STUDIES ON PREDOMINATION OF SELECTED BACTERIA OF SEWAGE ORIGIN

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CHAPTER I

INTRODUCTION

We are all participants in the most interesting and crucial journey ever undertaken. The journey began so long ago that its origin is a subject of unresolved debate. When and how it will terminate is no less nebulous; however, it seems the trip may be nearer to its end than to its beginning. Perhaps the most certain thing is that we are at a significant point in the journey and certain vital decisions must be made regarding the conduct of the passengers for the remainder of the voyage. The results of these decisions will be logged with the record of past occurrences and decisions in the book of man's history on Earth for we are the passengers and our spaceship is the planet Earth. What is recorded of our future should prove to be interesting reading. It will be interesting living. If we could look ahead and read of the result of our actions in what is now the future, what might we have done differently or what action might we have undertaken that was left undone or started at a late date? Such thoughts can provide a source of interesting speculation.

If we accept the concept of our planet and its atmosphere as a closed environment (our spaceship), another interesting analogy may be considered, one which may provide observations and lead to conclusions significantly related to our way of life. The proposed comparison is the macro-ecosystem of earth and its larger life forms with the

micro-ecosystem of a bacterial culturing vessel containing a medium rich in essential constituents for bacterial growth, yet not necessarily homogeneously distributed, and inoculated with a small quantity of a mixed culture of bacteria.

Our observations of the bacteria will likely reveal a pattern of moderate initial growth by most of the species. Then as nutrients are utilized the less fastidious bacteria may emerge into predominance, and migration to sources of additional food will occur. The composition of the environment and the population it supports is not likely to be homogeneous nor is it likely to be constant. Complex interactions may be seen to occur in the bacterial population. Competition for food and space become critical. Those organisms best able to compete for one or more reasons, e.g., greater growth rate, greater adaptability to the changing environment, or inhibition of competitors, will survive longer than those less able to compete successfully. Yet eventually even the best of competitors are finally reduced because the environmental reserves have been depleted or destroyed. The time required to progress through the various stages of growth in the micro-ecosystem is dependent on many factors, but a common observation may be made from numerous experiments; when the population becomes so large that the environmental reserves become depleted or when the waste products of growth destroy the environmental conditions which permit the growth to occur, the ecosystem begins to decline and accelerated death ensues. Can the preceding bear any relevance to man and earth? Are there any lessons to be learned from our observations? There may be certain conclusions drawn regarding our apparent fate and the means of approaching it that we are not ready to accept (e.g., population control); yet what

degree of overcrowding of the earth have we reached and what population-time relationship can our limited natural resources support? It is not intended here to proselytize anyone into advocating population control with its many ramifications. Many of nature's methods for population control are generally rejected by man as too direct or "inhuman" and a widely acceptable solution to population control is still being sought. However, it is suggested that man, by studying nature, may find answers to many of his problems.

With the foregoing presentation emphasizing the demands placed on our limited resources by an increasing population, hardly any greater justification can be made for conserving what resources remain from misuse, abuse, and contamination through pollution. It is almost inconceivable, when we know the fate of a bacterial population in a closed environment, that we should hasten our own decline by allowing our own waste products to destroy our "growth" environment.

Not only do the observations on bacterial communities help us to gain insights into our own ecological progression but they also serve to guide us toward a way and means to dissipate our organic waste materials. Only in relatively recent history have we recognized the great adaptability of microorganisms for treatment or degradation of our water-borne wastes. This recognition has led to the wide use of aerobic biological treatment processes. However usage does not necessarily indicate that the processes are well understood. With the present demands, made by an increasing population and diverse industrial development, for expansion and greater effectiveness in sewage treatment, it has become imperative that we gain a greater understanding of

the interactions which occur in the microbial ecosystem during sewage treatment.

Attainment of the goal of more complete understanding requires input from various fields such as microbiology, biochemistry, and engineering. Bioenvironmental Engineering, which combines these disciplines, represents a new approach toward achieving greater understanding of the complexities involved.

In regard to the magnitude of the problems faced in environmental protection and control, our present knowledge and its application may be considered to be at a very young stage in its development, an infant, hardly able to cope with anticipated requirements. However, when considering the growth potential existing in Bioenvironmental Engineering, one may recall an exchange between Michael Faraday and Benjamin Disraeli, then Prime Minister of England and usually credited with being quite perceptive in his observations. Faraday was performing some early experiments with electricity when Disraeli, after watching a few apparently insignificant sparks, skeptically asked, "What good is it?" In reply Faraday posed his own question, "What good is a baby?"

It is toward the goal of increasing the knowledge in the area of biological waste treatment and natural microbial ecosystems in general that the present study has been undertaken. It is realized that biological relationships other than those existing among bacteria are of importance in wastewater treatment; however, for purposes of this work only bacterial interactions were examined. The bacteria employed in the study were originally isolated from sewage. Their growth was

studied in pure and mixed cultures under continuous and discontinuous culture conditions.

CHAPTER II

LITERATURE REVIEW

In the search for the means by which the treatment of water-borne wastes may be accomplished in a more complete and efficient manner, the researchers and those who apply the research information to waste treatment have largely come to accept and proclaim the need for a greater interdisciplinary exchange of ideas and information among engineers and scientists. Such an interdisciplinary approach is a focal point of the Bioenvironmental Engineering Laboratories at Oklahoma State University.

Throughout its early history, progress in the design and application of biological waste treatment was on an empirical basis, and improvements in design concepts were not made until the significant relationship between the effect of the microbial populations existent in the treatment facility and the treatment process was realized. Today, even after the recognition of the importance of microbial activities in waste treatment has been recognized, the utilization of this important concept has yet to be fully realized (1).

The microbiology of water, sewage, and sewage treatment processes is a subject of considerable past and present study. The very nature of the problem is quite complex when one considers the great variety of microbial species present and the variations in the physical and chemical environment and available nutrients. With an understanding of the

broad scope of the areas of possible exploration into the understanding of biological waste treatment, one may see why it is important that each investigator and observer disclose his new information and add to the growing accumulation of knowledge in an attempt to clarify that which often has been nebulous.

While there have been numerous publications concerning microbial predomination or interaction, the following citations have been chosen as representative of those most closely related to the area of this study, i.e., the isolation and study of sewage bacteria with particular emphasis on their interactions and predomination in mixed cultures.

Isolation of Bacteria of Sewage Origin

It is generally acknowledged that no single medium or procedure is apt to be totally satisfactory for the enumeration and isolation of all the bacterial species which may be present in sewage. However, considerable effort has been spent in devising special media and in comparative studies to determine the most satisfactory medium.

Ferrer, Stapert, and Sokolski (2) developed an iron peptone agar and a procedure which employed membrane filtration rather than a poured agar plate. Incubation was conducted at room temperature for four days. This medium and procedure was found to yield higher counts of bacteria from water samples than those obtained by the use of Difco tryptone glucose agar.

van Gils (3), in his studies on the bacteriology of activated sludge, employed tryptone glucose agar and a sewage agar prepared from screened municipal sewage. The tryptone glucose agar yielded two to six times the number of colonies found on the sewage agar. In a

study of the relative numbers of predominant bacteria isolated from the various activated sludges examined with sewage agar and a mineral medium agar with glucose and ammonium sulfate, a high percentage of the species found on the sewage agar were not capable of growth on the other medium. The cultures were incubated at 25° C for six days.

A preparation of autoclaved and neutralized (H_3PO_4) sewage solidified with agar was used by Dias and Bhat (4) to study the dominant bacteria in activated sludge. Some 150 isolates were obtained from raw sewage. Upon transfer to proteose peptone yeast extract agar about 91 percent were found to survive. A 10 to 15 day incubation period at 16° C to 27° C (room temperature) was employed.

Prakasam and Dondero (5) prepared a sewage agar and an activated sludge extract agar for comparative use with nutrient agar and five other counting media. They noted an apparent difference between the predominant populations of sewage and activated sludge and the suitability of each medium for the respective sampling source. For the enumeration of activated sludge bacteria, the activated sludge extract agar medium yielded the highest counts with nutrient agar a close second best. The enumeration of sewage bacteria was best performed (highest counts) with nutrient agar. The activated sludge extract agar produced results not significantly different from nutrient agar for some sewage inocula. Sewage agar was judged to be a poor medium for growth and isolation. Both sewage agar and the sludge extract agar were found to vary in efficacy, i.e., their comparative effectiveness with other media was not constant. Dondero et al. (6) presented various isolation techniques employing sewage agar supplemented with growth factors for the isolation of Sphaerotilus from activated sludge.

Berg et al. (7) utilized the pour plate method with various media to determine the bacterial populations present in primary waste water effluents. Nutrient agar was found to yield higher counts than either brain-heart infusion agar or trypticase soy agar. Nutrient agar was also found to give higher counts than those obtained on yeast-extract agar or tryptone glucose yeast-extract agar in studies on the aerobic bacteria in waste stabilization ponds by Gann et al. (8).

Bacterial isolates were obtained from the waters of two rivers by Stumm-Zollinger (9) using three media for one river and nutrient broth agar for the other river. In the comparison of the three media, a medium consisting of casitone, sodium caseinate, starch, glycerol, potassium phosphate, and filter-sterilized river water was found to be superior for bacterial enumeration to brain-heart infusion medium or a medium of yeast extract, casein hydrolysate, and potassium phosphate. Nutrient agar provided about 50 isolates which were propagated in a nutrient broth, glucose, and sodium oleate medium for further studies on mixed microbial communities.

Lighthart and Oglesby (10) reviewed wastewater treatment plant ecology and noted the following: a) there has been no standardization of an analytical method for the isolation of bacteria from wastewater or its treatment processes; b) there are many types of organisms associated with the various treatment processes; and, c) to understand the functional capabilities of the bacteria and classify them taxonomically, a large number of organism characteristics must be determined. They selected tryptone-glucose-meat and yeast extract agar as the most satisfactory of the eight tested media for enumeration, differentiation, and isolation. However, the data which they presented indicated that

nutrient agar also appeared to be satisfactory for enumeration, with activated sludge extract agar being slightly less satisfactory. They tested 50 to 100 colonies selected at random without regard to predominant colony types. A 10 day incubation at 20° C was employed.

Cultivation

The methods for the determination of bacterial growth rates and the formulation of equations to describe the aspects of growth under various conditions have been the subjects for numerous publications. The purpose of this study was not directed toward a study of kinetics, but since the subject of growth rate has been felt to play such an important role in the determination of the predominant bacterial population in mixed cultures, some references to its calculation are in order.

The oft-cited work of Monod (11) (12) (13) on the determination of growth constants has met with general acceptance after experimental evidence frequently supported his proposals. He noted, in discussing the physiological significance of the growth constant, that there may be more than a single limiting factor in action and classified such growth limiting factors into three groups: a) exhaustion of nutrients, b) accumulation of toxic metabolic products, and c) changes in ion equilibrium, especially pH. Contois (14) has suggested that the growth rate is also a function of the population density.

Garret and Sawyer (15) have stated from their experiments using glucose and peptone as substrates that for practical purposes the relation between the rate of growth and the remaining soluble BOD is well represented by a discontinuous function. With high concentrations of

BOD the rate of growth is directly proportional to the remaining soluble substrate. The relative size of the inoculum and substrate concentration has been suggested to affect the growth rate. Fujimoto (16) noted that an inoculum of 10^3 or 10^4 cells/ml of Escherichia coli produced an apparent greater growth rate than did the inoculum concentration of 10^7 cells/ml. Therefore, when a comparison of growth rates is desired, a similar range of cell concentration in the inocula was recommended.

Meers and Tempest (17) accepted the Monod equation, but considered its form to be unsatisfactory when culture growth was limited by the accumulation of metabolic products in the environment. They recommended the addition of a population-product factor to the equation. A review of the Monod equations and their various modifications has been presented by Peil (18). He concluded that the hyperbolic single phase relationship described by the Monod equation, $[\mu = \mu_{max} \left(\frac{S}{K_s + S} \right)]$, could be employed to depict the effect of the substrate concentration on the logarithmic growth rate. The addition of Na^+ to the buffer salt mixture of the synthetic waste had little or no effect on the growth patterns of activated sludge; however, the addition of NaCl caused a slight increase in cell yield.

Herbert, Elsworth, and Telling (19) and Herbert (20) proposed formulations for use in describing the kinetics of microbial growth in continuous cultivation. They stated that, theoretically, in a completely mixed reactor at steady state the rate of change in cell concentration and substrate concentration is zero. The steady state was said to be closely approached for pure cultures.

Experimental results led Gaudy, Ramanathan, and Rao (21) to the conclusion that for a completely mixed once-through type reactor, a steady state with respect to the biomass (heterogeneous populations) could be approached, not attained.

Painter and Marr (22) have surveyed much of the current literature on the mathematical treatment of microbial growth. The reader concerned with such information can find numerous references in this review or in the bibliographies of several recent publications (23) (24).

Microbial Interactions

Gaudy and Gaudy (25) reviewed a significant quantity of literature on the microbiology of waste water purification. They stated that pure culture information may find engineering application if certain differences between pure culture studies and heterogeneous populations are kept in mind. For the time being, we must be content with the use of a range of design "constants" while we continue to gather data and information to use for further insight into the complexities of biological treatment.

A historical review of microbial interactions has been published by Waksman (26) which cites work beginning with the observation by DeBary in 1879 of the predomination of one of two organisms when they were grown together. Waksman described the development of organisms grown in natural substrates as being dependent upon a number of factors such as: a) food supply, both quantitative and qualitative; b) environmental conditions; c) the presence or absence of certain other organisms which through various modes of action may help or hinder the growth of the certain organisms of interest; and d) the presence of

organisms which are parasitic or phagocytic to the particular organisms in question.

Rahn (27) made the early observation, which has often been cited, that pure cultures in nature are very rare, and that generally nature works with mixed cultures. In many natural processes mixed microbial populations exist; one species may predominate, but it still may be influenced by the rest of the population. He stated that the results obtained with pure cultures were not sufficient to provide the information necessary to explain all the activity observed in natural microbial populations. Similar views regarding the inadequacy of pure culture studies were expressed by Fawcett (28). However, in order to minimize the complexities resulting from the use of heterogeneous microbial populations, he advocated a necessity for investigation of the effects of known culture mixtures in comparison with the effects of individual organisms alone in studies of plant diseases.

A discussion of the types of bacterial interactions which may occur and proposed definitions for describing the effect of the interaction(s) have been given by Brock (29) and Bungay and Bungay (30). The latter especially is informative in tracing a historical review of microbial interactions through recent research approaches. Bungay and Bungay conclude that research on mixed cultures is in its infancy and expanding research will require sophisticated work involving many types of talent and interdisciplinary cooperation.

Antagonistic relationships have been the subject of frequent study. Quite often the study has been limited to only two cultures in an effort to determine the nature of the cause of the antagonistic effect. Savage and Florey (31) could find no proof from their experiments that

bacteria can be induced to produce an antibiotic for any selected species. Charlton (32) concluded that the antagonism found to exist in his experiments was due to competition for gaseous nutrients in the medium rather than such other factors as antibiotic production, crowding, or direct contact between organisms.

Miller (33) argued against a requirement for cell contact in the exertion of antagonism and stated that with the exception of predation, grazing, and parasitism, one individual affects another through the environment and not directly from individual to individual.

Hetling et al. (34) determined growth yields in continuous culture with several pure cultures and mixtures of pure cultures using a variety of substrates. The yield varied with the substrate and organism; however, higher yields were obtained with complex rather than simple media and with mixtures of organisms rather than a single pure culture.

Shindala et al. (35) have reported a commensalism effect of the bacterium Proteus vulgaris with the yeast Saccharomyces cerevisiae in continuous cultures. By use of a medium capable of supporting the growth of the yeast but not the bacteria in pure cultures, it was shown that the bacteria could grow in mixed cultures in continuous flow because the niacin-like nutrient it needed was elaborated by the yeast. Steady state populations were established which could be upset and restored by the addition of niacin or NAD and its subsequent dilution. The numbers of yeast produced in the defined medium were the same in pure and mixed cultures; therefore, the interaction was a true commensalism. Additional observations of growth in mixed cultures followed this study (36).

Meers and Tempest (17) utilized six organisms (5 bacteria and 1 yeast) in pairs to study the influence of extracellular products on the behavior of mixed microbial populations in magnesium-limited chemostat cultures. As a general observation they found the Gram-negative bacteria outgrew the Gram-positive bacteria. The ability of Bacillus subtilis and Bacillus megaterium to outgrow one another depended on the size of the inoculum. This dependence resulted from the presence of specific extracellular products in the Bacillus cultures which stimulated their growth and uptake of magnesium. The concentration of extracellular products varied with population density. A decrease in magnesium concentration decreased the B. subtilis population density, but the addition of extracellular products from a dense B. subtilis culture stimulated growth in the low population. They concluded that magnesium assimilation by B. subtilis was more dependent on extracellular substance(s) than was magnesium assimilation by the yeast or Gram-negative organisms examined. Modification of the basic theory of microbial growth in a chemostat culture to take into account product-stimulated substrate assimilation was suggested.

Gann et al. (8) studied the dominant bacteria of waste stabilization ponds and discussed the effect of pH in attributing to the dominance of the genera Pseudomonas, Achromobacter, and Flavobacterium which together comprised 90 to 95 percent of the total bacterial count. These organisms were found to be able to produce an alkaline reaction in culture medium containing a minimal quantity of protein. Other references were cited to support these findings, such as the work of Bender and Levine on acid production from carbohydrates by strains of Pseudomonas, which was masked by alkali production if the medium also

contained protein. Delay found that organisms of the above predominant groups are highly resistant to alkaline conditions but are susceptible to acidic conditions. Their optimum pH for growth lies between 7.2 and 7.5 and they are killed at pH values below 5.5.

Adamse (37) (38) found that the bacterial populations in an activated sludge developed in an oxidation ditch and in a laboratory dairy waste were closely similar - consisting mostly of Arthrobacter, Pseudomonas, and Flavobacterium. He reported that the addition of a heavy load of dairy waste to the laboratory activated sludge unit resulted in a drop of pH and dissolved oxygen concentration in the aeration vessel. The decrease in pH was apparently due to the accumulation of acidic intermediates from carbohydrate metabolism. Oxygen transfer was concluded to be the limiting factor in the oxidation of organic matter from the waste.

Bacterial Predominance Studies

Fawcett (28) in 1931 expressed the need for studying mixed cultures and the predomination of certain bacteria within such mixtures. He felt the results from the study of a mixture of two or more organisms might be more significant than the effects of individual pure culture studies.

Gaudy (39) used viable count and oxygen uptake data to study the pure and mixed culture response of four known bacteria during growth on media which contained glucose, glucose with nutrient broth, fructose, sucrose, or maltose as the carbon source. The mixtures of the cultures were found to be more efficient in substrate utilization than the pure cultures. It was not always possible to predict predominance in mixed

populations from pure culture studies, since no indication of the potential interaction among cultures when grown together may be seen.

Engelbrecht and McKinney (40) studied activated sludge cultures and concluded that predomination was controlled by the chemical structure of the substrate provided other environmental factors were constant. They also concluded that the use of structurally related compounds as substrate produced sludges which were similar in morphological appearance and produced similar biochemical changes. Such conclusions do not appear to be wholly valid in view of the personal observations of this author and reported observations of predominance changes observed in batch operation by Rao and Gaudy (41) and in continuous flow by Cassell (42) and Cassell, Sulzer, and Lamb (43) when a single medium was fed and operational conditions were controlled.

Rao and Gaudy (41) noted variations in COD removal and solids concentration and variable cell yield during long term batch operations with heterogeneous populations. Such variations were observed to accompany changes in the predominant bacterial population.

Cassell (42) and Cassell et al. (43), from observations of a continuous flow operation with a heterogeneous population grown on a skim milk substrate, concluded that at various constant detention times all parameters which reflected biological activity fluctuated continuously. Thus, continuous mixed cultures were observed to be a dynamic systems resulting from the various microbial interactions which occurred within the system. No simple relationship was found which satisfactorily explained the observed population fluctuations. It was recommended that the terms "steady state" or "equilibrium" when applied to continuous mixed cultures should be defined as an average condition because

of fluctuations. An adequate characterization of an "average steady state" may require sampling over an extended time. Predominance changes were noted by a color change and microscopic observations. It is this author's contention that in view of the many factors which can influence pigment production and the development and accumulation of amounts of pigment large enough to be readily detected, the use of color changes to denote changes in microbial predominance have only limited use for notation of gross population alterations under special conditions.

The sequence of predominant groups of organisms that may exist in a plug flow type aeration tank in an activated sludge process has been described by McKinney (44). Alteration of the substrate and a decrease in available nutrients along with such direct effects as predation contribute to the determination of the predominant populations found at different locations within the tank.

The primary dominant organisms, Zoogloea and Comamonas, isolated from activated sludge by Dias and Bhat (4) were found to have stored polybetahydroxybutyric acid in concentrations of up to 40 percent of their dry cell weight. They suggested that the ability to store food probably acts as a factor which helps bacteria to dominate in a system where food is in short supply.

Jasewicz and Porges (45) observed a change in bacterial predominance in a batch operation utilizing a dairy waste substrate. About 74 percent of the bacteria in the assimilative phase were Bacterium or Bacillus but only about 8 percent of the endogenous phase population was composed of these organisms. The dominant bacteria in the

endogenous phase were 42 percent Alcaligenes and Pseudomonas and 48 percent Flavobacterium or Micrococcus.

Contois and Yango (46) established steady state populations on chemically defined minimal media using combinations of: a) bacterium - bacterium, b) bacterium - yeast, c) bacterium - phage, and d) bacterium - myxamoeba. In each system the second organism was dependent upon the first under the conditions imposed. They concluded that several microbial species will coexist in steady state in a chemostat only if a physiological interdependence between the coexisting organisms is operative. Usually when two organisms compete for the same substrate, the faster growing species displaces the slower growing species from the culture vessel.

Leal (47) employed a strain of Serratia marcescens and an unidentified bacterial species which produced a yellow colony on nutrient agar to study the growth of a mixture of cultures whose growth rates were nearly equal on the same medium (glucose minimal medium). Kinetic constants for predicting growth patterns in continuous flow were determined from batch operations. The substrate (glucose) concentration affected the exponential growth rate up to some critical value. From the mixed culture studies it was concluded that cell concentration and mode of predominance in mixed cultures cannot be predicted in a quantitative sense from pure culture studies of the same organisms. One possibly could predict the predominant species in a mixture from a comparison of pure culture growth rates. However, the population density of each organism and a more rapid dilution of some species from a continuous flow reactor could not be predicted. The mechanism(s) of interaction of the investigated species was not fully determined.

Jannasch (48) has been cited by Stumm-Zollinger (9) as stating that continuous flow systems, although in many respects simulating the dynamic nature of natural habitats, tend to enrich for single species populations. In studies on sequential substrate removal, Stumm-Zollinger (9) stated that such enrichments and selection appear to be common in natural communities of bacteria. The opinion was given that mixed bacterial cultures in the laboratory do not simulate natural populations, but that a mixed, acclimated laboratory culture could be reconstituted from pure cultures of natural isolates. The metabolic activity of a microbial community depends in a complicated way on: a) the species structure of the community and shifts in this structure; b) the physiological response of the individual microorganisms to a multisubstrate environment; and c) the interactions between the various microbial species. It was felt that shifts in the species structure of mixed bacterial communities during growth in laboratory culture media were influenced by the relative differences in growth rates of the individual isolates.

Hungate (49) also considered it reasonable to assume that the most active species in an ecosystem would be the most abundant species present.

A broad spectrum exploratory work into the study of bacterial predomination was accomplished by Bustamante (50). Six strains of bacteria were used in the study. Their pure culture kinetic constants were determined from batch studies. Mixtures of two or three species were grown in batch systems and their growth patterns observed. Continuous flow cultivation of one and two organisms was performed to ascertain the dilute-out characteristics which proved to be similar but

not identical to the theoretical dilute-out characteristics. A lack of intermediates in systems utilizing sorbitol as a substrate in contrast to the significant quantities of intermediates (largely acetic acid) which were found in systems metabolizing glucose tended to suggest that different metabolic pathways were operative for the two carbon sources. He concluded that gross predominance predictions may be made for mixed cultures from the growth rate values of pure cultures if antagonistic relationships between cultures do not develop. An interrelationship between Pseudomonas aeruginosa and other organisms was found to benefit P. aeruginosa in the presence or absence of exogenous substrate. In continuous flow operations, the predominance of a species or relative changes in numbers could result from quantitative, qualitative, or hydraulic shock loads applied to the system.

Zahradka (51) determined that a definite relationship existed between the mechanical energy supplied to an activated sludge plant and the purifying ability of the sludge. He demonstrated that increasing energy input to the system resulted in a shift of the population dynamics of the system away from the predominance of filamentous forms of organisms. Rickard and Gaudy (52) have also reported the development of filamentous forms of organisms in a glucose mineral salts medium. The population density of these forms was subsequently decreased as the mixing energy supplied to the continuous flow reactor was increased.

Thabaraj and Gaudy (53) reported the occurrence of a transient response, following a quantitative increase in feed to a continuous flow reactor, which consisted of two sequential responses: a) a metabolic or physiological reaction by the indigenous population with a greater

appearance of COD in the effluent at lowest dissolved oxygen levels; and b) a secondary response which involved an ecological shift or predominance change in the reactor. Both responses involved a release of metabolic intermediates into the mixed liquor.

Mateles and Chian (54) observed the kinetics of substrate uptake and growth in pure and mixed culture systems. With the use of a glucose-lactose substrate as much as 25 percent of the original substrate appeared as acetic acid. Triphasic growth was observed as glucose, lactose, and acetate were utilized. It was suggested that the growth rate of a bacterial species can strongly influence its ability to predominate. Studies with a species of Pseudomonas and a coliform growing in a dispersed state revealed that in batch operations the Pseudomonas constituted about 80 percent of the population; however, in continuous flow the coliform was dominant at low dilution rates. The experiments on population dynamics strongly indicate that the acclimatization process frequently used in preparing laboratory sludge should be carried out under flow conditions identical to those to be used in the actual experiments. Batch acclimated cultures need considerable time to become optimal for use in continuous flow systems, and it is important not to begin taking data until an approximate steady state in terms of populations has been reached. Their experiments revealed that a "steady state" was reached after approximately 10 to 25 residence times, which is considerably longer than the 3 to 6 residence times often accepted as providing "steady state" conditions in pure cultures.

CHAPTER III

MATERIALS AND METHODS

A. Analyses and Techniques

1. Chemical oxygen demand (COD)

The total COD was determined in accordance with the procedure prescribed in "Standard Methods for the Examination of Water and Wastewater" (55) with the addition of silver sulfate.

2. Biochemical oxygen demand (BOD)

During the preliminary studies involving the preparation of sewage agar, the BOD of fresh and concentrated sewage was determined according to the procedure given in "Standard Methods" (55).

3. pH

The determinations of pH values were made with a pH meter (Beckman-Zeromatic II).

4. Viable bacteria count (plate count)

Except for those studies involving the preparation of sewage agar, nutrient agar (Difco) with supplemental 0.5% Bacto agar (Difco) was used for the enumeration and differentiation of bacterial colonies. The spot plate technique investigated by Gaudy (39) and Gaudy et al. (56) was employed to determine the viable bacteria count in sewage and in later experiments with selected bacteria. A spread plate technique was also used for viable count determination with sewage.

The preparation of the plates differed only in the manner in which the bacterial suspension was applied. In both techniques, 15 ml of sterile liquified substrate-agar at approximately 45° C were carefully poured into a sterile 90 mm diameter Petri dish, covered, and cooled until solidified. The dishes were then inverted and incubated at 37° C for approximately 48 hours to remove excess moisture from the surface of the agar. The drying period permitted a more rapid absorption of the liquid inoculum and better dispersal and fixation of bacteria than on freshly prepared plates, especially for the spot plate technique.

Inoculation of the plates with an appropriate dilution of bacteria was performed as follows:

a. Spot plate technique. The outside bottom of the plate was bisected with a marking pen line to provide two counting units per plate. A total sample volume of 0.08 ml per half plate was inoculated in four 0.02 ml spots, i.e., 0.16 ml per plate (8 - 0.02 ml spots).

b. Spread plate technique. A total volume of 0.20 ml of diluted sample was inoculated onto the agar surface in the Petri dish. The inoculated dish was placed on a turntable and rotated while the inoculum was spread evenly over the agar with a bent glass rod. The rod was sterilized prior to spreading the inoculum by immersion in isopropyl alcohol, flaming and cooling.

After inoculation the plates were covered and allowed to stand until the liquid from the inoculum appeared to have been absorbed into the medium. The plates were then inverted and incubated at room temperature (about 25° C); some plates, in the preliminary work, were incubated at 37° C. A 48 hour incubation period was found to be sufficient for total enumeration and most colony differentiation. When

sewage was used as inoculum, observations were also made at 72 and 96 hours on colonies which developed slowly.

Upon sufficient development, the colonies were differentiated and enumerated using a Quebec colony counter. Duplicate (or more) plates were always prepared for each inoculum. From the colony count, the inoculum volume, and appropriate dilution factor the population density of the sample was determined.

5. Sterile techniques

All equipment, glassware, media, and dilution water used in the experiments that might have introduced contaminating microorganisms to the cultures under study were sterilized either by autoclaving for 15 to 120 minutes at 15 psi and 121^{0} C or by hot air oven for at least two hours at 160^{0} C.

6. Biological solids

Measurements of biological solids were performed either directly (gravimetrically) from the dry weight of solids retained on a 0.45 μ pore diameter membrane filter (Millipore Filter Co., HAWP 04700, 0.45 μ) from a measured sample volume or indirectly by determining the optical density of a suspension of solids at 540 mμ with a spectrophotometer (Bausch and Lomb, Spectronic 20).

7. Glucose

Glucose concentration was determined enzymatically with the Glucostat test in accordance with Method 1-A of the Worthington Biochemical Corporation (57).

8. Carbohydrate

Carbohydrate content of the substrate and filtered effluent was measured by the anthrone method recommended by Gaudy (58).

B. Special Equipment

Aside from the more common equipment and glassware the following were used in conducting the studies and experiments during the course of this research. Where necessary the technique involving their use will be given under the appropriate section of the experimental protocol (part D of this chapter).

1. Flash-evaporator (Buchler Instruments). This was used to concentrate sewage for the preparation of sewage agar.

2. Growth flasks (special design). Flasks of special design were used to culture and measure growth rate by optical density. The flasks were 250 ml capacity Erlenmeyer type with 24/40 ⧩ tops fitted with optically matched test tubes. A side-arm was attached to the side of the flask. Cotton was used to plug the side arm to maintain sterile flask contents or protect the desired culture from contamination. The optical density of the reaction liquor was determined by inverting the flask and inserting the test tube into the port of the spectrophotometer.

3. Water bath shaker (Research Specialties Company). The growth flasks and flasks containing acclimating or growing cultures were incubated in a water bath shaker. The temperature was controlled at 25^{o} C and the shaking rate was 90 oscillations per minute.

4. Chemostat and large volume batch tube for bubble aeration. A 2 liter capacity cylindrical glass resin kettle having a slightly flattened bottom was used as the reactor or culturing vessel for the continuous flow and large volume batch growth studies. The top of the reactor had a ground glass flange which was fitted with a matching cover. The cover contained four holes which were utilized for the necessary attachments for feeding the unit, aeration, effluent removal, and sampling.

