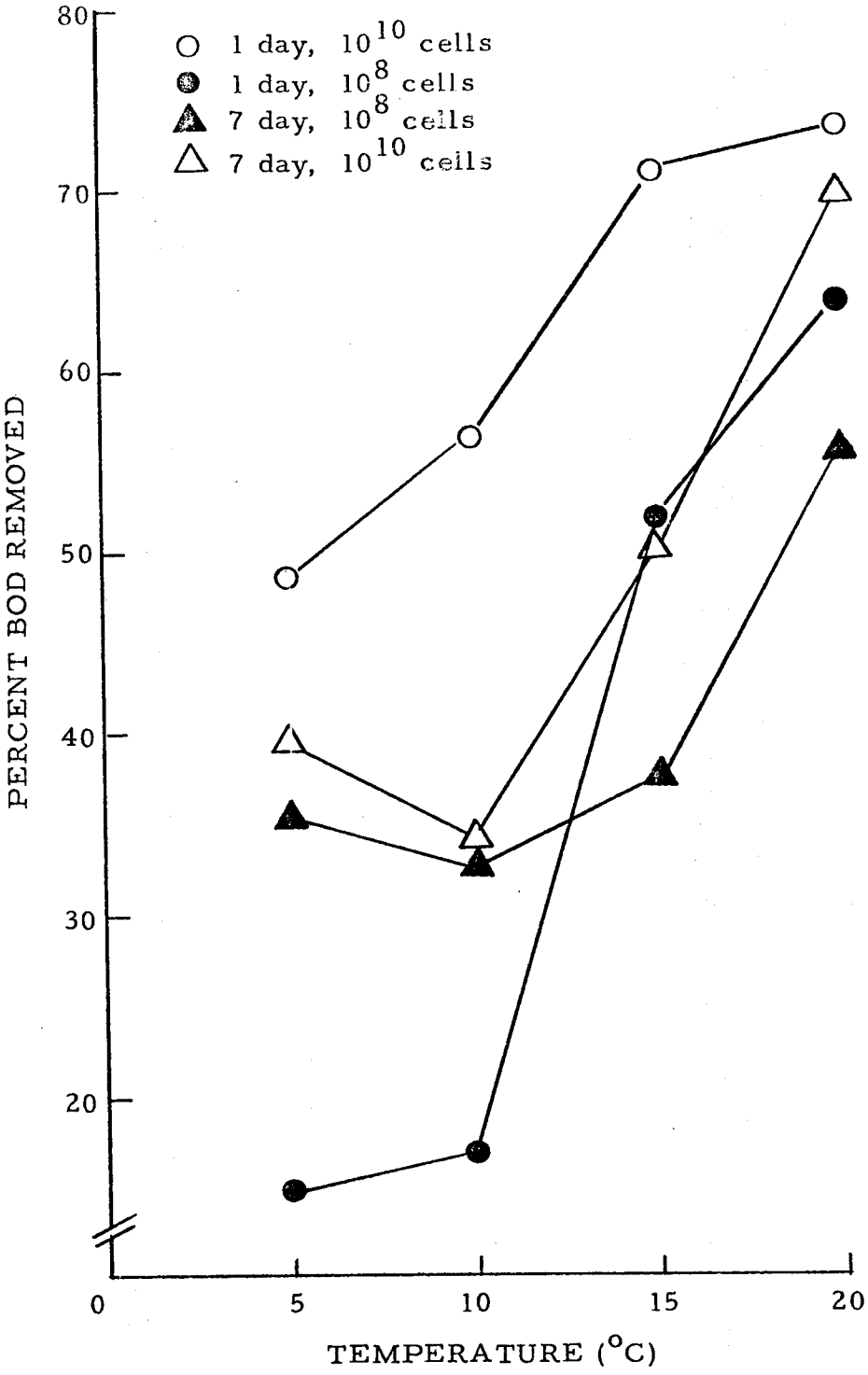


Figure 2. Average percent BOD removals by large and small quantities of 1 and 7 day old cultures of inocula at different temperatures.

Table 2. Percent BOD removal data, from heat sterilized sewage, for different age and size of four inocula at 5, 10, 15 and 20 C.

Temp. °C	Age	Organism							
		XVI-4		XXIV-1		T28B		MIXTURE	
		A	B	A	B	A	B	A	B
5	1 day	57.4	17.6	56.5	7.2	50.7	23.6	39.0	8.7
		51.0	13.5	50.9	17.6	52.5	19.3	32.3	8.2
		48.5	13.5	50.9	23.8	52.5	15.0	42.4	9.9
		(52.3)*	(14.9)	(52.8)	(16.2)	(51.9)	(19.3)	(37.9)	(8.8)
	7 days	35.4	20.4	51.0	35.9	50.7	38.1	20.4	37.9
		32.7	34.8	47.8	40.6	53.6	38.1	14.3	29.4
		32.7	25.8	44.5	49.9	50.7	41.8	35.1	35.2
		(33.6)	(27.0)	(47.8)	(42.2)	(51.7)	(39.3)	(23.3)	(34.2)
10	1 day	64.5	33.1	56.5	77.8	44.9	31.0	61.4	16.8
		58.7	9.7	59.6	66.1	46.2	4.8	58.8	10.9
		55.3	27.6	57.5	18.0	50.3	17.0	62.2	19.6
		(59.5)	(23.5)	(57.9)	(10.6)	(47.2)	(17.6)	(60.8)	(15.8)
	7 days	34.6	27.1	47.8	37.1	40.6	38.3	30.4	21.3
		11.0	60.8	47.8	46.7	39.0	32.7	32.5	1.5
		24.6	35.9	46.3	35.1	39.0	32.7	18.0	23.6
		(23.4)	(41.2)	(47.3)	(39.6)	(39.5)	(34.6)	(27.0)	(15.4)

Table 2. Percent BOD removal data, from heat sterilized sewage, for different age and size of four inocula at 5, 10, 15 and 20 C.

Temp. °C	Age	Organism							
		XVI-4		XXIV-1		T28B		MIXTURE	
		A	B	A	B	A	B	A	B
15	1 day	83.3	46.8	79.5	47.7	66.0	39.8	77.1	64.8
		47.4	53.2	78.7	51.0	60.9	38.6	86.0	63.6
		49.2	38.2	85.1	69.3	56.9	46.2	80.5	63.6
		(60.0)	(46.1)	(81.1)	(56.0)	(61.3)	(41.5)	(81.2)	(64.0)
	7 days	58.5	48.5	62.5	43.9	33.9	26.4	41.6	35.2
		57.8	49.4	69.5	39.5	29.0	27.1	51.5	36.1
		57.4	49.4	62.5	34.1	31.9	28.1	43.8	35.2
		(57.8)	(49.1)	(64.8)	(39.2)	(31.6)	(27.1)	(45.6)	(35.5)
20	1 day	69.9	72.4	76.0	79.4	62.8	55.9	85.2	52.9
		70.5	69.6	73.7	76.5	59.8	54.5	86.6	52.2
		71.0	63.2	76.9	78.9	61.0	55.3	86.6	55.5
		(70.4)	(68.4)	(75.3)	(78.3)	(61.2)	(55.2)	(86.2)	(53.2)
	7 days	63.3	45.3	70.8	67.7	57.6	45.6	84.4	79.0
		55.5	43.8	71.4	62.4	65.7	46.3	87.1	84.5
		52.6	44.5	74.8	27.7	67.2	44.0	85.5	79.6
		(57.1)	(44.6)	(72.3)	(52.6)	(63.5)	(45.3)	(85.6)	(81.0)

A - Initial inoculum of approximately 2×10^7 cells/ml

B - Initial inoculum of approximately 2×10^5 cells/ml

* - (Average value)

Table 3. Four-way analysis of variance of percent BOD removal data for different age and size of four inocula at 5, 10, 15, and 20 C.

SOURCE	d.f.	Sum Squares	Mean Square	F
Total	191	80,531.79		
Treatments	63	73,506.17		
Organisms (O)	3	2,133.40	711.13	12.96***
Temperature (T)	3	32,509.43	10,836.48	197.42***
Size (S)	1	13,227.71	13,227.71	240.99***
Age (A)	1	1,302.13	1,302.13	23.72***
Interactions				
O · T	9	5,867.90	651.99	11.88***
O · S	3	411.78	137.28	2.50
O · A	3	274.94	91.65	1.67
T · S	3	547.51	182.50	3.32**
T · A	3	3,291.58	1,097.19	19.98***
S · A	1	3,763.11	3,763.11	68.55***
O · T · S	9	1,327.98	147.55	2.69**
O · T · A	9	4,916.83	546.31	9.95***
O · S · A	3	499.74	166.58	3.03*
T · S · A	3	3,539.71	1,179.90	21.05***
O · T · S · A	9	1,194.55	132.73	2.42*
Residual	128	7,025.62	54.89	

Significance levels:
* 5.0%
** 2.5%
*** 0.5%

Table 4. Percent BOD removals from sterilized sewage by the psychrophile and mixed, mesophile inocula averaged over the other treatments.

Inoculum			
XVI-4	XXIV-1	T28B	Mixed Mesophile
45.6%	52.1%	43.0%	47.2%

Table 5. Percent BOD removals from sterilized sewage at different temperatures averaged over the other treatments.

Temperature (^{o}C)			
5	10	15	20
34.6%	35.1%	52.6%	65.7%

Table 6. Percent BOD removals from sterilized sewage by large and small quantities of inocula averaged over the other treatments.

Number of Cells Inoculated	
10^{10}	10^{8}
55.3%	38.7%

Table 7. Percent BOD removals from sterilized sewage by 1 and 7-day old cultures of the inocula averaged over the other treatments.

Culture Age of Inocula	
1 day	7 days
49.6%	44.4%

Table 8. Average percent BOD removals by the four inocula at different temperatures.

	Inoculum			
Temperature (^{o}C)	XVI-4	XXIV-1	T28B	Mixed
5	32.0%	39.7%	40.6%	26.0%
10	36.9%	38.9%	34.7%	29.7%
15	53.2%	60.3%	40.4%	56.6%
20	60.1%	69.6%	56.3%	76.6%

Table 9. Average percent BOD removals by large and small quantities of the four inocula.

Number of Cells Inoculated	Inoculum			
	XVI-4	XXIV-1	T28B	Mixed
10^{10}	51.8%	62.4%	51.0%	55.9%
10^{8}	39.6%	41.8%	35.0%	38.5%

Table 10. Average percent BOD removals by 1 and 7-day old cultures of the four inocula.

Culture Age	Inoculum			
	XVI-4	XXIV-1	T28B	Mixed
1 day	49.4%	53.5%	44.4%	51.0%
7 days	41.7%	50.7%	41.6%	43.5%

Table 11. Average percent BOD removals of large and small quantities of inocula at different temperatures.

Number of Cells Inoculated	Temperature (^{o}C)			
	5	10	15	20
10^{10}	43.9%	45.3%	60.4%	71.5%
10^{8}	25.2%	24.8%	44.8%	59.9%

Table 12. Average percent BOD removals by 1 and 7-day old cultures of inocula at different temperatures.

Culture Age	Temperature (^{o}C)			
	5	10	15	20
1 day	31.8%	36.6%	61.4%	68.6%
7 days	37.4%	33.5%	43.8%	62.8%

Table 13. Average percent BOD removals by large and small quantities of 1 and 7-day old cultures of inocula.

Number of Cells Inoculated	Culture Age of Inocula	
	1 day	7 days
10^{10}	62.3%	48.2%
10^{8}	36.9%	40.5%

Table 14. Average percent BOD removals by large and small quantities of 24 hour and 7 day old cultures of inocula at different temperatures.

Culture Age	Number of Cells Inoculated	Temperature (^{o}C)			
		5	10	15	20
1 day	10^{10}	48.7%	56.3%	70.9%	73.3%
	10^{8}	14.8%	16.9%	51.9%	63.9%
7 days	10^{10}	39.1%	34.3%	50.0%	69.7%
	10^{8}	35.7%	32.7%	37.7%	55.9%

The effect of culture age (Table 7) was relatively slight compared to the temperature and size treatments, but was still significant (Table 3).

The organism-temperature interaction data (Table 8, Figure 1) revealed that changes in temperature affected each organism's ability to remove BOD differently, with the mixed, mesophilic inoculum being affected the most and T28B the least. Although T28B removed the

least percent BOD over the entire temperature range studied, it removed the greatest percent at 5 C.

As seen in Table 9, showing the average percent BOD removed by two sizes each of four inocula, a smaller inoculum removed less BOD than a larger inoculum - a fact consistent with the data from Table 6. The effect of a smaller inoculum on BOD removed was similar for each organism, indicating that no organism-size interaction existed (a small, insignificant F value).

The data, presented in Table 12, for the temperature-age interaction showed that 7-day cultures removed more BOD at 5 C than did 1-day cultures, although, at 10, 15, and 20 C, 7-day cultures removed less than 24 hour cultures. The significance of this difference at 5 C was confirmed by the F value given for this interaction.

The size-age interaction disclosed that a small inoculum of 7-day old cultures removed more BOD than 1-day old cultures, but a large inoculum of 7-day old cultures removed much less percent BOD than old cultures. The significance of these findings, which opposed the general tendency of lower removals by 7-day cultures, could also be seen by the large F value.

The average percent BOD removed by two ages of two inoculum sizes at different temperatures, the temperature-size-age interaction, are shown in Table 14 and Figure 2. At 5 and 10 C, 1-day cultures of small inocula removed less BOD than corresponding 7-day cultures; however, at 15 and 20 C, 1-day cultures of small inocula removed more BOD. Relationships found in the two-way interactions (Table 11-13) could also be verified as shown in Table 14.

Table 15 shows percent BOD removals by 1 and 7-day old inocula of combinations of the three isolates at 5, 10, 15, and 20 C. Because only the large inoculum (10^{10} cells) was used, the data of Table 15 and the data for the large inoculum of Table 2 were statistically analyzed together. The results of the analysis (Table 16) disclosed that all treatments and their interactions were statistically significant. Table 17 shows that there was little detectable difference in percent removals between single and combined culture inocula.

The effect of temperature on percent BOD removals by the indigenous flora of raw sewage is given in Table 18. Low temperature (5 C) had

Table 15. Percent BOD removal data, from heat sterilized sewage for different aged combinations of inocula at 5, 10, 15, and 20 C.

Temp. (°C)	Age (days)	Organisms			
		A	B	C	D
5	1	50.4	11.2	34.2	68.5
		52.3	12.6	33.2	66.5
		51.4	9.7	31.1	63.6
		(51.4)*	(11.2)	(32.8)	(66.2)
	7	18.7	31.3	22.4	41.3
		21.5	27.8	16.7	36.2
		25.6	25.5	31.9	56.4
		(21.9)	(28.2)	(23.7)	(44.6)
10	1	58.7	58.7	73.3	70.8
		40.6	61.6	72.0	68.2
		46.3	57.8	89.2	68.9
		(48.5)	(59.3)	(78.2)	(69.3)
	7	45.6	47.3	34.2	36.1
		55.5	49.1	36.2	26.0
		50.0	46.4	34.2	30.5
		(50.4)	(47.5)	(34.8)	(30.9)
15	1	52.5	62.8	60.9	61.0
		49.1	61.0	59.3	50.0
		46.9	60.1	60.1	64.6
		(49.5)	(61.3)	(60.1)	(58.6)
	7	54.7	54.8	66.8	68.0
		54.1	55.7	62.4	65.0
		56.1	55.7	65.0	66.5
		(55.0)	(55.4)	(64.7)	(66.5)
20	1	54.9	61.7	71.8	70.6
		58.8	66.6	72.4	70.0
		83.6	63.0	73.0	71.6
		(65.8)	(63.8)	(62.4)	(70.7)
	7	67.4	76.1	74.6	73.9
		69.1	76.6	72.9	70.0
		70.2	76.6	74.6	71.6
		(68.9)	(76.4)	(74.0)	(71.8)

A - XVI-4 & T28B, B - XVI-4 & XXIV-1, C - T28B & XXIV-1,
D - XVI-4 & T28B & XXIV-1.
* - (average value)

Table 16. Three-way analysis of variance of the percent BOD removal data for different aged combinations of inocula at 5, 10, 15 and 20 C.

SOURCE	d.f.	Sum Squares	Mean Squares	F
Total	191	60,232.67		
Treatments	63	56,674.59		
Organisms	7	3,339.36	477.05	17.16***
Temperature	3	26,704.03	8,901.34	320.19***
Age	1	5,075.34	5,075.34	182.47***
Interactions				
O T	21	9,624.41	458.31	16.49***
O A	7	2,344.17	334.88	12.05***
T A	3	3,200.26	1,066.75	38.37***
O T A	21	6,387.02	304.14	10.94***
Residual	128	3,558.08	27.80	

Significance Levels: * 5.0%
** 2.5%
*** 0.5%

a pronounced adverse effect on percent removals, as removals at 5 C were much lower than at the other temperatures. There was little difference between removals at 10 and 15 C, but removals at 20 C were appreciably greater. It should be noted, however, that random selection of sewage samples provided initially higher oxygen demands for the 20 C samples. This may have influenced the percent BOD removals slightly.

II. Comparison of the BOD removal ability of psychrophiles inoculated into raw sewage to the removal ability of raw sewage's indigenous flora.

A. With local isolates.

Previous experiments had been performed using heat sterilized sewage, instead of raw sewage, so that the data represented BOD

Table 17. Percent BOD removals by two culture ages of single and combined culture inocula averaged over all temperatures.

Inoculum Type	Average Percent BOD Removed
Single culture	(55.1)*
XVI-4	51.8
XXIV-1	62.4
T28B	51.0
Combined culture	(54.2)*
XVI-4 & T28B	51.4
XVI-4 & XXIV-1	50.4
XXIV-1 & T28B	55.1
XVI-4 & XXIV-1 & T28B	59.8

*(Average)

removals attributable to treatment effects rather than to the indigenous flora effects. To determine whether an inoculum of psychrophiles could increase BOD removals in raw sewage at low temperatures, tests at 2 C on raw sewage inoculated with psychrophiles were compared to removal by a mesophilic inoculum and to raw sewage with no inoculum.

As shown in Table 19, the sewage inoculated with psychrophiles removed more BOD (%) than mesophile-inoculated or non-inoculated raw sewage. As seen in Table 20, this difference was statistically significant at the 0.5% level; however, the variance among replications was greater. Although the psychrophile-inoculated sewage had a greater percent BOD removed than the mixed or non-inoculated sewage, Table 21 shows that the initial and final BOD concentrations in inoculated sewage were greater than in non-inoculated sewage.

B. With Alaskan isolates

The effects of inoculated and indigenous flora on BOD over a five day holding period are expressed graphically in Figure 3, 5,

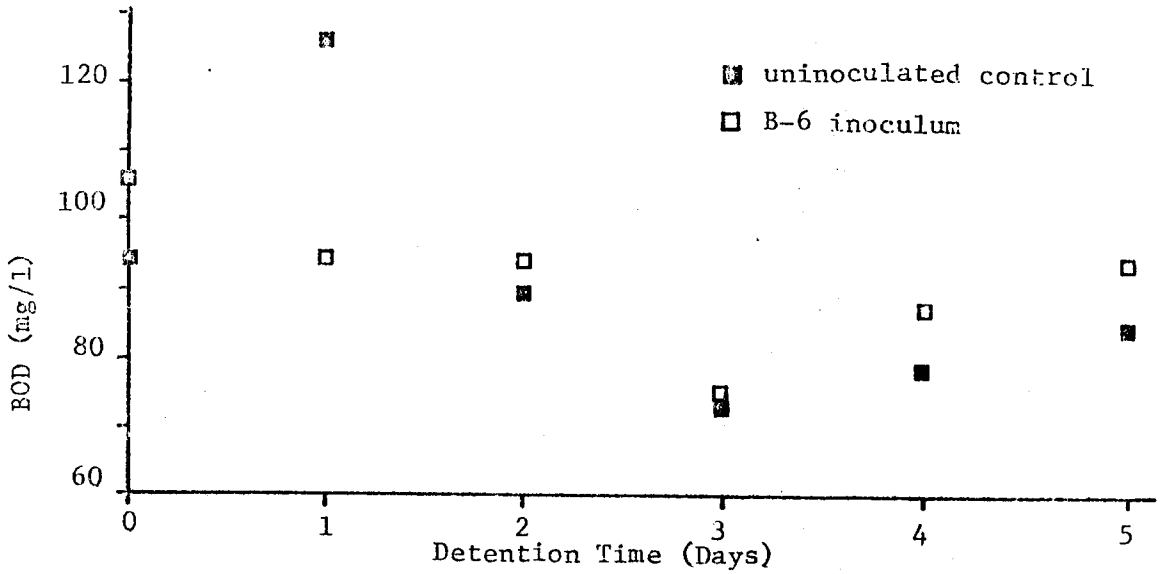


Figure 3. A plot of BOD in treated and untreated raw settled sewage at 5 C expressed as a function of time.

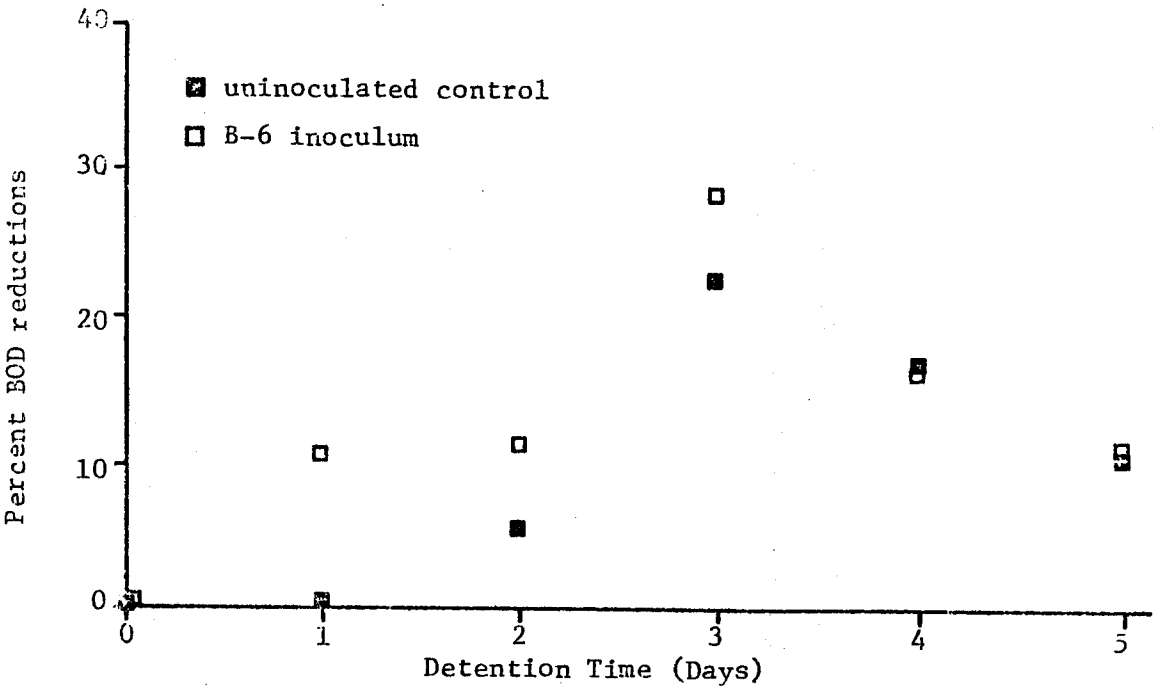


Figure 4. A plot of percent BOD reductions in treated and untreated raw settled sewage at 5 C expressed as a function of time.

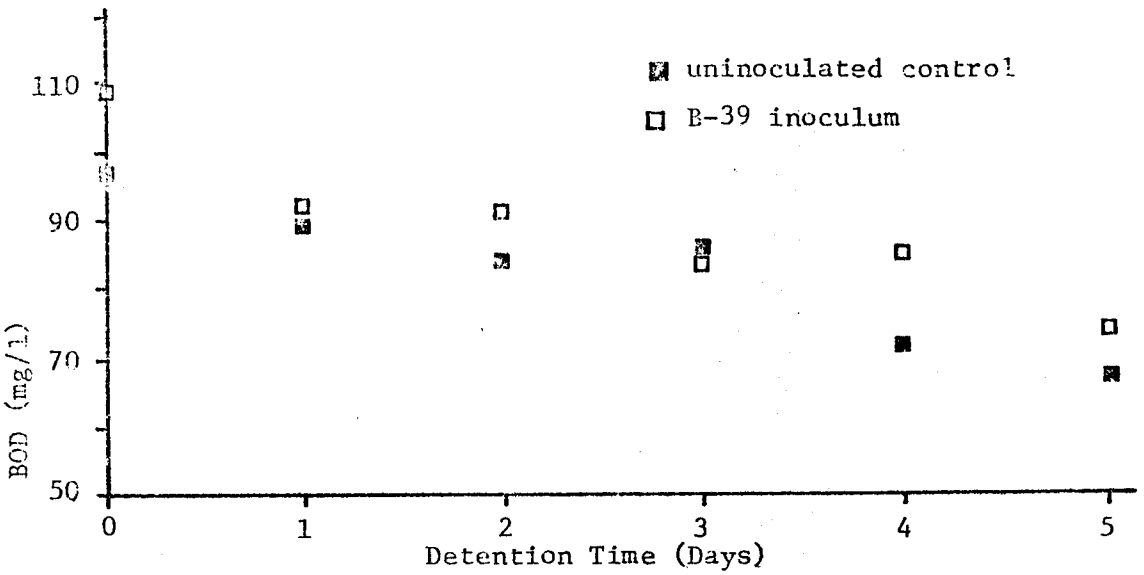


Figure 5. A plot of BOD in treated and untreated raw settled sewage at 5 C expressed as a function of time.

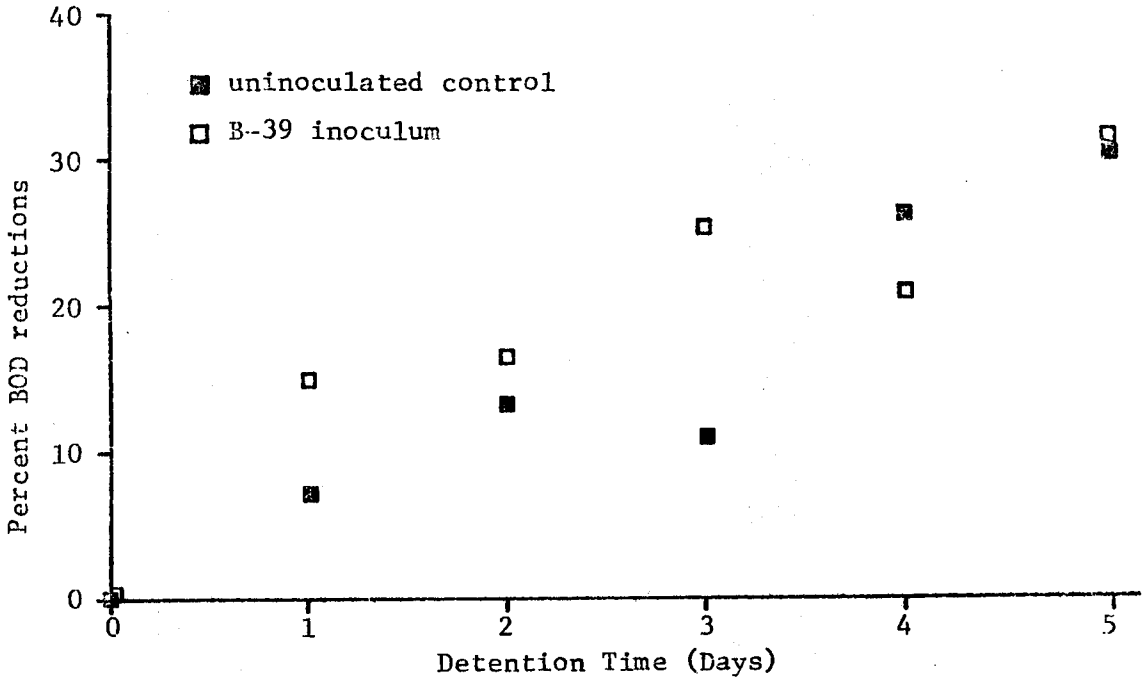


Figure 6. A plot of percent BOD reductions in treated and untreated raw settled sewage at 5 C expressed as a function of time.

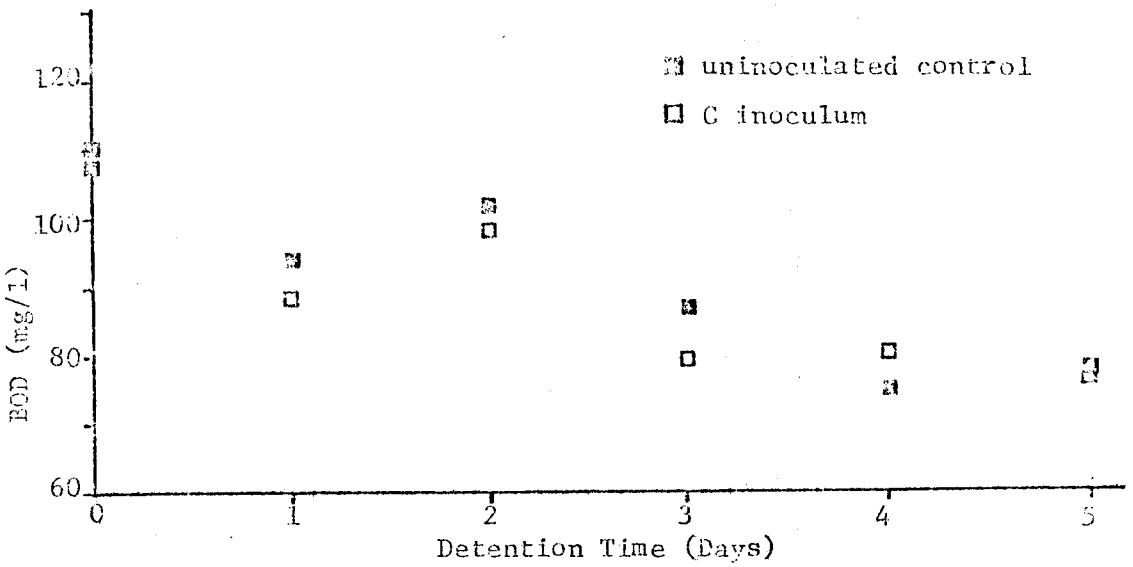


Figure 7. A plot of BOD in treated and untreated raw settled sewage at 5 C expressed as a function of time.

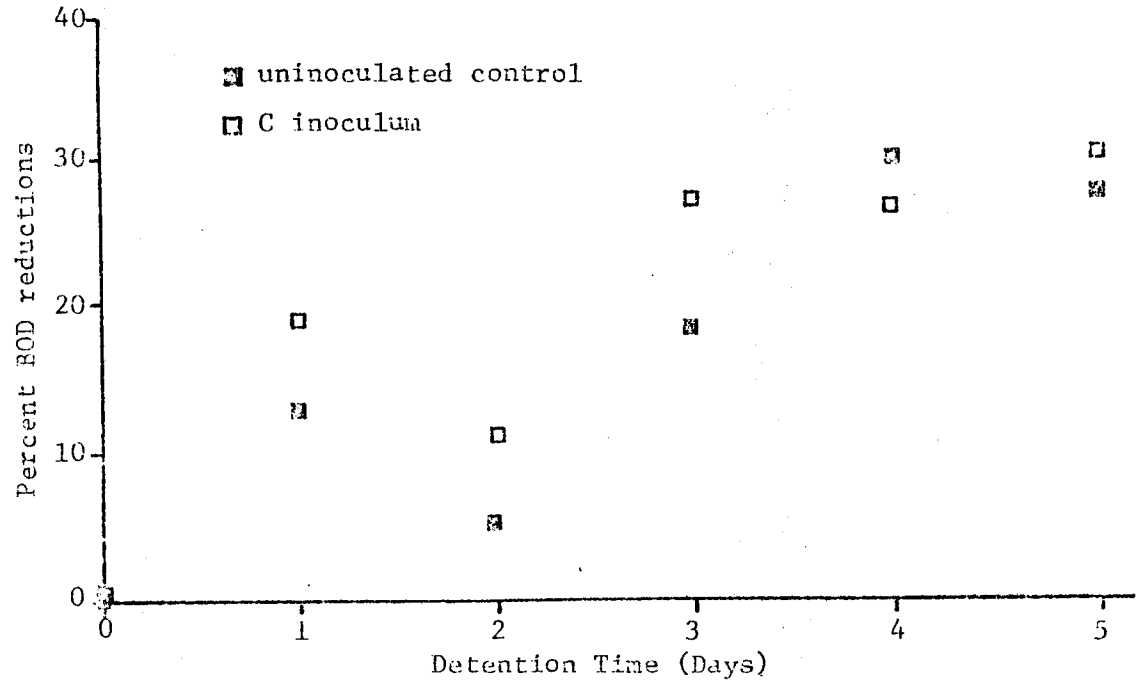


Figure 8. A plot of percent BOD reductions in treated and untreated raw settled sewage at 5 C expressed as a function of time.