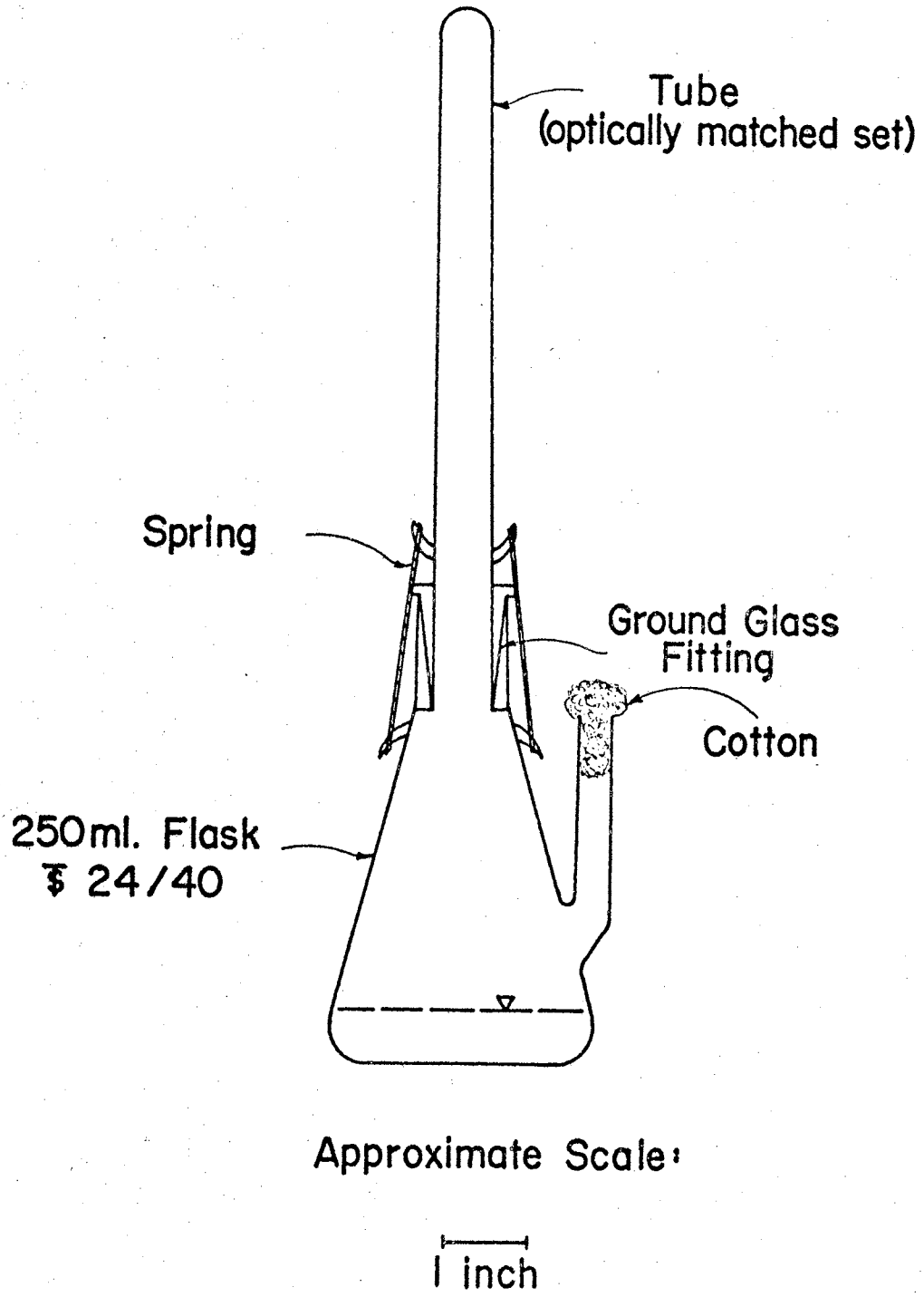
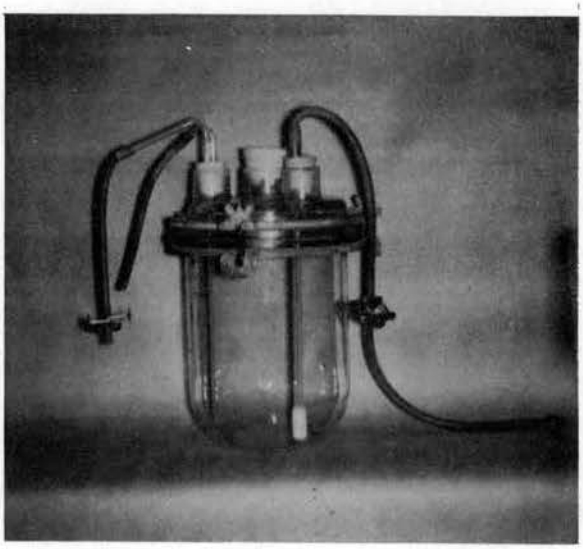


Figure 1. Diagram of shaken flask reactor.



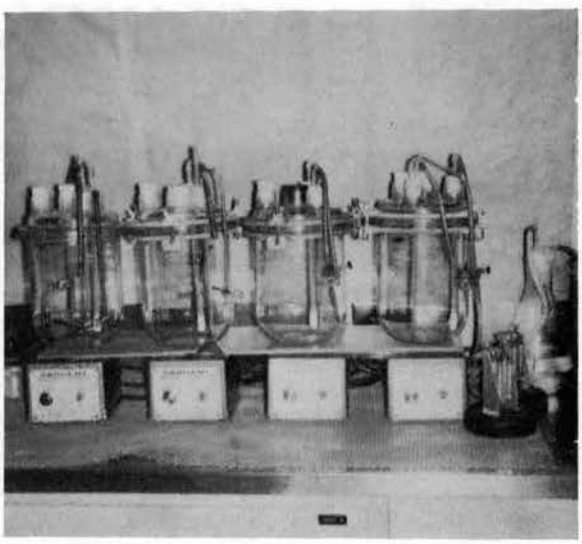
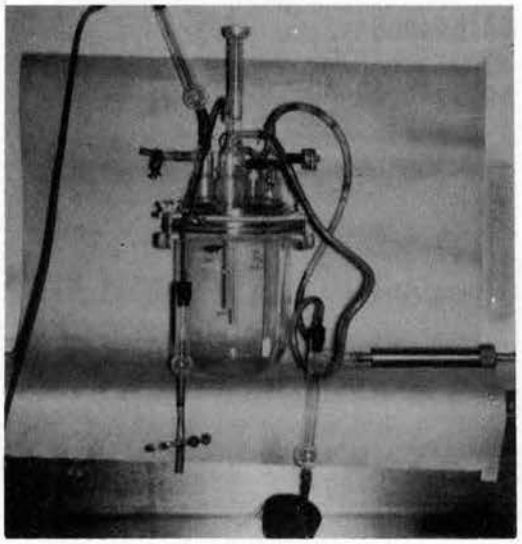
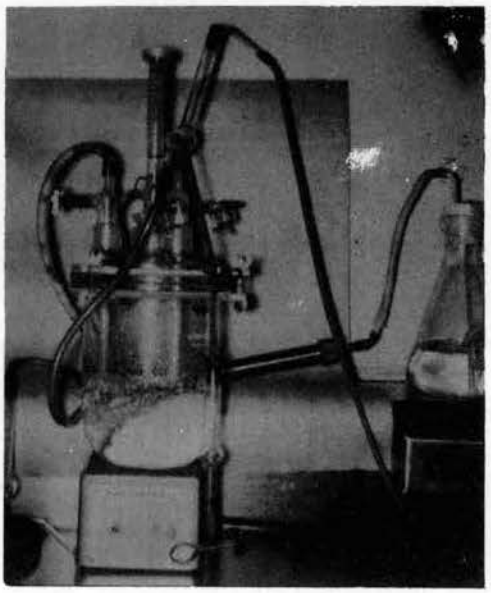


Figure 2. Bubble aeration batch reactor.





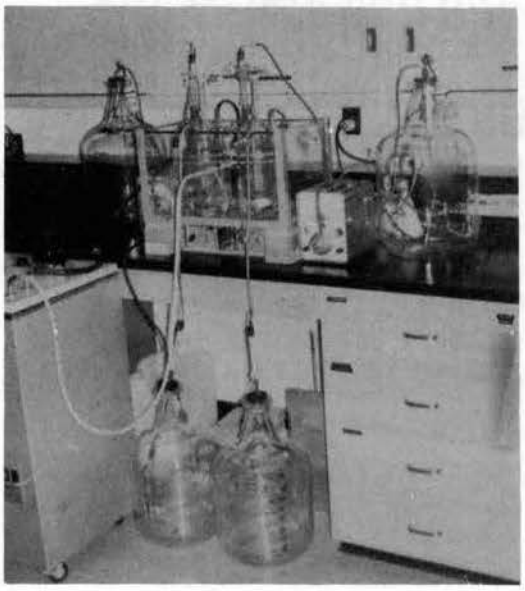


Figure 3. Continuous flow reactor.

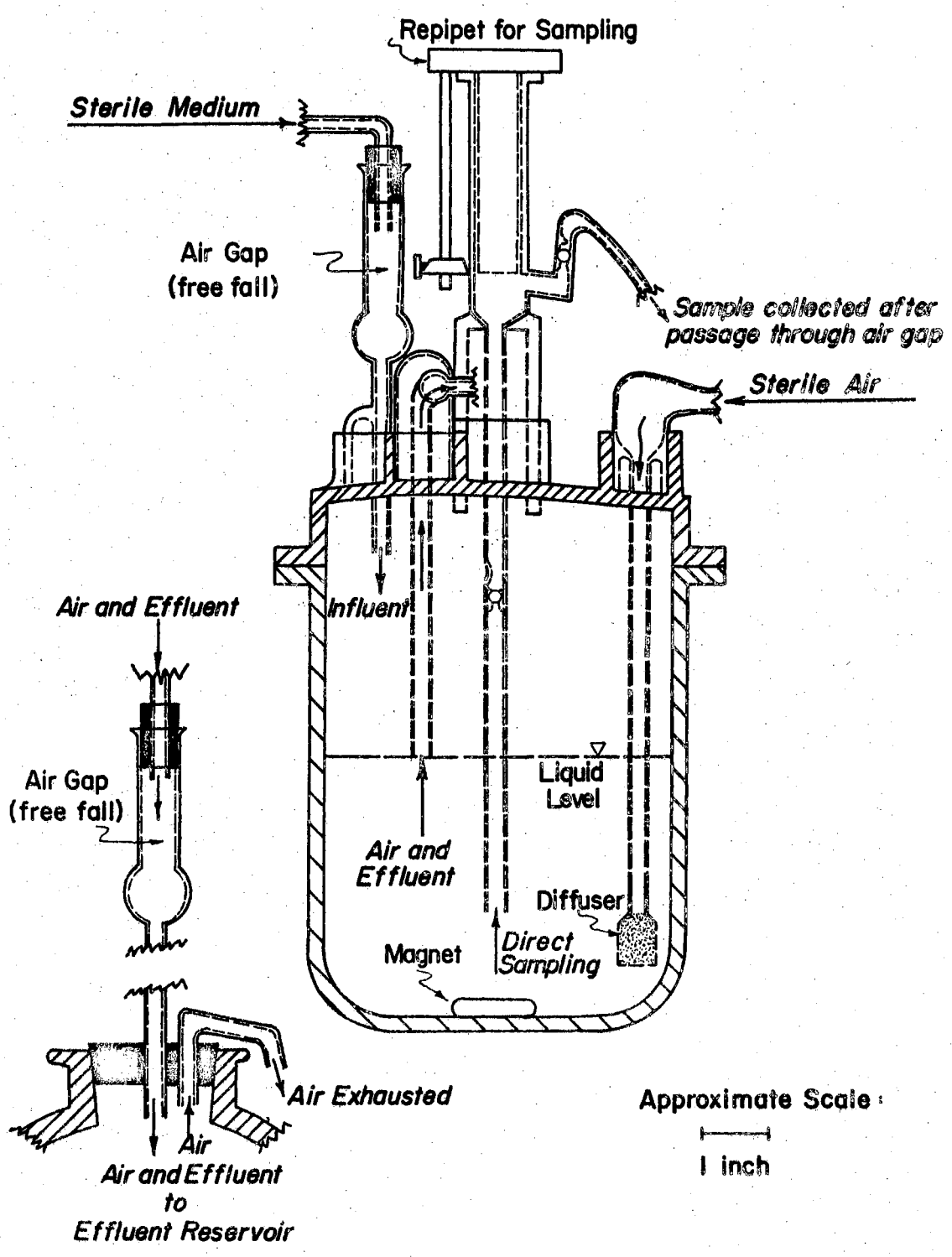


Figure 4. Diagram of the continuous flow reactor.

A 1.0 liter volume of medium was used in the reactor.

5. Pumps. A peristaltic pump (Sigmamotor, Inc., AL-4-E) was used to feed the influent medium at a controlled rate into the reactor during continuous flow studies.

6. Recirculating water bath (Precision Scientific, Lo/Temptrol). This was used to control the temperature in the reactors immersed in a water bath tank.

7. Magnetic stirrers (E. H. Sargent and Co., S 76490). Increased mixing of the reactor contents was achieved by using a magnetic stirrer to drive a Teflon coated stirring bar in the reactor bottom.

8. Vacuum pump (Gast Mfg. Corp.). This was used to assist in the filtration of samples for biological solids determinations and the collection of filtrate for analysis.

9. Centrifuges. A Servall Superspeed Centrifuge (Ivan Sorvall, Inc., Type SS-1A) was used for centrifugation of small samples. A Sharples Super Centrifuge (Sharples Equipment Div., Pennsalt Chemicals Corp., Type T-1) was used for centrifugation of sewage in the preparation of sewage agar.

C. Media

The synthetic medium used in these studies consisted of a minimal salts medium with glucose as the carbon source or a glucose minimal medium containing Bacto-peptone (Difco Laboratories). The composition of the medium is given in Table I. The concentrations of salts and buffer are those commonly used in the Bioenvironmental Engineering Laboratories of the Oklahoma State University.

TABLE I

CONSTITUENTS OF THE GROWTH MEDIUM

$(NH_4)_2SO_4$	500 mg/l
$MgSO_4 \cdot 7\ H_2O$	100 mg/l
$MnSO_4 \cdot H_2O$	10 mg/l
$FeCl_3 \cdot 6\ H_2O$	0.5 mg/l
$CaCl_2$	7.5 mg/l
KH_2PO_4	526 mg/l
K_2HPO_4	1070 mg/l
Tap water	100 ml/l
Distilled water	to volume
Glucose	variable
Bacto-peptone	variable

D. Experimental Protocol

This research undertaking on microbial species predominance was developed in various interrelated and interdependent phases. The experimental protocol and results are presented in the following major groups.

Phase 1. Development of sewage agar and its comparative use with nutrient agar.

Phase 2. Sewage sampling, selection of test bacteria, and growth medium selection.

Phase 3. Pure culture batch studies, mixed culture batch studies, and continuous flow mixed culture studies.

1. Phase 1

a. Development of sewage agar

Prior to the extensive sampling of sewage for viable bacteria count and determination of the predominant bacterial populations on nutrient agar (Difco Laboratories), a study was made to evaluate this medium as compared with a medium composed of or containing sewage as the source of nutrients and growth factors. Several inherent difficulties involved with the use of sewage as a substrate were initially anticipated. The nonuniform composition and concentration of domestic sewage constituents and its relatively low soluble substrate concentration were expected to present some problems in the preparation of a solid counting medium. However, the preparation of a sewage base counting medium and comparison of viable counts using it and nutrient agar were felt to be desirable in order to determine if the use of nutrient agar as a medium for the enumeration and differentiation of sewage bacteria would be permissible.

Primary clarifier effluent from the Stillwater, Oklahoma, wastewater treatment plant was used as the source of the sewage. This sewage was further clarified by centrifugation and in some instances filtration through a 0.45 μ pore diameter membrane filter. The centrifugate, or filtrate, was stored as briefly as possible at 3 to 5° C until it could be concentrated, then sterilized by autoclaving.

Concentration of the sewage was accomplished with a flash evaporator at approximately 60° C under vacuum by aspiration. Batches of 500 to 600 ml were concentrated to approximately 50 ml, collected, and refrigerated until final processing to the desired concentration.

Sewage which had been filtered prior to being concentrated was found to form flocculent particles during concentration, thereby nullifying attempts to produce a clear concentrate without filtration of the concentrate. In preparation of the sewage agar only centrifugation was used to remove initial particulate matter since autoclaving the filtered concentrate also produced flocculent particles. Incorporation of the floc particles was not found to cause appreciable difficulty in observing the bacterial colonies growing on the agar surface.

The BOD, COD, and pH of the sewage, concentrated sewage, and condensate were determined, to assess effects the concentrating process might have on these parameters.

Numerous variations of sewage media were examined to determine the requirements for the degree of concentration, pH adjustment, addition of supplemental salts, and the effect of filtration. The pH was adjusted to 7.2 with a phosphate buffer and the salts which were added were the first five listed in Table I. The types of media which were compared are given in Table II.

TABLE II

MEDIA TYPES FOR COMPARATIVE GROWTH STUDY

Type	Preparation*
1	C B F S
2	C B F
3	C B S
4	C B
5	C F S
6	C F
7	U B F S
8	U
9	U B S
10	U F
Nutrient agar	
Glucose agar	1000 mg/l glucose + B+S

*Code to notation:

C - concentrated sewage

U - unconcentrated sewage

B - phosphate buffer added to pH 7.2

F - filtered through 0.45μ pore diameter filter

S - addition of salts (first 5 from Table I)

b. Comparison of nutrient agar and sewage agar

Fifteen ml volumes of medium per Petri dish were used. A culture of Serratia marcescens was obtained from Dr. R. B. Bustamante for use as an initial test organism to evaluate the various media preparations. The spot plate technique was employed and the plates were incubated at 37° C for 48 hours or longer (up to 4 days) then counted.

After determining the need for sewage concentration, pH adjustment, and addition of supplemental salts, simultaneous inoculations of heterogeneous populations of sewage bacteria were made on sewage agar and nutrient agar. Inoculation was made using the spot plate technique with incubation at 37° C. After 48 hours of incubation comparative enumeration and colony type differentiation were made. Reexamination was made after 72 to 96 hours.

2. Phase 2

a. Sewage sampling

Weekly sampling of sewage was made at the Stillwater, Oklahoma, wastewater treatment plant from September, 1967, through February, 1969. The samples were collected in a sterilized bottle at the head of the preaeration basin. The sewage temperature was measured at the time of sampling. After collection the sample was promptly returned to the laboratory for dilution, plating, and pH determination. A dilution of 10^{-5} was found to be suitable for use as inoculum. Five plates each were prepared by the spot plate and spread plate techniques. Incubation at both 37° C and room temperature (approximately 25° C) were used until it was determined that incubation at room temperature consistently yielded greater numbers of colonies from duplicate inoculations.

Enumeration and differentiation of the colonies were performed after 72 to 96 hours of incubation. The enumeration might have been performed after 48 hours; however, a longer incubation period was required to differentiate certain colonies having latent morphological characteristics such as pigment development. When the density of the colonies developing on the plates was seen to be so great as to cause potential counting difficulty because of coalescence, the enumeration and differentiation were performed after approximately 48 hours of incubation and checked again 24 to 48 hours later.

Tabulation of the sampling times, sewage temperature and pH, bacterial population density, and approximate number of colony types as distinguished by their gross morphology appears in the following chapter.

Specimens of the predominating colony types were taken from each sewage sample and descriptively noted. The specimen was streaked on the surface of sterile nutrient agar and an isolated colony possessing the characteristics of the original colony was inoculated onto a sterile nutrient agar slant in a culture tube, capped, and labeled. Growth on the slant was allowed to develop before the slant was stored at 4^{o} C. Periodic transfers were made to fresh nutrient agar slants with checks made on the purity of the culture by streaking a sample of the inoculum on a sterile nutrient agar plate and observing the resulting nature and purity of colonies developed.

Each specimen isolated was given a code number for future reference and a record was kept of the number of such colony types per sewage sample.

b. Selection of test bacteria and growth media

In order to determine which of the 18 isolated "predominant" bacteria to use in conducting further studies, a visual evaluation was made of their relative growth in various liquid media. The media used were: nutrient broth, glucose minimal medium, glucose minimal medium with vitamin supplement, 0.1% Bacto-yeast extract (Difco), glucose minimal medium with 0.1% Bacto-yeast extract, and glucose minimal medium with 0.1% Bacto-peptone (Difco). Evaluation was made with respect to apparent quantitative growth, dispersed or flocculent growth, and freedom from adherence on the wall of the culturing flask. Flasks of 250 ml capacity containing 60 ml of medium were inoculated and incubated at 25^{0} C on a shaker at 90 oscillations per minute. Observations were made over at least five days after inoculation. Evaluation for selection was also based on the relative ease with which the developing colonies of each culture could be differentiated when grown in mixed cultures.

Based on the above criteria for evaluation, four cultures were selected which had apparent differences in growth rate in selected media. Each grew in a dispersed suspension in liquid media with no adherence to the flask wall, and each culture's colonies were sufficiently different in appearance to permit ready identification even at early stages of development. The isolated bacteria were not generically identified. The four selected cultures are reported herein by the identifying letters A, B, C, and D. They were readily distinguished from one another by colony color and morphology as indicated below:

Culture A - Colonies yellow, small, transparent, rounded smooth glossy surface; Gram negative cocci or very short rods, single cells or pairs, non-motile.

Culture B - Colonies white, moderate size, translucent, smooth glossy surface; cocci or very short rods, singular or in pairs, Gram positive changing to Gram negative in old cultures. Non-motile.

Culture C - Colonies colorless to iridescent, producing a reddish-brown soluble pigment upon aging in high substrate concentrations, translucent, slightly irregular surface and margin, large colony; rods, singles or short chains, Gram negative, motile (apparent polar flagella - fast movement - viewed in hanging drop), appear to contain Gram positive grana.

Culture D - Colonies colorless to buff or tan with color developing with age, more uniformly translucent than C and larger than C, surface and margin smooth in young colony becoming slightly ridged and irregular in late stages of development; rods, singular, Gram negative, motile (apparent polar flagella - slower movement than C - viewed in hanging drop).

Based on the growth characteristics of the selected bacteria in the test media, a choice was made to use glucose minimal medium (GMM) and GMM with Bacto-peptone (GMM + P) for studies on the growth and interaction of the selected bacteria.

3. Phase 3

a. Pure culture batch studies

The kinetic constants μ, μ_{max}, and K_s were determined experimentally for each of the four selected bacteria on both GMM (glucose concentration variable) and GMM + P (peptone concentration variable) with two types of batch operations, i.e., shaken flask and bubble aeration. The method used for the determination of these values was that proposed by Monod (12) with a comparative check by the method of Lineweaver and Burk (59). The notation is that of Herbert et al. (19) where:

μ = the exponential growth rate

μ_{max} = the maximum rate of growth

K_S = the "saturation constant" numerically equal to the substrate concentration, S, at which the growth rate, μ, is one half the maximum value, μ_{max}, (i.e., $\mu = \mu_{max}/2$)

A representation of these values is shown in Figure 5.

The value of μ for each culture and substrate concentration, S, was determined from a semi-logarithmic plot of optical density, O. D., versus time, T, in hours, with O. D. plotted on the logarithmic axis. The straight line portion of the plot with O. D. increasing with T represents the exponential growth phase used for the calculation of μ as follows:

$$\mu = \frac{\ln 2}{T} = \frac{0.693}{T}$$

where T = the doubling time or generation time as determined by a two-fold increase in O. D.

i) Shaken flask method

Prior to performing an experiment, the culture to be studied was acclimated on the appropriate substrate for at least three days. Daily transfers (2 ml) were made to fresh sterile media. Purity checks were made by spot plate technique at each transfer period. The temperature of acclimation and study was maintained at 25° C in a water bath shaker. The shaker was operated at 90 oscillations per minute. Both the acclimation flasks and the growth study flasks (as previously described) were of 250 ml volume and contained 60 ml total volume of liquid. The acclimation medium was either 2000 mg/l GMM or 2000 mg/l GMM with 250 mg/l Bacto-peptone. Growth flasks were seeded with 2 ml (4 ml for culture A because of low solids) of acclimated culture which had been transferred to fresh medium 18 to 24 hours prior to being seeded. Duplicate flasks were prepared for each substrate concentration. The

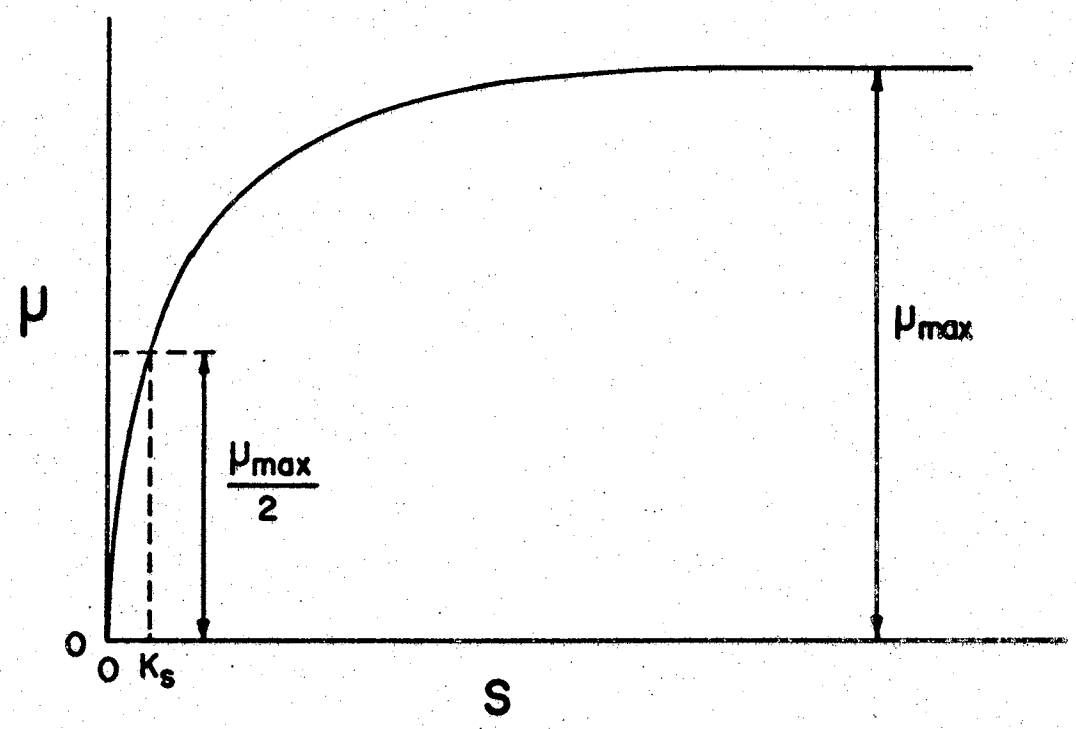
Figure 5. The relationship between the exponential growth rate, μ, and the growth limiting substrate concentration, S.

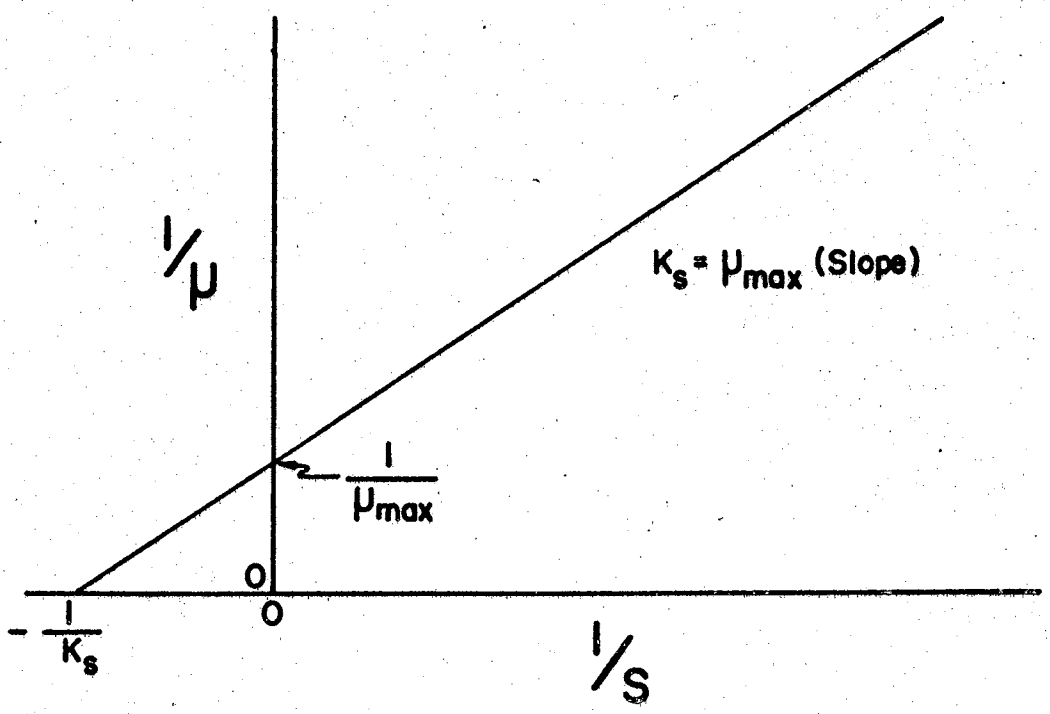
μ_{max} = the maximum growth rate

K_s = the saturation constant (numerically equal to S where $\mu = \mu_{max}/2$)

Top) Monod plot

Bottom) Lineweaver-Burk plot


μ
μmax
μmax/2
0
0 Ks
S


1/μ
Ks = μmax (Slope)
1/μmax
0
0
-1/Ks
1/S

initial optical density (O. D.) of the fluid in each reaction flask was determined at a wavelength of 540 mμ with the spectrophotometer. A sterile medium sample was used as a blank (O. D. = 0). Optical density values were measured at least hourly (occasionally at 30 minute intervals) until little or no change in O. D. occurred between readings. The purity of the cultures was checked by plating a sample of the suspension on nutrient agar following the run. Upon confirming the purity of the culture an inoculum from the plate was made into sterile liquid growth medium to acclimate for use in the next study or onto a sterile nutrient agar slant in a culture tube, labeled, grown, and then stored at 4^{o} C for future reference and use. Values of μ, μ_{max}, and K_s were determined as previously described.

ii) Bubble aeration method

The same procedure for culture acclimation as described for the shaken flask method was used to prepare the inoculum for this type of batch growth study. Calculation of the kinetic constants was made in the same manner as previously described. The major differences between this type of batch study and the prior one are the volume of culture, the method of aeration and mixing, the sampling methods, and the sample analysis.

The reactors used were the 2 liter cylindrical glass kettle type described earlier. Initial volume of the medium and inoculum was 1.0 liter. The aeration rate was 3 liters per minute (checked by a rotameter). The air was filtered through two tubes packed with glass wool (one of which had been autoclaved with the medium reservoir and reactor assembly) before being discharged through a diffuser into the medium in the reactor. More complete mixing than that resulting from

aeration alone was achieved by using a magnetic stirrer to drive a Teflon coated metal bar at the bottom of the reactor.

Sampling was performed at 30 to 60 minute intervals until nearly all growth had occurred with a final sample taken at 24 hours from the time of inoculation. The samples were removed from the reactor by closing the air outlet and opening a sampling tube which extended vertically into the reactor, thereby causing the air pressure increase in the reactor to force a portion of the reactor contents up and out through the sampling tube. When the required volume of sample had been withdrawn, the sampling tube was closed and the air outlet re-opened.

Samples were analyzed for viable count, optical density (and biological solids directly in some instances), and pH. The sample filtrate (0.45 μ membrane filter) was analyzed for total COD, glucose (by Glucostat method 1-A, calculated as COD), and carbohydrate (anthrone method).

The inoculum volume was 10 ml per reactor. The purity of the culture was checked through use of the plates prepared for viable count determinations. A specimen colony of the culture was inoculated into sterile liquid growth medium to acclimate for use in the next study or onto a nutrient agar slant in a culture tube, labeled, grown, and then stored at 4^{o} C for future reference and use.

A water bath was not used to maintain a temperature of 25^{o} C, but temperature checks were made and the operating temperature was found to be $27^{o} \pm 1^{o}$ C.

b. Mixed culture batch studies (shaken flask method)

The four test organisms and the eleven possible combinations of species were grown in shaken flasks as described earlier on the media selected for use in the continuous flow studies, i.e., 500 mg/l GMM and 500 mg/l GMM with 250 mg/l Bacto-peptone. The value of μ for all cultures was calculated for each medium. Viable count determinations by spot plate technique on nutrient agar were made of the initial inoculum and the flask contents approximately midway through and near the end of the logarithmic growth phase.

The observations were used to evaluate the mixed cultures of greatest potential interest for further study under continuous flow conditions. Four mixes were chosen: AB, BC, CD, and ABCD.

c. Mixed culture continuous flow studies

Prior to performing the studies with the bacterial cultures a check was made to assure that the reactor contents would be in a completely mixed state. A 1.0 liter volume of phenol solution (1000 mg/l) was placed in the reactor (2 liter glass kettle as previously described). Aeration (3 lpm) and mixing with a magnetic stirrer (setting 6 of 10) were set as proposed for later use if complete mixing was found to occur under these conditions. The results of the dilute-out check were satisfactory and these conditions were subsequently used. Dilute-out of the phenol concentration was performed by pumping tap water at a set rate from a glass carboy reservoir into the reactor. The effluent was removed from the reactor by the air escaping through a glass tube cut off so as to maintain the reservoir contents at 1.0 liter (essentially an air lift pump removal of effluent). Effluent samples were analyzed for COD and a plot of the actual COD was compared with the

theoretical COD for dilute-out at the established detention time. Two runs were made using detention times of 7.4 and 24 hours. Details of the dilute-out method are given in the appendix.

Sterilization of the reactor assembly, medium reservoir, and air filter was accomplished by autoclaving the entire assembly as a single unit to minimize potential contamination. The reservoir contained 20 liters of medium when sterilized. The phosphate buffer was sterilized in a separate bottle attached to the top of the reservoir and connected by tubing. When the medium had cooled after autoclaving, the buffer was tipped into the reservoir and thoroughly mixed prior to filling the reactor. Air gaps were provided in the influent line, effluent line, and sampling line, which allowed free fall of liquid droplets through vertical glass tubes and prevented bacterial growth in the influent feed line and potential contamination from entering the reactor via the effluent and sampling lines.

The volume of inoculum placed in the reactor varied from 4 to 8 ml depending on the culture and experiment. Except where an initial numerical advantage was purposely desired, an attempt was made to have approximately equal initial populations of each organism. After inoculation the reactor was operated as a batch unit for 9 to 18 hours (usually 10 to 12 hours) before starting the pumps to establish the continuous flow operation. In all experiments an 8 hour detention time ($D = 0.125\ hr^{-1}$) was employed. A water bath was used to maintain the temperature at 25^{o} C.

Samples were removed directly from the reactor with a 10 ml Repipette (Labindustries) set in a port on the reactor cover as a part of the reactor assembly. The sample effluent was analyzed for optical

density, viable count, pH, and biological solids. The sample filtrate (0.45 μ membrane filter) was analyzed for total COD, glucose (Glucostat), and carbohydrate (anthrone).

Observation of the viable count plates served as a check for contamination. No particular difficulty was experienced from external bacterial contamination; however, on one occasion experimental results have been reported in which an apparent "back-inoculation" of the reservoir contents from the reactor occurred, i.e., bacterial growth observed in the reservoir was found to be caused by an organism which appeared to be like one of those under cultivation in the reactor.

As an extension of the continuous flow studies the sampling was continued after utilization of the medium in the reservoir with the units operating under batch conditions without additional feeding (imposition of endogenous conditions).

Two reactors were operated simultaneously with the same initial inoculum. One reactor was fed 500 mg/l GMM and the other reactor was fed 500 mg/l GMM plus 250 mg/l Bacto-peptone. In certain experiments the feed lines from the reservoir to the reactor were exchanged so as to switch the influent to the reactor after about three days of continuous flow operation. Assessment of the response of the culture to the change in substrate was performed using the same procedures and analyses employed before the change was made.

CHAPTER IV

RESULTS

A. Phase 1

1. Development of sewage agar

The sewage concentrate, after processing in the flash evaporator, was a turbid yellowish liquid which contained greyish colored settleable flocculent particles. The concentrate filtrate (0.45 μ pore size membrane filter) had a yellow-orange color, the intensity of which deepened with increasing degrees of concentration. The material had a urine-like odor. The residue retained on the filter was grey to yellow-brown in color with a slick grease-like consistency.

Evaluation of the effect of processing upon the COD of the sewage was performed on each sewage sample. Three general ranges of concentration were used for comparison with the original unconcentrated sample.

a. low concentration (approximately 10:1 to 20:1)

b. medium concentration (approximately 30:1 to 40:1)

c. High concentration (approximately 45:1 to 75:1)

Each sample was subdivided into the following treatment fractions:

a. concentrate

b. autoclaved concentrate

c. filtered concentrate

d. autoclaved and filtered concentrate

e. condensate

Table III shows the COD values for each subdivision. Table IV shows the percentage of theoretical COD (% COD_T) for the concentrate in each subdivision. The values for the condensate are not given as they were always less than 1 percent of the COD_T. The theoretical COD (COD_T) for the concentrate was determined as follows:

COD_T = C_f x COD of the unconcentrated sewage

where

C_f = the concentration factor

Table V indicates the average percent COD_T values for the principal subdivisions of the three concentration ranges.

Figure 6 shows the effect of concentration, filtration, and autoclaving on percent COD_T recovered in each fraction of concentrated sewage.

Concentration of the sewage was found to cause an increase in pH. For example, analysis of two separate sewage samples indicated the pH increased from 7.5 to 8.9 and from 8.1 to 8.7 when concentrated to 30:1.

Figure 7 shows the effect of concentration on COD and BOD for the same sewage sample.

A check was made on the concentration technique to examine the unaccounted loss in COD for the concentrated sewage. A glucose solution was concentrated in the same manner as the sewage. The following

TABLE III

COD (MG/L) DATA FOR PROCESSED SEWAGE

Sewage Sample Date	Concentration Range																					
	Unconcentrated				Low						Medium						High					
	Raw	Autoclaved	Filtered	Filtered and Autoclaved	Concentration Factor	Concentrate	Autoclaved	Filtered	Filtered and Autoclaved	Condensate	Concentration Factor	Concentrate	Autoclaved	Filtered	Filtered and Autoclaved	Condensate	Concentration Factor	Concentrate	Autoclaved	Filtered	Filtered and Autoclaved	Condensate
3/21/67	207	279	80	108							30		3784		2635	41						
5/14/67	136	130	54	60							30	2864	2900	758	537	5						
8/6/67	78	102	67	80	12	1310	1263	471	463	8	30	3561	3169	1090	980	12	77.4	6687	8863	2628	2573	8
					20.3	1510	1526	716	698	10	37	3655	3624	1412	1412	4						
Rerun of 8/6	109	113	64	70							30	3146	3223	1049	1049							
					20.3	1825	1748	622	583		37	3690	2058	1204	1165		77.4	6952	6214	2175	2019	
8/14	113	107	82	72	14.8	1464	1446	605	597	12	30.8	3030	3107	1278	1250	10	43.6	3930	3915	1962	1875	5
Rerun 8/14	113		91		14.8	1480	1382		552		30.8	3060	2719		1145		43.6	4260	3806			
8/18	91	92	66	67	10	780	780	388	384	1	30	2313	2175	1153	1126	4	50	3968	3813	1843	1804	6
8/31	96	96	63	80	17.2	1380	1349	713	706	2	41.7	3116	3254	1686	1715	6	60	4058	4175	2264	2249	10

TABLE IV

PERCENTAGE OF THEORETICAL COD FOUND IN THE FRACTIONS OF CONCENTRATED SEWAGE AFTER INDICATED TREATMENT

Concentration Range	Concentration Factor, C_f	COD_t Theoretical COD of the Concentrate (mgCOD/l)	% of Theoretical COD, % COD_t (100% COD_t = C_f x COD of Unconcentrated Sewage			
			Concentrated	Concentrated Autoclaved	Concentrated Filtered	Concentrated, Filtered & Autoclaved
Low	10	910	85.8	85.8	42.8	42.2
	12	1310	100	96.6	36.0	35.3
	14.8	1675	87.5	86.3	36.1	35.7
	14.8	1675	88.3	82.5	--	33.0
	17.2	1657	83.3	81.4	43.0	42.6
	*20.3	1585	95.2	96.1	45.1	44.0
	†20.3	2215	81.6	78.1	27.8	26.1
Medium	30	6200	--	61.0	--	42.5
	30	4080	70.2	71.1	18.6	13.2
	*30	2340	152.2	135.4	46.5	41.8
	†30	3270	96.2	98.6	32.1	32.1
	30	2730	84.7	79.7	42.3	41.3
	30.8	3475	87.3	89.5	36.8	36.0
	30.8	3475	88.0	78.2	--	33.0
	*37	2890	126.5	125.5	48.8	48.8
	†37	4040	91.3	89.7	29.8	28.8
	41.7	4010	77.7	81.1	42.1	42.8
High	43.6	4935	79.7	79.3	39.8	38.0
	43.6	4935	86.4	77.2	--	--
	50	4550	87.3	83.8	40.6	39.7
	60	5760	70.5	72.5	39.3	39.1
	*77.4	6040	110.8	146.8	43.5	42.6
	†77.4	8440	82.5	73.7	25.8	23.9

*The theoretical COD for these concentrations is based on a raw unconcentrated sewage COD which appears to be in error.

†This is a rerun for those concentrations marked *. It is these values which are used in the following plot of concentration factor vs. % theoretical COD.

Note: The COD of the condensate collected in the concentration process is not indicated in this table. Its value was always less than 1% of the theoretical COD for the concentrate.

TABLE V

AVERAGE PERCENTAGE OF COD_T VALUES

Concentration Range		Concentrate	Autoclaved Concentrate	Filtered Concentrate	Filtered and Autoclaved Concentrate
Low	$\leq$ 20:1	86.3	82.8	37.4	35.9
Medium	$\geq$ 30:1 $\sim$ $\leq$ 40:1	85.1	76.3	33.6	33.7
High	$\geq$ 45:1 $\sim$ $\leq$ 75:1	81.3	77.3	36.4	35.2
Average (entire range)		84.2	78.8	35.8	34.9

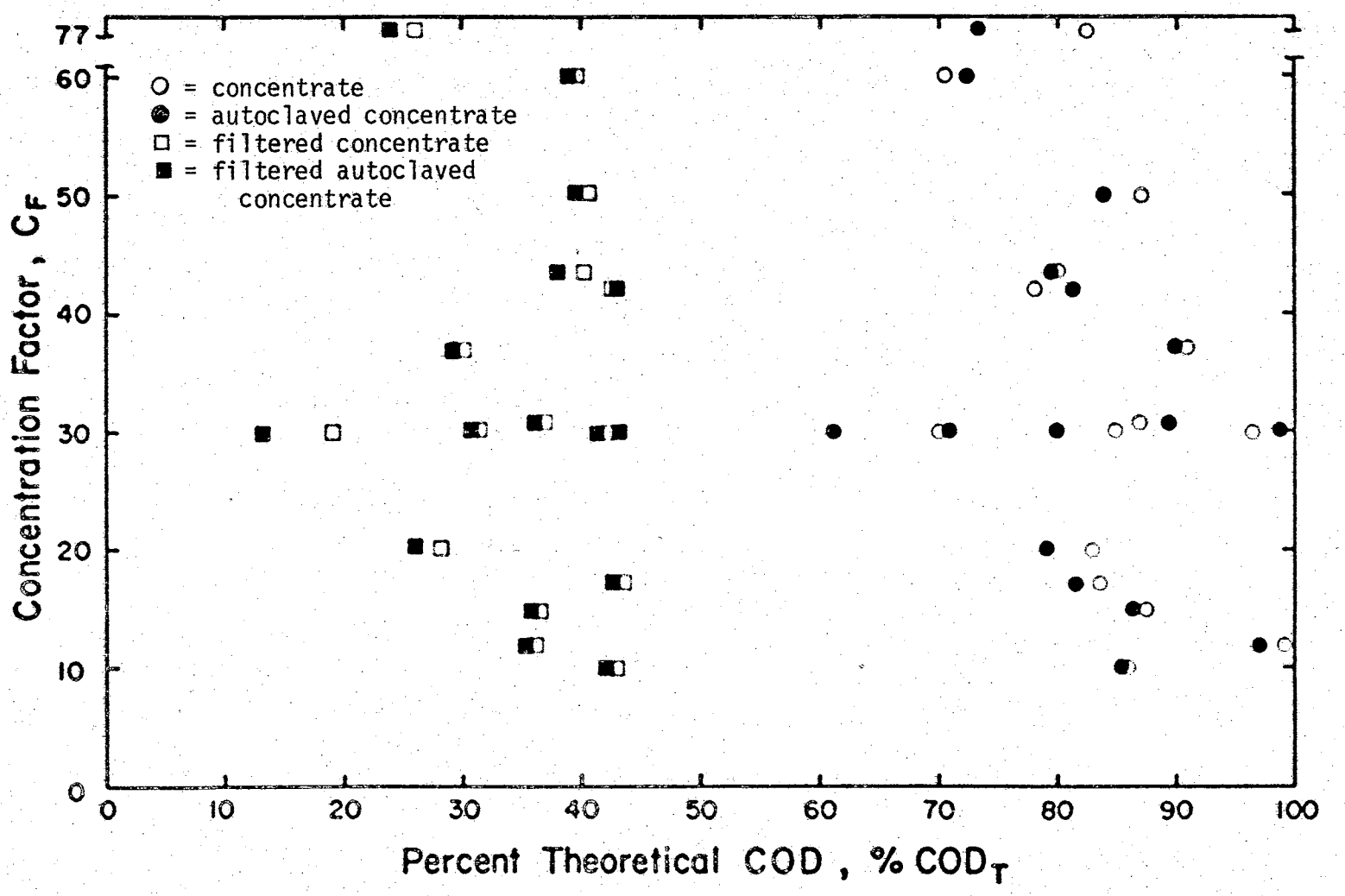
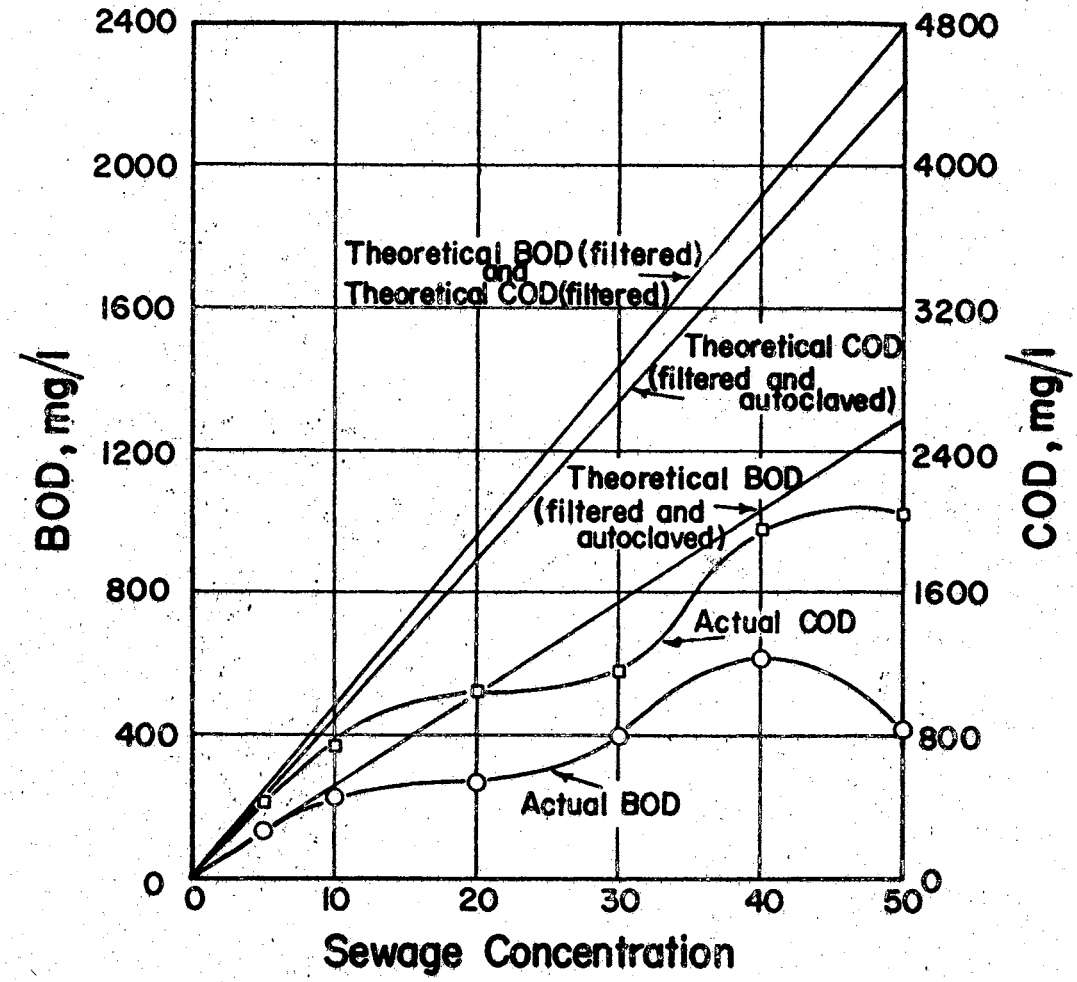


Figure 6. Concentration factor vs. percent theoretical COD.



BOD and COD of Processed Sewage

Sample	BOD mg/l	COD mg/l
Primary effluent	116	208
Primary effluent filtrate	48	96
Primary effluent filtrate autoclaved	26	90
5:1 concentrate filtrate autoclaved	131	437
10:1 " " "	231	755
20:1 " " "	268	1044
30:1 " " "	402	1153
40:1 " " "	628	1968
50:1 " " "	420	2028

Figure 7. The effect of concentration, filtration, and autoclaving on the BOD and COD of processed sewage.

results indicate the concentration technique was satisfactory if volatile compounds measured as initial COD are not lost in the concentration process or their loss is small compared to the total COD.

Sample	COD_T, mg/l	COD_{actual}, mg/l	% COD_T
Original glucose solution	--		--
Concentrate (10.75:1)	3480	3398	97.7
Concentrate (20.83:1)	6750	6795	100.1
Condensate (each sample)	--	0	0

The total initial volume was recovered as concentrate and condensate; therefore, no apparent volume was lost by evaporation.

2. Comparison of sewage agar with nutrient agar

The first attempt at using concentrated sewage in an agar base as an enumeration medium was unsuccessful. Sewage concentrations ranged from 5:1 to 50:1. The test bacteria, Serratia marcescens, grew well on nutrient agar, but no growth occurred on the sewage agar. Failure to grow was tentatively attributed to an unfavorable pH (8.9 to 9.3) of the concentrated sewage. No buffer or supplemental salts had been added to the sewage agar.

In the second test the effects of filtration, buffering, and addition of supplemental salts were evaluated. The COD values for the media were:

Medium or Processed Sewage Fraction	COD, mg/l
Standard nutrient broth	8100
Primary clarifier effluent (PCE)	207
PCE - filtered	80
PCE - autoclaved	279
PCE - filtered and autoclaved	108
Condensate	41
Concentrate (30:1) autoclaved	3784
Concentrate (30:1) filtered and autoclaved	2635

The results of the plate counts on the various media are given in Table VI. S. marcescens was used as the test organism. The spot plate technique was employed with incubation at 37° C for 48 hours.

A statistical analysis of the medium types was made to determine if the various preparations of sewage agar yielded colony counts significantly different from those obtained on nutrient agar using the same inoculum of the test bacterium (S. marcescens). Incubation at 37° for 48 to 72 hours was employed. Counts were made at 48 hours and checked at 72 hours because of the small size of the colonies on those plates having a low substrate concentration (unconcentrated sewage agar).

Significance of the difference in mean colony counts on nutrient agar and other media were tested at the 5 percent level of significance using a Studentized t distribution (60). The following equations and notations were used to calculate t. The subscript 1 denotes the standard of comparison, the nutrient agar. The negative sign that results in some instances where the term $\bar{x}_1 - \bar{x}_2$ appears may be disregarded, as for this purpose the term could as well have been reversed to $\bar{x}_2 - \bar{x}_1$ thereby resulting in a positive value.

Equation 1: $\bar{d} = \bar{x}_1 - \bar{x}_2$

TABLE VI

PLATE COUNTS OF *SERRATIA MARCESCENS* FOR THE COMPARISON OF NUTRIENT AGAR AND SEWAGE AGAR

Medium	Number of Colonies			Colony Characteristics
	½ Plate	½ Plate	Total Plate	
Nutrient Agar	95	83	178	large, red-orange
" "	99	83	182	" "
" "	91	82	173	" "
" "	90	86	176	" "
" "	96	84	180	" "
" "	98	88	186	" "
Sewage Agar, CBFS	103	93	196	medium, med. red
" " CBF	83	82	165	med., white-pink
" " CBS	98	74	172	medium, dark red
" " CB	No Growth			
" " CFS	92	91	183	medium, med. red
" " CF	No Growth			
" " UBFS	106	85	191	very small, white
" " U	89	84	173	very small, white
" " UBS	106	101	207	small, pink
" " UF	105	100	205	very small, white

Note: C = concentrated sewage (30:1)

U = unconcentrated sewage

B = buffered to pH 7.2 with phosphate buffer

F = filtered through 0.45μ pore size membrane filter

S = supplemental salts (first 5, Table I, Chapter III)

Equation 2a: $$S^2 = \frac{(n_1 - 1)(S_1^2) + (n_2 - 1)(S_2^2)}{n_1 + n_2 - 2}$$

Equation 2b (where $n_1 = n_2$): $$S^2 = \frac{\Sigma X_1^2 + \Sigma X_2^2}{2n - 2}$$

where: $$\Sigma x^2 = \Sigma(x - \bar{x})^2 = \Sigma x^2 - (\Sigma x)^2/n$$

Equation 3a: $$S_{\bar{d}} = \sqrt{S^2 \left(\frac{1}{n_1} + \frac{1}{n_2}\right)} = \sqrt{S^2 \frac{n_1 + n_2}{n_1 n_2}}$$

Equation 3b (where $n_1 = n_2$): $$S_{\bar{d}} = \sqrt{\frac{2 S^2}{n}}$$

Equation 4: $$t_{calc.} = \frac{\bar{d}}{S_{\bar{d}}}$$

x = the value (count) of an individual unit (½ plate)

$\bar{x}$ = the mean value of all units of one type ($\Sigma x/n$)

n_2 = the number of units (x) counted

S^2 = the mean square or variance

S = the standard deviation

If $t_{calc.}$ was found to be less than $t_{tabulated\ .05}$, then the null hypothesis that the tested means were equal at the 5 percent level of significance was accepted. If the value of $t_{calc.}$ was greater than $t_{tabulated\ .05}$, the null hypothesis of equality of means was rejected and the alternate hypothesis that they were not equal was accepted, i.e., a significant difference in means existed at the 5 percent level.

The results of the test for significant difference in mean colony count on various media are given in Table VII.

The COD and pH values for the sewage preparations used in the statistical study are given below.

Medium Base	COD, mg/l	pH (before buffering)
Unconcentrated sewage	136	8.1
Unconcentrated sewage - filtered	54	8.1
Unconcentrated sewage - autoclaved	130	8.0
Unconcentrated sewage - filtered and autoclaved	60	8.0
Concentrated sewage (30:1)	2864	9.1
Concentrated sewage (30:1) - filtered	758	9.1
Concentrated sewage (30:1) - autoclaved	2900	8.7
Concentrated sewage (30:1) - filtered & autoclaved	537	8.7
Condensate	5	10.1
Nutrient agar (nutrient broth)	8100	6.8
Glucose agar (glucose solution)	1067	7.0

A comparison was made between nutrient agar and sewage agar (30:1 concentrate, buffered, with supplemental salts) using a 10^{-5} dilution of raw sewage for the inoculum. The COD values of the unconcentrated and concentrated sewage were 105 mg/l and 2740 mg/l, respectively. The respective pH values were 7.2 and 8.4. The pH of the concentrate was adjusted to 7.2. Ten plates of each medium were prepared, inoculated by the spot plate technique, and incubated at 25° C for 48 to 72 hours. Enumeration was initially made at 48 hours and checked at 72 hours. Colony differentiation was also made at the time of counting and rechecked at 96 hours. The nutrient agar yielded a mean of 59.6 colonies per plate and the sewage agar yielded a mean of 53.3 colonies per plate. Subjection of the colony count data to a Studentized t test at the 5 percent level of significance indicated that the two media

TABLE VII

SUMMARY OF COLONY ENUMERATION AND SIGNIFICANT DIFFERENCE (5% LEVEL) IN NUTRIENT AGAR, GLUCOSE AGAR, AND VARIOUS SEWAGE AGAR PREPARATIONS (USING SERRATIA MARCESCENS AS A TEST ORGANISM)

Medium	n	$\bar{x}$	S^2	S	$t_{calc.}$	$t_{tab.05}$	$\bar{x}$ Significantly Different From Nutrient Agar
Nutrient Agar	40	224.3	194.80	13.95	--	--	--
Glucose Agar	40	129.5	301.26	17.35	11.86	1.995	Yes
Sewage Agar CBFS	20	274.7	493.99	22.2	4.50	2.39	Yes
" " CBF	40	No Growth	--	--	--	--	Yes
" " CBS	20	259.9	251.61	15.8	3.26	2.00	Yes
" " CB	20	No Growth	--	--	--	--	Yes
" " CFS	40	No Growth	--	--	--	--	Yes
" " CF	20	No Growth	--	--	--	--	Yes
" " UBFS	6	240.3	995.07	31.6	0.80	2.020	No
" " U	40	260.9	234.13	15.3	4.65	1.995	Yes
" " UBS	40	112.0	187.33	13.7	14.4	1.995	Yes
" " UF	40	46.8	61.08	7.8	23.4	1.995	Yes

U = unconcentrated sewage

C = concentrated sewage (30:1)

B = buffered to pH 7.2

F = filtered through a 0.45μ pore size membrane filter

S = supplemental salts (first 5, Table I, Chapter III)

were significantly different ($t_{calc.} = 2.917$, $t_{tab.05} = 2.101$). Generally the same predominant colony types appeared on both media. A few more yellow colored types appeared on the nutrient agar. No particular difficulty was encountered in counting or differentiating the colonies on the sewage agar even though it contained numerous small flocculent particles from the sewage preparation.

B. Phase 2

1. Sewage sampling

The data for time of sampling, sewage temperature, pH, bacterial population density by plate count, and approximate number of different colony types per sample are given in Table VIII. It should be noted that for the period from September 11, 1967, to February 10, 1968, the plates were incubated at 37° C for 48 hours, whereas, after a period from February 17, 1968, to April 6, 1968, when both 37° C and ambient temperature (approximately 25° C) were used for incubation, only ambient temperature was subsequently used, as larger colony counts and often a greater variety of colonies developed at this temperature.

It may be seen from Table VIII that the pH of the sewage varied little. Values of 7.4 ± 0.1 were common except for the first three months when several samples had a pH of slightly less than 7.0.

The bacterial population density of the sewage (colonies/ml) is given for both spread-plate and spot plate technique for each of the 75 samples of sewage. It may be noted that the spot plate technique yielded a greater population density than did the spread plate technique in 58 of the 75 samples examined. The average bacterial population density in the sewage ranged from approximately 1×10^7 / ml to 5×10^7 / ml. Some indication of a slight seasonal trend toward

TABLE VIII

DATA FOR RAW SEWAGE FROM THE STILLWATER, OKLAHOMA, TREATMENT PLANT

Date	Day	Hour	Temp. °C	pH	Bacterial Density, col./ml Sewage, (10^{-7}) By Spot Plate	By Spread Plate	Avg. All Plates	Approx. No. Colony Types	COD, mg/l	Remarks
9/11/67	M	0900	24.5	6.9	2.47	1.37	1.67	15		37°C incubation
9/19	Tu	0900	24.5	6.95	2.01	2.05	2.03	23		"
9/23	Sa	1100	24.5	7.0	1.83	1.76	1.79	19		"
9/30	Sa	1400	24.0	6.9	4.33	3.90	4.12	28		"
10/8	Su	1130	23.5	7.0	3.18	2.21	2.69	25		"
10/14	Sa	1400	23.5	6.95	3.25	2.67	2.96	24		"
10/21	Sa	1245	23.0	6.85	3.50	2.78	3.14	24		"
10/28	Sa	1230	23.0	7.0	2.96	3.14	3.05	28		"
11/4	Sa	1130	22.5	6.85	1.97	2.31	2.12	27		"
11/11	Sa	1030	22.5	6.8	0.96	0.89	0.93	15		"
11/18	Sa	0915	22.0	6.5	1.81	1.82	1.82	19		"
11/28	Tu	0930	21.0	7.3	1.14	1.05	1.09	21		"
12/2	Sa	1330	21.0	7.4	2.72	2.09	2.41	19		"
12/9	Sa	1015	21.0	7.3	1.52	1.58	1.55	20		"
12/16	Sa	0930	19.0	7.6	1.44	1.48	1.45	18		"
12/23	Sa	1100	18.0	7.45	0.86	0.74	0.81	16		"
12/30	Sa	1010	18.0	7.50	1.68	1.26	1.49	19		"
1/6/68	Sa	1100	17.0	7.3	0.88	0.73	0.79	18		"
1/13	Sa	1000	15.5	7.55	0.77	0.65	0.70	22		"
1/21	Su	1230	17.0	7.55	1.46	1.15	1.30	30		"
1/27	Sa	0830	16.5	7.3	1.15	1.08	1.13	30		"
2/3	Sa	1000	17.0	7.4	0.80	0.71	0.76	40		"
2/10	Sa	0900	17.5	7.4	1.89	1.45	1.67	50		"
2/17	Sa	0900	17.5	7.5	3.44 5.15	3.65 4.43	3.54 4.79	65 70		incubation temp. 37° C 25° C
2/24	Sa	1045	17.0	7.4	0.99 2.04	1.08 1.71	1.03 1.86	40 45		" "
3/2	Sa	0830	17.5	7.15	3.07 5.36	2.68 5.15	2.88 5.76	70 80		" "
3/9	Sa	0930	18.0	7.4	1.76 4.24	1.86 3.95	1.80 4.09	50 60	107	" "
3/16	Sa	1000	18.5	7.45	3.83 4.19	2.13 3.79	3.07 4.01	60 70	173	" "
3/23	Sa	1330	17.0	7.45	1.23 2.96	1.05 2.30	1.15 2.63	50 60	344	" "
3/30	Sa	0930	18.5	7.4	0.64 1.94	0.86 1.90	0.75 1.92	25 45	136	" "
4/6	Sa	1100	18.5	7.5	1.25 3.23	1.90 3.23	1.61 3.25	40 45	240	" "
4/13	Sa	0830	20.5	7.45	2.94	2.17	2.55	20	196	25°C Incubation
4/20	Sa	1300	20.5	7.55	1.30	1.29	1.30	30	--	"
4/27	Sa	0900	21.0	7.35	2.06	1.95	2.01	60	--	"
5/4	Sa	0930	22.0	7.4	2.90	2.31	2.61	40	--	"
5/11	Sa	1000	22.0	7.4	2.85	2.72	2.78	50	--	"
5/18	Sa	1000	22.5	7.4	3.65	3.05	3.35	30	133	"
5/24	F	1930	24.0	7.4	5.08	3.85	4.46	35	471	"

TABLE VIII (Continued)

Date	Day	Hour	Temp. °C	pH	Bacterial Density, col./ml Sewage, (10^{-7}) By Spot Plate	By Spread Plate	Avg. All Plates	Approx. No. Colony Types	COD, mg/l	Remarks
6/1/68	Sa	0900	22.5	7.35	2.03	2.24	2.13	50	--	25°C Incubation
6/8	Sa	1200	24.0	7.5	4.02	3.50	3.76	40	254	"
6/15	Sa	1000	24.0	7.25	1.66	1.18	1.42	50	--	"
6/22	Sa	1000	24.5	7.45	2.53	2.38	2.45	65	133	"
6/29	Sa	1000	25.0	7.4	2.84	2.35	2.60	60	407	"
7/6	Sa	1100	25.5	7.35	3.30	2.96	3.14	50	355	"
7/13	Sa	1000	26.0	7.4	2.96	2.30	2.63	45	89	"
7/19	F	1400	26.0	7.4	4.86	3.94	4.40	65	369	"
7/26	F	2000	27.5	7.25	4.72	4.55	4.64	60	399	"
8/12	M	1500	28.5	7.25	3.43	3.13	3.28	60	538	"
8/16	F	1320	27.5	7.35	5.20	4.32	4.76	70	403	"
8/23	F	1600	28.0	7.3	11.00	9.91	10.46	75	227	"
8/30	F	1000	26.5	7.3	2.23	2.07	2.15	45	--	"
9/8	Su	1420	27.0	7.15	4.81	5.05	4.93	50	391	"
9/14	Sa	1300	27.0	7.25	4.83	4.52	4.67	30	575	"
9/20	F	1335	27.0	7.0	4.60	3.75	4.18	55	413	"
9/27	F	1900	27.0	7.05	4.72	4.58	4.65	60	310	"
10/4	F	1900	26.5	7.05	3.03	3.90	3.47	65	316	"
10/11	F	1900	26.0	7.1	4.25	4.15	4.20	70	365	"
10/19	Sa	1330	25.0	7.2	4.05	4.91	4.53	70	468	"
10/27	Su	1400	24.5	7.4	4.12	4.45	4.31	75	415	"
11/4	M	1130	24.0	7.2	5.96	5.42	5.69	50	465	"
11/9	Sa	1030	23.0	7.5	5.54	4.70	5.12	80	70	"
11/16	Sa	1200	22.0	7.4	4.69	4.80	4.74	80	225	"
11/23	Sa	0900	21.0	7.5	4.06	3.13	3.60	60	85	"
11/29	F	1800	19.0	7.4	1.73	1.88	1.80	35	60	"
12/5	Th	1145	20.0	7.5	6.12	5.65	5.89	60	352	"
12/14	Sa	1015	19.0	7.5	5.06	5.18	5.12	60	158	"
1/3/69	F	1015	16.0	7.45	2.26	2.07	2.47	40	148	"
1/11	Sa	1100	16.5	7.45	2.61	2.25	2.43	50	125	"
1/18	Sa	1020	17.0	7.5	2.96	2.32	2.64	40	115	"
1/21	Tu	0820	16.5	7.45	2.23	2.52	2.36	45	162	"
1/25	Sa	1000	16.0	7.5	2.94	2.47	2.70	35	160	"
1/31	F	1600	17.0	7.2	4.20	3.32	3.76	40	390	"
2/7	F	1645	18.0	7.05	4.80	4.45	4.63	35	426	"
2/15	Sa	1115	17.0	7.5	2.39	2.15	2.27	30	145	"
2/22	Sa	1030	16.0	7.5	1.19	0.90	1.04	25	195	"

greater population density occurred in the summer and fall months when a warmer sewage temperature was recorded. Figure 8 shows the bacterial population density and temperature for each sample of sewage.

The COD of the sewage was quite variable, ranging from less than 100 mg/l to more than 500 mg/l. Generally samples taken in the afternoon had greater COD values than those taken in the morning.