Table 18. Initial BOD, change in BOD, and percent BOD removed for the raw sewage control (no inoculum) at 5, 10, 15, and 20 C.

Temp. (°C)	Initial BOD		Change in BOD		Percent BOD removed	
5	125.0	190.0	62.5	62.0	50.0	32.6
	140.0	240.0	32.5	136.0	23.2	56.8
	180.0	155.0	98.0	39.0	54.4	25.1
	240.0	185.0	124.0	65.0	51.7	35.1
	165.0	164.0	49.0	92.0	29.7	56.1
	115.0	222.9	43.0	134.9	37.4	60.5
	(176.8)*		(78.2)		(42.7)	
10	195.0	130.0	101.0	94.0	51.8	72.3
	175.0	205.0	95.0	165.0	54.3	80.5
	175.0	175.0	141.0	121.0	80.6	69.1
	165.0	190.0	105.0	150.0	63.6	79.0
	175.0	265.0	147.0	189.0	84.0	71.3
	190.0	195.0	148.0	205.0	77.9	69.5
	(194.6)		(138.4)		(71.2)	
15	150.0	200.0	104.0	104.0	69.3	74.0
	70.0	205.0	28.0	161.0	40.0	78.5
	170.0	140.0	122.0	115.0	71.8	82.1
	210.0	227.5	143.3	169.2	68.2	74.4
	160.0	275.0	106.0	213.0	66.2	77.4
	(180.8)		(131.0)		(70.2)	
20	350.0	330.0	298.0	301.7	85.1	91.4
	300.0	230.0	272.0	174.0	90.7	75.6
	579.0	260.0	561.0	216.0	96.7	83.1
	186.8	145.0	154.8	115.0	87.7	79.4
	180.0	165.0	142.0	115.0	78.9	69.7
	210.0	235.0	158.0	171.0	75.1	72.8
	(264.2)		(223.2)		(81.8)	

*(Average)

Table 19. Percent BOD removals from raw sewage at 2 C using no inoculum, a mixed (mesophilic) inoculation, and a psychrophilic inoculum.

Replications	Inoculum Type					
	None		Mixed		Psychrophilic	
1	24.4 23.0	(23.7)*	15.9 17.4	(16.7)	23.7 27.4	(25.6)
2	13.0 9.4	(11.2)	10.7 12.4	(11.5)	15.9 25.1	(20.5)
3	17.8 15.6	(16.7)	5.9 5.9	(5.9)	23.6 14.9	(19.3)
4	17.7 7.2	(12.5)	7.6 9.6	(8.6)	12.5 14.6	(13.5)
5	2.6 2.6	(2.6)	2.8 2.8	(2.8)	18.7 14.7	(16.7)
6	17.8 22.2	(20.0)	24.8 24.8	(24.8)	22.9 20.8	(21.9)
7	0.0 1.7	(0.9)	6.9 2.2	(4.5)	8.1 8.1	(8.1)
8	0.1 5.1	(2.6)	5.1 19.8	(12.5)	14.7 16.7	(15.8)
9	29.8 29.8	(29.8)	25.7 22.1	(28.6)	17.7 19.8	(18.8)
10	25.4 31.8	(28.6)	22.6 34.4	(28.6)	34.9 31.0	(33.0)
Sums	279.3	(14.0)	296.8	(14.8)	385.9	(19.3)

*(Average)

Table 20. Analysis of variance for the effect that a psychrophilic, a mesophilic, and no inoculum have on BOD removals from raw sewage within 5 hr at 2 C.

Source	df	Sum Squares	Mean Squares	F
Total	59	5,145.98		
Treatments (Inoculum)	2	327.12	163.56	6.55***
Replications	9	3,625.19	402.80	16.12***
Residual	48	1,193.67	24.87	

Significance levels: * 5.0%
** 2.5%
*** 0.5%

and 7. Each point on the graph was the arithmetic average of three experimental determinations. The trend exhibited by both inoculated psychrophiles and indigenous flora present in the uninoculated raw sewage was generally that of a gradual decline. The exception to this can be seen in Figure 3 where the BOD decreased up through the first three days of incubation then increased for the remaining two days. Differences between the BODs of inoculated and uninoculated systems were slight.

Percent BOD reductions represented in Figures 4, 6, and 8 indicate a trend toward increasing removals with increasing periods of detention. However, at no time during the five day incubation did any of the systems examined approach reductions to the 80 percent level, the minimum usually required. Differences between percent BOD reductions of inoculated and uninoculated systems were also slight as could be expected in view of the fact that differences between their BOD values were small.

Psychrophilic cell growth in the raw settled sewage substrate is described in Figures 9 through 11 for both inoculated microorganisms and those naturally present in the uninoculated sewage.

Table 21. Initial and final BOD concentrations (mg/1) raw sewage with and without inocula.

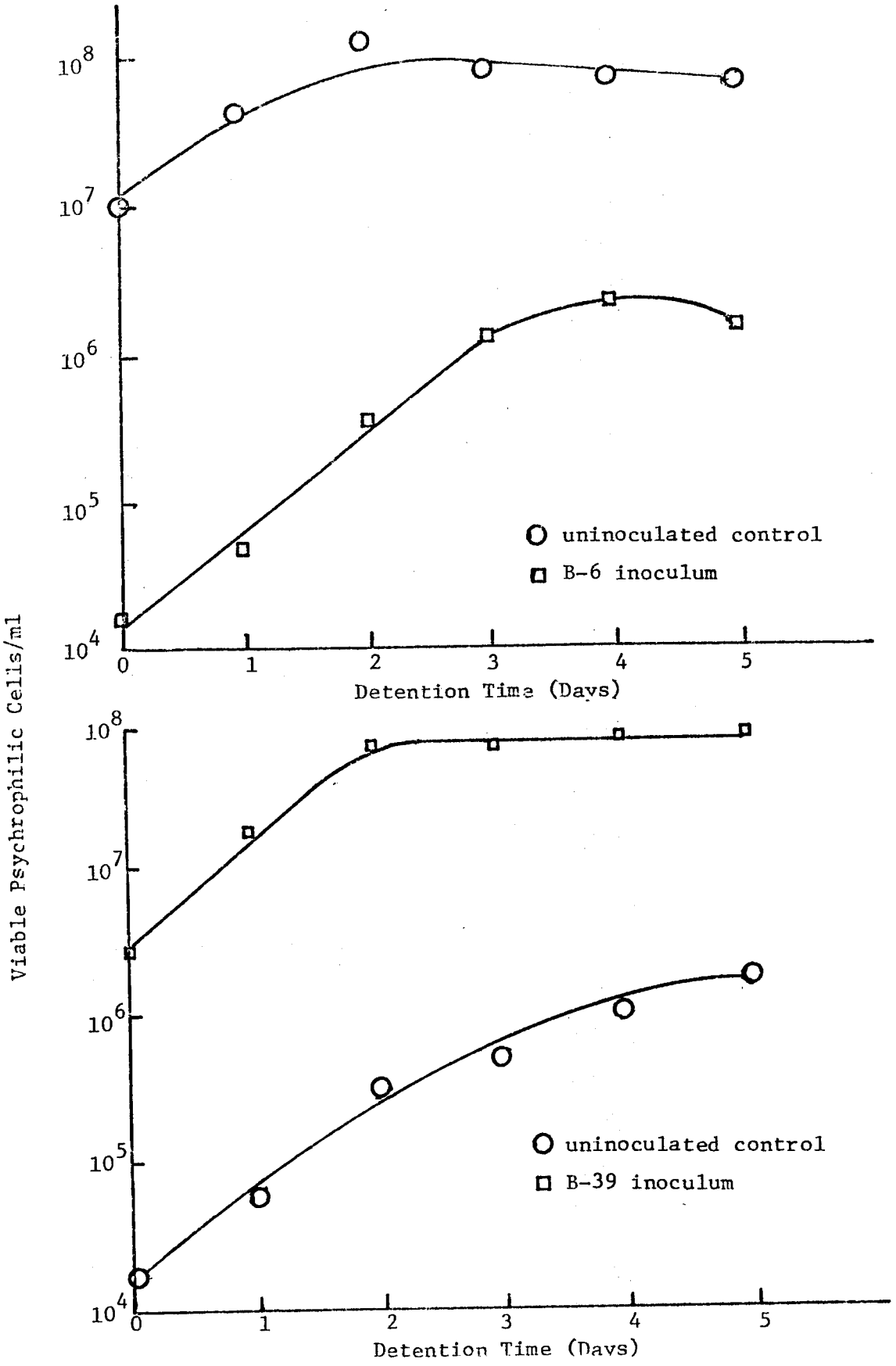
Mixed Inoculum		No Inoculum		Psychrophilic Inoculum	
Initial	Final	Initial	Final	Initial	Final
170	160 160	154	127 130	192	147 163
166	153 150	158	130 146	160	140 137
144	140 140	130	127 127	168	137 143
164	123 123	150	123 117	160	123 127
136	127 133	156	183 153	156	143 143
158	150 127	144	143 137	172	147 143
154	197 167	210	157 143	256	167 177
230	193 190	238	183 180	271	207 197
194	173 170	184	160 167	218	183 163
184	137 142	152	107 107	158	130 127
(180)	(153)	(168)	(142)	(191)	(152)

Figure 9

Viable psychrophilic cell number in treated and untreated raw settled sewage at 5 C expressed as a function of time.

Figure 10

Viable psychrophilic cell number in treated and untreated raw settled sewage at 5 C expressed as a function of time.


Viable Psychrophilic Cells/ml
10^8
10^7
10^6
10^5
10^4
0
1
2
3
4
5
Detention Time (Days)
uninoculated control
B-6 inoculum
10^8
10^7
10^6
10^5
10^4
0
1
2
3
4
5
Detention Time (Days)
uninoculated control
B-39 inoculum

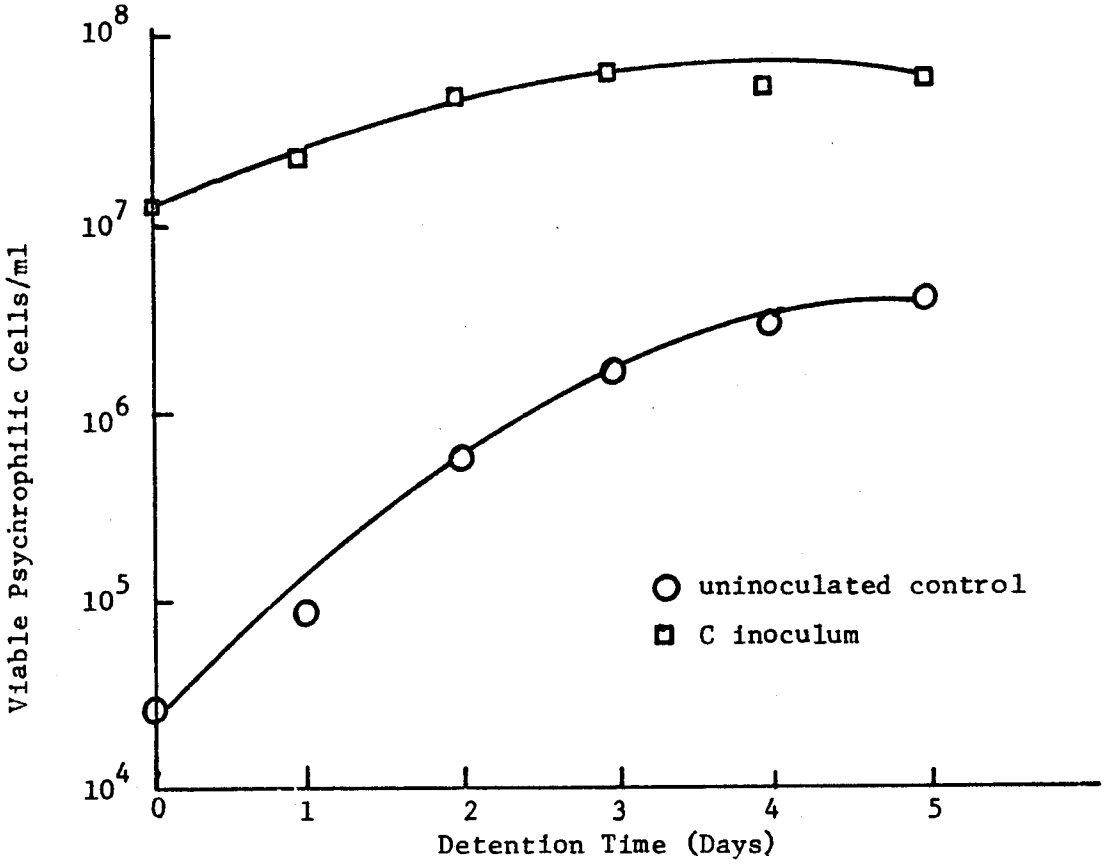


Figure 11. Viable psychrophilic cell number in treated and untreated raw settled sewage at 5 C expressed as a function of time.

These counts were made simultaneously with BOD determinations in an effort to correlate cell growth with BOD removals. All inoculated psychrophiles exhibited slow exponential growth for the first two days of detention, after which the cell numbers became stationary. However, with the exception of the uninoculated system shown in Figure 9, the psychrophilic cells indigenous to raw sewage never reached the stationary phase of growth. As could be expected, the indigenous flora of the uninoculated sewage was also found to be present in much lower numbers than inoculated psychrophiles. Viable cell numbers of three experimental runs were averaged to obtain each graphical value.

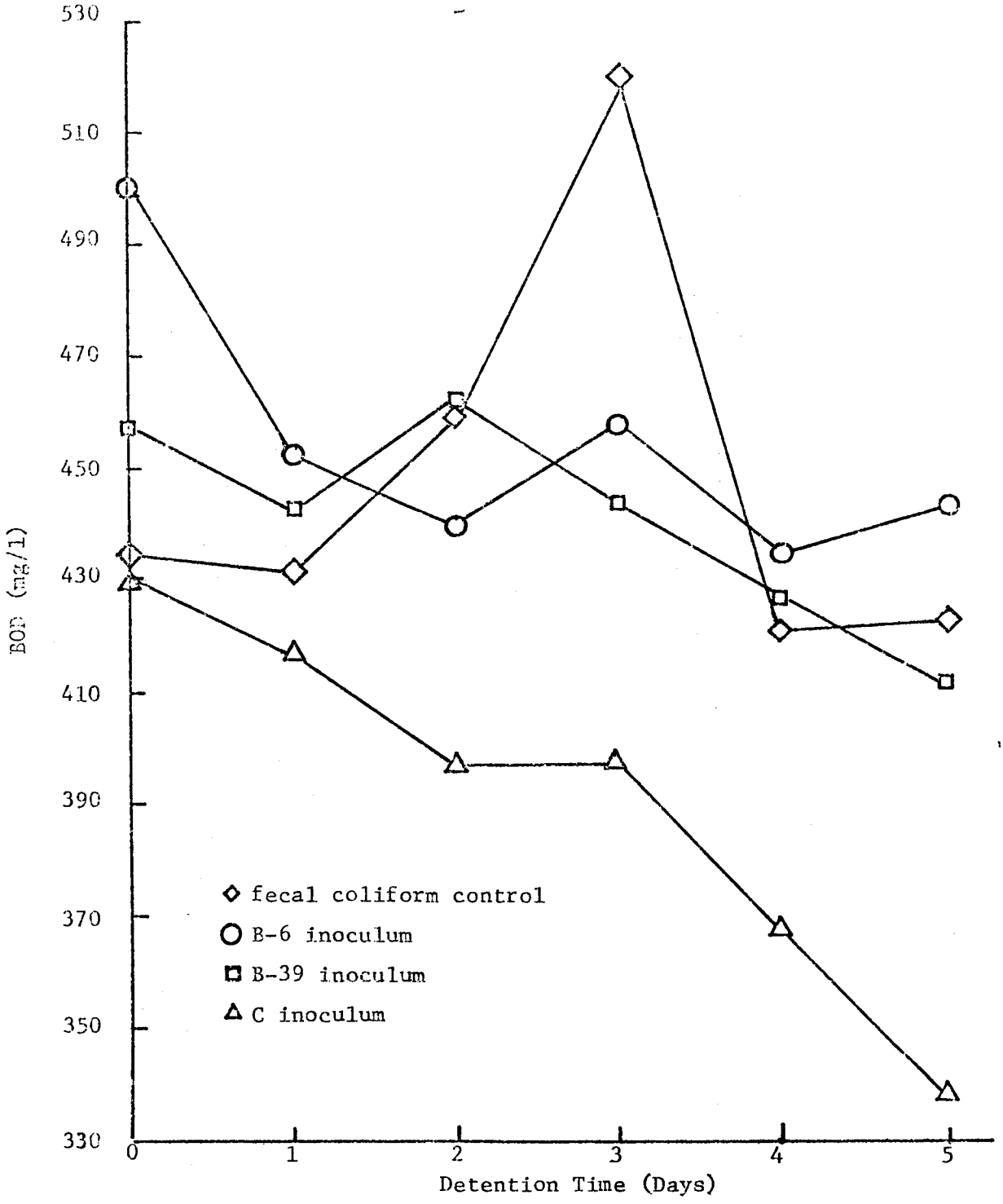


Figure 12. The effects of inoculated microorganisms on the BOD of concentrated synthetic sewage at 5 C expressed as a function of time.

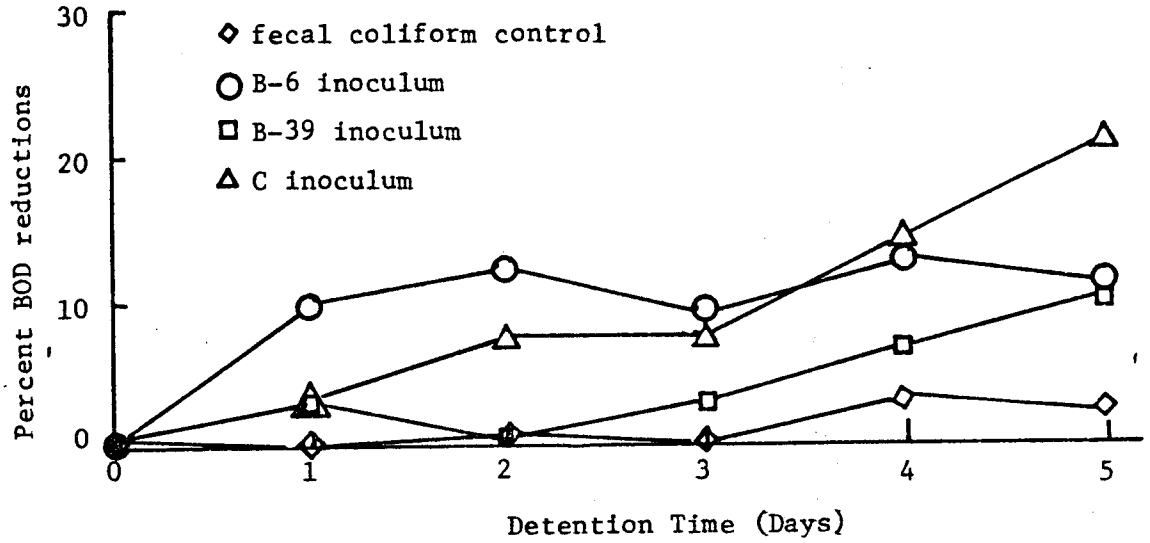


Figure 13. The effects of inoculated microorganisms on percent BOD reductions in concentrated synthetic sewage at 5 C expressed as a function of time.

III. Comparison of the ability of Alaskan psychrophilic isolates to stabilize a concentrated synthetic sewage, measured by BOD and percent BOD reduction, with the stabilization ability of a known mesophile (fecal coliform) in the same substrate.

The effects of inoculated psychrophiles on the BOD of a concentrated synthetic sewage as compared to effects exerted by a fecal coliform inoculated in like manner as a mesophilic control are presented in Figure 12.

Systems inoculated with organisms B-39 and C show trends toward decreasing BOD values. The system inoculated with organism B-6 exhibited a relatively rapid reduction of BOD within the first two days of holding after which very little appears to have been removed. The fecal coliform system demonstrated rather extreme increases in BOD at the second and third days of detention; however, after the third day of detention the BOD dropped back to slightly below initial values. As expected, the trend exhibited by the fecal coliform did not appear to be one of a decreasing BOD.

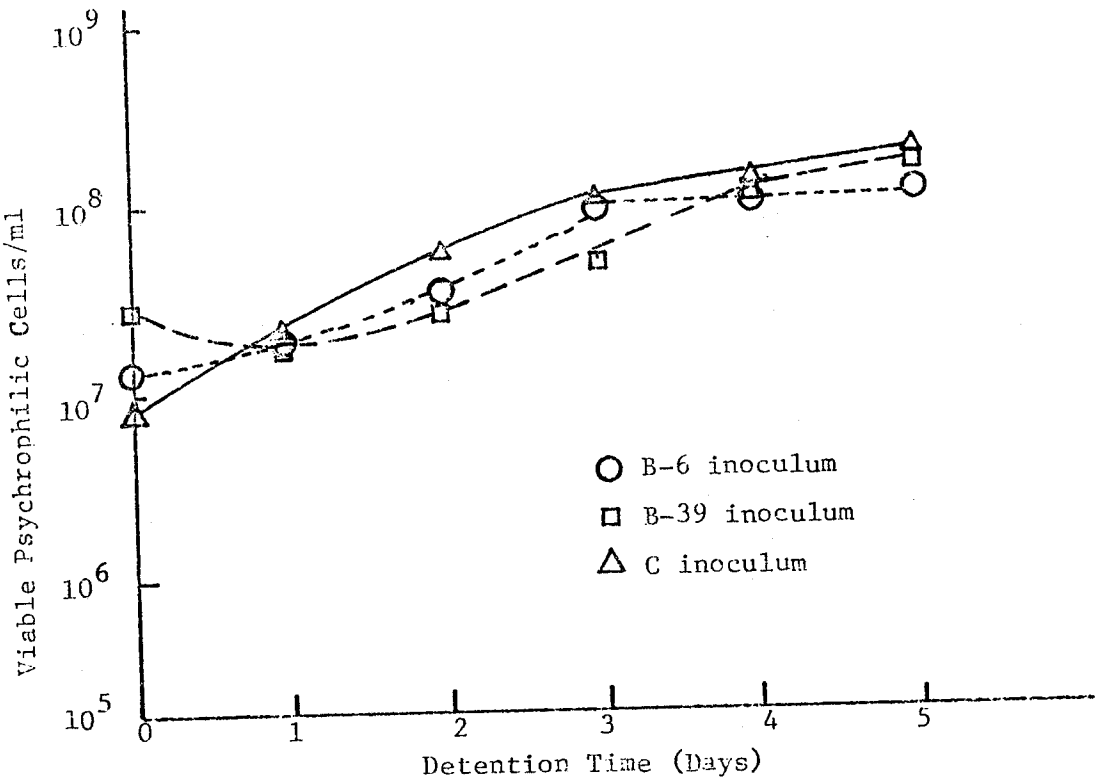


Figure 14. Viable psychrophilic cell number in treated and untreated concentrated synthetic sewage at 5 C expressed as a function of time.

Figure 13 gives percent BOD reduction in the psychrophilic and mesophilic systems. Data contained in this Figure indicates that proportionately less reductions in BOD were obtained in the concentrated synthetic sewage by each psychrophile than in the raw settled sewage substrate previously described, although more absolute BOD was removed by each organism in the synthetic system as shown in Figure 12. Percent BOD reductions in the mesophilic fecal coliform system were very low grade, never exceeding 3 percent.

Viable psychrophilic cell numbers in the concentrated substrate are given in Figure 14. These curves resemble the curves produced by each psychrophile in the raw settled sewage in that cell numbers increased only slightly more than one log unit over the five day

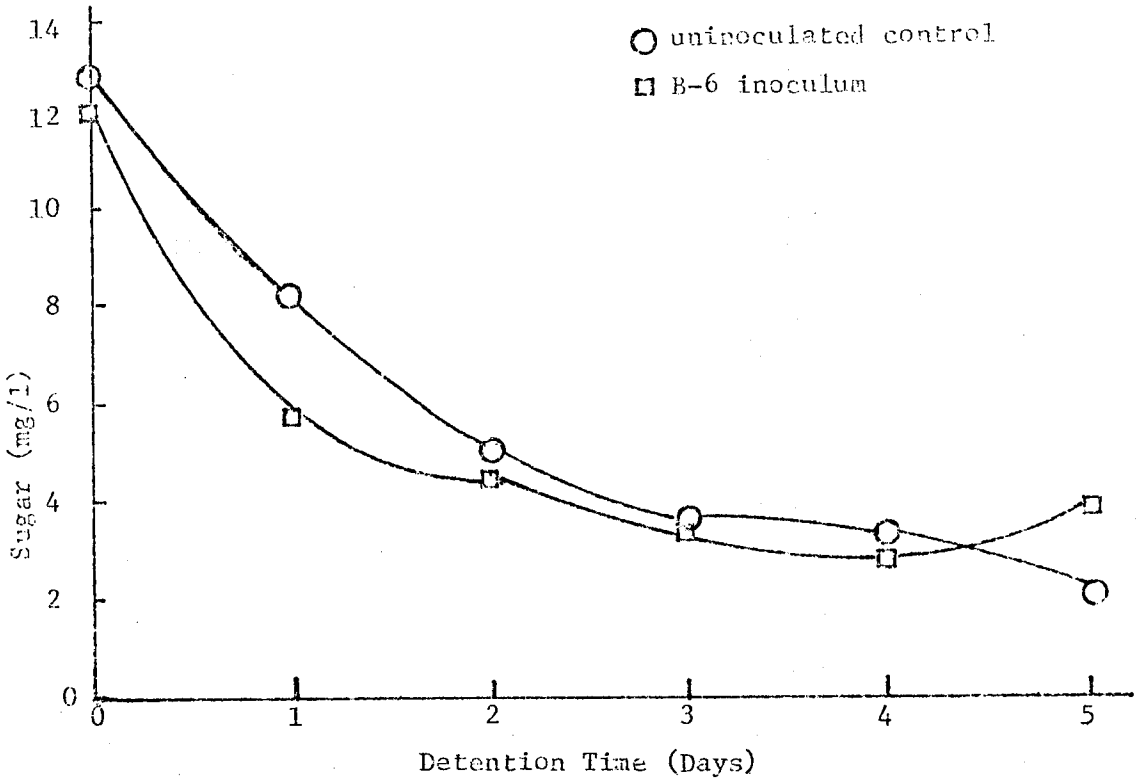
period of incubation and cell growth was very slow. However, organisms B-39 and C were still increasing in number at the end of the five day period as contrasted to their growth in raw settled sewage where they reached the stationary phase of growth in two days. Organism B-6 reached the stationary phase of growth in three days in the concentrated synthetic sewage as compared to two days required to attain the same stage of growth in the raw settled sewage. Since the fecal coliform was mesophilic in nature, no growth was obtained in the flasks containing this organism due to the low incubation temperature and for this reason Figure 14 contains no data for this organism.

IV. Comparison of the effects of inoculated Alaskan psychrophiles on the chemical and physical composition of the wastewater substrate with effects exhibited by the raw sewage's indigenous flora.

In order to determine what effects were being exerted upon the chemical and physical composition of the wastewater by inoculated psychrophilic microbes and psychrophiles naturally present in the raw sewage, analyses for carbohydrates in the form of sugar, nitrate-nitrite nitrogen, and orthophosphate were done in the incubation milieu at daily intervals over a five day period. Viscosity determinations were also made initially and at the end of the five day detention.

Uptake of sugar by psychrophilic organisms is indicated in Figures 15 and 16. These figures indicate that sugars are rapidly removed from the raw sewage by both inoculated and indigenous psychrophiles. Inoculated psychrophiles seem to remove the sugar faster than the natural psychrophilic flora of the raw sewage within the first one to two days detention; after that differences between inoculated and uninoculated systems become insignificant. The results expressed in these figures are averaged data from three separate analyses, as were the numerical values obtained in the nitrate-nitrite nitrogen analysis, the analysis for orthophosphate and viscosity determinations.

Table 22-A lists the results of the analysis for nitrate-nitrite nitrogen. The data indicates that very little variation occurred in each system over the five day detention period. Neither inoculated psychrophiles nor indigenous psychrophiles appear to have exerted


uninoculated control
B-6 inoculum
Sugar (mg/l)
Detention Time (Days)

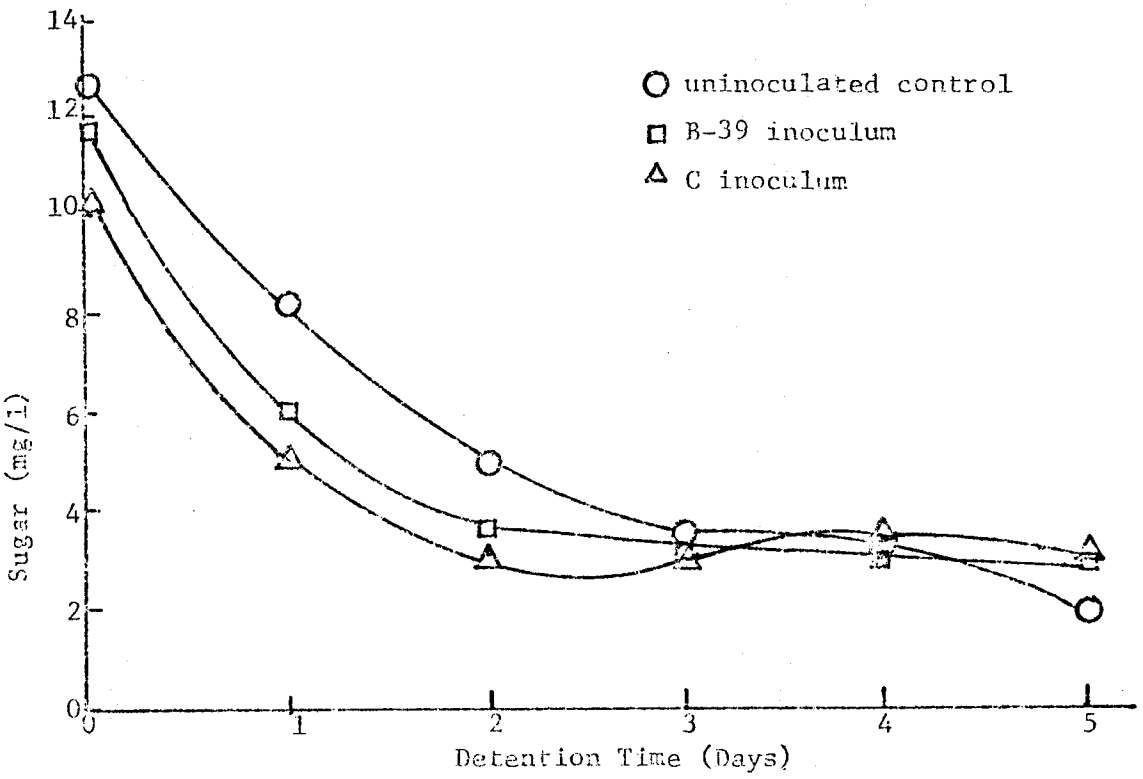

uninoculated control
B-39 inoculum
C inoculum
Sugar (mg/l)
Detention Time (Days)

Table 22. Analysis of treated sewage effluents

A. Nitrate & Nitrite Nitrogen (mg/l)

Detention Time (days)	Treatment			
	B-6	B-39	C	Raw Sewage Control
0	0.86	0.85	0.82	0.88
1	0.85	0.79	0.90	0.84
2	0.92	0.78	0.87	0.83
3	0.86	0.78	0.85	0.90
4	0.87	0.85	0.87	0.79
5	0.82	0.81	0.85	0.86

B. Orthophosphate (mg/l)

Detention Time (days)	Treatment			
	B-6	B-39	C	Raw Sewage Control
0	4.0	4.0	4.0	4.1
1	3.6	3.9	3.7	4.4
2	2.9	3.0	2.9	3.7
3	3.5	3.2	3.3	3.7
4	3.1	3.7	3.3	3.5
5	3.8	3.6	3.4	3.5

C. Viscosity (centipoise*)

Detention Time (days)	Treatment			
	B-6	B-39	C	Raw Sewage Control
0	0.98	1.00	1.00	0.99
5	1.02	1.01	1.02	1.02

*centipoise = 10^{-2} (gram mass)/(cm)(sec)

any effect upon the concentration of the nitrate-nitrite nitrogen present in the wastewater.