The number of colony types having readily distinguishable morphological differences generally ranged from fewer than 30 for plates incubated at 37° C to 30 to 70 for plates incubated at room temperature (approximately 25° C). No definite pattern of colony type variation was established from the samples taken. Although there are exceptions, some trend toward greater colony variation may be seen during periods of warmer temperatures and for samples taken in the afternoon.

During the first five months of sampling, isolates were made from most of the colonies which exhibited different morphological characteristics. This large selection of isolates was continued until frequent recurrence of certain colony types was noted. Over the sampling period a total of 18 readily identifiable recurrent predominant colony types were noted. The isolates of these 18 cultures were tested as indicated in the following section for the selection of 4 suitable bacteria to use in further studies. The descriptive colony morphology for the 18 principal isolates is given in Table IX.

2. Selection of test bacteria and growth media

The results of the observations on the growth of the bacteria isolated from sewage in various liquid media are given in Table X. Where no growth or very slight growth was observed for a particular isolate in a specific medium, the test was repeated up to four times

Figure 8. Sewage temperature and bacterial population density (September 1967) - February 1969). Influent at the head of the preaeration basin (Stillwater, Oklahoma, treatment plant).

Key to symbols:

Sewage temperature	●
Population density (37^{o} C incubation)	■
Population density (25^{o} C incubation)	▲

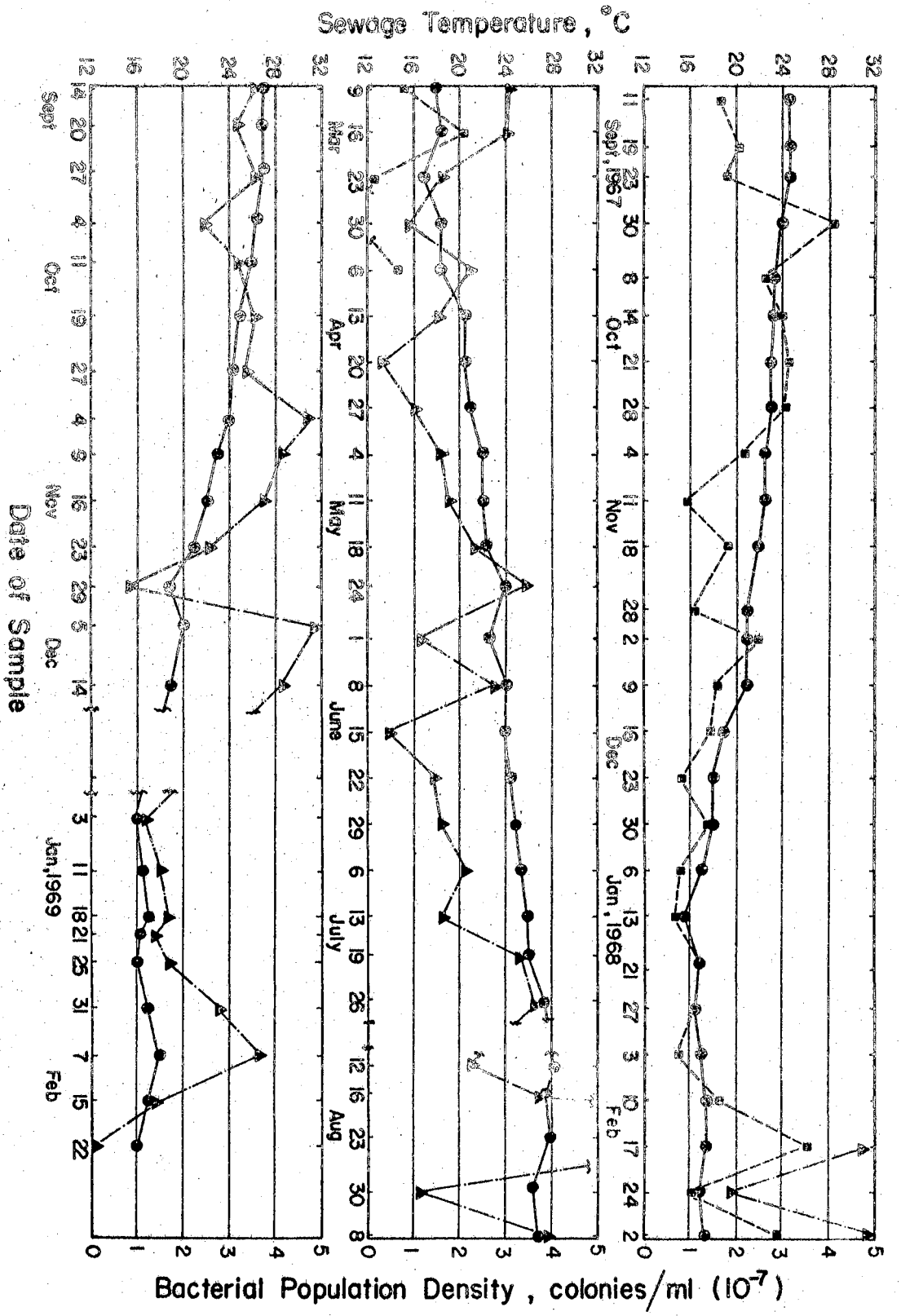

Sewage Temperature, °C
Date of Sample
Bacterial Population Density, colonies/ml (10⁻⁷)
Sept
Oct
Nov
Dec
Jan, 1969
Feb
Mar
Apr
May
June
July
Aug
Sept, 1967
Oct
Nov
Dec
Jan, 1968
Feb

TABLE IX

DESCRIPTION OF COLONY MORPHOLOGY FOR THE PRINCIPAL SEWAGE BACTERIA ISOLATES. OBSERVATION ON NUTRIENT AGAR.

Culture	Relative Size	Form	Elevation	Surface	Edge	Optical Characteristics	Remarks
1	small	circular	conical	radially ridged	irregular	transparent edge translucent center	colorless to white at center
2	small	circular	conical	smooth	entire	transparent	colorless
3	small	circular	convex	smooth	entire	translucent	grey-white color
4	medium	circular	convex	smooth	entire	transparent	bright dark yellow
5	medium	circular	convex	smooth	entire	translucent to opaque	yellow-white
6	medium	circular	umbonate	smooth	entire	transparent edge translucent center	colorless edge bright yellow center
7	small	circular	umbonate	smooth	irregular	transparent edge opaque center	colorless edge pink center
8	medium-large	circular	convex	smooth	entire	opaque	white
9	very small	circular	convex	smooth	entire	transparent	colorless
10	medium	circular	umbonate	smooth	irregular	transparent edge opaque center	colorless edge white center
11	large	circular to irregular	irregular convex	irregular smooth	entire to irregular	translucent edge opaque center	colorless flat edge pink convex irregular center
12	large	circular	raised	rough to smooth concentric rings	entire	translucent	grey-white to colorless
13	large	circular to irregular	irregular convex	irregular smooth	entire to irregular	transparent edge opaque center	colorless edge, white center similar to 11 except color
14	medium-large	circular	convex	smooth	entire	translucent, iridescent	colorless to light pink turning red-brown, soluble pigment
15	medium-large	circular	flat	smooth	entire	transparent	bright yellow, semi-glossy
16	small	circular	convex	smooth	entire	opaque	pink
17	medium	circular	conical	smooth	entire	translucent	whitish
18	large	circular	raised-conical	smooth	entire	transparent	colorless to light brown

TABLE X

GROWTH OF SEWAGE BACTERIA ISOLATES IN VARIOUS MEDIA

Culture Number	Medium				
	Glucose Minimal Medium (GMM)	Nutrient Broth	GMM + Vitamin Supplement	GMM +0.1% Yeast Extract	GMM +0.1% Bacto-Peptone
1	-	±	-	±	-
2	-	±	-	+	+
3	-	±	-	+	+
4	±	+	-	+	+
5	+	+	+	+	+
6	-	±	±	+	+
7	±	±	±	±*	+*
8	+	+	+	+	+
9	-	-	-	-	-
10	-	+	±	+*	+*
11	±*	+		+	+
12	±	+		+*	+*
13	+	+		+	+
14	+	+		+	+
15	±*	+		+	+*
16	±	±		+	+
17	±	+		+	+
18	+	+		+	+

\+ good growth
± slight growth
\- no growth (apparent)
* floc formation or wall adherence

with fresh medium and new inoculum. Major considerations in assessing the degree of acceptability of each culture for use in further experiments are given in Table XI. The following factors generally governed selection: the ease of distinguishing different colony types in mixed populations, apparent quantitative growth in the tested media, dispersed or flocculent growth, and freedom from adherence on the wall of the culturing flask.

Cultures 4, 8, 14, and 18 (redesignated A, B, C, and D, respectively) were chosen for further study. Glucose minimal medium (GMM) and GMM with Bacto-peptone (GMM + P) were selected as media.

C. Phase 3

1. Pure Culture Batch Systems

a. Shaken flask growth studies

The four selected cultures were grown in the special flasks described previously at 25° C in a water bath shaker operated at 90 oscillations per minute. The two media used for the studies were glucose minimal medium (GMM) with glucose concentrations of 100, 300, 500, 700, and 1000 mg/l and glucose minimal medium with Bacto-peptone (GMM + P) which contained a glucose concentration of 500 mg/l and variable peptone concentrations of 25, 50, 100, 250, and 500 mg/l. These concentration ranges were sufficient to establish values for μ_{max} and K_s as described in Chapter III.

Figures 9, 10, 11, and 12 illustrate the Monod plots and Lineweaver-Burk plots for the determination of μ_{max} and K_s in each medium for cultures A, B, C, and D, respectively. The values of μ_{max} and K_s were found to be in close agreement for each culture when calculated from the two types of plots. The tabulated values of μ_{max}

TABLE XI

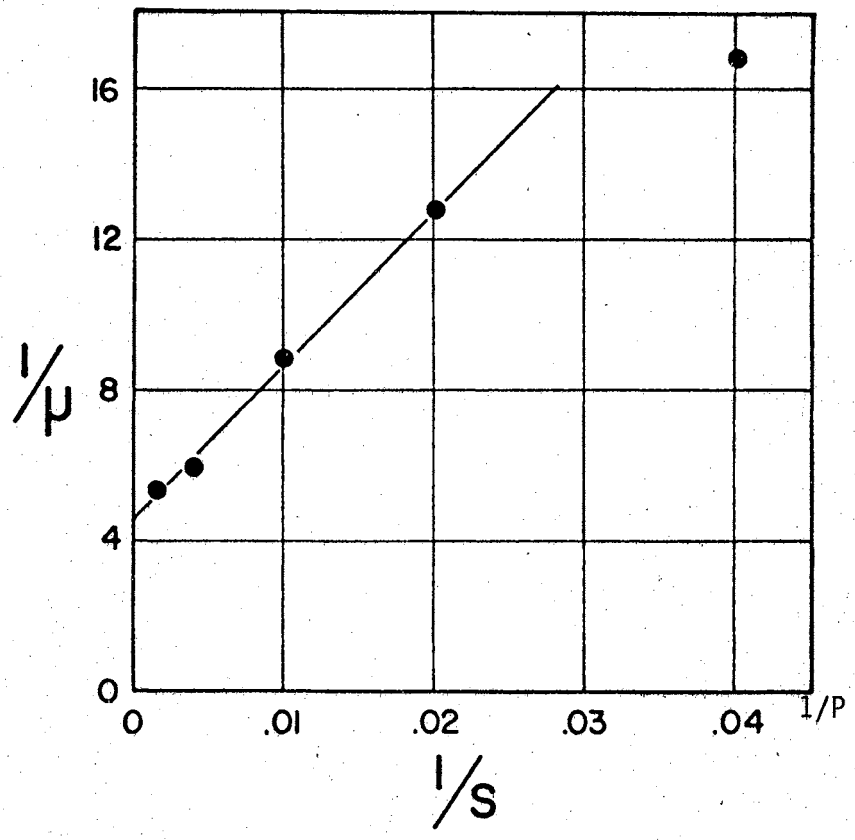
ACCEPTABILITY OF SEWAGE BACTERIA ISOLATES FOR FUTURE STUDY

Culture Number	Remarks
1	Not acceptable - very poor or no growth
2	Limited acceptance - no growth on GMM
3	Limited acceptance - no growth on GMM
4	Limited acceptance - very poor growth on GMM
5	Not acceptable - coloration variable (white-yellow)
6	Limited acceptance - no growth on GMM
7	Not acceptable - flocculent growth
8	Acceptable
9	Not acceptable - no apparent growth
10	Not acceptable - floc formation and wall adherence
11	Not acceptable - floc formation
12	Not acceptable - floc formation
13	Not acceptable - inconsistent colony morphology
14	Acceptable
15	Not acceptable - floc formation
16	Limited acceptance - very poor growth on GMM
17	Limited acceptance - poor growth on GMM, similar to 2
18	Acceptable

Figure 9. Determination of μ_m and K_s for culture A, top) Lineweaver-Burk plot, bottom) Monod plot. (Note: no growth occurred on glucose minimal medium, GMM,) Shaken flask (90 osc/min), 25° C.

	L-B plot		Monod plot	
	GMM	GMM+P	GMM	GMM+P
μ_m	0	0.22	0	0.20
K_s	0	89	0	70

P = peptone, mg/l (with 500 mg/l glucose)


16
12
8
4
0
1/μ
0
.01
.02
.03
.04
1/P
1/S

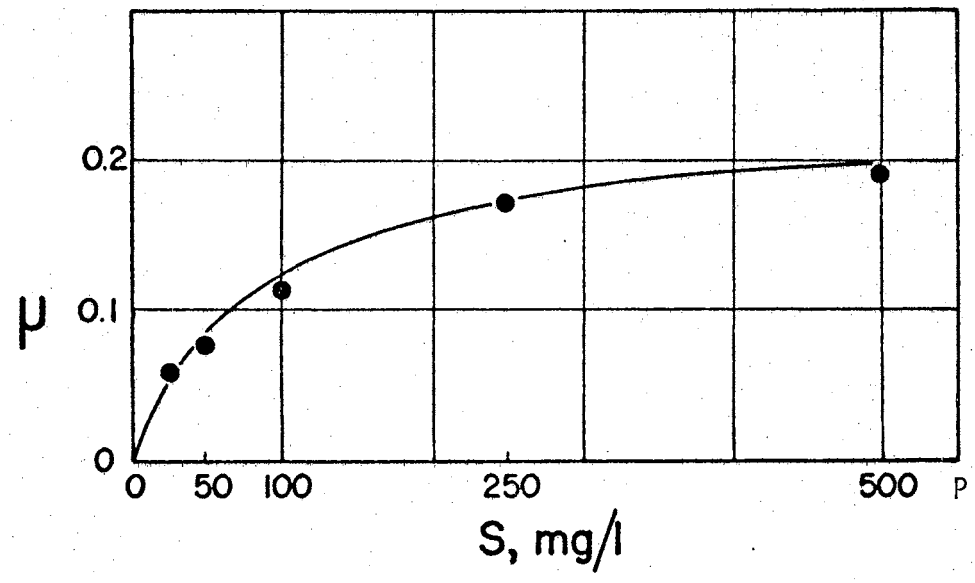

0.2
0.1
0
μ
0
50
100
250
500
P
S, mg/l

Figure 10. Determination of μ_m and K_s for Culture B, top) Lineweaver-Burk plot, bottom) Monod plot. Shaken flask (90 osc/min), 25° C.

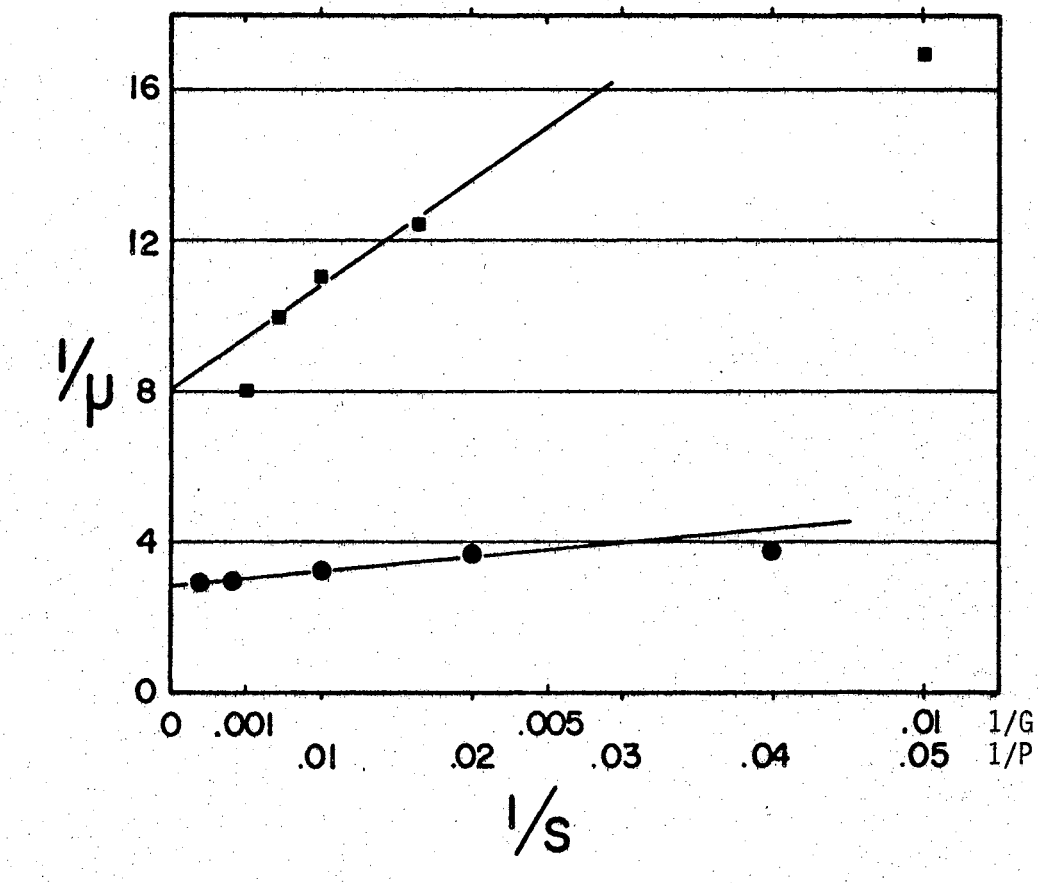
	L-B plot		Monod plot	
	GMM	GMM+P	GMM	GMM+P
μ_m	0.13	0.35	0.12	0.35
K_s	175	15	100	15

Key to symbols:

	L-B plot	Monod plot
GMM unit	■	■
GMM+P unit	●	●

G = glucose, mg/l

P = peptone, mg/l (with 500 mg/l glucose)


16
12
1/μ
8
4
0
0 .001 .005 .01 1/G
.01 .02 .03 .04 .05 1/P
1/S

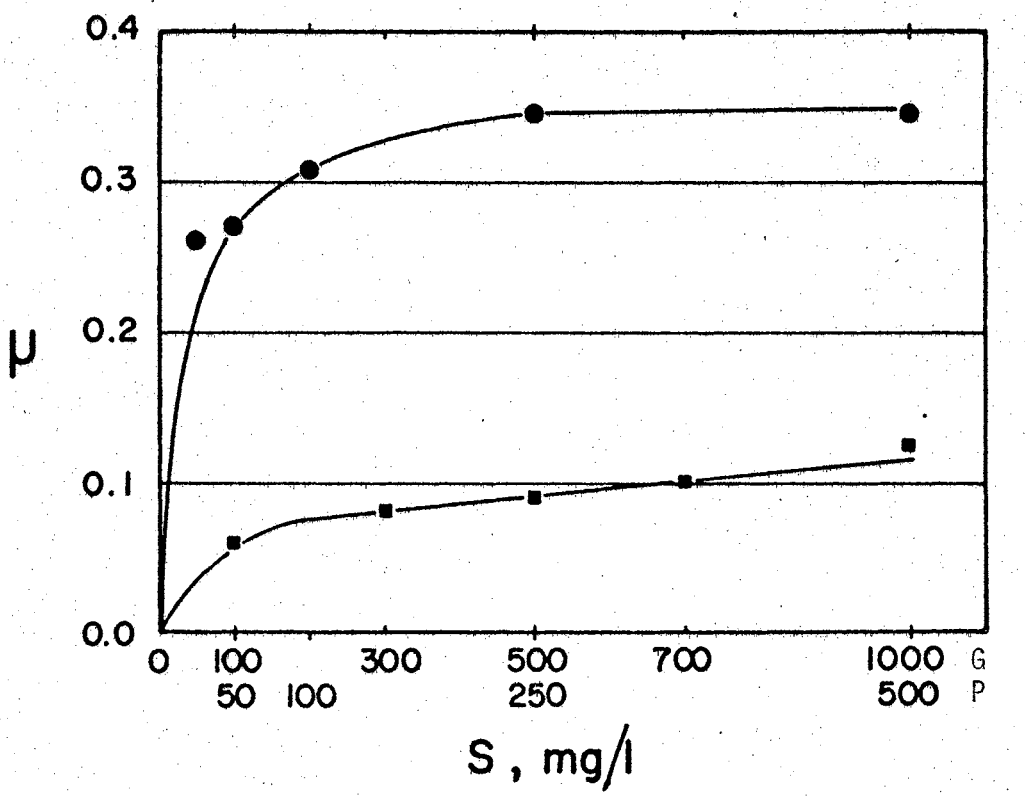

0.4
0.3
μ
0.2
0.1
0.0
0 100 300 500 700 1000 G
50 100 250 500 P
S , mg/l

Figure 11. Determination of μ_m and K_S for culture C, top) Lineweaver-Burk plot, bottom) Monod plot. Shaken flask (90 osc/min), 25° C, Bubble aeration (3 lpm/l), 27° C.

	L-B plot			Monod plot		
	GMM*	GMM	GMM+P	GMM*	GMM	GMM+P
μ_m	0.45	0.35	0.40	0.45	0.35	0.39
K_S	18	12	24	20	20	10

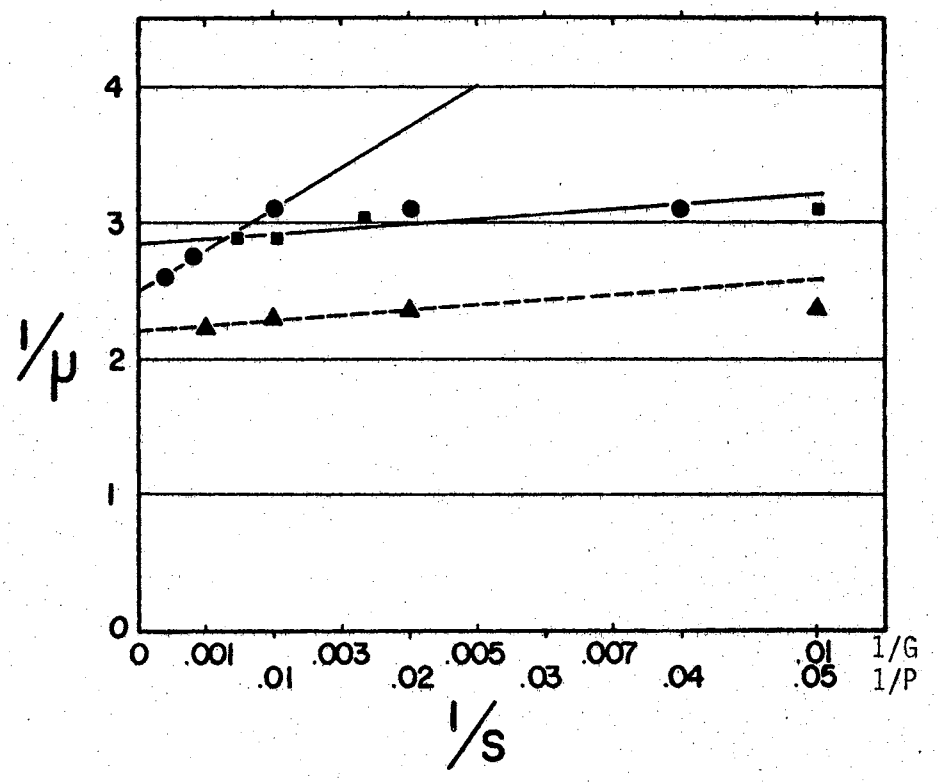
Key to symbols:

	L-B plot	Monod plot
GMM unit	■	■
GMM unit*	▲	▲
GMM+P unit	●	●

G = glucose, mg/l

P = peptone mg/l (with 500 mg/l glucose)

*Indicates value or symbol for aerated (3 lpm) and stirred unit.


4
3
2
1
0
1/μ
0
.001
.003
.005
.007
.01
1/G
.01
.02
.03
.04
.05
1/P
1/S

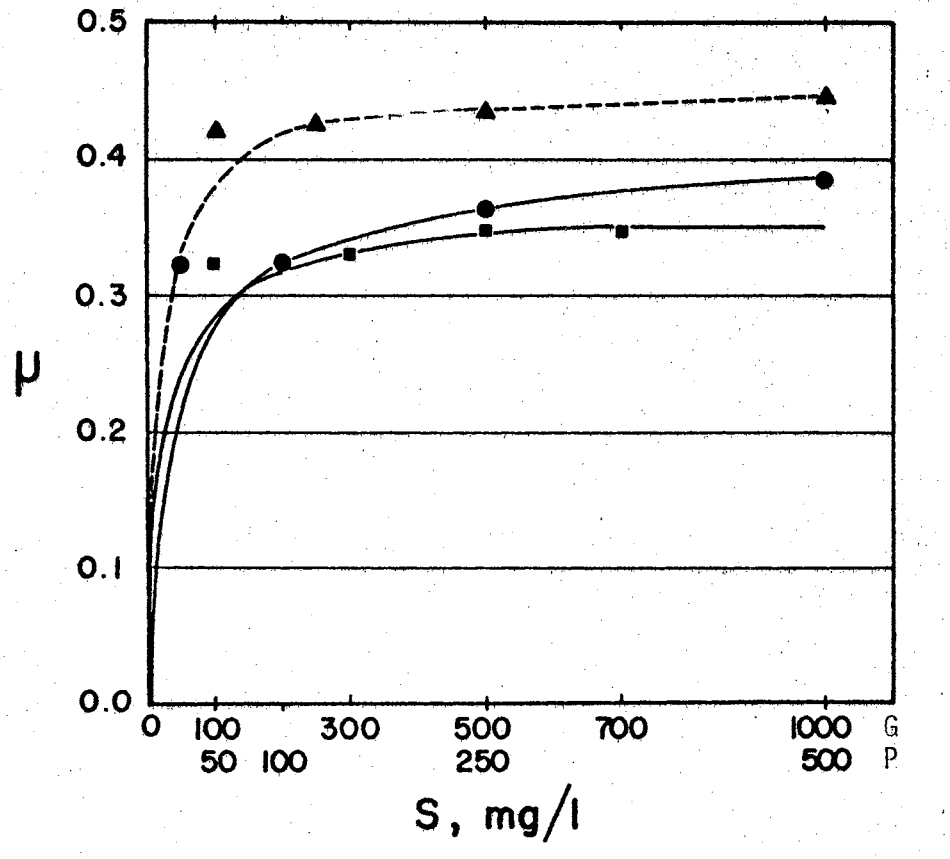

0.5
0.4
0.3
0.2
0.1
0.0
μ
0
100
300
500
700
1000
G
50
100
250
500
P
S, mg/l

Figure 12. Determination of μ_m and K_s for culture D, top) Lineweaver-Burk plot, bottom) Monod plot. Shaken flask (90 osc/min), 25° C. Bubble aeration (3 lpm/l), 27° C.

	L-B plot			Monod plot		
	GMM*	GMM	GMM+P	GMM*	GMM	GMM+P
μ_m	0.57	0.56	0.63	0.58	0.56	0.63
K_s	29	3	4	20	15	5

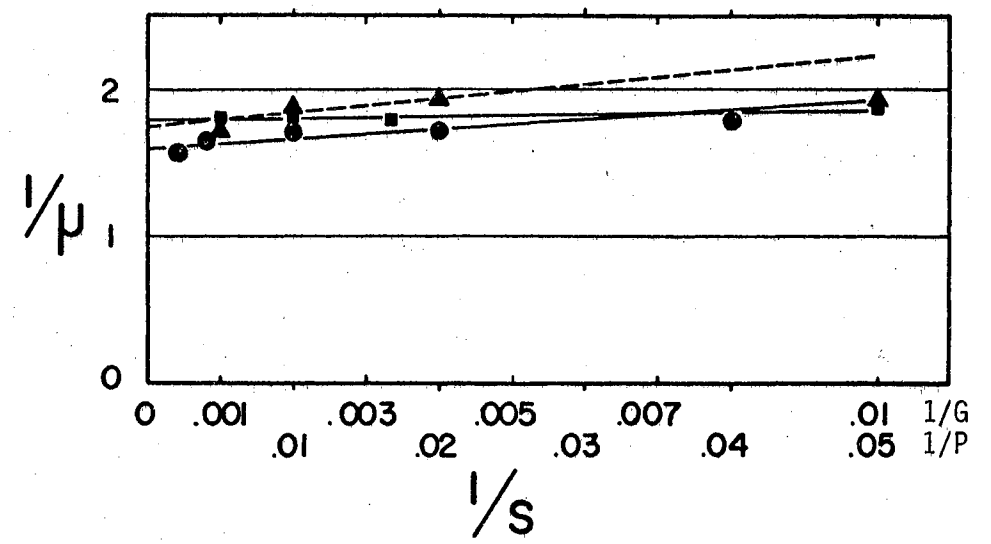
Key to symbols:

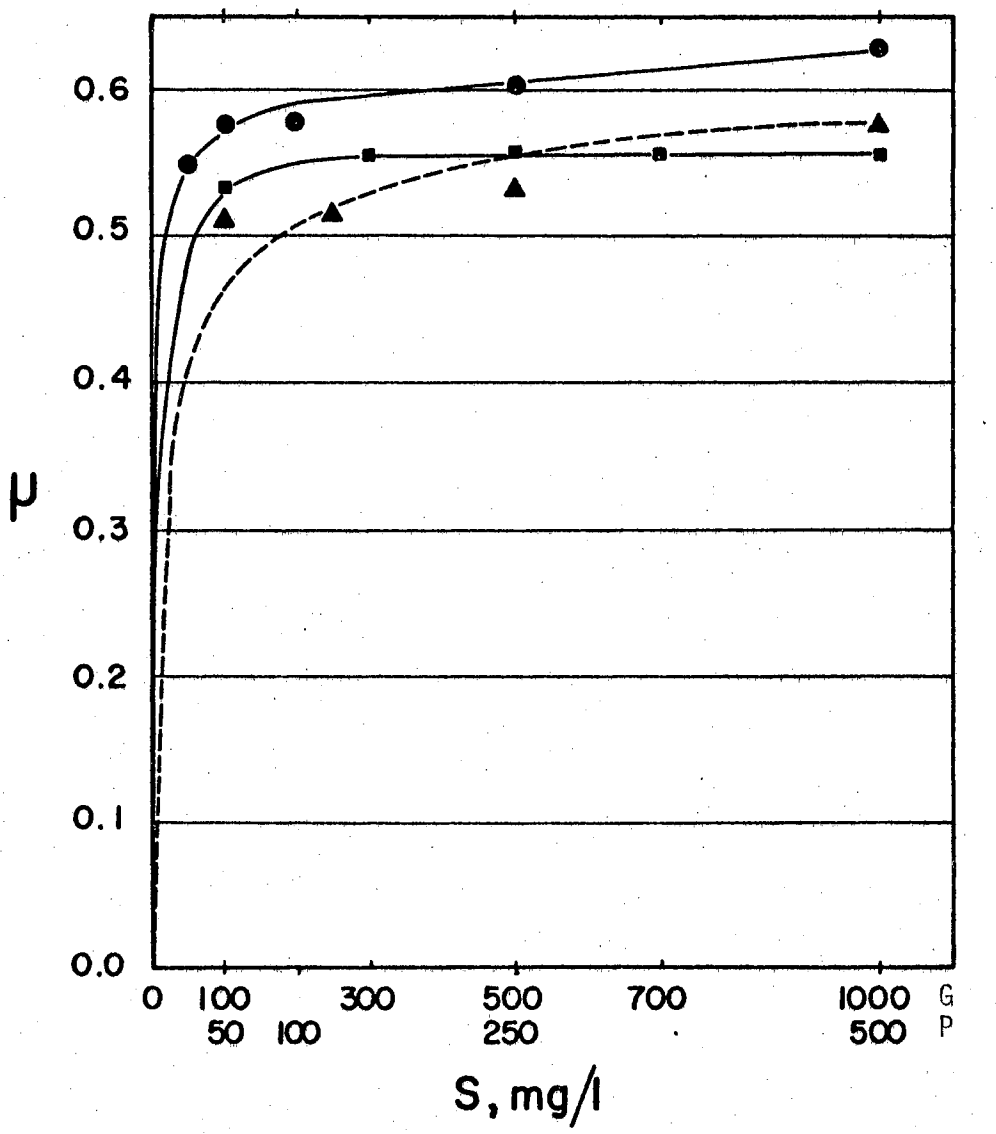
	L-B plot	Monod plot
GMM unit	■	■
GMM* unit	▲	▲
GMM+P	●	●

G = glucose, mg/l

P = peptone, mg/l (with 500 mg/l glucose)

*Indicates value or symbol for aerated (3 lpm) and stirred unit.


1/μ
2
1
0
0 .001 .003 .005 .007 .01 1/G
.01 .02 .03 .04 .05 1/P
1/S


μ
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0 100 300 500 700 1000 G
50 100 250 500 P
S, mg/l

and K_s for each culture in both media and the value for each culture in a specific medium concentration are given in Tables XIIa and b. Also indicated are values of μ_{max}, K_s, and μ obtained in the 1.0 liter reactor with bubble aeration. Table XIIa shows, in summary, the values substantiated by most of the available data. All values obtained from experimental determinations are shown in Table XIIb.

As may be noted from Table XIIa, the four selected cultures were found to possess a wide range of growth rates when grown under the given conditions. The μ_{max} in GMM may be noted to increase by approximately 0.2 hr^{-1} per culture from B through D. With GMM + P medium, the same sequence of μ_{max} values for the cultures was found (A<B<C<D) but with less differential in μ_{max}, especially for cultures B and C which had very similar values for μ_{max}.

Bacto-peptone was a necessary supplement to GMM for the growth of organism A. The maximum rate of growth in 500 mg/l GMM was attained with the addition of approximately 500 mg/l peptone. The addition of 250 mg/l of peptone to 500 mg/l GMM resulted in a three-fold increase in μ_{max} for culture B. The same concentration of peptone increased μ_{max} for cultures C and D by approximately 12 percent over that found in GMM alone. Medium concentrations of 500 mg/l GMM and 500 mg/l GMM plus 250 mg/l peptone produced maximum or near maximum growth rates for each culture; therefore, these concentrations were chosen for use in certain future studies, e.g., continuous cultivation.

The effect of the shaking rate (oscillations per minute) on the value of μ_{max} was determined for culture C. Two shakers, each holding a duplicate set of flasks, were operated at rates of 90 and 120 oscillations per minute. The faster rate was observed to provide a

TABLE XIIa

VALUES OF μ, μ_{max}, AND K_s FOR SELECTED CULTURES

Reactor		Shaken Flask						1 Liter (Bubble Aeration)				
Medium		GMM			GMM + Peptone			GMM				GMM + Peptone
Values		μ_{max} (1000 G)	K_{s_G}	μ (500 G)	μ_{max} (500 G + 250 P)	K_{s_P}	μ (500 G + 250 P)	μ_{max} (1000 G)	K_{s_G}	μ (500 G)	μ (1000 G)	μ (1000 G + 250 P)
Culture	Date											
A	4/10	0	0	0	0.20	70	0.17					
	11/4										0.04	0.30
B	4/10	0.12	100	0.09	0.35	15	0.35					
	10/24										0.13	0.50
C	1/5 from 9/12	0.35	20	0.35	0.39	10	0.37	0.45	10	0.43		
	10/22										0.58	0.87
D	12/16	0.56	15	0.56	0.63	5	0.60	0.58	20	0.53		
	10/29										0.77	0.77

Note: The letters G and P denote glucose and peptone, respectively. Numerical values of G and P have units of mg/l.

TABLE XIIb

VALUES OF μ, μ_{max}, AND K_s FOR SELECTED CULTURES

Medium		Glucose Minimal Medium			Glucose Minimal Medium + Peptone		
Culture	Date	μ_{max}	K_{s_G}	μ (500 G)	μ_{max}	K_{s_P}	μ (500 G + 250 P)
	4/10	0	0	0	0.20	70	0.17
	4/18	0	0	0	0.22	90	0.18
	4/30	0	0	0			
A	7/1						0.30
(formerly	7/2						0.33
4)	7/8			0			
	7/						0.20 a
	7/			0			
	11/4			0.04*†b			0.30*†c
	4/10	0.12	100	0.09	0.35	15	0.35
	4/21	0.12	150	0.09	0.38	25	0.34
	7/1						0.38
B	7/2						0.38
(formerly	7/8			0.12			
8)	7/						0.21 a
	7/			0.08			
	7/			0.10			
	10/24			0.13*†b			0.50*†c
	4/2	0.29	30	0.28	0.34	5	0.32 c
	7/1						0.51
	7/2						0.40
	7/8			0.35			
	7/			0.39			
	7/			0.42			
C	7/						0.35 a
(formerly	12/6	0.35	30	0.35	0.35	5	0.33
14)	12/11	0.46*		0.46*			
	12/13	0.39*	15*	0.39*			
	12/16	0.46	20	0.45	0.48	8	0.43
	1/5(12/16)	0.37	10	0.35	0.39	5	0.37
	1/5(9/12)	0.35	20	0.35	0.39	10	0.37
	1/5(9/12)	0.45*†	10*	0.43*†			
	10/22			0.58*†b			0.87*†c
	4/3	0.53	30	0.51	0.53	3	0.53
	7/1						0.87
	7/2						0.61
D	7/8			0.58			
(formerly	7/						0.48 a
18)	7/			0.46			
	7/			0.87			
	10/29			0.77*†b			0.77*†c
	12/16	0.58*†	20*	0.53*†			
	12/16	0.56	15	0.56	0.63	5	0.60

Note: G and P denote glucose and peptone. Numerical values of G and P have units of mg/l. * for values from bubble aeration. † value calculated and in agreement by optical density and viable count, e.g., see Figure 13. a = 100 P, b = 1000 G, c = 1000 G + 250 P.

much more vigorous mixing of the flask contents although the slower rate appeared to be sufficient to produce thorough mixing. The same concentrations of GMM and GMM+P were used as in the prior growth studies. The values of μ_{max} were similar at each rate of shaking (μ_{max} = 0.35 hr^{-1} at both rates in GMM and μ_{max} = 0.37 hr^{-1} and 0.35 hr^{-1} at 90 and 120 oscillations per minute, respectively, in GMM + P) and similar to values of μ_{max} previously presented for culture C (0.35 in GMM and 0.39 in GMM + P). Therefore, a shaker rate of 90 oscillations per minute appeared sufficient to yield the maximum μ obtainable on the given medium by this method for this culture.

b. Bubble aeration growth studies

The values of μ obtained for each culture when grown in a 1.0 liter volume with bubble aeration are given in Table XII. Figures 11 and 12 and Table XII show the values of μ_{max}, K_s, and μ for cultures C and D when grown in this manner. When grown on GMM the same sequence of relative μ values was found as had been observed in the shaker flask studies, i.e., A < B < C < D. When grown in GMM + P, however, the sequence of μ values was altered to A < B < D < C. Values of μ determined from each type of cultivation are given below.

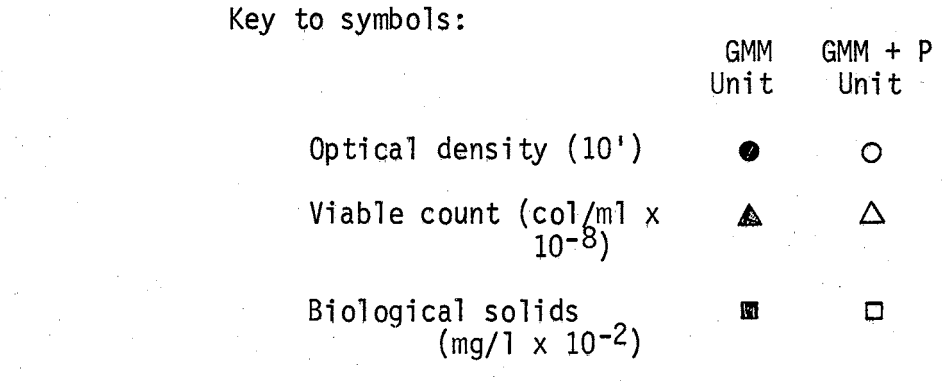
	MEDIUM			
	1000 mg/l GMM	1000 mg/l GMM	500 mg/l GMM + 250 mg/l P	1000 mg/l GMM + 250 mg/l P
Culture	μ by Shaker	μ by Bubble Aeration	μ by Shaker	μ by Bubble Aeration
A	0	0.04	0.17	0.30
B	0.12	0.13	0.35	0.50
C	0.35	0.58	0.37	0.37
D	0.56	0.77	0.60	0.77

The increase in μ for cultures A and B with the addition of peptone was more marked with bubble aeration than when grown in the shaker flask. Culture C responded to the addition of peptone with a large increase in μ. The addition of peptone had no apparent effect on μ for culture D. Growth with bubble aeration resulted in an increase in μ which varied with the culture and substrate.

All of the preceding values of μ were calculated from optical density data. Optical density was verified as a valid means for the determination of μ for each culture by comparison with μ values calculated from viable count and biological solids data from the same experiment (Figure 13). The values of μ as calculated by each method follow.

	Medium					
	1000 mg/l GMM			1000 mg/l GMM + 250 mg/l P		
Culture	μ O.D.	μ V.C.	μ Solids	μ O.D.	μ V.C.	μ Solids
A	0.04	0	0	0.30	0.30	0.30
B	0.13	0.15	0.13	0.50	0.53	0.46
C	0.58	0.60	0.46	0.87	0.99	0.87
D	0.77	0.77	0.77	0.77	0.77	0.77
	500 mg/l GMM					
	μ O.D.	μ V.C.				
C	0.43	0.46				
D	0.53	0.63				

Figure 13. Comparison of μ values from data for optical density, viable count, and biological solids for culture D.



Calculated μ values for culture D.

	GMM Unit	GMM + P Unit
Optical density	0.77	0.77
Viable count	0.77	0.77
Biological solids	0.77	0.77

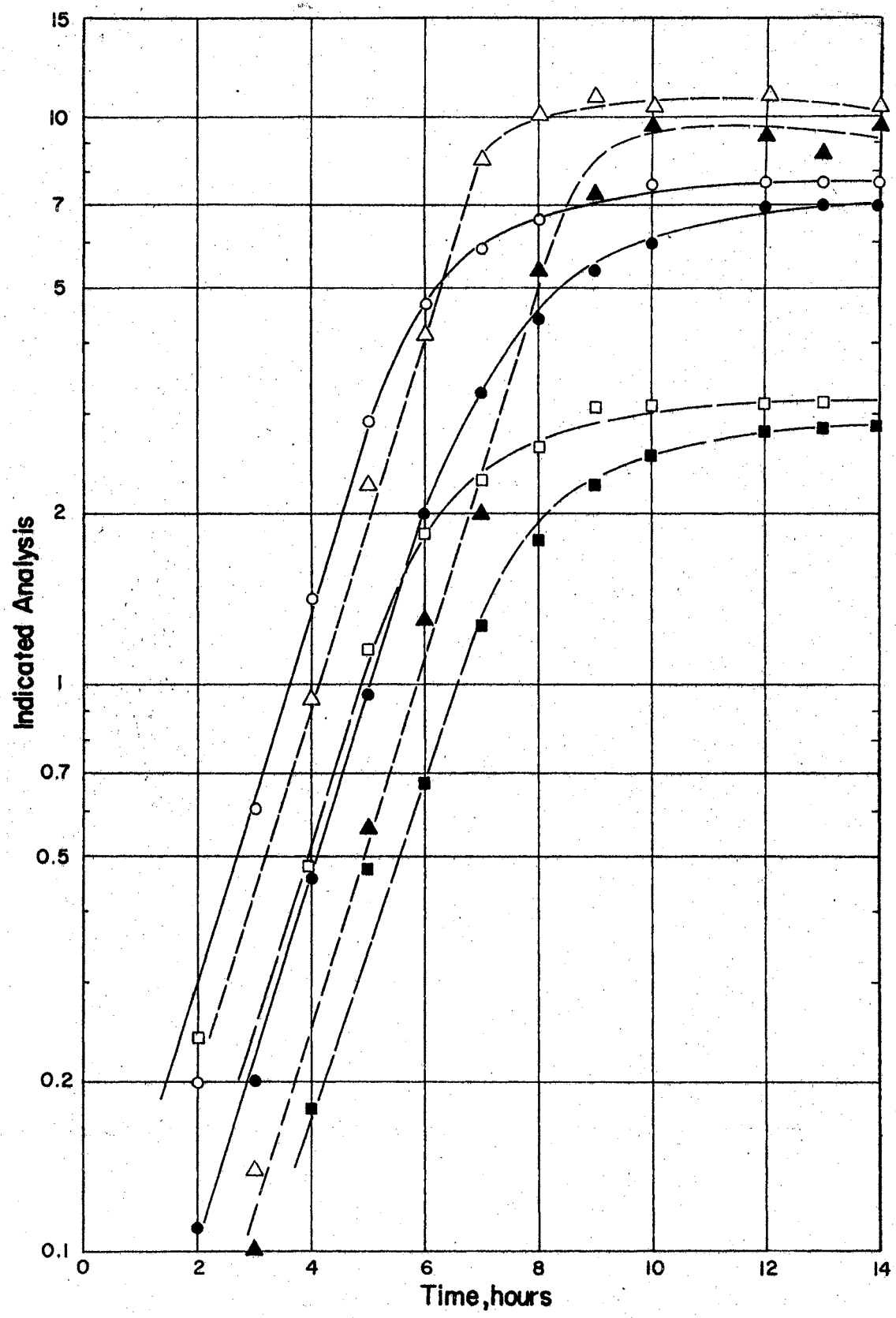

15
10
7
5
2
1
0.7
0.5
0.2
0.1
Indicated Analysis
0
2
4
6
8
10
12
14
Time, hours

Figure 13 shows the data used to calculate μ by each of the three methods for culture D.

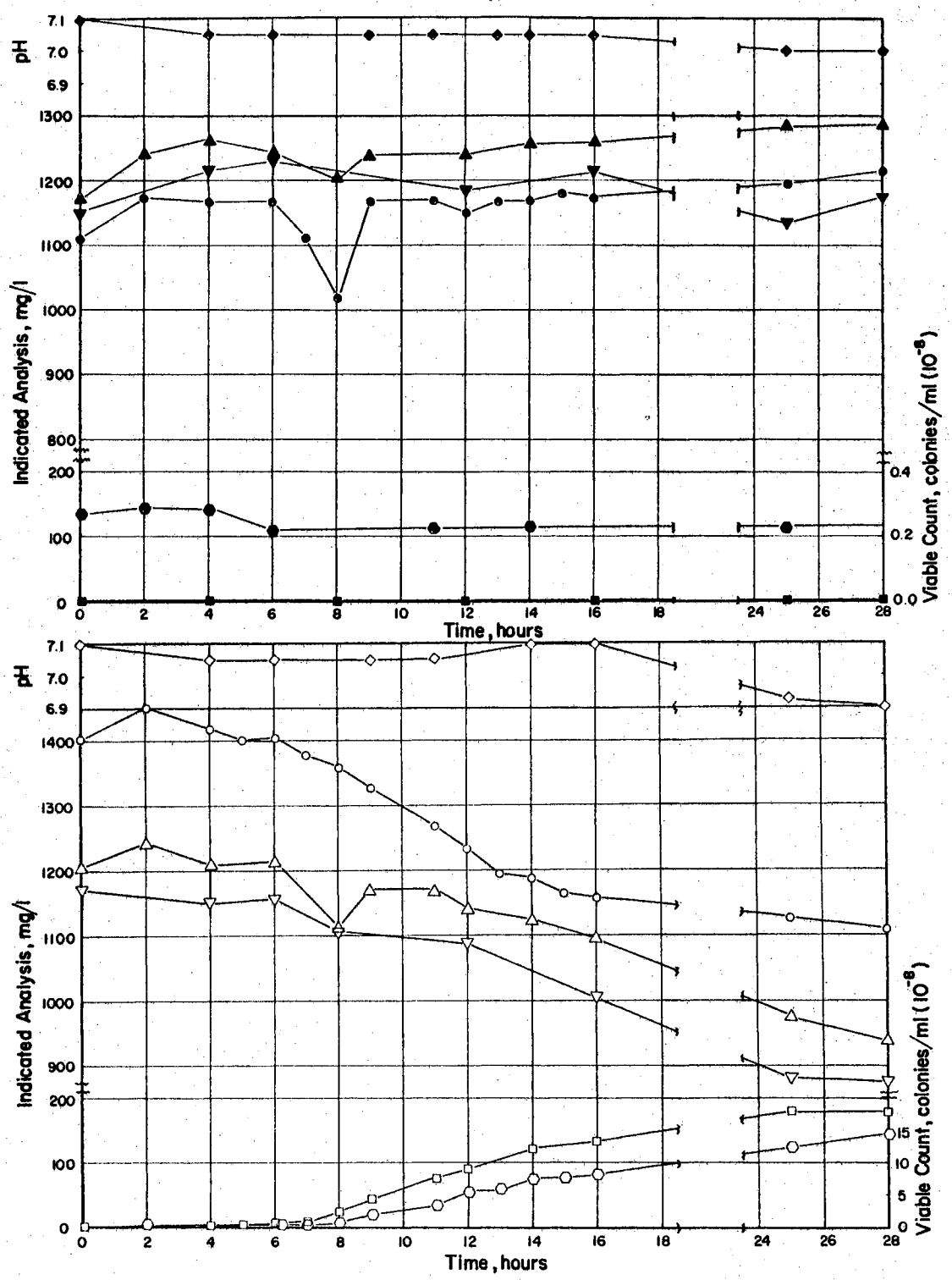
Figures 14 through 20 show the growth and substrate removal patterns for each pure culture when grown in the 1 liter batch reactor with bubble aeration at an air flow rate of 3 liters per minute and supplemental magnetic mixing.

From Figure 14 it may be seen that culture A exhibited no apparent growth in GMM and only a very slow response in GMM + P. Although the culture had been acclimated on GMM + P, there was a lag of approximately 6 hours before the initiation of significant glucose and peptone utilization with a concomitant increase in biological solids and viable count. The rates of increase in biological solids, optical density, and viable count were found to be similar. Much of the decrease in total COD during the early phase of growth (e.g., to hour 13) seems to be attributable to peptone COD as the decrease in glucose COD accounts for only about one third of the COD removal. After growth had become established, the difference in total COD and glucose COD increased from approximately 65 mg/l at 14 hours to approximately 170 mg/l at 28 hours. This increase in COD differential may indicate a release of metabolic intermediates and/or end products into the mixed liquor or less utilization of peptone COD. An accompanying decrease in pH (from 7.1 to 6.9) occurred as the differential COD increased, possibly indicating an accumulation of acidic products in the mixed liquor. Culture A apparently utilized much of the peptone before it increased its utilization of glucose and the accumulation of intermediate products increased. The discrepancy between the values for glucose COD and the value for total COD cannot be explained by any means except analytical

Figure 14. The growth of culture A in GMM (top) and GMM + P (bottom).
T = 27° C, Aeration = 3 lpm.

Key to symbols:

	GMM Unit	GMM + P Unit
Total COD	●	○
Glucose COD	▲	△
Carbohydrate	▼	▽
Biological solids	■	□
Viable count	⬢	⬡
pH	◆	◇


pH
Indicated Analysis, mg/l
Viable Count, colonies/ml (10⁻⁸)
Time, hours
pH
Indicated Analysis, mg/l
Viable Count, colonies/ml (10⁻⁸)
Time, hours

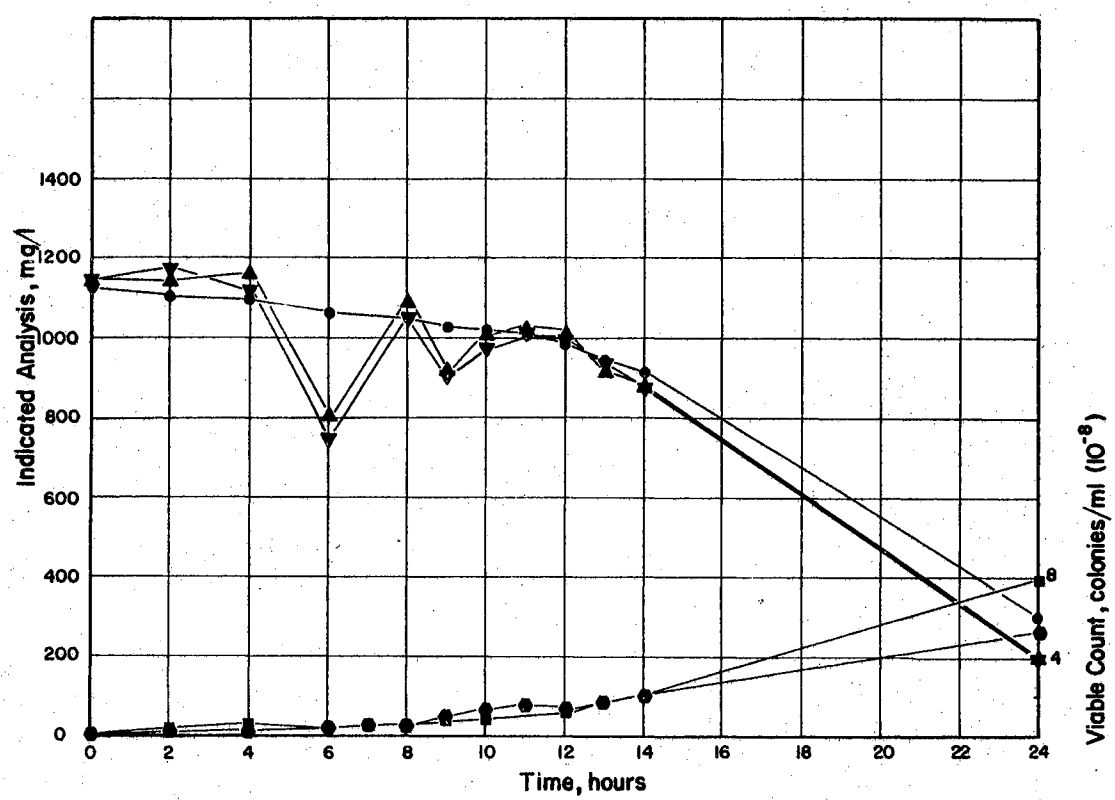
error. It should be noted that the differential between the glucose COD and carbohydrate analyses is due to the calculated conversion of glucose to glucose as COD (glucose, mg/l x 1.067 = glucose COD, mg/l). Carbohydrate (by anthrone test using glucose as a standard) was not converted to COD.

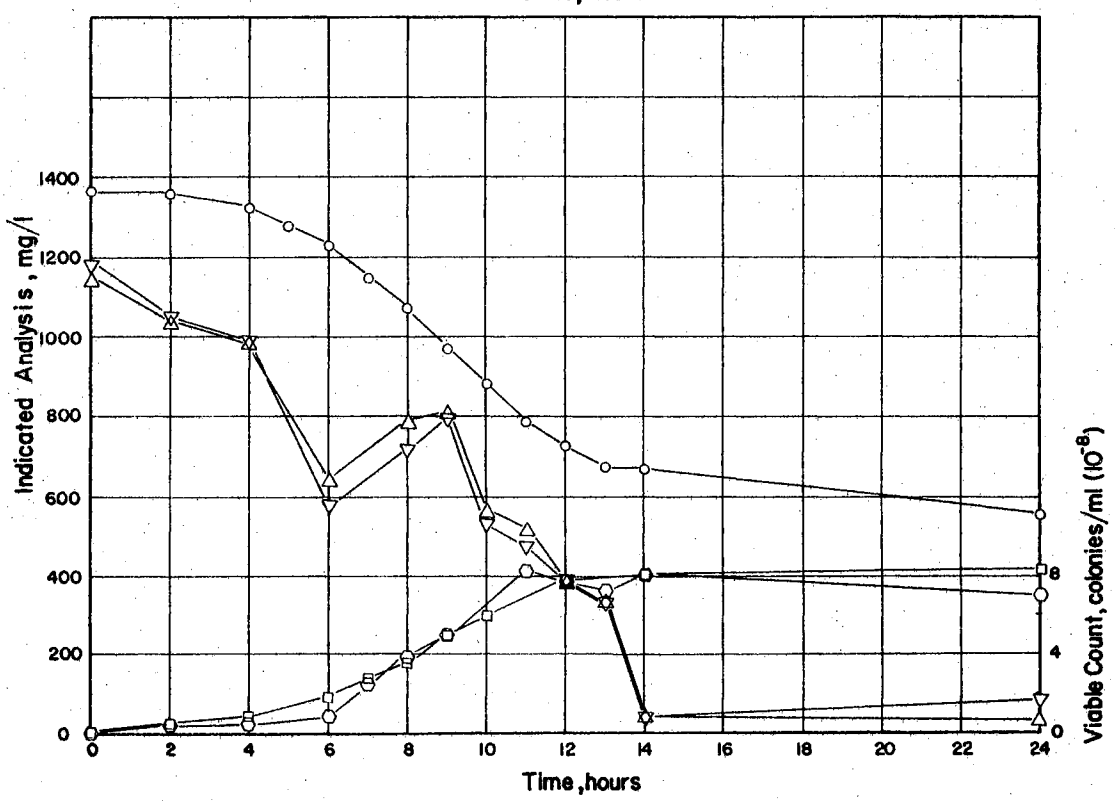
Culture B, like culture A, was able to utilize glucose more rapidly and after a shorter lag period when grown in the GMM + P medium (Figure 15). In the GMM + P medium the total COD remained high after the utilization of the glucose was nearly completed. At 24 hours the total COD was 560 mg/l of which no more than 230 mg/l may be attributed to peptone (as that was its initial contribution to the total COD); approximately 40 mg/l COD was attributable to glucose, thereby indicating approximately 290 mg/l COD as metabolic intermediates or end products. In order to enable the calculation of the approximate concentration of intermediate or end product COD in the GMM + P reactor in this experiment and in subsequent experiments, little or no peptone COD utilization was assumed. It is realized that this assumption may produce some error in the calculated intermediate COD values, however, the values found by this means of calculation will represent minimum intermediate COD concentrations. Small differences in intermediate COD values between experiments are not considered as significant as instances of relatively large differences, especially when the pH is affected. At 24 hours for the GMM medium a total COD of approximately 300 mg/l was found. Glucose COD accounted for approximately 200 mg/l; therefore, about 100 mg/l COD was attributable to intermediates or end products. Measurements of growth response by optical density, biological solids, and viable count gave comparable results for culture B in

Figure 15. The growth of culture B in GMM (top) and GMM + P (bottom).
T = 27° C, Aeration = 3 lpm.

Key to symbols:

	GMM Unit	GMM + P Unit
Total COD	●	○
Glucose COD	▲	△
Carbohydrate	▼	▽
Biological solids	■	□
Viable count	⬢	⬡
Initial pH	7.10	7.10
Final pH	4.70	4.95


Indicated Analysis, mg/l
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time, hours
8
4
Viable Count, colonies/ml (10⁻⁸)


Indicated Analysis, mg/l
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
22
24
Time, hours
8
4
0
Viable Count, colonies/ml (10⁻⁸)

both media. Increasing growth was accompanied by the removal of COD. A decrease in pH occurred in both units from an initial value of 7.10 to values of 4.70 for GMM and 4.95 for GMM + P at 24 hours.

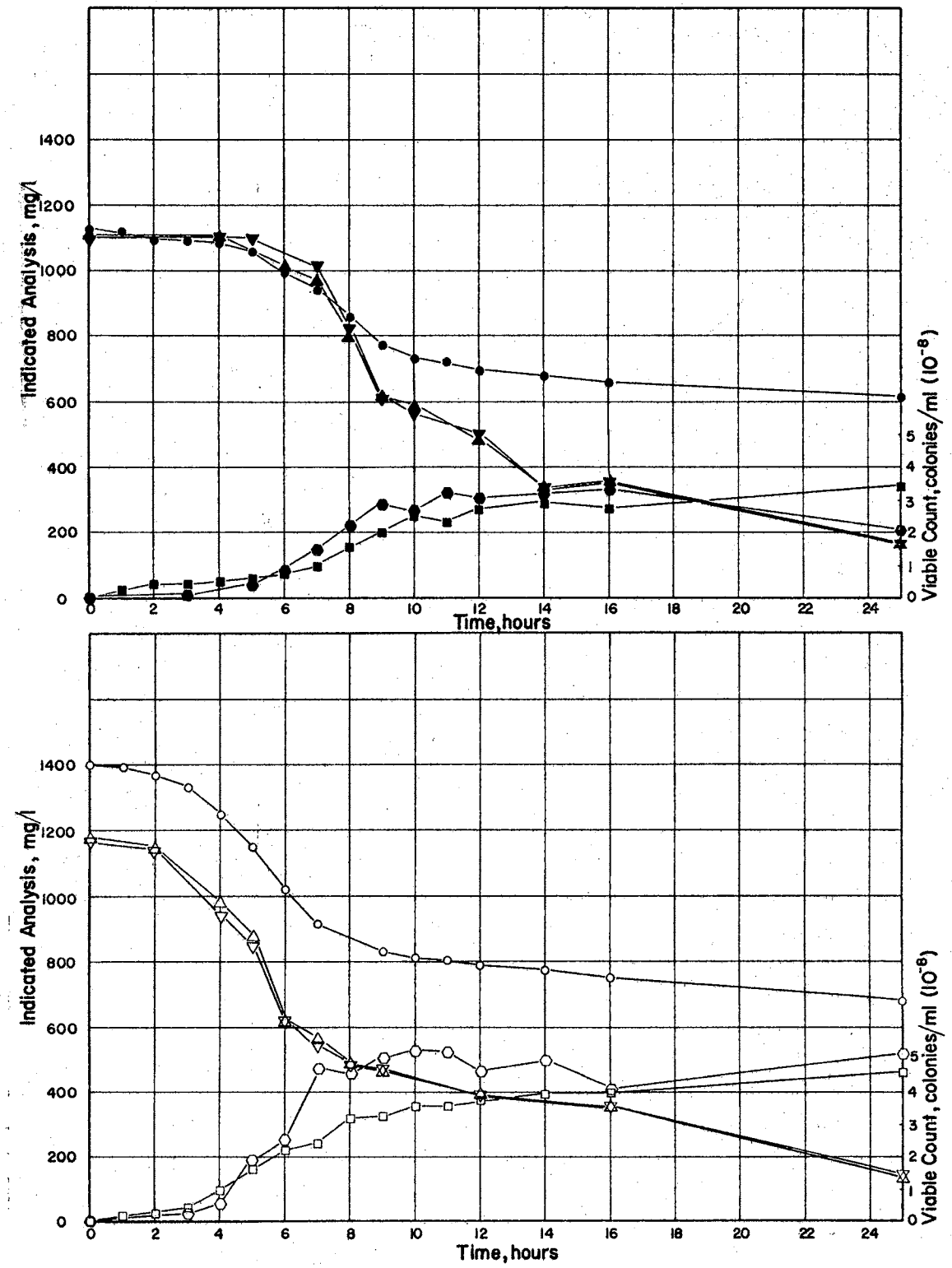
Figure 16 shows the response of culture C when grown in GMM and GMM + P with bubble aeration. As with cultures A and B, the growth response of C in GMM + P medium occurred more quickly following inoculation and proceeded at a more rapid rate than in the GMM medium. After 12 to 14 hours of operation the total COD became nearly stable in both media. After 25 hours of operation the remaining total COD was quite high in both units (616 mg/l in GMM and 681 mg/l in GMM + P). By allowing a deduction for the remaining glucose COD (and peptone COD in GMM + P, assuming no significant peptone COD utilization) the intermediate or end product COD in the units was found to be approximately 455 mg/l in GMM and at least 230 mg/l in GMM + P. The pH after 25 hours had decreased from an initial value of 7.10 in each unit to 4.80 in the GMM medium and 5.40 in the GMM + P medium. Reference to the preceding list of μ values as given for each medium with culture C shows that μ as calculated from data for optical density, viable count, and biological solids varied slightly according to the method of calculation. However, at least two of the three values of μ in each medium were the same or nearly so, thereby lending credibility to having established the proper value of μ for the experiment.