Concentrations of orthophosphate found in the systems treated by psychrophilic inoculation and in the uninoculated raw sewage control over a five day period are given in Table 22-B. There appears to be a slight uptake of orthophosphate by inoculated psychrophiles in the first two days of holding, but after the second day orthophosphate levels in the filtered effluent began to increase on the whole, over the given five day contact time, a substantial alteration of orthophosphate concentration apparently did not occur. Slight losses of orthophosphate in the uninoculated raw sewage were suggestive of a very low grade uptake by the sewage's natural flora, but once again the reduction in orthophosphate concentration was so small that it cannot be considered significant.

Changes in viscosity of the wastewater substrate are tabulated in Table 22-C. Slight increases in viscosity were observed in all systems studied. The viscosity in all systems studied, including sewage inoculated with treatment psychrophiles and uninoculated sewage maintained as a control, fell within the $\pm$ one percent error inherent in the instrument.

V. Comparison between growth rates and oxygen uptake rates of the three local psychrophilic isolates in heat sterilized sewage.

Figure 17 shows the Arrhenius plots of growth rates in sterilized sewage versus the inverse of temperature (^{o}K) for the three psychrophilic isolates. Isolates T28B and XVI-4 showed optimum growth rates between 15 and 25 C, but the optimum rate of growth for XXIV-1 could not be determined as there was no decline in growth rate, within the range plotted, as temperature increased. The figure shows that T28B was only slightly affected by decreasing the temperature from 10 to 5 C, but a corresponding temperature drop affected the other isolates greatly.

The Arrhenius plots of oxygen uptake rates in sterilized sewage by the three psychrophilic isolates grown in sterilized sewage supplemented with 1/5 m-PCB for less than 1 day are shown in Figure 18.

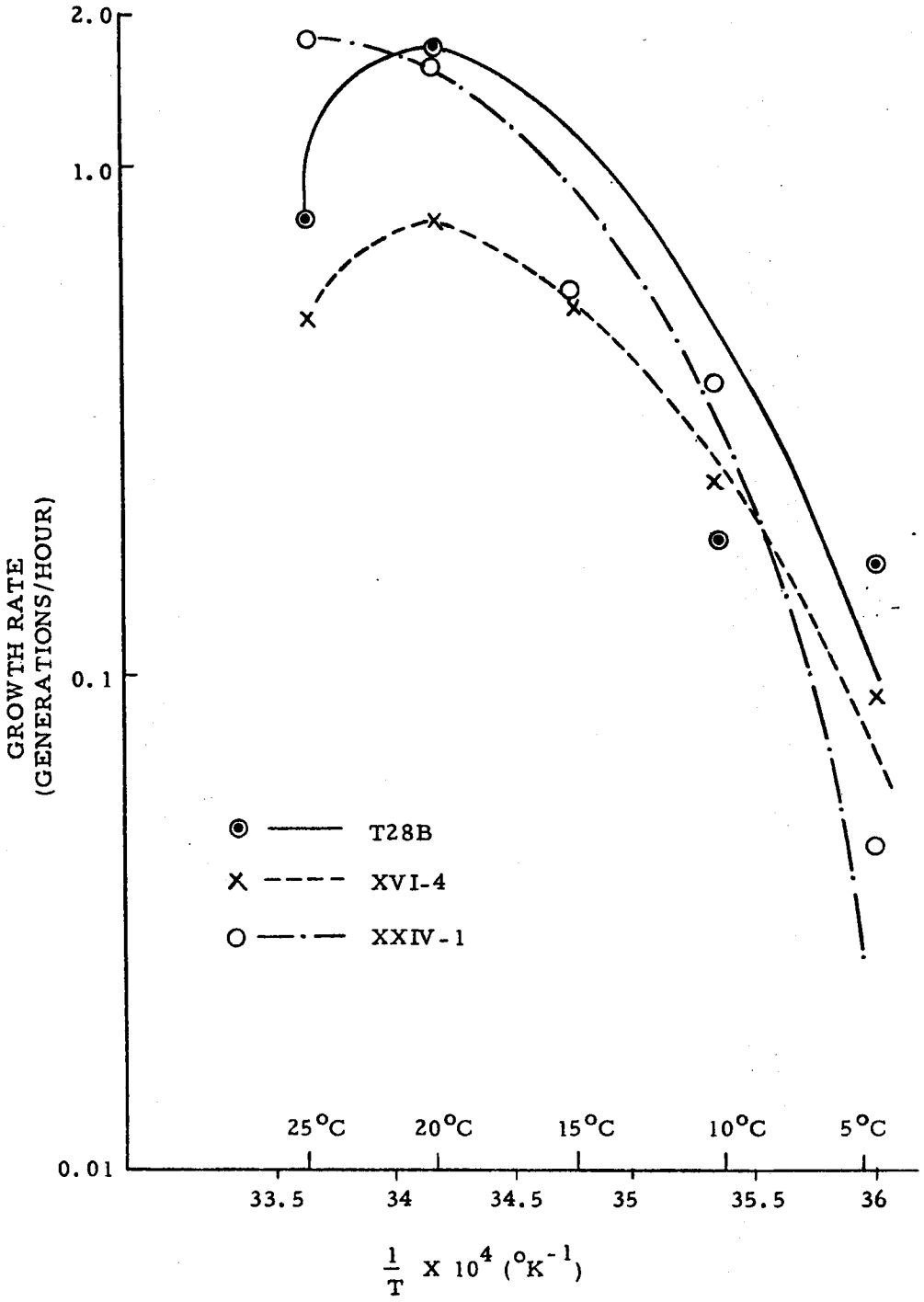


Figure 17. Arrhenius plots of growth rates (generations/hour) in sterilized sewage versus the inverse of absolute temperature for the three psychrophilic isolates.

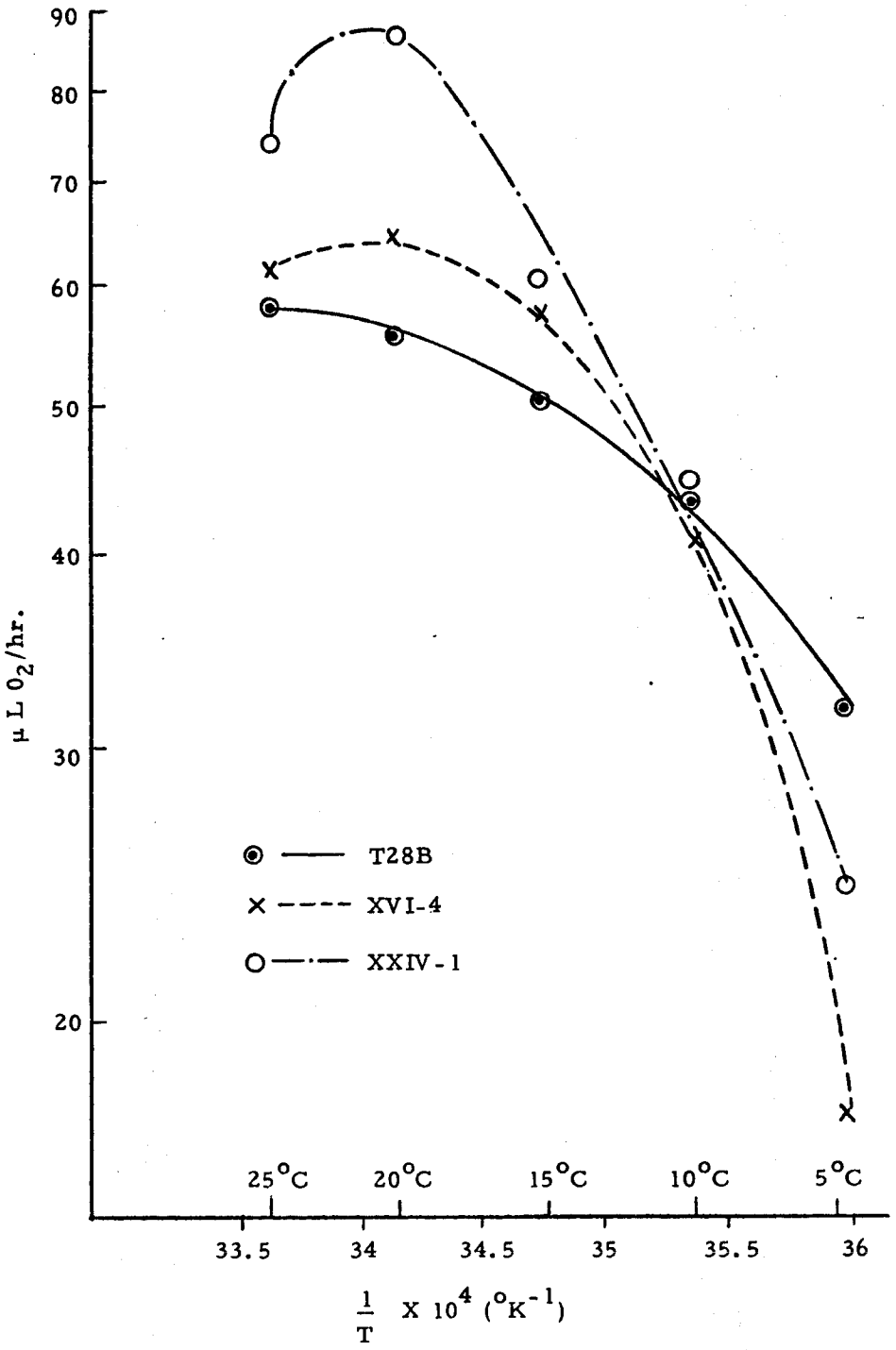


Figure 18. Arrhenius plot of oxygen uptake rates in sterilized sewage versus the inverse of absolute temperature for the three psychrophilic isolates grown for 1 day in sterilized sewage supplemented with 1/5 m-PCB(Difco).

The plot showed linearity only for isolate XXIV-1, and the plot was linear, with a negative slope, only between 5 and 20 C; at 25 C, the uptake rate was less than that at 20 C. Isolate T28B was the only isolate which showed no decline in uptake rate, within the range plotted, as temperature increased. Although T28B showed no decline in uptake rate at 25 C, its rate was still less than corresponding rates of XXIV-1 and XVI-4, but T28B had the greatest uptake rate at 5 C.

Table 23 shows that, with few exceptions, oxygen uptake rates by 1-day cultures were greater than uptake rates by 7-day cultures and that uptake rates by cultures grown in sterilized sewage supplemented with 1/5 m-PCB was greater than uptake by cultures grown in 1/2 m-PCB only.

Figure 19 shows oxygen uptake, at 5 and 20 C, versus time for 24-hr cultures of the three isolates grown in 1/2 m-PCB, and Figure 20 shows oxygen uptake, at 5 and 20 C, versus time for 7-day cultures of the isolates grown in sterilized sewage supplemented with 1/5 m-PCB. These figures were representative of oxygen uptake data in general.

VI. The effect of pH on the growth and oxidative ability of the Alaskan isolates in sterile sewage supplemented with 0.05 percent yeast extract.

The effects of pH on the growth of treatment organisms B-6, B-39, and C are given graphically in Figures 21, 23, and 25, respectively. Results given in these figures indicate that all of these organisms grow best in a pH range of approximately 6.8 to 8.6. The effects of pH on the oxidative ability of each psychrophile (given in Figures 22, 24, and 26) yielded largely the same results. A good correlation was observed between pH ranges at which maximal growth densities and substrate oxidation occurred for each psychrophile studied. The variation observed in different determinations of the same test for each bacterium was presumably due to fluctuations in the constituents of different sewage samples utilized by each organism as substrate. Three consecutive experimental determinations were plotted for Figures 21 through 26 with the exception of Figure 25 which contains the data obtained in only two consecutive experimental runs.

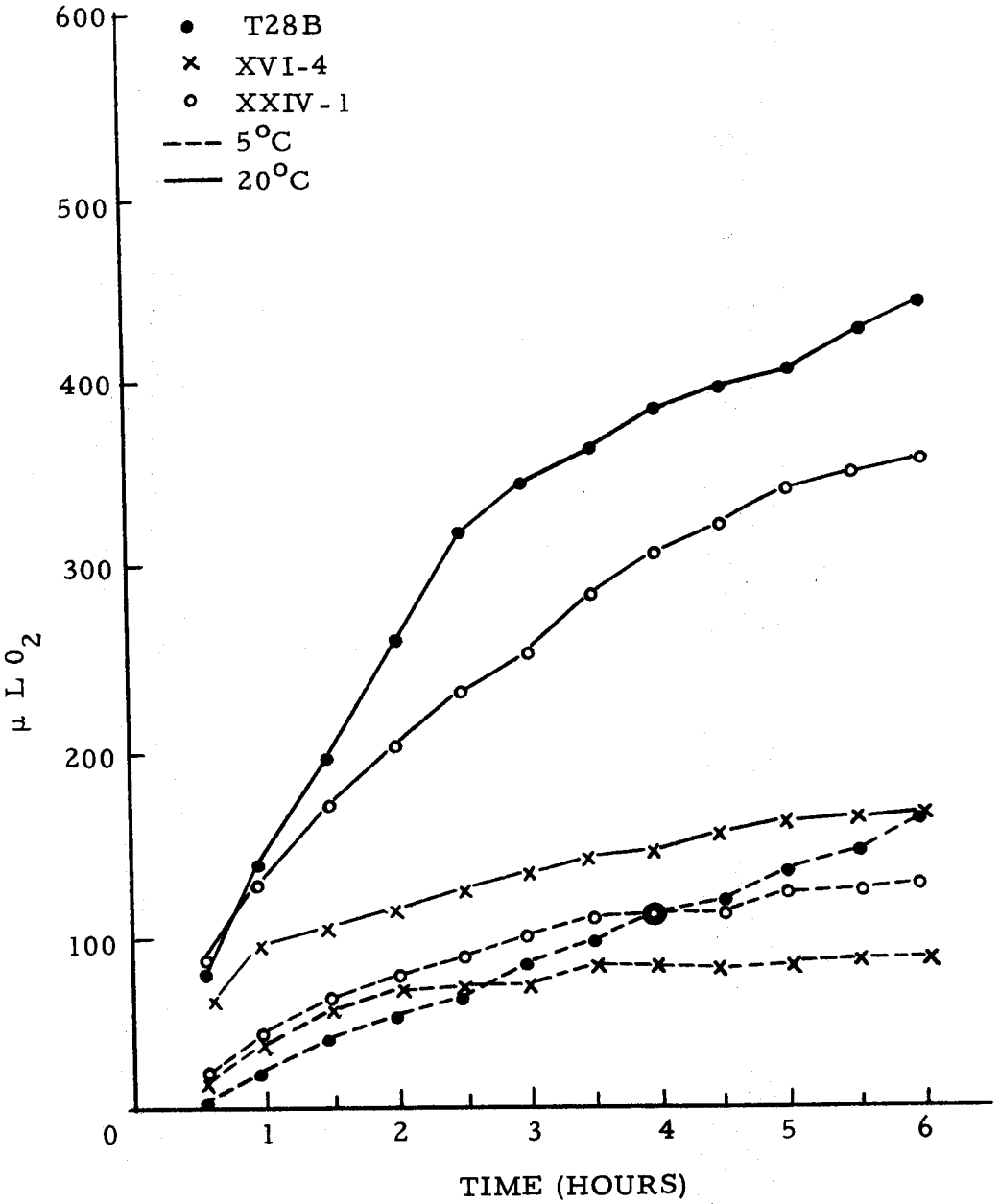


Figure 19. Oxygen uptake at 5 and 20 C in sterilized sewage by the psychrophilic isolates grown for 1 day in ½ m-PCB(Difco).