To ascertain whether the decrease in pH produced the incomplete glucose uptake and the high residual total COD observed in Figure 16 and in particular to observe the response of culture C in the GMM + P medium at the concentration employed in continuous flow operation, an experiment was performed wherein two units were used which were alike

Figure 16. The growth of culture C in GMM (top) and GMM + P (bottom).
$T = 27^{o}$ C, Aeration = 3 lpm

Key to symbols:

	GMM Unit	GMM + P Unit
Total COD	●	○
Glucose COD	▲	△
Carbohydrate	▼	▽
Biological solids	■	□
Viable count	⬢	⬡
Initial pH	7.10	7.10
Final pH	4.80	5.40


Indicated Analysis, mg/l
Viable Count, colonies/ml (10⁻⁸)
Time, hours
0
200
400
600
800
1000
1200
1400
2
4
6
8
10
12
14
16
18
20
22
24
1
3
5

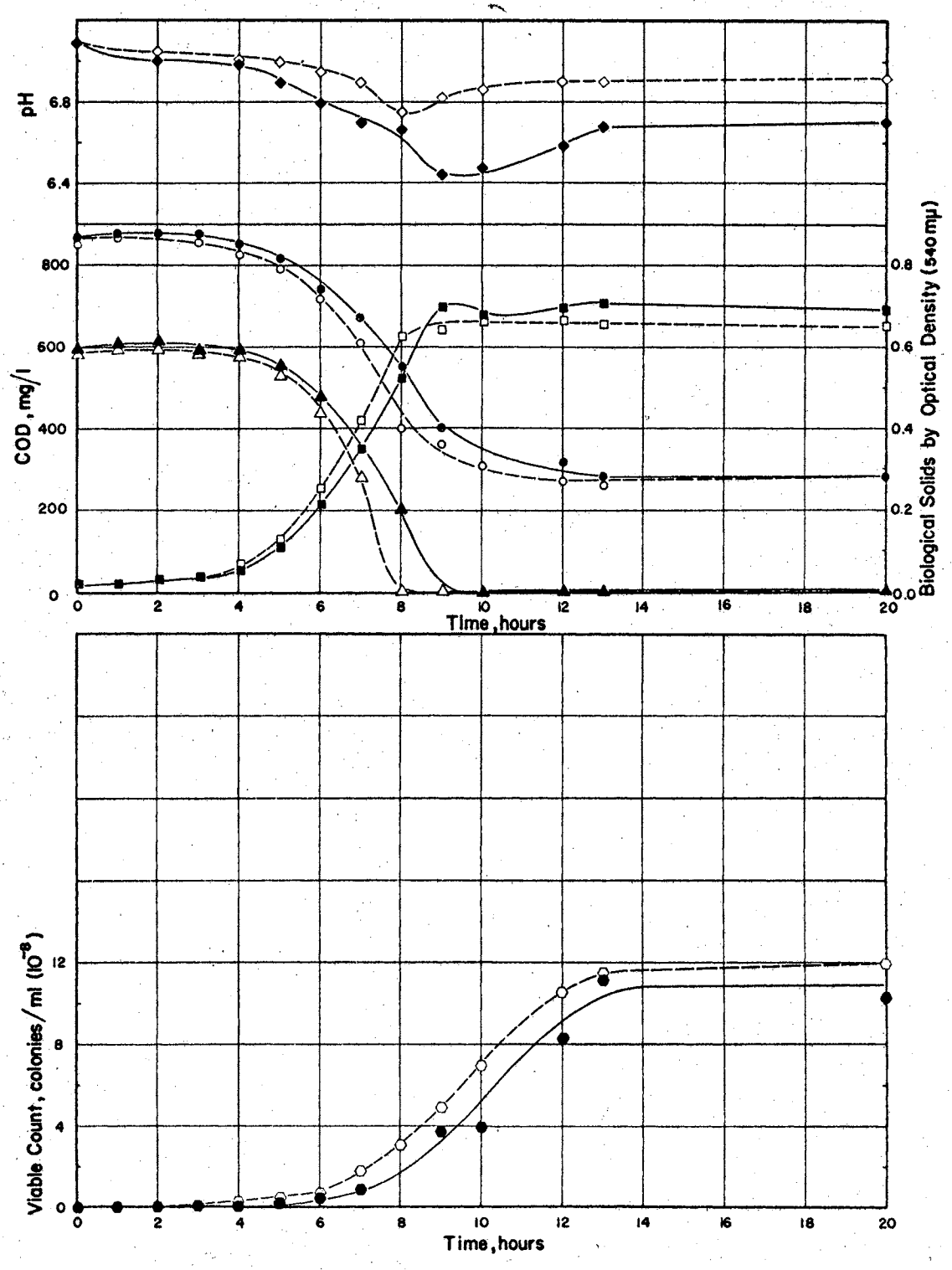
in all respects except for the buffer concentration. Unit I contained the normal buffer concentration and in Unit II the concentration of buffer was increased threefold. The results of the experiment are shown in Figure 17. Calculation of μ from optical density and viable count revealed the same value of μ for each culture. The growth response in the reactor which contained the normal buffer concentration lagged behind the response in the three-fold buffer reactor. A greater decrease in pH was noted in the unit with the lower buffer concentration (minimum of 6.45 compared to 6.75). Each unit exhibited an increase in pH, or a reversal in the pH trend, upon the exhaustion of the glucose supply. The pH in each unit appeared to stabilize at values of approximately 6.70 and 6.90 for the normal buffer unit and the three-fold buffer unit, respectively, after approximately 13 hours. The glucose was completely removed from the medium in each unit. The residual COD in each unit after approximately 13 hours was approximately equal to the initial COD attributable to the peptone, therefore it appears that little residual intermediate or end product COD was present. However, this is not to say that such products were not produced. Evidence of their production and liberation into the mixed liquor may be found by noting the pH change and the differential between the total COD and the COD attributable to glucose and peptone, e.g., an increase in differential (intermediate) COD from approximately 55 mg/l at 7 hours to 165 mg/l at 8 hours in the three-fold buffer unit (Unit II). The change in pH reflected the change in intermediate COD, decreasing as the intermediate COD increased and then rising as the intermediate COD decreased until both became approximately stable when the intermediate COD apparently was exhausted.

Figure 17. Growth of culture C in GMM + P with normal buffer concentration and 3 x normal buffer concentration.

T = 27° C, Aeration = 3 lpm.

Key to symbols:

	Normal Buffer Unit	3 x Buffer Unit
Total COD	●	○
Glucose COD	▲	△
Biological solids	■	□
Viable count	◍	⬡
pH	◆	◇


pH
6.8
6.4
800
600
400
200
0
COD, mg/l
0.8
0.6
0.4
0.2
0.0
Biological Solids by Optical Density (540 mμ)
0
2
4
6
8
10
12
14
16
18
20
Time, hours
Viable Count, colonies/ml (10^{-8})
12
8
4
0
Time, hours

An observation of interest with culture C which is clearly shown in Figure 17 and Figure 18 is the continued increase in viable count after the exhaustion of the glucose supply. While the growth parameters of optical density and biological solids appeared to stabilize at a time which coincided with glucose exhaustion the viable count continued to increase by approximately two-fold without an apparent lag or discontinuity in the growth curve. The viable count increased to its maximum value at a time which coincided with the stabilization of the total COD.

Figure 18 shows the growth response of culture C in GMM medium without peptone. An increase in viable count followed the coincident exhaustion of glucose and stabilization of optical density similar to that previously observed in Figure 17. The change in pH pattern followed a trend similar to that in Figure 17, i.e., it reached a minimum (6.35) at approximately the time of glucose exhaustion, then rose slowly as the intermediate COD decreased.

From the observation of the data presented in Figures 16, 17, and 18 it appears that the presence of peptone in the medium permits culture C to minimize the residual COD attributable to intermediates and/or end products produced from GMM.

Figure 19 depicts the response of culture D when grown in GMM and GMM + P. Both units experienced a major decrease in pH from an initial value of 7.1 to 5.20 for GMM and 5.05 for GMM + P. The occurrence of minimal pH coincided (approximately) with the stabilization of total COD. Considerable residual intermediate or end product COD was found in each unit (approximately 530 mg/l in GMM and 600 mg/l in GMM + P, assuming no significant peptone utilization). Glucose continued to be

Figure 18. Growth of culture C in GMM.
$T = 27^{o}$ C, Aeration = 3 lpm.

Key to symbols:

Total COD	○
Glucose COD	△
Biological solids	□
Viable count	⬡
pH	◇

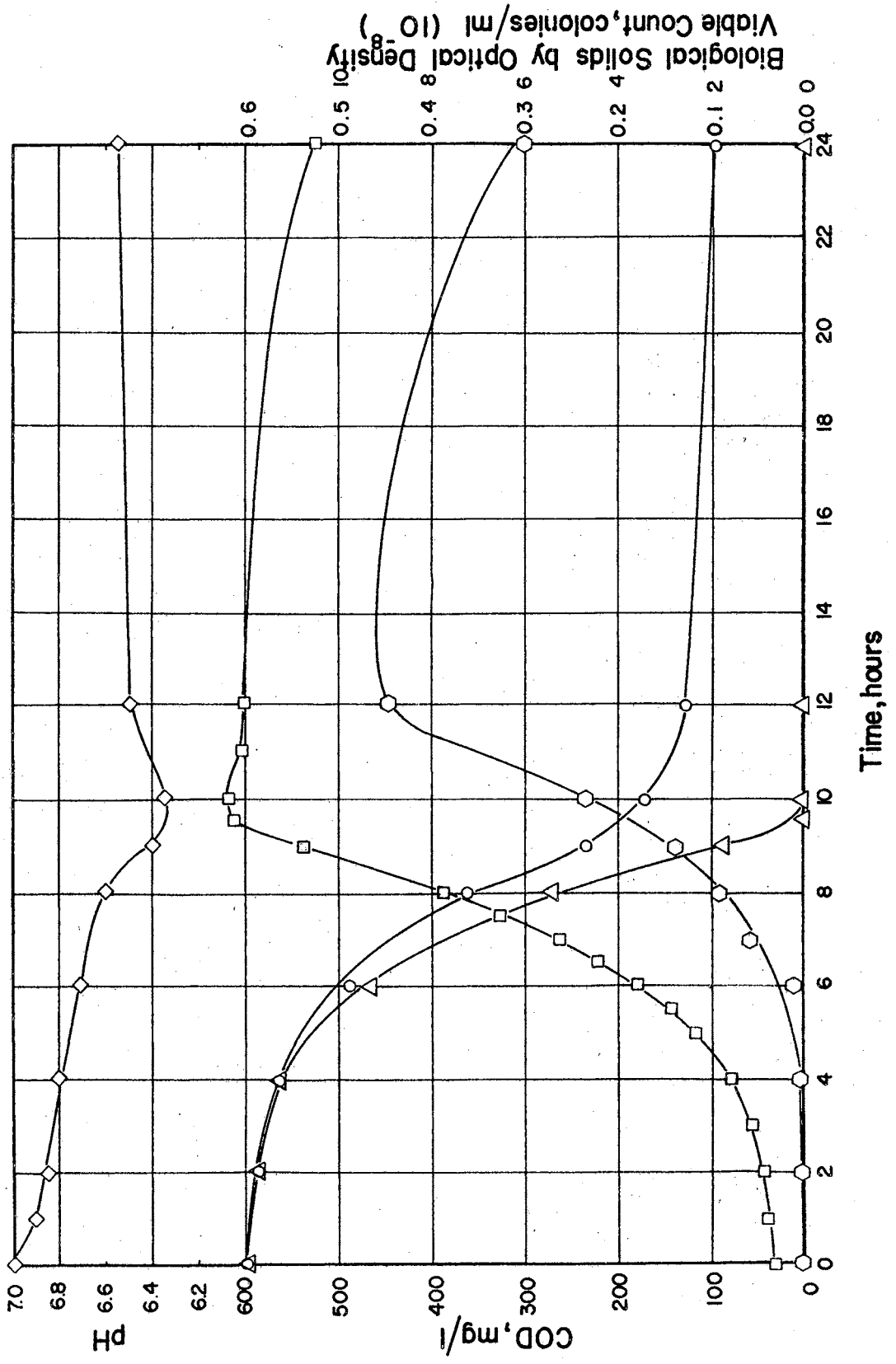
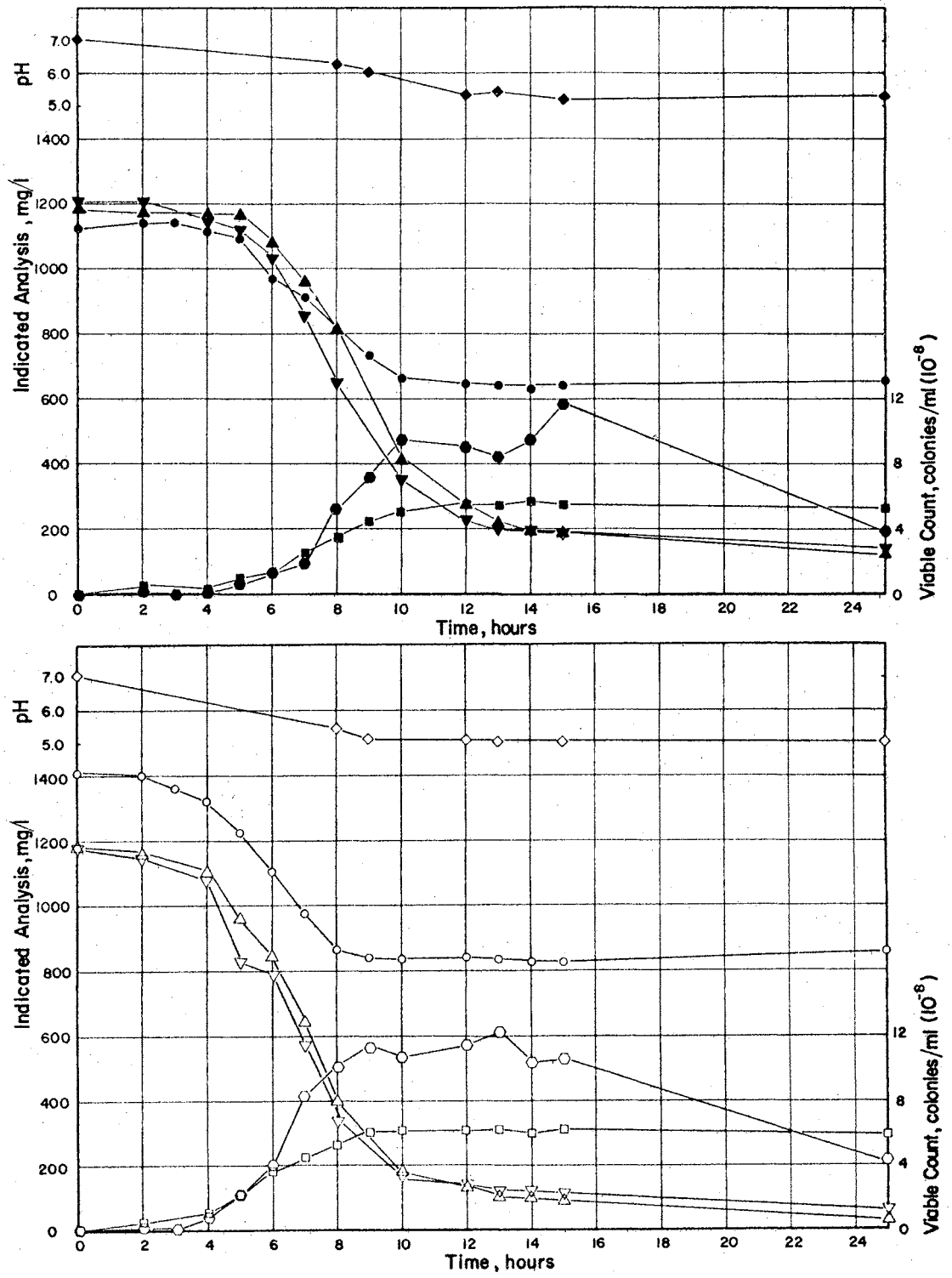

pH
COD, mg/l
Biological Solids by Optical Density
Viable Count, colonies/ml (10^{-8})
Time, hours
7.0
6.8
6.6
6.4
6.2
600
500
400
300
200
100
0
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
18
20
22
24

Figure 19. Growth of culture D in GMM (top) and GMM + P (bottom).

T = 27° C, Aeration = 3 lpm

Key to symbols:

	GMM Unit	GMM + P Unit
Total COD	●	○
Glucose COD	▲	△
Carbohydrate	▼	▽
Biological solids	■	□
Viable count	⬢	⬡
pH	◆	◇


pH
Indicated Analysis, mg/l
Viable Count, colonies/ml (10^{-8})
Time, hours
7.0
6.0
5.0
1400
1200
1000
800
600
400
200
0
12
8
4
0
2
4
6
8
10
12
14
16
18
20
22
24

removed from each medium after the total COD became nearly stable, thereby indicating a near equalization of rates for glucose COD uptake and intermediate COD liberation. In both units growth, as measured by all three parameters, apparently ceased and the total COD approached a degree of stability as the pH decreased into the approximate range of 6.0 to 5.5. The minimum pH in each unit was reached after approximately 1000 mg/l of glucose COD had been utilized.

When culture D was grown on a lower concentration of glucose (approximately 600 mg/l glucose COD) in GMM as shown in Figure 20 the pH was found to have a lesser decrease (5.9 compared to 5.2) than when grown on approximately 1200 mg/l glucose COD as in Figure 19, and the culture appeared to utilize the intermediate COD after a lag of 1 to 2 hours. The utilization of intermediate COD is indicated by the pattern of decline in total COD after glucose COD had been utilized and the concomitant rise in pH, viable count, and optical density.

2. Mixed Culture Shaken Flask Studies

All possible combinations of the four selected cultures were grown in shaken flasks in the same manner as was performed for the pure culture studies. The growth rate, μ, of each mixture was determined by optical density and is given in Table XIII. Both GMM (500 mg/l glucose) and GMM + P (500 mg/l glucose + 250 mg/l peptone) media were employed. The viable count was determined for pure cultures and for each culture type in each mixture at the time of initial inoculation and again near the end of the logarithmic growth phase. From the viable count data thus obtained a comparison was made of the growth response of each culture in the mixed populations with the growth response of the pure culture over the same period. The viable count

Figure 20. Growth of culture D in GMM.
$T = 27^{o}$ C, Aeration = 3 lpm.

Key to symbols:

Total COD	○
Glucose COD	△
Biological solids	□
Viable count	⬡
pH	◇

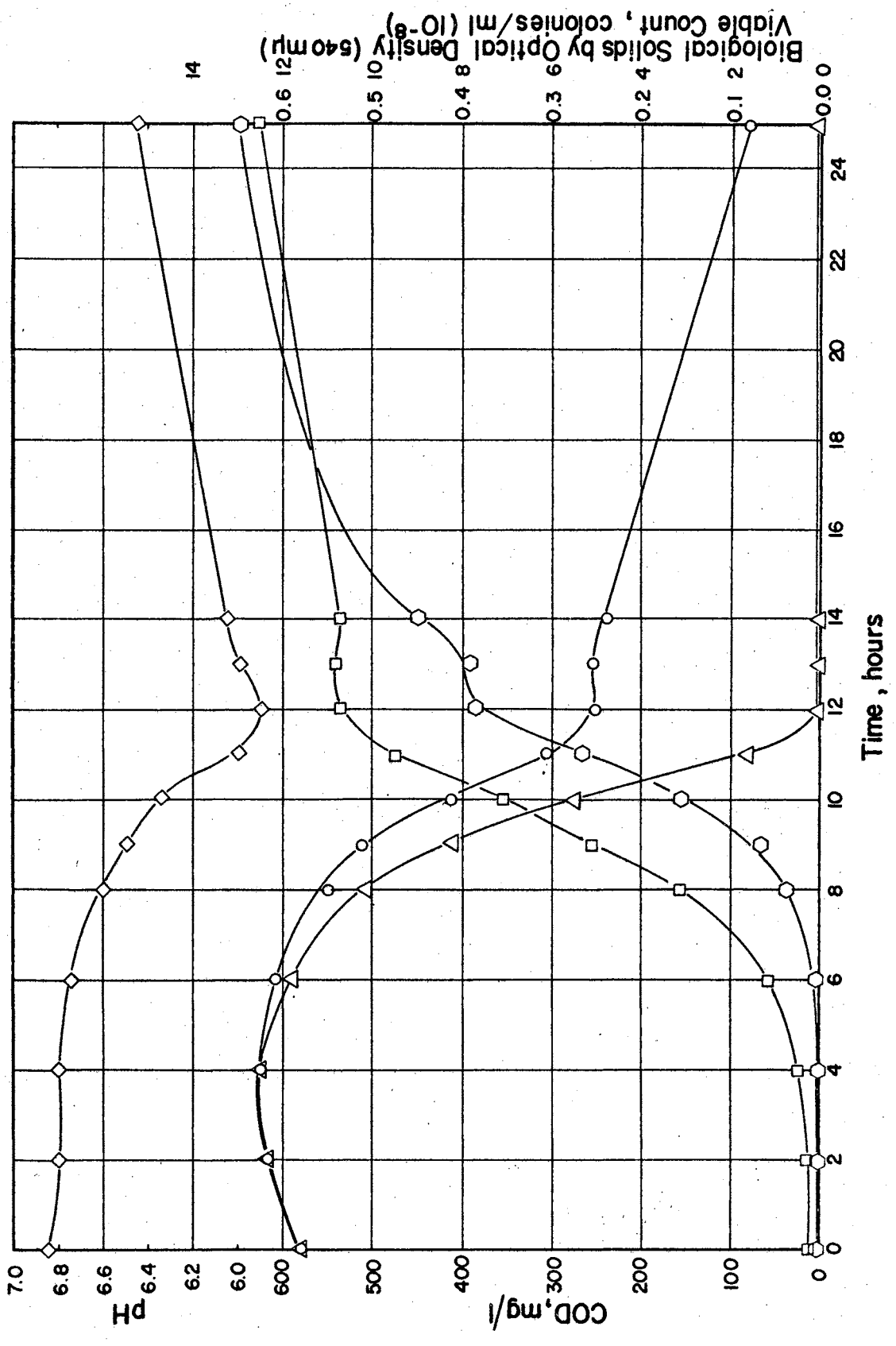

Biological Solids by Optical Density (540 mμ)
Viable Count, colonies/ml (10-8)
pH
COD, mg/l
Time, hours

TABLE XIII

VALUES OF μ FOR MIXED POPULATIONS (SHAKEN FLASK)

Cultures	Medium			
	500 mg/l GMM		500 mg/l GMM + 250 mg/l P	
	Date	μ	Date	μ
AB	7/ a	.28	7/1	.36
	7/ b	.36	7/2	.39
	7/8	.32		
AC	7/ a	.38	7/1	.53
	7/ b	.37	7/2	.41
	7/8	.33		
AD	7/ a	.48	7/1	.69
	7/ b	.63	7/2	.69
	7/8	.43		
ABC	7/ a	.32	7/1	.40
	7/ b	.33	7/2	.31
	7/8	.31		
ABD	7/ a	.30	7/1	.45
	7/ b	.43	7/2	.37
	7/8	.32		
ACD	7/ a	.35	7/1	.46
	7/ b	.39	7/2	.38
	7/8	.39		
ABCD	7/ a	.27	7/1	.41
	7/ b	.32	7/2	.30
	7/8	.33		
BC	7/ a	.30	7/1	.42
	7/ b	.33	7/2	.35
	7/8	.35		
BD	7/ a	.30	7/1	.51
	7/ b	.41	7/2	.37
	7/8	.36		
BCD	7/ a	.29	7/1	.42
	7/ b	.33	7/2	.30
	7/8	.36		
CD	7/ a	.35	7/1	.60
	7/ b	.39	7/2	.37
	7/8	.39		

Note: a and b represent two separate experiments for which the exact calendar date was not recorded.

data are given in Table XIV wherein each number for the culture population increase given in the table represents the factor by which the population existent at the time of sampling had increased from the initial inoculum population.

Table XV summarizes the findings of this study. For each medium and each mixed population, the relative degree by which each individual culture comprising the mixed population increased relative to the increase in population of the pure culture for the same medium and time period has been ranked. Also the apparent effect on the growth rate (stimulated or inhibited or no apparent effect) for each culture as a result of the mixing of cultures has been indicated. In all mixtures at least one of the cultures indicated an increased growth response and all cultures experienced a stimulatory effect in several mixtures. The only culture which appeared to be inhibited regularly in mixed populations was culture D. Such inhibition may be seen to have occurred when culture C was present in the mixed population.

Also given in Table XV are the values of μ for the mixed population and each of the pure cultures comprising the mixture.

After the period of logarithmic growth with the attendant optical density and viable count determinations, the flasks were allowed to continue incubating on the shaker for a total elapsed time of 24 to 36 hours. After this time observations were made in regard to the formation of flocculent particles or growth adherence to the sidewall of the flask. As noted in Table XV several flasks were observed to contain either floc particles or sidewall growth at the upper margin of liquid contact as the mixed liquor oscillated in the flask. It may be noted

TABLE XIV

INDIVIDUAL CULTURE POPULATION INCREASE IN MIXED CULTURE SYSTEMS (POPULATION AFTER ELAPSED TIME/INITIAL POPULATION)

Culture	Medium									
	500 mg/l GMM					500 mg/l GMM + 250 mg/l Peptone				
	Run # (elapsed time, hr)	Culture Population Increase				Run # (elapsed time, hr)	Culture Population Increase			
		A	B	C	D		A	B	C	D
Pure Cultures	1 (7) (10) 2 (8) 3 (8)	1.3 0.8 1.6 0.6	2.2 1.7 2.0 2.8	5.6 8.3 14.0 37.0	2.0 37.0 20.0 33.0	4 (8) 5 (6) 6 (8)	4.0 1.7 2.8	11.0 1.8 16.0	17.0 10.0 12.0	25.0 33.0 21.0
Mixed Cultures										
AB	1 (7) 2 (8) 3 (8)	1.0 2.1 2.6	7.6 5.8 14.0			4 (8) 5 (6) 6 (8)	11.0 1.8 5.2	17.0 1.8 12.0		
AC	1 (7) 2 (8) 3 (8)	1.8 1.3 2.8		5.7 24.0 27.0		4 (8) 5 (6) 6 (8)	6.1 2.8 2.0		20.0 16.0 61.0	
AD	1 (7) (10) 2 (8) 3 (8)	0.8 1.6 1.4 2.0			9.4 38.0 24.0 34.0	4 (8) 5 (6) 6 (8)	6.0 2.2 6.9			21.0 96.0 38.0
ABC	1 (7) 2 (8) 3 (8)	1.6 1.9 5.4	13.0 5.4 12.0	12.0 23.0 44.0		4 (8) 5 (6) 6 (8)	4.3 4.5 1.7	10.0 5.0 13.0	17.0 21.0 20.0	
ABD	1 (7) 2 (8) 3 (8)	1.7 2.7 3.1	12.0 7.6 33.0		7.8 40.0 77.0	4 (8) 5 (6) 6 (8)	6.0 1.8 2.3	12.0 2.2 12.0		25.0 86.0 60.0
ACD	1 (7) 2 (8) 3 (8)	4.2 1.0 1.8		14.0 12.0 26.0	10.0 33.0 22.0	4 (8) 5 (6) 6 (8)	3.9 3.3 5.5		22.0 14.0 10.0	19.0 48.0 12.0
ABCD	1 (7) 2 (8) 3 (8)	2.8 4.8 3.2	5.9 5.5 14.0	17.0 24.0 23.0	7.4 34.0 34.0	4 (8) 5 (6) 6 (8)	6.7 4.1 4.8	11.0 5.0 7.0	12.0 28.0 20.0	18.0 69.0 8.2
BC	1 (7) 2 (8) 3 (8)		5.3 15.0 14.0	6.6 15.0 38.0		4 (8) 5 (6) 6 (8)		8.0 6.4 9.2	10.0 23.0 40.0	
BD	1 (7) (10) 2 (8) 3 (8)		8.0 15.0 20.0 17.0		6.8 29.0 38.0 39.0	4 (8) 5 (6) 6 (8)		14.0 -- 12.0		35.0 100.0 49.0
BCD	1 (7) 2 (8) 3 (8)		10.0 -- 12.0	24.0 -- 22.0	16.0 -- 14.0	4 (8) 5 (6) 6 (8)		15.0 3.9 8.6	22.0 21.0 9.6	23.0 42.0 12.0
CD	1 (7) (10) 2 (8) 3 (8)			8.2 17.0 10.0 48.0	5.6 15.0 10.0 27.0	4 (8) 5 (6) 6 (8)			17.0 25.0 14.0	17.0 39.0 18.0

TABLE XV

REMARKS ON MIXED CULTURE SHAKEN FLASK STUDIES

Culture	Degree of Culture Population Increase Compared to Population Increase in Pure Culture		Comparative Values of μ (500 mg/l Glucose, 250 mg/l Peptone)		Remarks
	GMM	GMM + P	GMM	GMM + P	
AB	B* $\gtrsim$ A*	A* $\gtrsim$ B	.32 mixed 0 for A .09 for B	.39 mixed .17 for A .35 for B	slight sidewall adherence - easily shaken off in GMM after 24 hours
AC	A* > C	A* > C	.37 mixed 0 for A .35 for C	.41 mixed .17 for A .37 for C	
AD	A* > D	A* > D	.63 mixed 0 for A .56 for D	.69 for mix .17 for A .60 for D	
ABC	B* > C* > A*	A* ≃ B* ≃ C*	.31 mixed 0 for A .09 for B .35 for C	.40 mixed .17 for A .35 for B .37 for C	small floc formed after 24 hours in GMM + P
ABD	B* > A* $\gtrsim$ D*	D* > A $\gtrsim$ B	.32 mixed 0 for A .09 for B .56 for D	.45 mixed .17 for A .35 for B .60 for D	large floc formed after 24-36 hours in GMM + P
ACD	D* > A* > C	A* > C > D†	.39 mixed 0 for A .35 for C .56 for D	.38 mixed .17 for A .37 for C .60 for D	
ABCD	A* > B* > D* ≃ C*	A* > B* $\gtrsim$ C ≃ D	.33 mixed 0 for A .09 for B .35 for C .56 for D	.41 mixed .17 for A .35 for B .37 for C .60 for D	slight small floc after 36 hours in GMM + P
BC	B* > C	B* ≃ C*	.33 mixed .09 for B .35 for C	.35 mixed .35 for B .37 for C	slight sidewall adherence in GMM after 24 hours - easily shaken off
BD	B* > D*	D* > B	.41 mixed .09 for B .56 for D	.51 mixed .35 for B .60 for D	sidewall adherence after 24 hours in GMM + P
BCD	B* > D $\gtrsim$ C	B* ≃ C* > D†	.36 mixed .09 for B .35 for C .56 for D	.42 mixed .35 for B .37 for C .60 for D	sidewall adherence after 24 hours in GMM + P
CD	C* > D†	C* > D†	.39 mixed .35 for C .56 for D	.37 mixed .37 for C .60 for D	

*Stimulated †Repressed

that only those mixtures which contained culture B experienced either of these conditions.

3. Mixed culture continuous flow studies

All continuous flow studies were performed in the same type 1 liter reactor as had been used for the bubble aeration batch studies with the pure cultures. Certain modifications were made to allow for the addition of substrate and the removal of effluent and to facilitate sampling directly from the reactor rather than collecting effluent. These modifications have been illustrated and discussed in Chapter III. In all experiments a detention time of 8.0 hours (D = .125 $hour^{-1}$) was employed and a constant 25^{o} C temperature was maintained. Two reactors were operated simultaneously and were inoculated identically. One reactor received minimal medium containing approximately 500 mg/l glucose (GMM) while the other reactor received a feed of approximately 500 mg/l GMM supplemented with approximately 250 mg/l Bacto-peptone (GMM + P). These concentrations were employed in the shaken flask growth studies and were found to be sufficiently high to permit the maximum growth rate (or to closely approximate it) for each pure culture. This is not meant to imply that substrate concentration will control the growth rate during the continuous flow operation. Under steady-state conditions for continuous flow, $\mu = D$ if no other limits are imposed. D = 1/detention time. Also, it was not considered to be advantageous to achieve a high concentration of biological solids as this might result in difficulty in performing optical density and viable population determinations should flocculation or adherence to the reactor wall occur.

Sampling was performed at intervals of approximately 8 hours and occasionally more often (especially for viable population determinations). Analyses were performed to determine the viable population, optical density, biological solids, and pH. The filtrate was analyzed for total COD, glucose (by Glucostat and adjusted to glucose COD), and carbohydrate (anthrone).

The selection of the mixed populations to be studied under continuous flow conditions was based on observations made during the mixed population batch studies. While none of the cultures, when grown individually, produced flocculent growth or showed a tendency to adhere to the reactor wall, certain mixed populations did exhibit these properties (see Table XIV). Since such properties were considered to be potentially detrimental to the measurement of viable populations, these mixtures were generally considered as unsatisfactory for further investigation. It was noted that only those mixtures which contained culture B exhibited the undesirable types of growth. Therefore, it would have been desirable to minimize the use of culture B; however, because of the apparent stimulatory effect culture B had on the growth of culture A and the desirability of observing its response with culture C it was felt that further investigation was warranted. It should be noted that when the mixed populations containing culture B were cultivated in the continuous flow reactor, a well-dispersed growth resulted with no noticeable adherence to the reactor wall.

The following mixed populations were chosen for study under continuous cultivation: AB, BC, CD, and ABCD. Combination AB was selected because of the aforementioned mutual benefit of A and B when grown together, especially in GMM where a more than three-fold increase

in μ was observed for the mixture over μ for culture B. Mixed population BC was selected because of the dissimilarity of growth rates as pure cultures in GMM and the similarity of growth rates as pure cultures in GMM + P. From the use of pure culture μ values, one might reasonably predict a predominance of organism C in GMM and a near balance in the relative populations of B and C in GMM + P. As will be seen, the latter prediction would have been erroneous even when, by accident, in one experiment, organism B contaminated the substrate in the reservoir and was continually pumped into the reactor. While this markedly increased the population of organism B in the reactor its population still was less than one half that of organism C.

Mixture CD was selected because of the apparent stimulation of organism C and inhibition of organism D. Mixture ABCD was used to allow comparison of the observations made on the pure cultures, the two-culture mixed systems and the same four-culture batch system with the four-culture continuous cultivation system.

Other mixed populations were not studied under continuous cultivation conditions either because of the anticipated nature of the growth (flocculent or adherent) or the probability that the study would add little information to that gained from studies of the selected culture combinations.

At the initiation of the studies, it was anticipated that by allowing the reactors to come to equilibrium, or steady-state, then exchanging the feed lines from the reservoirs to the reactors (GMM reservoir to GMM + P reactor and GMM + P reservoir to GMM reactor) a change in the relative populations might be induced and the effect upon the substrate and COD removal could be observed. However, after

noting that true steady-state conditions were usually not achieved, the substrate exchange was abandoned in favor of a longer period of study with the same substrate. Upon exhaustion of the substrate supply in the reservoir (about 16 to 17 detention times) the pumping of substrate ceased, the feed lines were clamped, and the reactor contents were allowed to "endogenate" for approximately 48 hours while periodic sampling continued.

a. Mixed population AB

The results of the first study on the mixed population AB are shown in Figure 21. This study is one of those in which the feed lines from the reservoirs to the reactors were exchanged. Since it was anticipated that organism B would predominate, a larger inoculum (approximately 2X) of culture A was used to insure an initial numerical advantage for organism A. Following the initial inoculation both units were operated as batch reactors for 18 hours to establish the growth of the populations before pumping commenced. This period of batch operation was deemed desirable to prevent the initial dilution of organisms A and B from the GMM unit since the dilution rate ($D = .125\ hr^{-1}$) exceeded the pure culture (shaken flask) growth rates of each culture ($\mu_A = 0$, $\mu_B = .09$).

Operational difficulties were initially experienced in establishing the desired flow rate, therefore, some early sampling periods were passed over with only optical density and viable count determinations having been made.

Observation of the viable population data at the end of the initial batch operation indicated that culture A in the GMM unit had benefited from its association with culture B by approximately a

Figure 21. Continuous cultivation of mixed cultures A and B.
$D = .125 \text{ hours}^{-1}$ Temperature = 25^{o} C

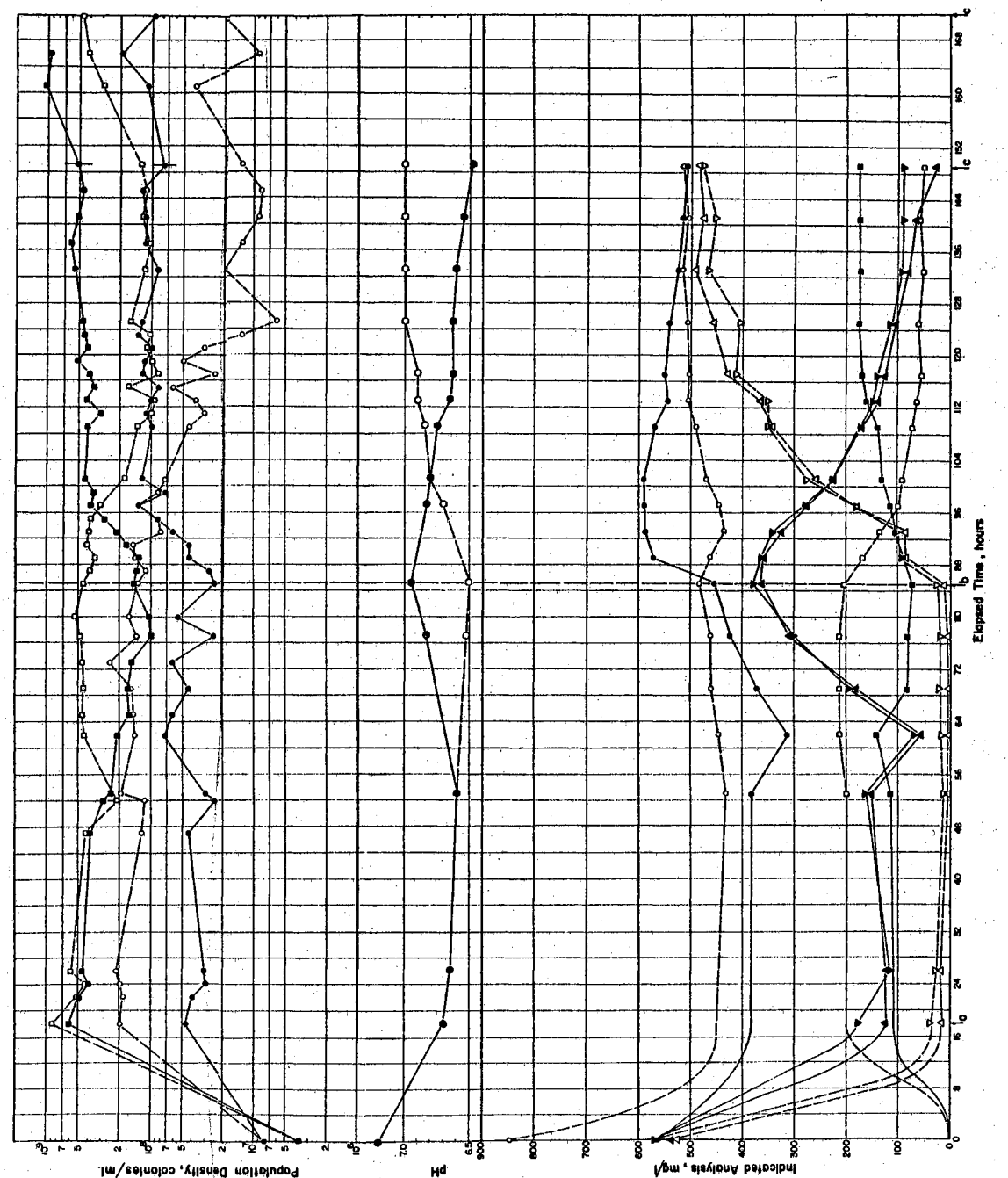
Legend:

	GMM Unit	GMM + P Unit
Total COD	●	○
Glucose COD	▲	△
Carbohydrate	▼	▽
Biological solids	■	□
pH	⬢	⬡
Viable count		
A	●	○
B	■	□

a = start of continuous flow operation

b = substrate exchanged

c = influent shut off


Population Density, colonies/ml.
pH
Indicated Analysis, mg/l
Elapsed Time, hours

six-fold increase in its population. This was significant in view of the fact that culture A exhibited no growth as a pure culture in GMM. After the flow was established, the population density of culture A in GMM became relatively stable (approximately 5 x 10^7 cells per/ml) until the change in substrate occurred. After receiving the GMM + P substrate the population of A increased to a relatively stable population of 1 x 10^8 cells/ml. Little change in this population occurred in the 24 hour period which followed the exhaustion of the substrate supply in the GMM + P reservoir (c).

Culture B may also be seen to have benefited from its association with culture A during the batch operation in GMM when its population increased approximately 160-fold. Such population increases appear to substantiate the increase in growth rate of the mixed population compared to pure culture growth rates of A and B obtained in the shaken flask studies. After flow was established the population density of organism B declined from its peak (approximately 6 x 10^8 cells/ml) at the end of the batch operation until it reached its minimal population (approximately 1 x 10^8 cells/ml) prior to the substrate change. After receiving GMM + P, the population of organism B gradually rose to a relatively stable value (approximately 4.5 x 10^8 cells/ml). During the 24 hour period of operation as a batch unit after the feed was shut off (c) the population of culture B again was seen to increase (to approximately 1 x 10^9 cells/ml).

In the reactor which initially had the GMM + P substrate the growth of both cultures A and B was more rapid than in the GMM reactor. This was expected from the previous results which indicated an increased growth response was experienced by each culture in the

presence of peptone. Culture A was seen to have experienced the greatest growth benefit from this association in the GMM + P medium as its population was found to become relatively stable at a level only slightly below that of culture B (approximately 1.5×10^8 cells/ml for culture A and approximately 4.5×10^8 cells/ml for culture B). When this nearly stable population began to receive the GMM medium, upon the exchange of substrate feed lines, the population of organism A experienced approximately a 10-fold decrease to about 1×10^7 cells/ml. Culture B decreased about 4-fold to approximately 1×10^8 cells/ml which was the population of organism B in the original GMM reactor. After the feed was shut off, ($\uparrow$c), the population of culture B gradually rose to a density which approached its former population prior to the substrate change.

From these observations, it appears that the pure culture μ values of A and B may be used to predict the predominant culture in a mixture of the two cultures but not the relative population levels.

During the first phase of the continuous flow operation the analysis of the filtrates revealed a marked difference in the operational behavior of the two systems. Each system received a feed of 530 mg/l (approximately) glucose COD and the GMM + P system's feed also contained 320 mg/l COD as peptone. The original GMM + P reactor had become nearly stable before the substrate exchange. At the time of substrate change almost complete glucose utilization occurred and produced a biological solids concentration of approximately 250 mg/l. The total COD was approximately 460 mg/l. By assuming a minimal use of peptone and the deduction of the small residual glucose COD (approximately 5 mg/l) and the peptone COD (approximately 320 mg/l) from the

total COD, an unspecified intermediate COD of approximately 125 mg/l was calculated. The pH declined from an initial value of 7.2 to about 6.5. When the substrate lines were exchanged so that the GMM medium without peptone was the substrate a severe disruption of the former conditions occurred. The decrease in both culture populations has been previously noted and may be confirmed by the decrease in biological solids (210 mg/l to 50 mg/l). Perhaps more striking was the rise in glucose COD concentration in the mixed liquor filtrate from approximately 10 mg/l COD to about 475 mg/l COD. This indicated that only about 60 mg/l of glucose COD was being utilized. It is quite possible in view of the fact that peptone aids the growth of organisms A and B that some peptone was also utilized before being washed out. This may in part, help to account for the maintenance of the biological solids. The total COD decreased slightly after the substrate exchange then rose about 40 mg/l and became nearly stable at approximately 505 mg/l. The rise in pH during this period is undoubtedly the result of dilution by the influent medium and lowered level of biological activity in the reactor.

In the original GMM reactor glucose utilization was incomplete (residual of approximately 125 to 150 mg/l COD) during the first period of nearly stable conditions. At this time about 230 mg/l COD existed as intermediates or end products of metabolism. The accompanying decrease in pH (7.2 to 6.6) indicated that these products were, at least in part, of an acidic nature. After about 62 hours (total elapsed time) a significant increase in the glucose COD, total COD and pH began to occur and the increase continued to about 365 mg/l glucose COD and 450 mg/l total COD, at which time the substrate feed lines were

exchanged. The rate of increase in total COD was less than the rate of increase for glucose COD. Upon receiving glucose plus peptone, the trend was reversed; the glucose COD steadily declined until the experiment was terminated. The biological solids increased (75 mg/l to 175 mg/l). The initial increase in total COD may be attributed for the most part to the addition of peptone. The increasing differential between glucose COD and total COD (with allowance for peptone COD) and the concomitant decline in pH indicates the release of acidic products into the mixed liquor. At the termination of the experiment (149 hours) approximately 165 mg/l COD existed as intermediates or end products of metabolism which is approximately the same intermediate COD value estimated for the original GMM + P reactor at the time of substrate exchange (assuming minimal peptone utilization in each case). Quite likely some peptone was used and the actual intermediate COD should be increased accordingly.

In order to assess the response of the mixed population AB during the early portion of the study (which was missed in the previous experiment; see hours 26-52 of Figure 21), an additional experiment was conducted without an exchange of substrate. The response of these systems is shown in Figure 22.

For this experiment approximately the same initial density of viable populations were used as in the previous experiment. The period of initial batch operation was shortened from 18 hours to 10 hours to decrease the concentration of acidic intermediates which appeared to accumulate in the reactors during this period. This was accomplished; however, after flow was established, the pH continued to decrease in both reactors until it reached a level (6.65) similar to that formerly

Figure 22. Continuous cultivation of mixed cultures A and B.

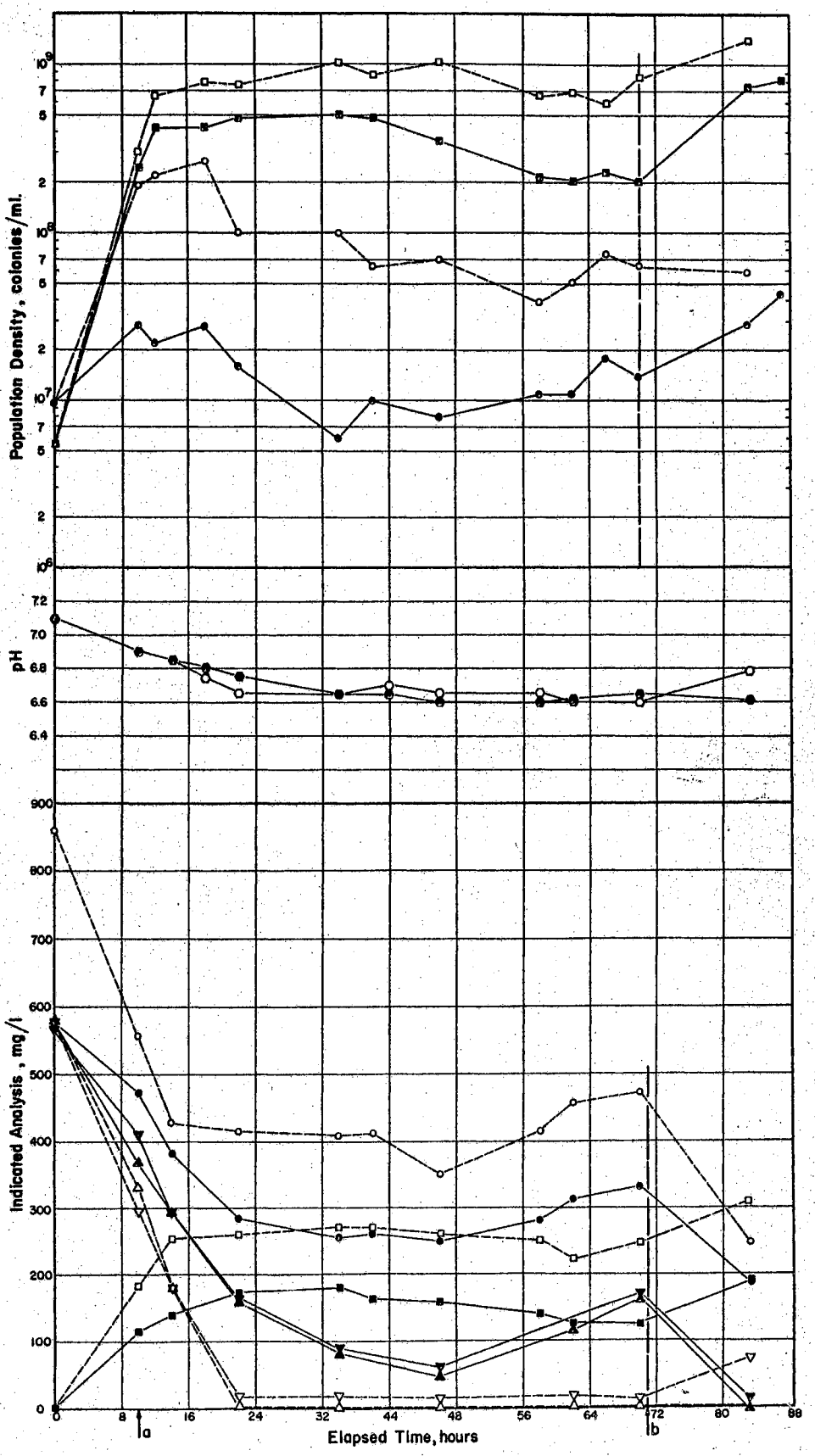
D = .125 hours^{-1} Temperature = 25^{o} C

Legend:

	GMM Unit	GMM + P Unit
Total COD	●	○
Glucose COD	▲	△
Carbohydrate	▼	▽
Biological solids	■	□
pH	⬢	⬡
Viable count		
A	◍	○
B	■	□

a = start of continuous flow operation

b = influent shut off

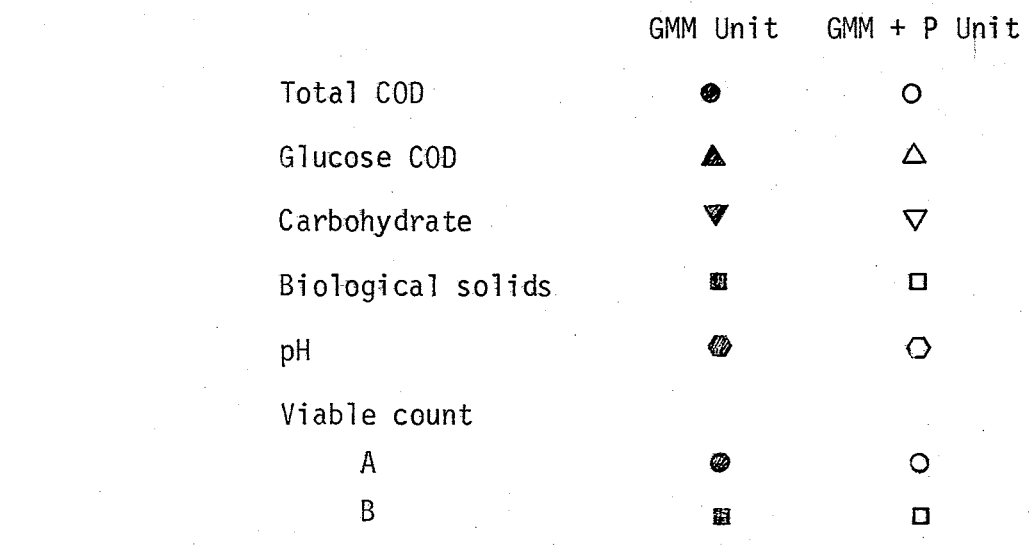

Population Density, colonies/ml.
pH
Indicated Analysis, mg/l
Elapsed Time, hours
a
b

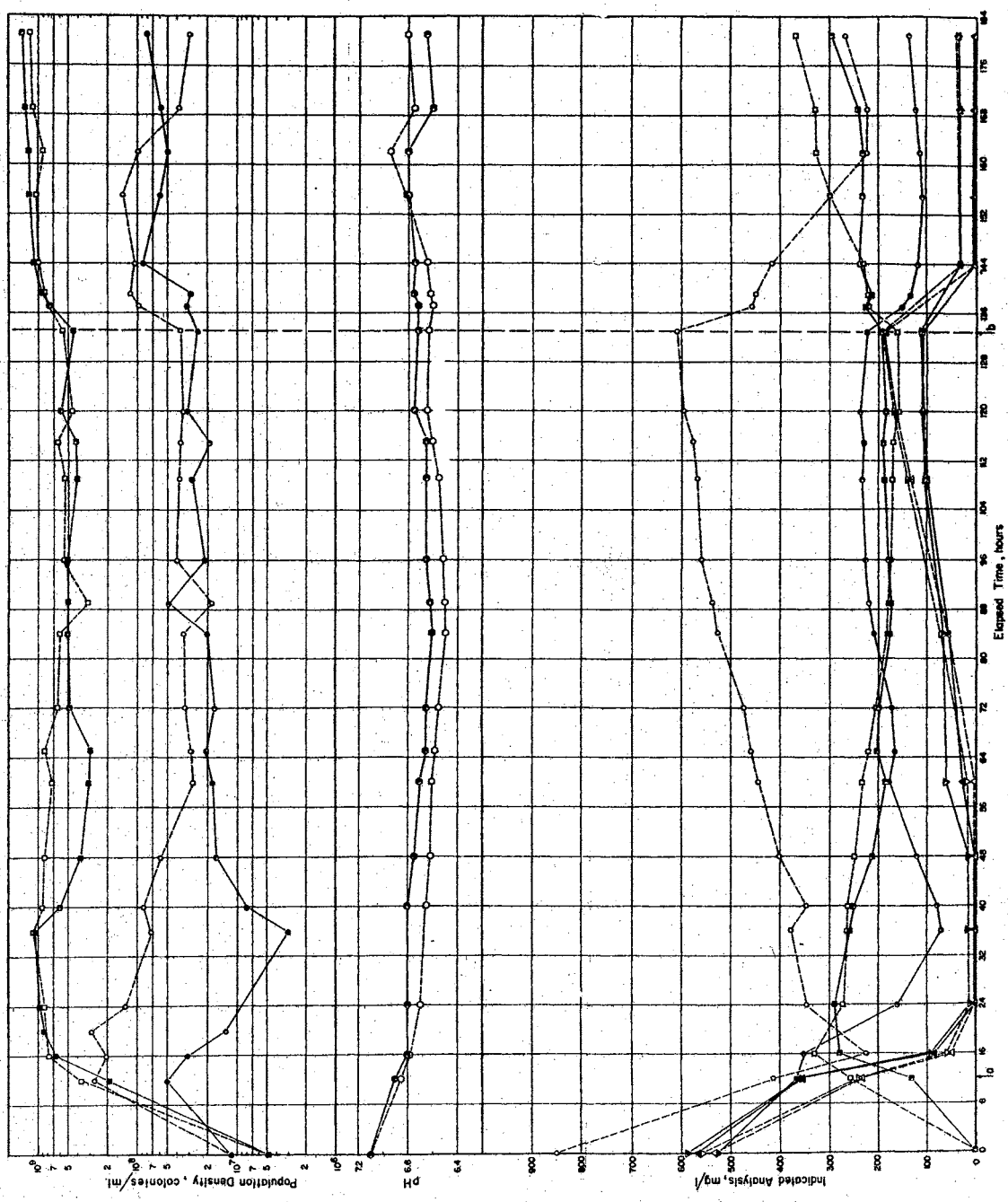
observed (6.60). With the exception of organism A in GMM, all populations continued to increase slightly after the initiation of flow. After approximately 8 hours of continuous cultivation (18 hours total time) some evidence of dilute-out of culture A was noted from both reactors. The population of organism B was relatively stable in each reactor until a decline was observed after about 40 hours in GMM and after about 48 hours in GMM + P. The population decline of organism B and the population level established correspond, in general, with those previously observed. The decline in population occurred at approximately the same time as the occurrence of an increase in total COD in both reactors and an increase in glucose COD in the GMM reactor. Since these declining population densities occurred during the time of lowered pH, it seems reasonable to suggest that organism B may be highly sensitive to changes in pH. The trends observed for total COD, glucose COD, and biological solids were similar to those from the previous experiment. Again the decreased glucose utilization in the GMM reactor was particularly noted.

A third experiment was performed with the mixed population of cultures A and B in which continuous cultivation was maintained through approximately 15 detention periods (8 hour detention) followed by 48 hours of batch operation after the influent was shut off. The results are shown in Figure 23.

The initial population densities were approximately the same as those for the previous experiment. Batch operation was maintained for 12 hours before flow was established. The initial population trends were similar to those observed previously. Nearly stable populations were established in both reactors after continuous flow periods of

Figure 23. Continuous cultivation of mixed cultures A and B.
D = .125 hours^{-1} Temperature = 25^{o} C




Population Density, colonies/ml.
pH
Indicated Analysis, mg/l
Elapsed Time, hours
Tb
To

approximately 48 hours (6 detention periods) for culture A and 60 hours (7.5 detention periods) for culture B. The approximate relative population concentrations of cultures A and B at apparent equilibrium in each reactor were: 1 A:20 B (GMM) and 1A:15 B (GMM + P). As seen from prior batch data culture A benefited from the presence of the peptone; however, it is significant to note that culture A also benefited from the association with culture B in that it was able to establish an equilibrium population in GMM and was not washed out of the reactor as would have been predicted from its pure culture μ value (0 on GMM). A similar statement may be made for culture B whose μ on GMM was 0.09 in shaken flask studies.

When the influent substrate was shut off after 133 hours the populations in each reactor increased temporarily in a manner which indicated the utilization of the residual glucose. After the glucose was removed in the GMM + P reactor there was evidence (increased pH) for the removal of intermediate COD. Some peptone COD may also have been utilized. No significant evidence for the removal of intermediate COD was found in the GMM reactor. The somewhat higher values for the last sample taken from this reactor are thought to be the result of a concentrating effect due to evaporation.

A feature of the substrate removal response in this longer experiment, which was not noted in the earlier runs, was the significant leakage or decreasing utilization of glucose in the GMM + P reactor which followed a period of apparent complete glucose utilization. The glucose was removed from each reactor within 11 hours from the time substrate pumping ceased. Intermediate COD in the GMM reactor increased from about 75 mg/l at 36 hours to 150 mg/l at 60 hours and 175 mg/l at

84 hours then decreased to 150 mg/l at 96 hours, 115 mg/l at 109 hours, and 105 mg/l at the end of the continuous flow period of operation. When flow was stopped to the GMM + P reactor apparent sequential removal of glucose COD and intermediate COD (possibly some peptone COD) occurred with glucose COD removed first. The same apparent intermediate COD concentration existed in the GMM and GMM + P reactors at 36 hours (70 mg/l) and 60 hours (140 mg/l) then became nearly stable at 125 to 150 mg/l. When the influent flow ceased in the GMM reactor the residual glucose was used but no appreciable amount of intermediate COD appeared to have been utilized. Therefore, it appeared that the presence of peptone aided in the utilization of intermediate COD and that peptone may also have been used.

As noted with the two prior experiments on mixed populations of cultures A and B, the pure culture μ values are of questionable value for population prediction for the cultures A and B except to predict the predominance of culture B, which was verified by the experiments. Estimates of the relative populations in the reactors and the response of culture A observed in GMM with culture B could hardly have been accurately predicted from growth rate values alone.

b. Mixed population BC

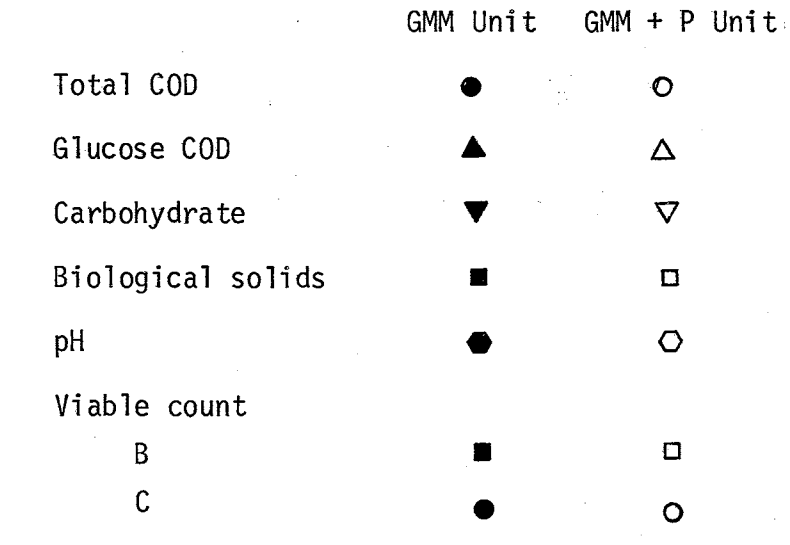
The continuous cultivation of the mixed cultures B and C proved interesting with regard to the prediction of population predominance by pure culture μ values. Based on the pure culture shaken flask μ values (0.09 for organism B and 0.35 for organism C in 500 mg/l GMM; 0.35 for organism B and 0.37 for organism C in 500 mg/l GMM + 250 mg/l P) it might have been predicted that a gross predominance of organism C would occur in GMM and nearly equal populations of cultures B and C

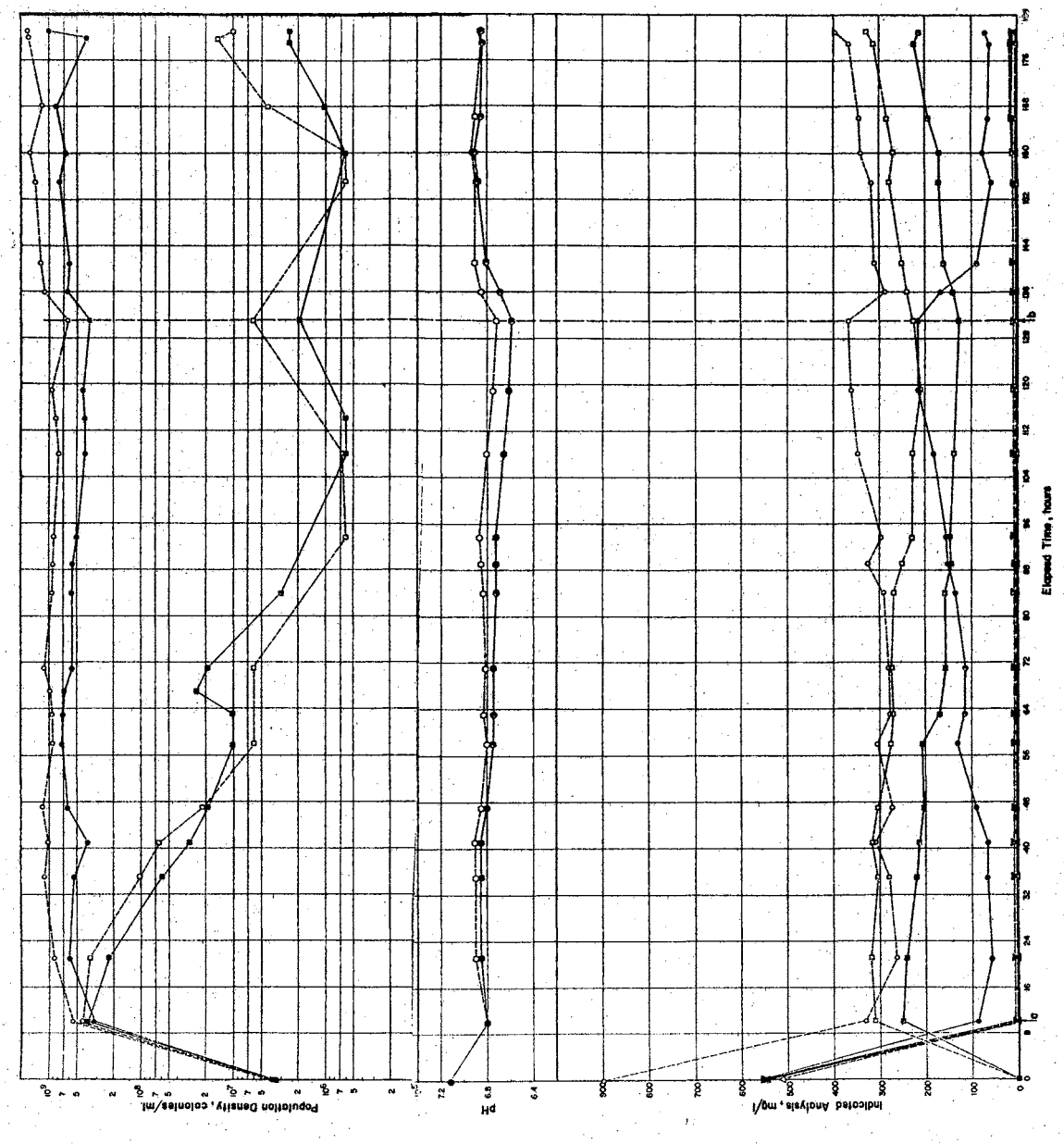
would exist in GMM + P. Values of μ from bubble aeration studies on the pure cultures also would have tended to foster the prediction of the predominance of culture C over culture B in both media, but by a greater margin in GMM + P than predicted from shaken flask μ values.

The results of the experiment are shown in Figure 24. Nearly equal populations of each culture were inoculated into the reactors which were then operated under batch conditions for 10 hours before flow commenced. After the initiation of flow the population of cultures B and C in each reactor exhibited divergent growth characteristics, i.e., culture B exhibited a rather steady decline, whereas, culture C increased slightly until a nearly stable population existed in each reactor, then it experienced a slight gradual decline until the flow of substrate was shut off. In the period of batch operation following the feed shut off culture C responded by apparent utilization of the intermediate COD and increased its viable population in each reactor. The viable count for culture B increased in each reactor after approximately 29 hours of the batch operation had elapsed. This increase of culture B in the batch operation was accompanied by an increase in biological solids concentration; however, no concomitant COD reduction was observed. Therefore, a concentrating effect due to evaporation is strongly suggested as the primary cause of this apparently incongruous observation as approximately 15 and 25 percent of the initial volumes were unaccounted for at the end of 181 hours in the GMM and GMM + P reactors, respectively.