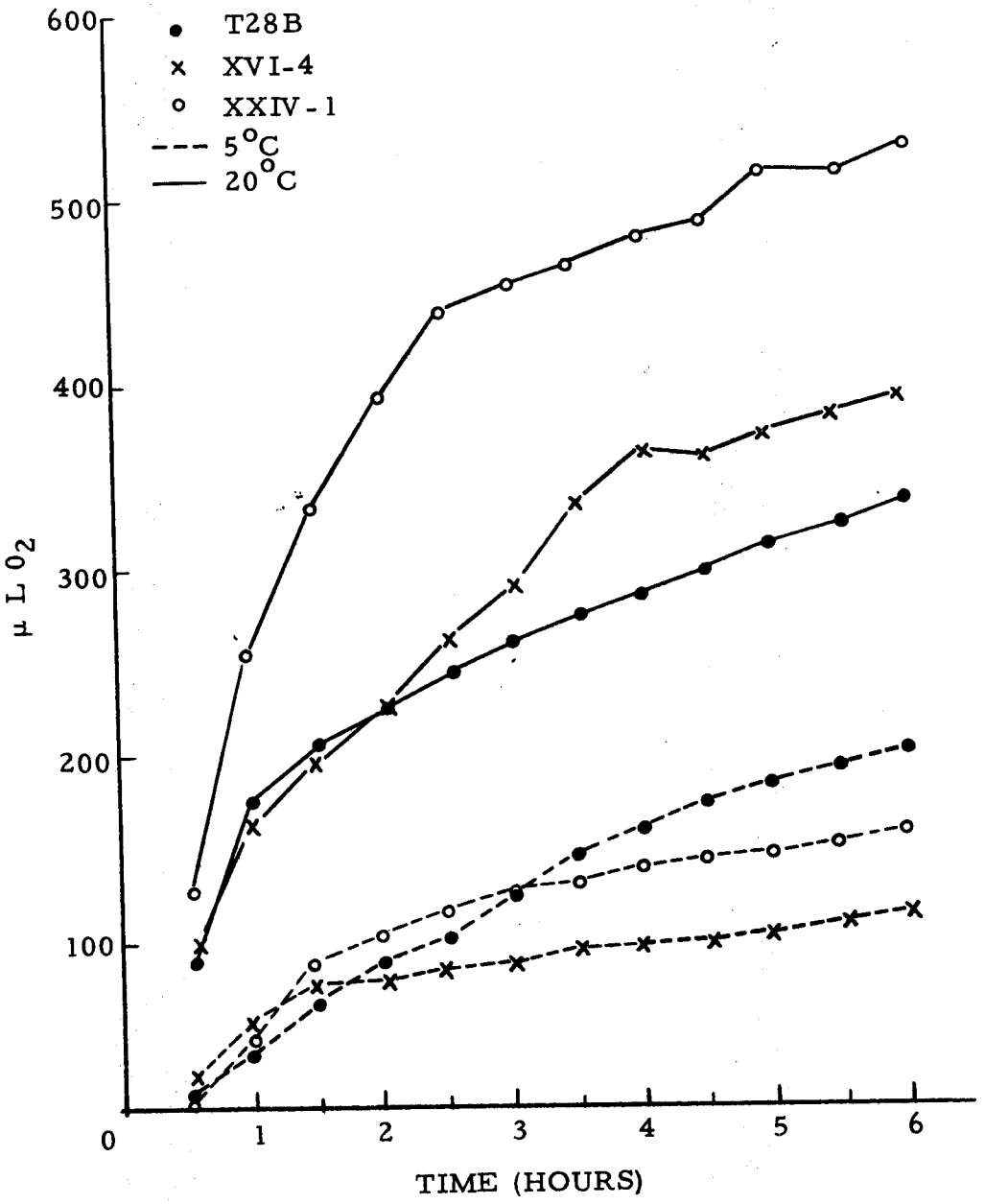
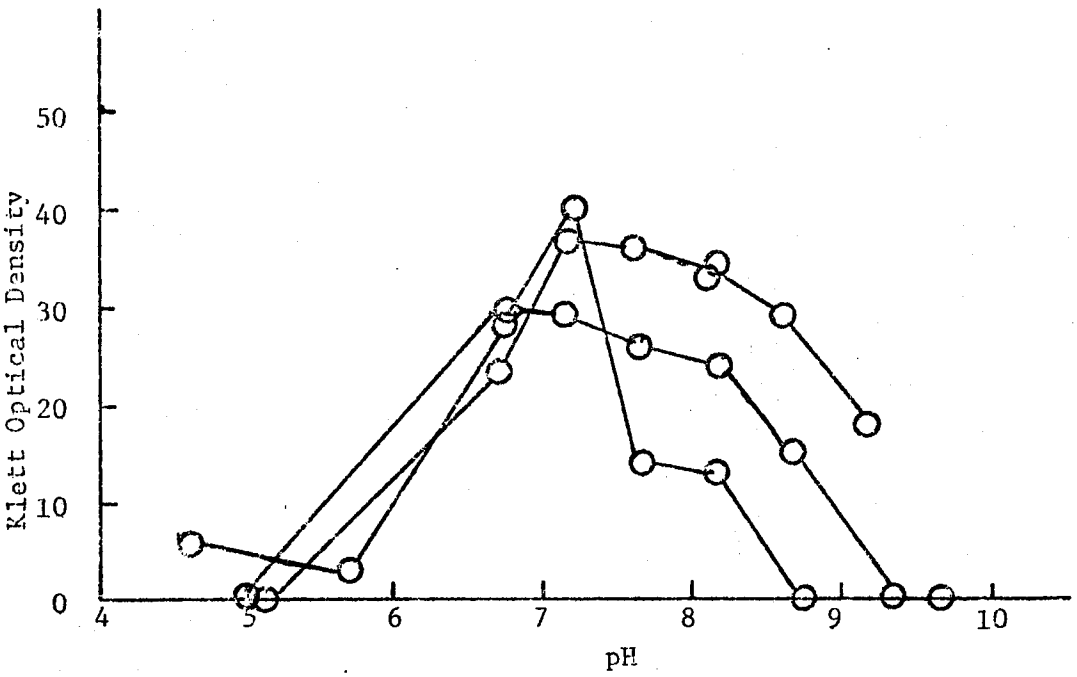
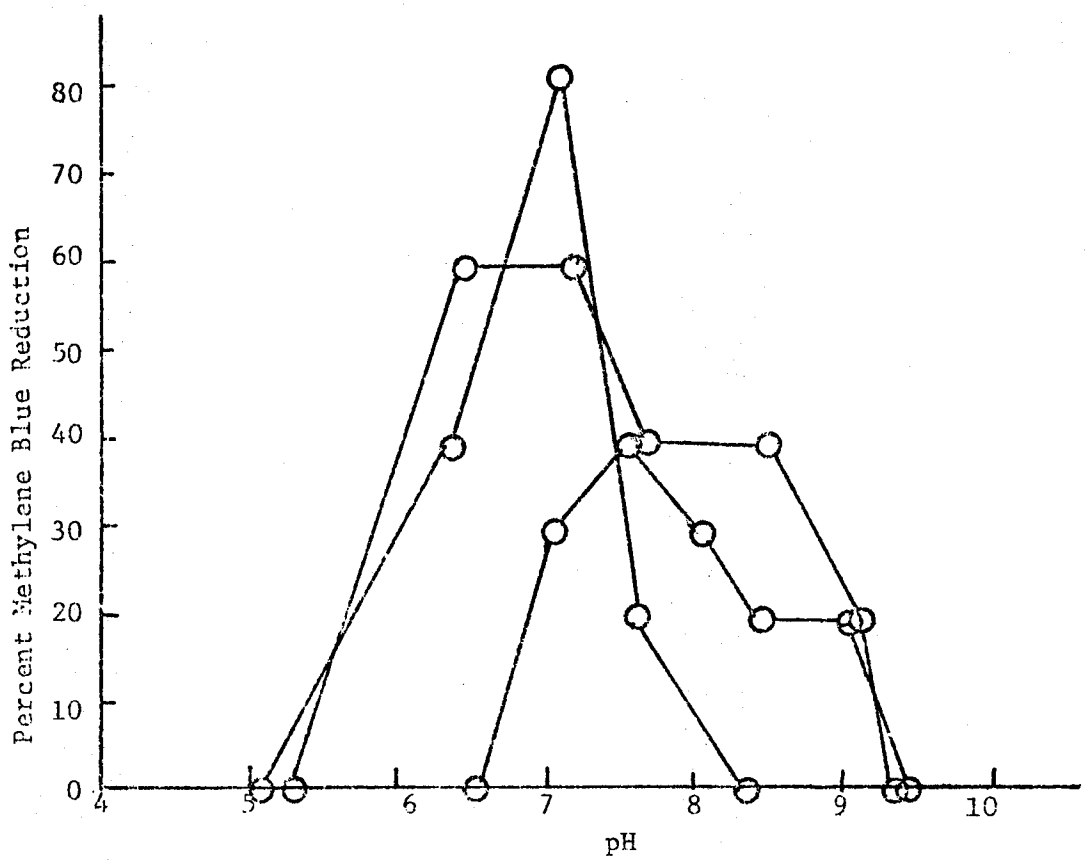


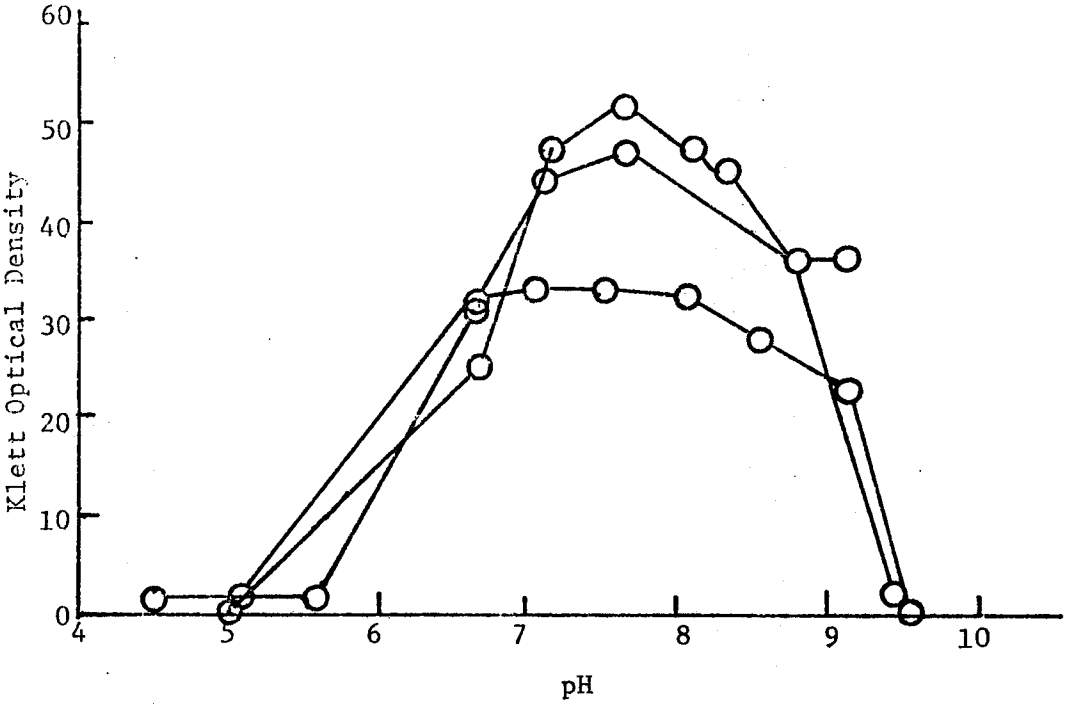
Figure 20. Oxygen uptake at 5 and 20 C in sterilized sewage by the psychrophilic isolates grown for 1 day in sterilized sewage supplemented with 1/5 m-PCB(Difco).

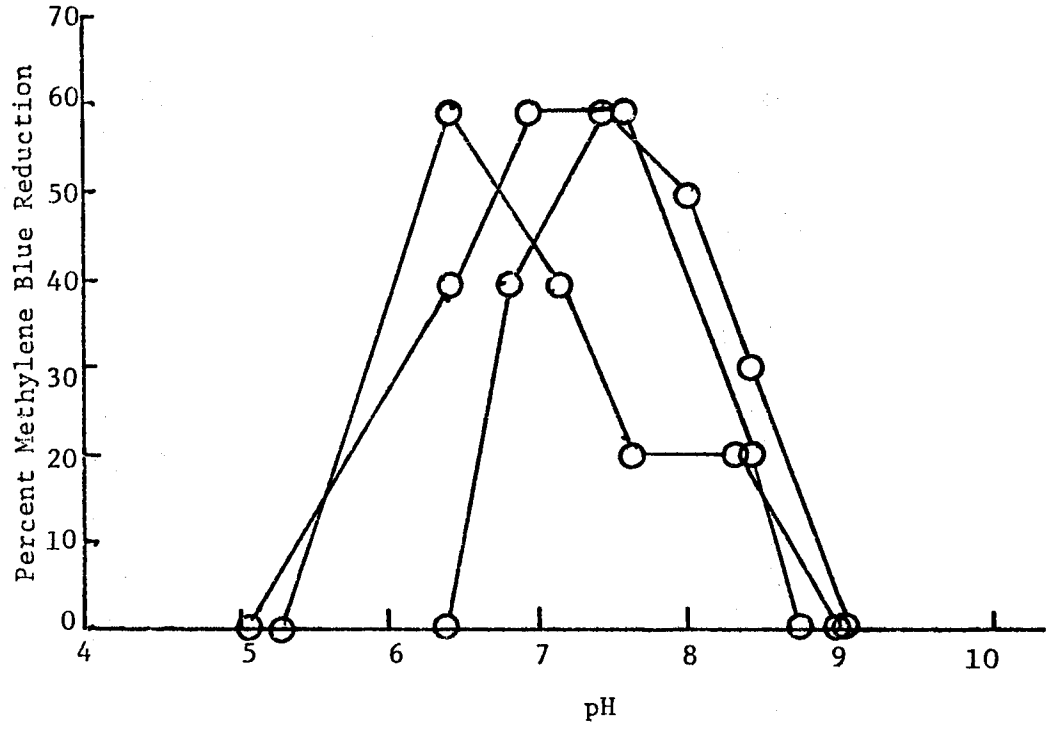
Table 23. Oxygen uptake at 10 C for XVI-4 cultured for 1 and 7 days in sterilized sewage supplemented with 1/5 m-PCB.

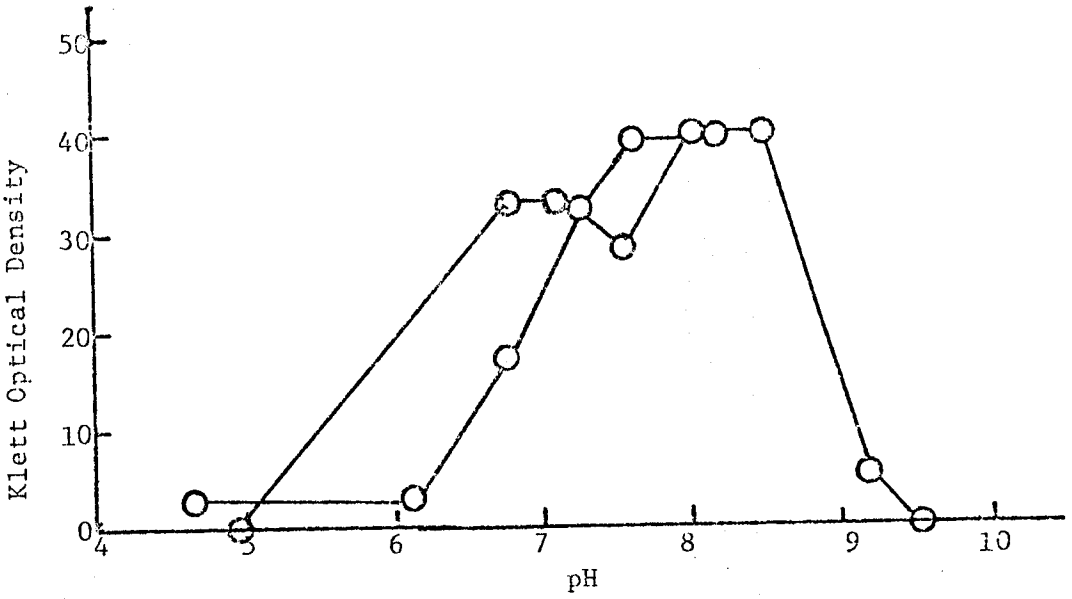
Uptake by 1-day Cultures ($\mu L\ O_2$)	Time (min)	Uptake by 7-day Cultures ($\mu L\ O_2$)
45.5	30	2.1
75.5	60	2.7
114.2	90	5.5
125.4	120	11.1
152.0	150	14.7
163.3	180	23.5
184.3	210	27.8
196.6	240	28.5
207.0	270	27.9
219.9	300	37.4
230.0	330	35.0
243.8	360	39.8

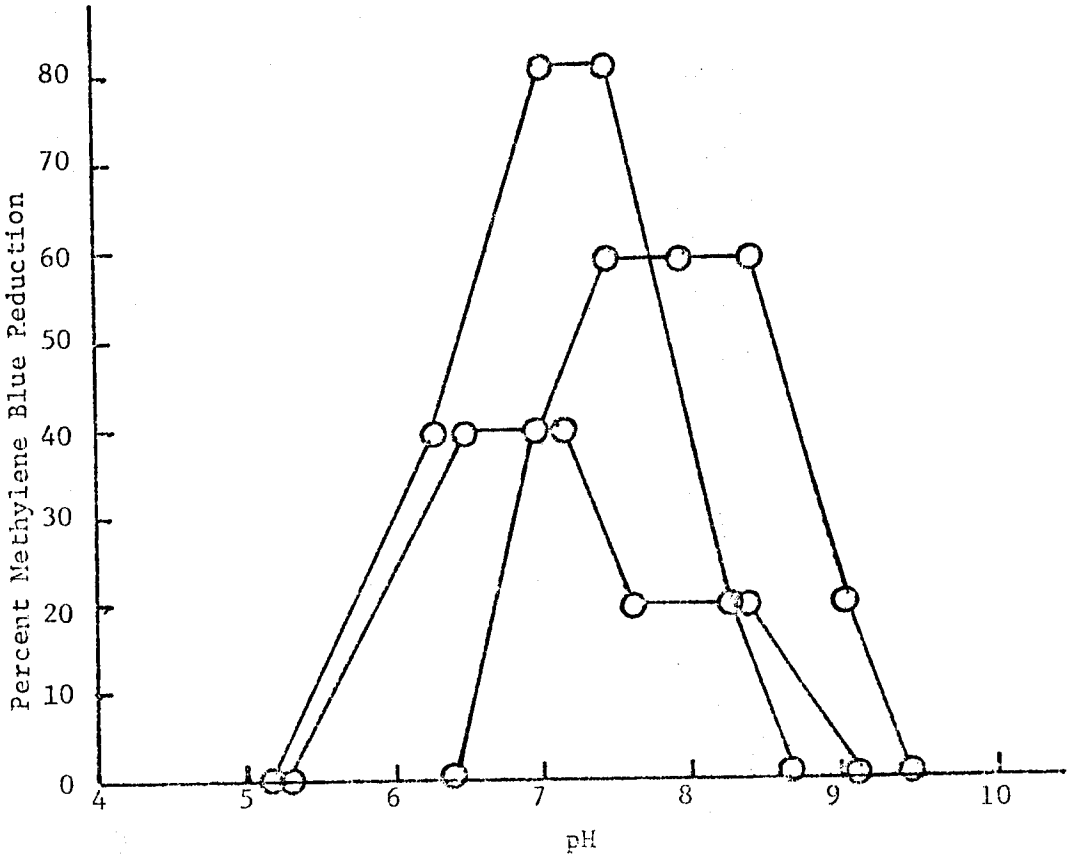

Klett Optical Density
0
10
20
30
40
50
4
5
6
7
8
9
10
pH


Percent Methylene Blue Reduction
0
10
20
30
40
50
60
70
80
4
5
6
7
8
9
10
pH


Klett Optical Density
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH


Percent Methylene Blue Reduction
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH


Klett Optical Density
50
40
30
20
10
0
4
5
6
7
8
9
10
pH


Percent Methylene Blue Reduction
80
70
60
50
40
30
20
10
0
4
5
6
7
8
9
10
pH

DISCUSSION

Many contributions have been made in the field of pollution control, but one area which has been noticeably lacking in development is that of low temperature wastewater treatment. If population centers located in areas which commonly experience long periods of cold weather are to meet effluent standards imposed by governmental agencies, some method of adequate sewage treatment at low temperatures will have to be found. Although several approaches to the solution of this problem are available, the one investigated in this study was a biological approach, i.e., the use of inocula of psychrophilic bacteria to degrade raw domestic sewage. If a psychrophilic inoculum could be used to reduce oxygen demands to practical levels in a relatively short period of time on a laboratory scale, the potential for adapting similar organisms for use in conventional treatment systems in cold climates would be very good.

Although psychrophiles were used to remove BOD from sewage, they were initially selected on the basis of their relative growth rates in a nutrient medium rather than in sewage. Since the temperature characteristic of growth is independent of the growth medium (42), if all growth requirements were present in sewage, relative growth should be the same in sewage as it is in a nutrient medium. Isolates with relatively high growth rates in a nutrient medium should also have relatively high growth rates in sewage.

Many of the locally collected psychrophilic isolates were irradiated with UV in an attempt to increase growth rates of mutant strains at low temperature. Although conversions from psychrophily to mesophily (90, 91, 92), and from mesophily to psychrophily (5, 67) have been reported, the basis for such conversions have been changes in optimal or minimal growth temperatures rather than changes in growth or metabolic rates. Therefore, reported conversions from mesophily to psychrophily do not, necessarily, imply an accompanying increased rate of growth at a specific low temperature.

Whereas shifts in minimal and optimal temperatures of growth may result from minor changes in cell wall composition or in temperature sensitivity of an essential enzyme, changes in growth rates within

the range between growth optimum and minimum likely involve changes in more than one cellular system. Mutations could cause minor alterations resulting in shifts in optimal and minimal growth temperatures, but mutagenic treatment probably would not cause the many beneficial alterations necessary for increasing growth rates at low temperature without also causing a lethal mutation. The UV irradiation of the psychrophilic isolates did not yield any mutants with faster growth rates at low temperatures, possibly reflecting the difficulty of creating such a mutant. However, only preliminary efforts were expended toward obtaining mutants and more intensive work would have to be done before the phenomenon of increasing growth rates at low temperature could be definitely supported or refuted.

Following the results, the remaining discussion will be presented under the following headings:

I. Local isolates in sterilized sewage.
II. Raw sewage degradation.
    A. By local isolates.
    B. By Alaskan isolates.
III. Alaskan isolates in concentrated synthetic sewage.
IV. Chemical analysis of effluent after treatment with Alaskan isolates.

I. Local isolates in sterilized sewage.

Of the five local isolates which grew relatively well in sterilized sewage, two that were isolated from different environments were chosen to be used in these experiments and studies. A third isolate was chosen because it was a soil isolate selected for its relatively rapid oxygen uptake rate at 10 C. These three isolates were then used in the subsequent experiments and studies in raw and sterilized sewage.

Because the culture age of the inocula and the number of cells inoculated would be relatively easy to control and could influence BOD removals, the effects of these two treatments, as well as the effects of temperature and specific isolates used as inocula, were studied. Since each of the variables within the four treatments may have an influence on other treatments' variables, it was also necessary

to study the effects that the treatments had on each other. To minimize the effects of extraneous variables which were not measurable, heat sterilized sewage was the medium used in these preliminary experiments.

The complexity of determining the effects of treatments on each other made it necessary to utilize statistics to evaluate the significance of the treatments and treatment interactions. A treatment or treatment interaction was considered to influence BOD removals if it was statistically significant at the 0.5% level.

A significant effect required that the variables within the treatment, or treatment interaction, gave BOD removals which varied among the others, and, although the statistical approach defined which treatments affected BOD removals, it did not disclose why the variables within treatments differed. The BOD removal data with which the analysis was determined, and not just the analysis of variance, should, thus, be inspected for biologically explainable phenomena.

In experiments using inocula in heat sterilized sewage, the single treatment effects of organisms used as inocula, size of inocula, age of inocula and temperature were statistically significant (Table 3). The reasons for their significance, however, were not discovered in the analysis and conclusions drawn from the single treatment data (Tables 4-7) could not be made without also analyzing their interactions with the other treatments.

On the basis of the organism treatment data alone (Table 4), one would conclude that organism XXIV-1 was the best to use as an inoculum. A significant organism-temperature interaction (Table 8), however, indicated that the effect of temperature on BOD removals differed among organisms, reflecting differences in temperature characteristics of growth or oxygen uptake among the different organisms. Organism XXIV-1 may have removed the greatest overall percent BOD, but, because changes in temperature affected each organism differently, T28B removed more at 5 C.

Since the temperature of actual treatment plants does not remain constant, the selection of an organism to be used as the inoculum should be made considering the changes in BOD removals as temperature varies,

rather than BOD removals at a single low temperature. If more data were available, empirical equations relating BOD removals to temperature could be formulated, and such formulas could be used to determine which inoculum would result in the maximum BOD removals, if reasonable predictions of the waste's temperatures could be made.

The influence of temperature on percent BOD removals observed in these experiments, using single culture inocula, was different than the reported slight effect on actual treatment plants at temperatures above 10 C but serious effects below 10 C (39, 48, 79). Because of the diversity of the waste's natural biota, adaptation to changes in temperature is possible; thus, in a temperature range in which the biota can adapt, treatment efficiencies should be similar. In experiments using single inocula in sterilized sewage, the BOD removals should parallel the effects of temperature on growth or metabolism. Because growth and oxygen uptake rates of the isolates decreased as temperature decreased from 20 C (Figures 1 and 2), percent BOD removals observed in these experiments also decreased as temperature decreased.

It was also observed (Table 5) that, although removals decreased as temperature decreased from 20 to 10 C, the removals at 5 C were nearly the same as those at 10 C, despite the fact that Arrhenius plots of growth and oxygen uptake rates continued to decrease. The reasons for the unexpectedly high observed percent removals at 5 C are not known.

As expected, the largest difference in BOD removals came from the size treatment (Table 6). It is well recognized that the rates of biochemical enzyme reactions are proportional to the enzyme concentration, if substrate is in excess (21). Although there was a hundredfold difference between the number of cells in the large and small inocula, rather than a hundredfold difference in BOD removed, there were less than one and one-half times as much percent BOD removed by the large than by the small inocula; consequently, percent BOD removals must also be influenced by factors other than the number of cells inoculated.

The BOD removal data includes both the enzymatic phenomenon as well as some non-biochemical processes. The BOD removed biologically is related to the concentration of enzymes present. Although there was a hundredfold difference in initial cell numbers between the two

inocula sizes, the relative difference in cell numbers, and presumably the same relative proportion of active enzymes, after the 24-hr incubation may have been considerably less. Since growth rates decrease as cell concentrations increase, the large inoculum would grow slower than the small inoculum, resulting in less than a hundredfold difference between large and small inocula's final bacterial concentration and an accompanying relative increase in BOD removed by the small inoculum.

A relative increase in BOD removed by small inocula would be even greater if the large inocula did not grow at all. In the raw sewage studies, discussed later, it was observed that an inoculum of cells increased the initial BOD (Table 21). This increase in BOD can only be accounted for by assuming that cells add to the BOD by dying and subsequently becoming utilizable nutrients. Normal sewage contains about 2×10^7 cells per ml (57) and there were about 6×10^6 cells per ml inoculated into the raw sewage. Assuming McGauhey's estimate to be correct, the final viable bacterial population in inoculated raw sewage was probably less than 2.6×10^7 cells per ml, as some of the cells were contributing to the increased BOD. In experiments with heat sterilized sewage, when the large inoculum was used, the sterilized sewage initially was inoculated with 2×10^7 cells per ml; consequently, growth of the large inoculum would be limited.

The non-biochemical removal of BOD is due to settling, chemical oxidation, and volatilization. Since flocculation was not observed for either size of inoculum, the amount of BOD removed by settling should be constant and independent of inoculum content. The BOD removed by chemical oxidation and other non-biochemical methods should also be constant and independent of inocula size. By percentage, the amount of BOD removed by these non-enzymatic processes would be a larger portion of the total BOD removed when a small bacterial load was present. Therefore, it is reasoned that the experimentally unexpected slight differences in BOD removed between large and small inocula were caused by: 1) growth of the cells after small numbers inoculation while the large inoculum's cells only exhibit slight growth and 2) the effects of non-biochemical forms of BOD removal which have a proportionately greater influence on the observed percent BOD removed by the small than on that removed by the large inoculum.

The reasons for relatively slight differences in BOD removed by 1 and 7-day old cultures (Table 7) were not disclosed until the temperature-age (Table 12) and temperature-size-age (Table 14) interaction data were inspected. Whereas the removals by the 1-day averaged over all other treatments were slightly greater than removals by the 7-day old cultures, 1-day old cultures removed less BOD at 5 C than did the 7-day (Table 12). It was observed, however, that only the small inocula of 1-day old organisms removed less BOD at 5 C than corresponding 7-day old cultures; large inocula of 1-day old cultures at 5 C and both large and small inocula of 1-day seed at 10, 15, and 20 C removed more BOD than corresponding 7-day old cultures (Table 14). Since the organisms were grown at room temperature, the observed influence of temperature on BOD removals by different culture ages and sizes of inocula might be explained on the basis of cold shock.

Many authors reported that "physiologically young" cells from late lag or early exponential phases were sensitive to abrupt temperature changes (82, 89). Epthimion and Corpse (22) reported, however, that growth phase susceptibility to cold shock differed among strains. While some strains were sensitive if cells were old, others were sensitive if cells were young, and in other cases, cells of any age of growth phase were insensitive to cold shock. Although Epthimion and Corpse (22) reported that cold shock susceptibility could occur in any phase, depending on the organism, small inocula of 1-day old cultures of all isolates removed less BOD than corresponding small inocula of 7-day old cultures at 5 C (Table 2). It was also observed that the mixed, mesophilic population was more susceptible to cold shock than any of the three psychrophilic strains, a finding consistent with Farrel and Rose (24).

Although cold shock would affect cells of both large and small inocula of 1-day old cultures, only the 1-day old small inocula were observed to be adversely affected by cold shock (Table 14). Because a substantial part of the BOD removed by the small inocula is due to an increase in cell numbers with an accompanying, though not necessarily proportional, increase in oxidation rate, any physiological stress, such as cold shock, which decreases the viability and growth capability of the inoculated cells would adversely affect the inoculum's degradative

abilities. Cold shock, which affects growth of 1-day old cells, would have little influence on the large, non-growing, inoculum's oxidation of sewage.

Cold shock was not a factor influencing BOD removals when large inocula were used or when the experimental temperature was greater than 5 C. When cold shock did not influence waste degradation, the 7-day old cultures removed less BOD than 1-day old cultures, possibly because there were probably fewer viable cells in 7-day cultures and because the old cells would be partially depleted of necessary enzymes due to their prolonged starvation.

Averaged over all treatments, the 1-day old cultures were more efficient than 7-day old cultures (Table 7), but, since cultures were grown at room temperature, small inocula of 7-day old cultures were less efficient than corresponding 1-day old cultures at 5 C (Table 14), because of the adverse effects of cold shock on growth of the cells after inoculation. On the basis of these results, if inocula of psychrophiles were employed to increase treatment efficiency at low temperatures, stationary phase cultures, rather than exponential growth phase cultures, should be used as relatively fewer cells are capable of continued active growth in sewage. This is fortunate, since it would be more economical to use a small inoculum and easier to harvest cells not in exponential growth phase, as careful monitoring of the cultures to determine growth phase of cells would not be practical.

Because microbial interactions, such as synergism, competition or inhibition (30) could influence microbial activities, combinations of the three psychrophilic isolates were used and their percent BOD removals were compared to the percent BOD removed by single culture inocula (Table 17). Although there were no significant experimental differences found between removal abilities of single cultures and combinations of cultures, the phenomenon is worth additional study using a larger selection of organisms.

Since experiments thus far discussed were performed in heat sterilized sewage, extending the results to raw sewage is questionable, because sterilization alters the chemical composition of the sewage

and destroys it biota. Studies in sterilized sewage were necessary, however, to minimize the effects of extraneous variables resulting from the chemical and biological differences among separate raw sewage samples. Although gamma irradiated (46) or filter-sterilized sewage would probably be more similar, chemically, to raw sewage, heat sterilization was used because irradiation and filtration were not feasible. To decrease variation caused by fluctuations in the composition of the sewage samples, raw sewage, sampled and frozen as collected over a period of approximately 3 months, was pooled at the end of the 3 months, frozen to preserve its characteristics (28) and heat sterilized as needed.

II. Raw sewage degradation.

A. By local isolates.

Due to limitations involved with using heat sterilized sewage, it was necessary to perform experiments using inocula of locally isolated psychrophiles in raw sewage. In such studies 19% of the BOD was removed within 5 hours at 2 C by the psychrophile-inoculated sewage, but 15% was removed with no inoculum used. Although the absolute difference (19% - 15% = 4%) appears rather insignificant, the relative difference (19% - 15%/15% = 27%) is quite appreciable. If however, retention times were of sufficient duration to permit 80-90% removals, the absolute difference between BOD by inoculated or non-inoculated raw sewage would increase, but it is likely that the relative difference would decrease.

Although the psychrophilic inocula removed more percent BOD than the mesophilic or non-inoculated sewage, with the 5-hr retention times used for this study, the final BOD concentrations were greater for the inoculated sewages than for the non-inoculated ones, because the inoculation of a cell mass added more BOD to the system than could be compensated for by the increased removal ability of a psychrophilic inoculum. If retention times were longer than 8.5 hr and the removal rates were linear with respect to time, the activities of the psychrophiles at low temperatures would more than compensate for the added BOD. Even if rates were non-linear, increasingly longer retention times should result in an increasingly

better effluent BOD for the psychrophile-inoculated sewage relative to the non-inoculated raw sewage. If sufficiently long retention times were used, the final BOD concentrations for psychrophile-inoculated raw sewage would be appreciably less than the final concentrations in non-inoculated raw sewage, and the "inocula" method of improving treatment efficiency at low temperature would appear practical.

Because the selection of the isolates to be used as inocula could be accomplished on the basis of either growth or oxygen uptake rates, studies were performed to determine which method of selection corresponded better with observed BOD removals. A comparison of Arrhenius plots of growth and oxygen uptake rates for the three isolates used (Figures 1-2) with the BOD removed by the isolates at different temperatures (Table 8), revealed that oxygen uptake rates closely corresponded to BOD removals, but growth rates corresponded poorly. Although two of the isolates used in this study were selected because they showed rapid growth at low temperatures, the evidence from these studies indicate that it is more valid to select isolates on the basis of oxygen uptake rather than on growth.

Cells used as inocula for oxygen uptake studies were grown in either ½ strength m-PCB or in sterilized sewage supplemented with 1/5 strength m-PCB, because it was thought that cells grown in a sewage medium would physiologically adapt to its nutrients and oxidize sewage faster than cells grown in a nutrient medium only. The sewage medium was supplemented with only 1/5 strength m-PCB to supply adequate growth factors, with minimal stimulatory additions of utilizable carbonaceous compounds, to initiate growth and ensure that the cells would physiologically adapt to sewage nutrients. Results of this study indicate that adaptation to sewage nutrients does occur at all temperatures, as cells grown in the sewage medium nearly always had greater oxygen uptake rates than had cells grown in the nutrient medium.

B. By Alaskan isolates.

Raw settled sewage inoculated with each of the three selected Alaskan-psychrophiles and incubated at 5 C over a five day period

indicated that BOD values were being reduced at a very slow rate (Figures 3, 5 & 7); however, there was very little variation between the BODs in the inoculated sewage and the uninoculated controls. Percent BOD reductions in Figures 4, 6 and 8 present a mirror image of the BOD values. All reductions were very low grade never exceeding approximately 30 percent. Paired t tests used to evaluate differences between treatments and controls in both BOD and percent BOD reductions indicated that, statistically, differences did not exist. Viable psychrophilic cell counts, made concurrently with BOD determinations (Figures 9, 10 & 11), indicated that a psychrophilic population of approximately 10^4 cells per ml was present in uninoculated sewage controls, a concentration equivalent to that reported to have been found in rivers (32). Flasks seeded with treatment cell suspensions initially contained about 1000 times more viable psychrophiles per ml than the uninoculated substrate. Accordingly, since both treatments and their respective controls contained psychrophilic populations, both would be expected to reduce BOD, but because seeded systems contained much denser populations, a more rapid rate of BOD reduction was expected in these flasks. This did not prove to be the case within the five day detention. Dias and Bhat (19) theorized that a single bacterial species would likely be limited in its ability to utilize organic substances that occur in sewage. This may be part of the reason that substantially greater BOD reductions in inoculated sewage were not observed over the uninoculated controls.

If the treatment organisms were able to utilize only a small portion of the organic constituents present in the sewage, a heavy inoculum would only result in a relatively rapid depletion of these materials, leaving most of the remaining carbonaceous compounds to be degraded by the varied sewage psychrophile population at their own rate. When the compounds utilized as substrate became limiting, the treatment cell numbers would become stationary, but because degradation would still be occurring by indigenous cells, the BOD would be expected to continue to drop. This is largely what was observed. Inoculated cell numbers became stationary within 2 days; yet the BOD continued to drop to the end of the five day incubation

at approximately the same rate as that observed in the uninoculated control. However, with the exception of the B-6 control, indigenous cell numbers did not become stationary within the same period. The rapid reduction expected in the growth-limiting nutrient during the two days that the cells were actively metabolizing and growing may not have been reflected in the BOD due to the natural sewage psychrophile population degrading a much wider range of carbon compounds.

III. Alaskan isolates in concentrated synthetic sewage.

Support for the limited-growth concept comes from a series of experiments using a synthetic sewage to determine the effects of substrate concentration at 5 C. Concentrating the sewage would increase the amounts of the nutrient components available to the inoculated cell mass and cell numbers would be expected to either not become stationary at all or become stationary at a later period. B-6 cell numbers became stationary one day later than in the raw settled sewage, and B-39 and C cell numbers never became stationary at all in the five day test period (Figure 14).

It is also interesting to note that bacterial activity in the concentrate generally resulted in a greater absolute BOD reduction (Figure 12) than in the natural sewage substrate, but proportionately less BOD was removed in the synthetic medium (Figure 13). The fecal coliform inoculum utilized as a control exhibited a behavior quite different from what was expected. The BOD of the medium containing the mesophile increased to a rather high level at three days detention, then rapidly dropped to initial BOD levels. Because fecal coliforms do not survive low temperatures well, the release of protoplasm into the medium by cell death and lysis could explain the BOD increase. However, the most penetrating question to answer is not,"Why did the BOD increase?",but rather,"Why did the BOD decrease?". There is no good apparent answer to this question. The reason could not be metabolic activity, so the only alternative is some unknown physical phenomenon.

The greatest difficulty in evaluating the results of the concentrated synthetic sewage experiments lies in the fact that the sewage

was artificial, and therefore, bacterial responses are almost certain to vary from those obtained in raw domestic sewage to which the cells were originally adapted.

IV. Chemical analysis of effluent after treatment with Alaskan isolates.

The analysis for sugar in the raw settled sewage, depicted in Figures 15 and 16, also lends support to the limited-growth hypothesis. This type of carbohydrate was utilized very rapidly by both the Alaskan psychrophilic isolates and those naturally present in the sewage. The greatest differences between sugar utilized by inoculated systems and their uninoculated controls occurred within the first two days of incubation at 5 C. Slightly over half of the existing sugar was depleted in inoculated systems in the first day of detention as compared to a little over one-third being removed by the uninoculated control. Although greater amounts of sugar were likewise removed in inoculated flasks during the second day, differences between treatments and controls were not as pronounced. Beyond two days of detention, differences between treatments and control were virtually nil. If the inocula cells became limited at the end of the two day period due to the lack of availability of essential nutrients, differences between their respective abilities to remove sugars and the ability of the uninoculated control to do the same would be expected to disappear. It is conceivable, but by no means certain, that the inocula psychrophiles may be capable of degrading only sugars as a carbon source.

The results of an analysis for nitrate-nitrite nitrogen (Table 22-A) indicate that no significant change or alteration occurred in amounts present during the five day contact period in the filtered effluent of either inoculated or control flasks. This conforms to the findings of earlier workers that psychrophiles are unable to either denitrify (12) or nitrify (14, 83) at low temperatures. The nitrogen source utilized by these organisms must obviously be in some form other than nitrate or nitrite, possibly ammonia.

Table 22-B lists concentrations of orthophosphate observed in the fluid filtrates of psychrophile inoculated and control flasks. The data obtained suggested that a low grade phosphate uptake at 5 C was occurring in the inoculated systems for the first two days of detention

but increases occurred in phosphate concentration beyond two days. The control exhibited what appeared to be a gradual uptake in phosphate at a much lower rate than observed in the inoculated systems during the first two days of incubation. The high phosphate value obtained in the control flask at the second day of detention is likely due to experimental error. Although the reason that phosphate concentrations increased in treated systems in the last three days of detention is uncertain, MacKelvie, Campbell and Gronlund (55) found that carbon limited Pseudomonas aeruginosa excreted both ammonia and ultraviolet absorbing materials into incubation medium. It is possible that nutrient limited cells may also release certain amounts of phosphate as well.

Many psychrophiles are characterized by their ability to produce storage polymers which greatly increase the viscosity of the medium in which the organism is being grown. An increased viscosity in raw sewage would produce the undesirable effect of holding matter in suspension and thereby rendering primary settling difficult at best. For this reason, viscosity measurements of the filtered wastewater in both inoculated and uninoculated sewage were made to determine whether or not the organisms being used would exert such a deleterious effect (Table 22-C). Initial as well as final values of viscosities in treatments and control all fell within the ± 1 percent error of the viscosimeter. Most findings regarding the psychrophilic production of these viscous polymers indicate that the molecule produced is often a polysaccharide such as dextran (25, 29); if such a substance were being produced it should have been detected in the analysis for sugar. The fact that increases in sugar content were not observed tends to support the finding of the viscosity data that significant increases did not occur in any of the systems examined.

The effects of pH on treatment cell growth and substrate oxidation are given in Figures 21-26. As expected, the data obtained suggests a definite correlation between oxidation and cell growth. Ranges of pH which resulted in the greatest amount of oxidation also provided for maximal rate of growth. All three treatment organisms appeared to function well at 5 C in the pH range of 7-8, well within values found in the raw sewage substrate. The variation observed between each of the three curves obtained for each test can be accounted for as being due to

differences in each sewage sample used as a substrate. The chemical composition of raw sewage varies greatly from sample to sample. Lamanna and Mallette (49) point out that for any given exzyme an optimal pH exists for its activity; this optima is not fixed, but changes with ". . . the nature and concentration of salts present." Undoubtedly, bacterial enzyme systems undergo the same fluctuation in optima with changes in surrounding chemical composition that individual enzymes do.

SUMMARY AND CONCLUSIONS

Bacterial isolates capable of growing near 0 C were used as inocula for experiments in heat sterilized, raw and concentrated synthetic sewage to determine whether BOD removals could be enhanced at low temperatures. Growth and oxygen uptake rates in sterilized sewage of these isolates were also determined as was a chemical analysis of raw sewage before and after treatment.

The percent BOD removals from heat sterilized sewage at temperatures ranging from 5 to 20 C by two culture ages (1 and 7 days) and two quantities of inocula (10^{10} and 10^{8} cells/200 ml) of the three psychrophilic isolates and of a mixed mesophilic population were analyzed by 4 X 4 X 2 X 2 factorial analysis of variance (Table 3). Culture age, quantity of cells inoculated, organisms inoculated, temperature, and many of the interactions among single treatments affected BOD removals.

One-day old cultures generally removed more BOD than 7-day old cultures (Table 4). Further inspection of the data revealed that 7-day cultures were more efficient only when the small inoculum was used (Table 14) or when at 5 C (Table 12). It was thought that these interactions of culture age with inoculum size and temperature were caused by the effects of cold shock on the inoculated cells.

The effect of number of cells inoculated on percent BOD removed was very significant, as seen by the large F value found in Table 3. The large (10^{10} cells) always removed more percent BOD than the small inoculum (10^{8} cells). Although the number of cells in the large inoculum was a hundredfold greater than the smaller, they removed only about one and a half times as much BOD as the small inoculum (Table 6). This relatively slight, though statistically significant, difference was thought to be caused by active growth of the cells of the small inoculum after inoculation while the cells of the large inoculum did not grow.

The differences in BOD removals among locally isolated organisms used as inocula were relatively slight, although statistically significant, probably because the organisms were psychrophiles having similar oxidative abilities. The isolates were affected differently

by changes in temperature (Table 8). As expected, the mesophilic population was more adversely affected by a decrease in temperature than the psychrophiles.

A decrease in temperature brought a general decrease in percent removals. It was observed, however, that the overall removals at 5 C were not much less than those at 10 C, although growth or oxygen uptake rates were much less at 5 C (Figures 17 and 18). It was suspected that, at the lower temperatures, non-biological removals of BOD (such as settling) were more significant than the oxidation of the organic wastes; thus, the adverse effect of low temperatures on biological oxidation rates were masked.

A comparison between BOD removed by single inocula and combinations of the single organisms revealed that combining the isolates did not increase oxidative abilities. Although this study did not show increased efficiency by using combinations of organisms as inocula, it is possible that synergisms and successive substrate utilizations by combinations of the appropriate bacteria could result in the oxidation of more substrate.

Because heat sterilized sewage is distinctly different from raw sewage, experiments were performed to determine whether inocula of psychrophiles could also improve BOD removal efficiency in raw sewage at low temperatures. The experiments showed that sewage inoculated with pure cultures of psychrophiles had approximately 19% of the BOD removed within 5 hr at 2 C, whereas the sewage inoculated with a mixed population of mesophiles had 15% removed and the raw sewage with no inoculum had 14% of the BOD removed. Although the inoculated sewages had the greatest percent BOD removed, they also had a higher final BOD, because BOD was added with the inoculum. Increased retention times might allow the more efficient psychrophile inoculated sewage to remove enough BOD and result in a lower absolute BOD concentration.

Low temperature stabilization of raw domestic wastewater already containing a heterogenous psychrophilic population is not substantially affected by the addition of a pure culture psychrophilic inoculum. Viable psychrophilic cell counts suggest that treatment psychrophiles became growth limited in two days at 5 C. Since the substrate apparently never became limited with respect to either nitrogen or phosphorus,

it seems likely that these organisms became carbon limited because they were only able to degrade a small portion of the non-carbohydrate organic matter present. Although the BOD itself is a carbon limited concept, its usefulness as a parameter of wastewater treatment efficiency is dependent upon a microbial population capable of oxidizing a wide range of organic compounds. Hydrogen ion concentrations apparently did not present a problem in the growth of the psychrophiles. Future studies into the psychrophilic degradation of wastewater might prove more fruitful if emphasis were placed on mixed culture inocula utilizing psychrophiles screened not only for rapid growth, but growth on a wide variety of carbon sources.

Oxygen uptakes in heat sterilized sewage were generally higher when cells were grown in a culture medium containing heat sterilized sewage rather than in ½ m-PCB. Uptakes by 1-day old cultures were greater than 7-day old cultures. Oxygen uptake rates by 1-day old cultures grown in a medium containing sewage more closely corresponded to BOD removals than did growth rates.

On the basis of these experiments it was concluded that:

1. Psychrophiles increased BOD removals at low temperatures, but it was also found that an inoculum increased the initial BOD. Long retention times will be needed to overcome the higher initial BOD and result in an acceptable effluent BOD.
2. Cultures in stationary growth phase were not susceptible to the effects of cold shock. Older cells would, therefore, probably make a better inoculum when added to a low temperature wastewater.
3. Because BOD removals more closely corresponded to oxygen uptake rates than to growth rates, the selection of psychrophiles to be used as inocula would probably be best if based on oxygen uptake rates at low temperatures rather than on growth rates.
4. The stabilization of wastewater by psychrophilic microorganisms is not effectively enhanced by concentrating the sewage substrate.

5. Future studies on psychrophilic degradation of domestic sewage should emphasize mixed culture inocula of organisms capable of growth on a wide variety of carbon sources.

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Appendix 1. Relation between number of cells/ml and Klett 1/50 reading.