During continuous flow the COD curves showed essentially complete glucose utilization, a slight increase in intermediate COD in the GMM + P reactor after about 94 hours of operation, and a gradual rise

Figure 24. Continuous cultivation of mixed cultures B and C.
D = .125 hours^{-1} Temperature = 25^{o} C



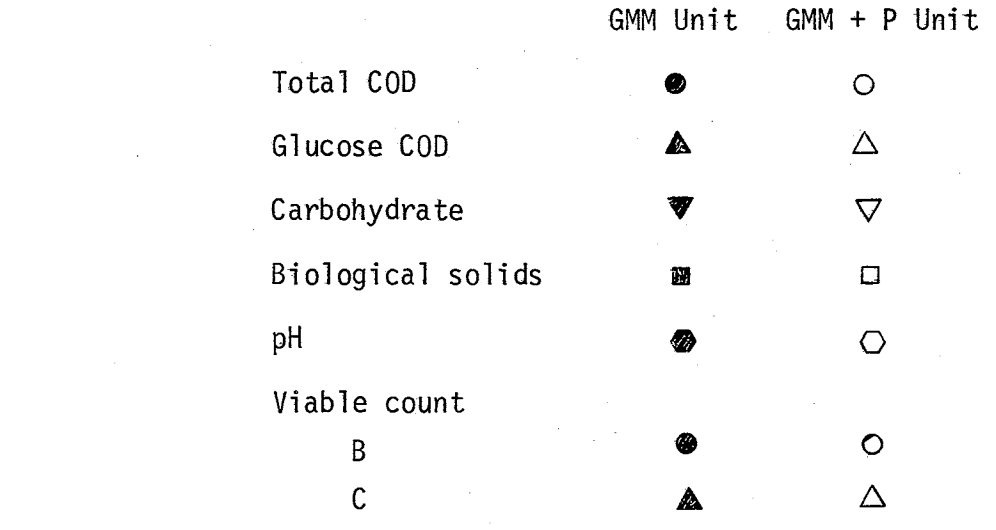

Population Density, colonies/ml.
pH
Indicated Analysis, mg/l
Elapsed Time, hours

in intermediate COD in the GMM unit after about 40 hours. Considering the relative populations of cultures B and C it can be speculated that culture C may be primarily responsible for the production of the intermediate COD and its subsequent utilization. During continuous flow the utilization of the intermediate COD was more readily achieved if peptone was also present in the medium.

The results of a preliminary experiment with the mixed cultures B and C under the same cultivation conditions employed for the experiment shown in Figure 24 were not intended to be presented here because of an apparent "seeding" or reinoculation effect of culture B in the GMM + P reactor, but have been included (Figure 25) to make possible certain comparisons and further substantiate the results shown in Figure 24. By accident a small inoculum of culture B reached the GMM + P reservoir (back contamination) where it grew, and cells were subsequently returned to the reactor with the influent substrate, i.e., a "seeding" or continuous reinoculation of culture B occurred in the GMM + P reactor. Evidence of growth was noted in the reservoir 71 hours after the initial inoculation of the reactor. A viable count made at 88 hours elapsed time indicated the population density of culture B in the GMM + P reservoir was 2.8×10^8 cells/ml. A comparison of the initial and final (139 hour) GMM + P reservoir contents revealed a decrease in total COD of 247 mg/l (848 mg/l to 601 mg/l) and a 245 mg/l decrease in glucose COD (549 mg/l to 304 mg/l).

The principal reasons for the inclusion of this experiment were to show the effect of the continuous inoculation of culture B into the GMM + P reactor and the similarity of results for the GMM reactor to those of Figure 24. Although culture B was initially the predominant

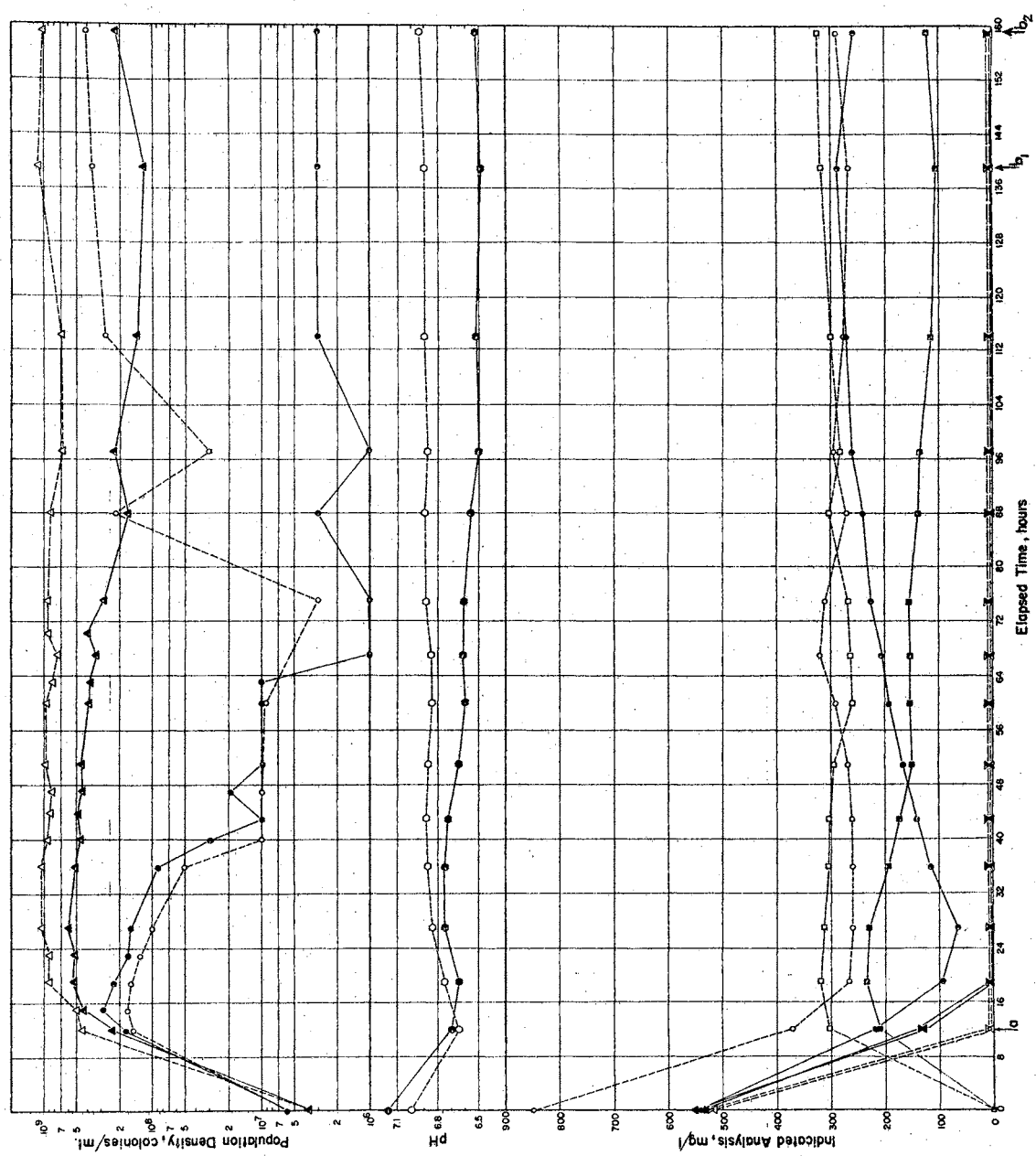
Figure 25. Continuous cultivation of mixed cultures B and C.
D = .125 hours^{-1} Temperature = 25^{o} C



a = start of continuous flow operation

b_1 = exhaustion of feed in GMM + P reservoir

b_2 = exhaustion of feed in GMM reservoir

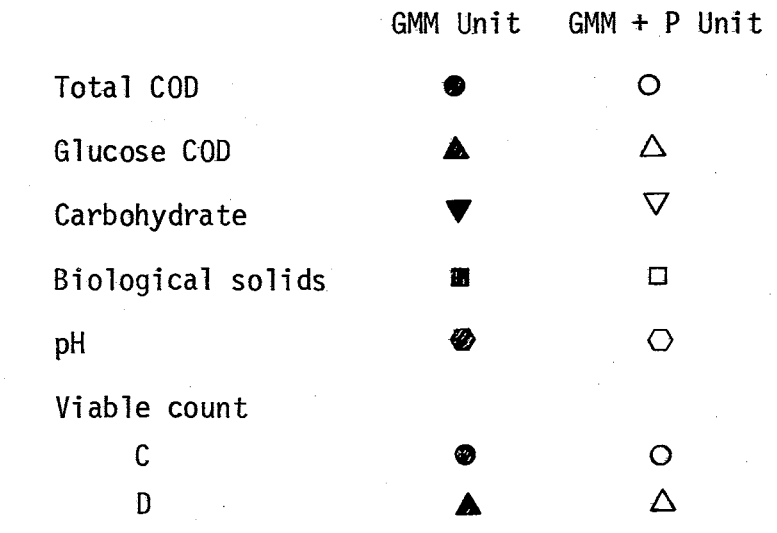

Population Density, colonies/ml.
pH
Indicated Analysis, mg/l
Elapsed Time, hours
t_a
t_{b_1}
t_{b_2}

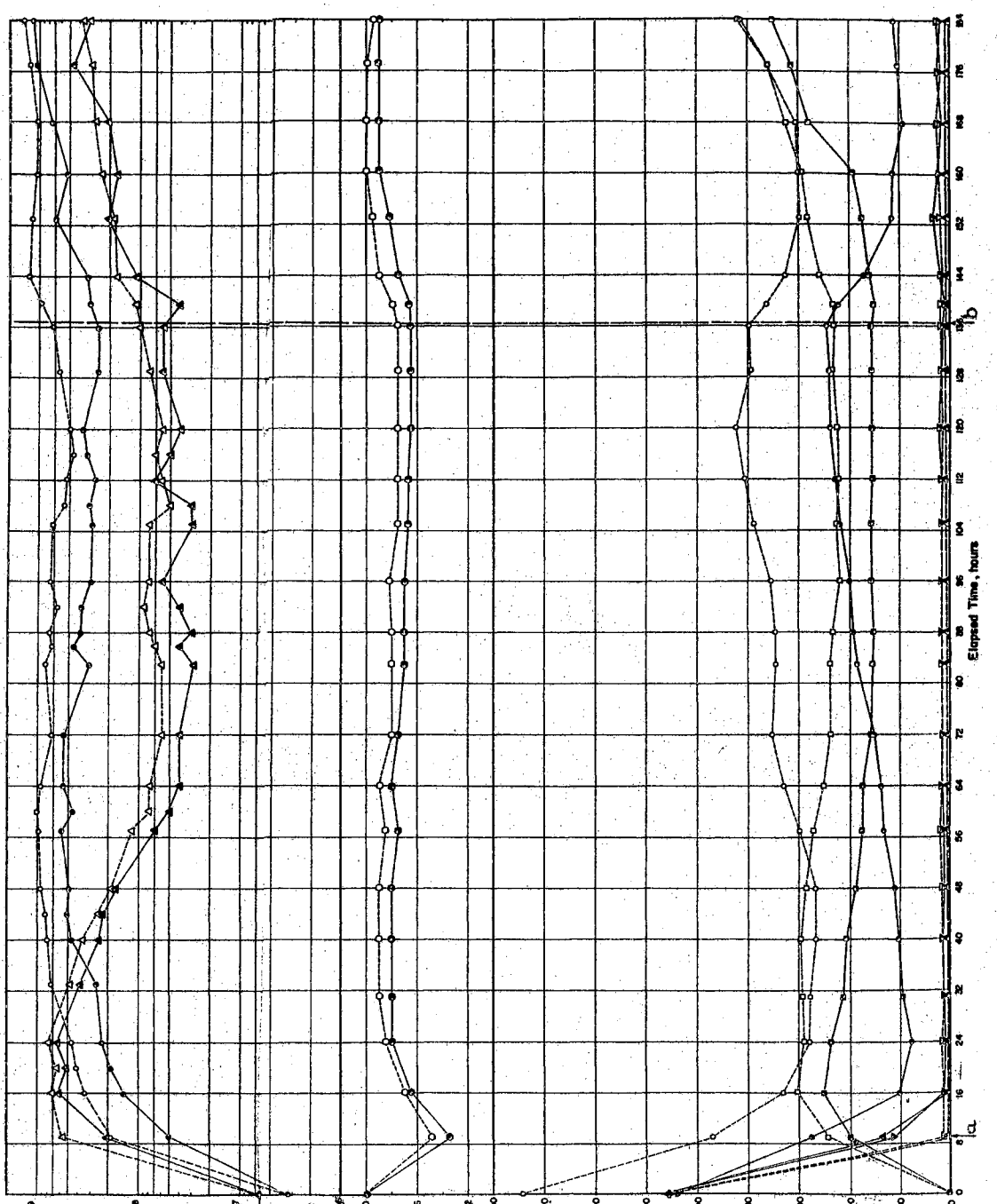
organism in the reactors its predominant position was taken over by culture C at the end of the 12 hour batch operation and was never regained. The observed predominance of culture C would likely have been predicted because its μ value in bubble aeration was greater than μ for culture B; however, if shaken flask values of μ were used a more nearly equal population of cultures B and C would have been predicted. The population density for culture C in the GMM + P reactor was the same in both experiments. The decline in the initial population of culture B was also similar for both experiments. The population of B in the GMM + P reactor increased through continuous reinoculation (see Figure 25) and very small amounts of intermediate products appeared to be present as determined from the intermediate COD calculations and the comparatively high pH.

c. Mixed population CD

The results of the study with the mixed cultures C and D are shown in Figure 26. The initial inoculum of culture D was double that of culture C for the following reason. The results of the shaken flask and bubble aeration studies had indicated conflicting information in regard to the prediction of population predominance by the use of growth rate values. The former indicated culture D as the probable predominant organism (by virtue of its greater μ), and the latter method indicated C possessed the greater μ value, thereby causing it to be favored as the probable predominant organism. In general, the author anticipated that culture C would eventually predominate; therefore, it was desired to afford culture D an initial quantitative advantage to be certain of culture C's ability to become the predominant species.

Figure 26. Continuous cultivation of mixed cultures C and D.
$D = .125 \text{ hours}^{-1}$ Temperature = 25^{o} C




Population Density, colonies/ml.
pH
Indicated Analysis, mg/l
Elapsed Time, hours
CL
D

The viable count data (Figure 26) indicated that organism D predominated in both reactors at the end of the initial batch operation period (9 hours) and continued its predominance until hour 29 in the GMM + P medium reactor and hour 36 in the GMM medium reactor at which times culture C became predominant. After 24 hours (elapsed time) culture D began to decline in population in both reactors and continued to decline until about hour 72 when it became nearly stable at a density approximately one tenth its former maximum. From the data shown here, as well as previous batch data, it may be surmised that culture D is chiefly responsible for the initial decrease in pH.

The growth of organism C followed the pattern observed previously in mixed population BC. After culture C attained a predominant status a decrease in its population density occurred which coincided with an increase in intermediate COD and a decrease in pH.

After the substrate flow was discontinued the viable count of both organisms in both reactors increased and there was a corresponding decrease in COD and increase in pH.

In this experiment with mixed cultures C and D, as noted before with organism C in pure culture (Figures 16, 17, and 18) and in the mixed culture of organisms B and C (Figures 24 and 25), a greater quantity of calculated intermediate COD was present in the reactor with the GMM medium than was found when peptone was also present in the medium (assuming minimal peptone utilization). This appeared to be further evidence that culture C either does not produce as large an amount of intermediate products or is better able to utilize such intermediates when peptone is present in the medium. Culture D may be seen to be capable of intermediate utilization (see Figure 20); however,

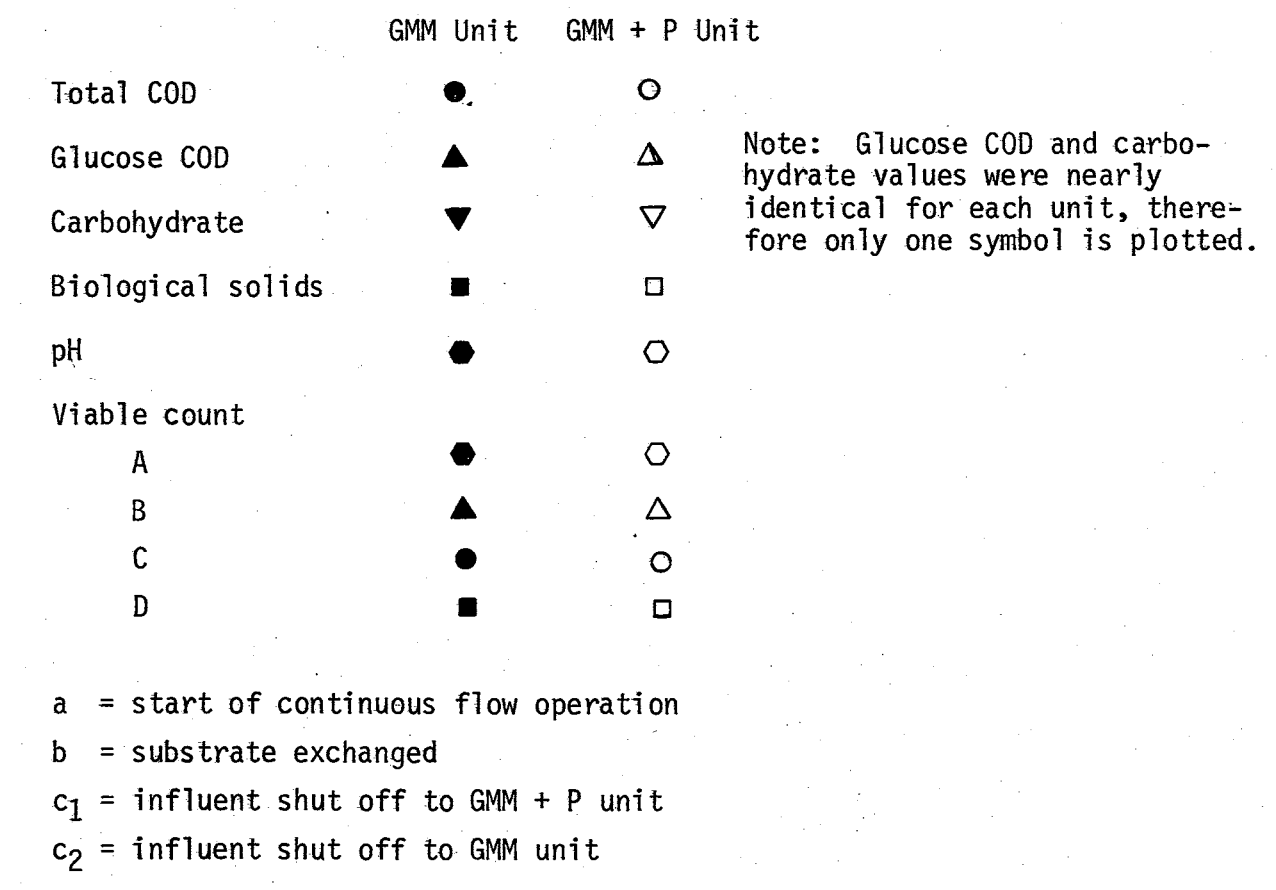
it was the minor population in this experiment and the decrease in its density reflects the increase in total COD.

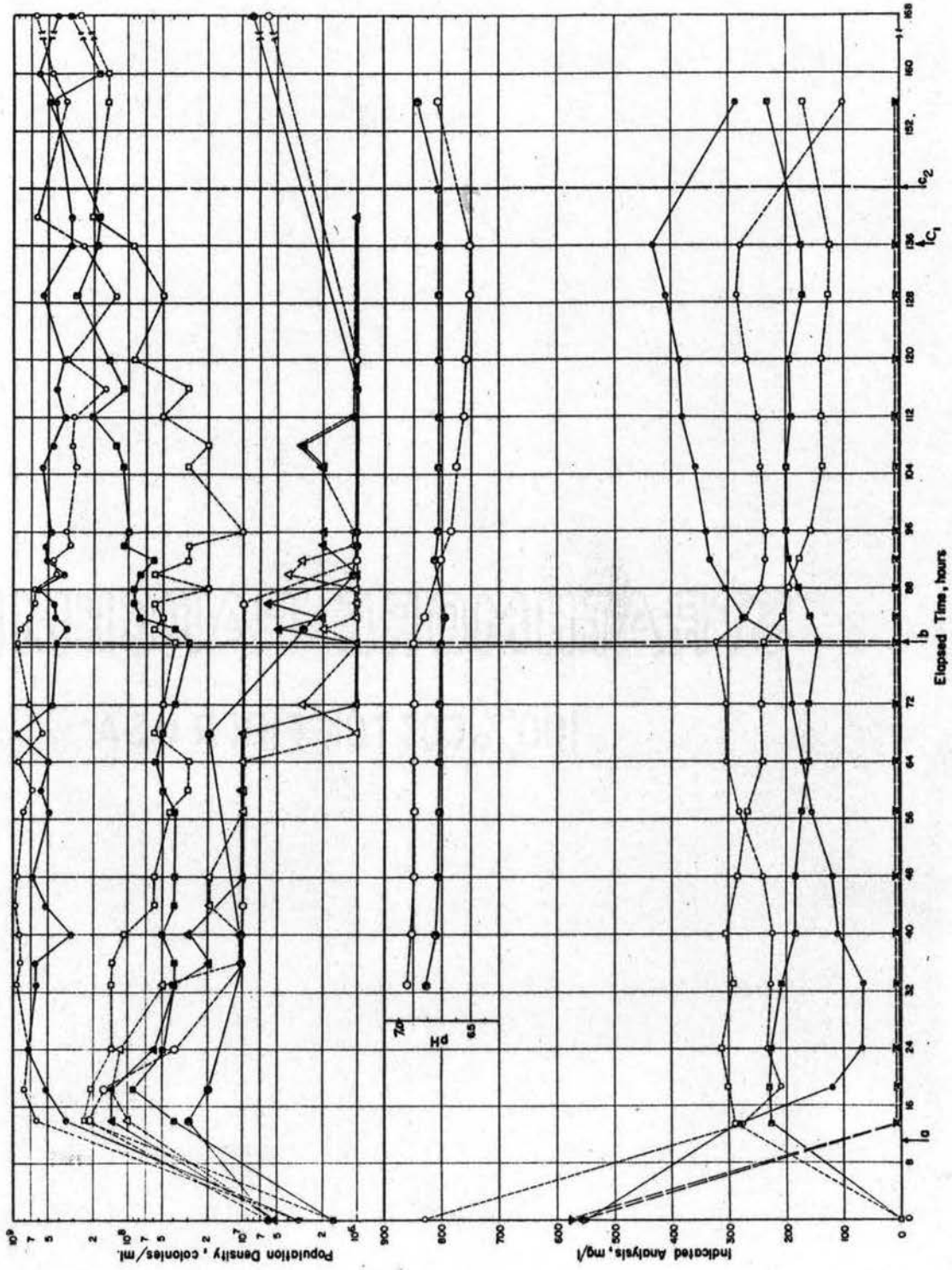
The question concerning the usefulness of employing pure culture growth rate values to predict predominance in this experiment was not wholly resolved. Culture D which was given an initial population density advantage was seen to predominate at the end of the initial period of batch operation and for a relatively short period afterward before culture C became the predominant organism throughout the remainder of the experiment. Some evidence has been presented previously which supports the contention that the growth of organism C may be slowed by low pH (Figure 17). In this instance, the production of acidic intermediates by both cultures C and D may have caused a retardation in the growth of culture C until dilution and/or utilization of the intermediates occurred with a lapse of time during which culture C continued growing and eventually predominated. The cause for culture D's loss of the predominant position, while not fully explainable, does not appear to be a result of adverse pH as an active population of D has been found to exist at a lower pH (see Figure 20) than that which occurred in this experiment.

d. Mixed population ABCD

Figure 27 shows the results obtained when all four cultures were present in the reactors. This experiment was conducted similarly to the experiment with mixed cultures A and B, i.e., the influent medium was exchanged during the run (Figure 21). Culture C had established its predominance in each reactor by the end of the initial batch operation. After the flow was initiated a continued brief increase in the viable count was noted for cultures C and D in the GMM medium and for

Figure 27. Continuous cultivation of mixed cultures A, B, C, and D.
D = .125 hours^{-1} Temperature = 25^{o} C




Population Density, colonies/ml.
pH
Indicated Analysis, mg/l
Elapsed Time, hours
t_0
t_b
t_{c_1}
t_{c_2}

cultures C and B in the GMM + P medium. The other cultures in each reactor experienced a general decrease in population density after continuous cultivation began. The relative population densities began to stabilize after about 4 detention periods (32 hours of continuous flow, 43 hours total elapsed time). The order of culture predominance was C > D > B > A. Late in the experiment culture A was present only in low concentrations. Culture A continued to be present in each reactor, but in its relatively low population it was difficult to detect in sufficient numbers to insure an accurate count for each sample.

Prior to the exchange of influent medium cultures C and D followed patterns similar to that previously observed with culture mixtures BC (Figures 24 and 25) and CD (Figure 26) wherein a slight gradual decrease in the population density was noted. Culture B experienced a very marked population decrease in each reactor which resembled its pattern in the mixed culture BC experiments (Figures 24 and 25). Although the data are sparse, culture A appeared to approach an apparent population density in each reactor similar to that formerly found for culture A in the GMM medium with culture B (Figures 21, 22, and 23). Among the mixture of other cultures in the GMM + P reactor, culture A did not attain as great a density as when mixed with culture B only. Culture A was not found to benefit from the association with the additional cultures over that beneficial effect found with culture B.

After exchange of the influent medium was accomplished the most significant changes in the relative population density involved cultures C and D. In the former GMM reactor, which now received GMM + P influent, the population of C remained nearly constant but culture D gradually increased in population density, thus narrowing the

differential in relative populations by nearly five-fold. In the reactor which began to receive GMM the population of culture C experienced a three-fold decrease, while the density of culture D fluctuated but generally exhibited a tendency to increase, thus closing the population differential from nearly twenty-fold to about three-fold. No great changes occurred in populations A and B in either reactor to indicate the formation of a definite trend immediately following the substrate exchange; however, after hour 112, the density of culture B became so low as to make difficult the determination of its population density. In like manner it became difficult to measure viable count for culture A after hour 116. After the exhaustion of the substrate supply in the reservoirs, the populations of cultures A, C, and D increased, in part due to a concentrating effect due to evaporation.

Analyses for glucose, total COD, pH, and biological solids exhibited trends similar to those previously observed with the other mixed cultures up to the time of medium exchange. Especially comparable was the response of the system consisting of cultures C and D. This result might be expected as these were the dominant cultures in the four-culture mixture. As noted before, essentially complete glucose removal occurred in each reactor throughout the experiment. After a period of temporary stability the total COD increased in each reactor. With allowance for peptone COD (assuming minimal peptone COD was utilized) more intermediates appeared to be produced in the GMM reactor. This is substantiated by the lower pH in the GMM reactor. An adjustment in the level of the total COD followed the exchange of influent medium in part because of the dilute-in and dilute-out of peptone COD in the former GMM and GMM + P reactors, respectively.

CHAPTER V

DISCUSSION

A. Phase 1

1. Development of sewage agar

To provide sufficient substrate for adequate bacterial colony development in an agar base sewage counting medium it was necessary to concentrate the primary effluent from the Stillwater wastewater treatment plant. This concentration process was successfully accomplished by means of flash evaporation. The principal disadvantage with this method was the rather lengthy period of time required to prepare sufficient amounts of concentrate at the desired degree of concentration (approximately 1 hour was required to reduce an initial batch charge to one tenth of its original volume). Flocculent material was formed during evaporation, but it posed no difficulty in colony counting when a surface plating technique was employed. An increase in pH caused by the concentrating process was adjusted by means of a phosphate buffer.

A 30:1 concentration of the primary clarifier effluent was adequate for the development of readily countable colonies. Such concentrates usually possessed a COD in excess of 3000 mg/l (compared with about 8000 mg/l for standard nutrient broth) or about 85 percent of the theoretical COD for the concentrated sewage (based on the COD of the primary clarifier effluent). After being autoclaved for sterilization and incorporation into the counting medium such concentrates

(approximately 30:1) contained about 76 percent of their theoretical COD.

Filtration of the concentrate was not advantageous for the removal of particulate material formed during concentration as some particulate material reformed upon autoclaving the filtered concentrate and the original particulate material did not interfere with the enumeration of colonies on the medium surface. Filtration of the concentrate through 0.45 μ pore size membrane filters reduced the COD to approximately 35 percent of the theoretical value for the concentrate.

The results of the studies on the effect of varying degrees of concentration upon the percent theoretical COD recovered indicated that within the range of concentrations employed (up to about 75:1) the percent theoretical COD recovered in the concentrate may be reasonably estimated at 80 to 90 percent. The average percent theoretical COD recovered for the high range of concentration was only 5 percent lower than the average recovery for the low range of concentration (86.3% to 81.3%, see Table V).

The loss of theoretical COD in the concentrate may be attributed to incomplete recovery of solids which adhered to the walls of the evaporation flask and/or the loss of volatile material during concentration. If volatile materials were lost they were not recovered in the condensate. A check on the concentration technique using a glucose solution indicated essentially complete recovery of the glucose in the concentrate.

2. Comparison of sewage agar with nutrient agar

Serratia marcescens was employed as a test organism to evaluate the comparability of sewage agar and nutrient agar as a counting medium.

It was determined that in order to obtain colonies of sufficient size for ease of detection and differentiation on sewage agar the following criteria should be observed:

a. The sewage should be concentrated sufficiently to possess a COD of at least 3000 mg/l. Weaker substrate may be used for enumeration but differentiation would be difficult because of the limited colony development.

b. The pH of the concentrate should be adjusted to neutral.

c. The addition of supplemental salts to the medium may be necessary for proper colony development and pigmentation.

Statistical evidence indicated that when Serratia marcescens was employed as a test organism there was a statistically significant difference (5 percent level, Studentized t test) in the higher colony counts obtained on sewage agar when prepared according to the noted criteria. However, the use of an inoculum of diluted raw sewage as a test for significant difference in media favored the use of nutrient agar. In addition to the statistical test results in which sewage was used to provide a heterogeneous bacterial inoculum, other considerations were used in the selection of nutrient agar as the medium for the isolation and enumeration of bacteria in sewage. Aside from difficulty anticipated in meeting the requirements for the preparation of the sewage agar using a non-uniform source of supply, other potential difficulties were considered after the failure to obtain growth on one batch of sewage agar. A check of the pH of the medium did not indicate this factor as a possible cause for growth failure. Reinoculation of the plates also failed to produce growth. Therefore, the presence of a toxic material was considered a possible reason for colony growth

failure. Since such unpredictable and unanticipated failures would negate a regular sampling program, it was decided that nutrient agar would be used as the enumeration, differentiation, and isolation medium for sewage bacteria. Lighthart and Oglesby (10) and van Gils (3) have also demonstrated that their preparations of activated sludge extract agar and sewage agar did not produce the maximum number of colonies found when other media were employed. Lighthart and Oglesby chose to use tryptone-glucose-meat and yeast extract with supplemental vitamins (TGEVA) in preference to other examined media including nutrient agar for their study of activated sludge bacteriology. The data which they presented were incomplete for enumeration of bacteria from raw wastewater on TGEVA, but were more complete for nutrient agar. While it is understood that they were not necessarily attempting to select and recommend a medium for the isolation of bacteria from sewage, the data which they have presented show that nutrient agar (Difco) could well be selected for such a use.

B. Phase 2

1. Sewage sampling

Incubation at ambient temperature (approximately 25^{o} C) produced greater population counts and occasionally greater variations in bacterial colonies from sewage inoculum than did incubation at 37^{o} C. The spot-plate technique was found to yield greater population density values from sewage inoculum than the spread-plate technique. This may be due to the retention of some cells on the glass spreading rod.

The temperature of the sewage was found to have a seasonal variation ranging from approximately 16^{o} C (January) to 28^{o} C (August). Considerable variation in the bacterial count data was noted; however,

sufficient data were available to detect the occurrence of greater population densities in afternoon samples and a trend for greater population densities during the seasons of warmer sewage temperature. A range of approximately 30 to 70 colony types per sewage sample was found. Generally more colony types were found in afternoon samples and those taken during warm seasons. The pH of the sewage had only slight variations, with pH values of 7.4 being common.

2. Selection of test bacteria and growth media

Eighteen readily identifiable colony types were noted to occur frequently in the sewage samples and isolates of these colony types were prepared for selection of certain cultures for experimental purposes. By the use of various media and the observation of the growth characteristics of each culture, four organisms and two media were selected. The cultures were readily identifiable in mixed populations and grew in the selected media (glucose minimal medium and glucose minimal medium plus peptone) in a dispersed manner without adherence to the reactor walls.

Of the 18 isolates examined, only 5 were observed to grow well on glucose minimal medium at 25^{o} C. Nearly all isolates were found to grow in varying degrees on nutrient broth or glucose minimal medium supplemented with either yeast extract (Difco) or Bacto-peptone (Difco). The addition of a multi-vitamin supplement to glucose minimal medium did not produce a significant growth increase in those cultures which exhibited no apparent growth or only slight growth in glucose minimal medium. The growth of those cultures which responded poorly to glucose minimal medium seemed to be possibly dependent upon a nitrogen source other than ammonium sulfate and the choice was made to use

Bacto-peptone, a typical analysis of which is given in the Difco Manual (61), as a supplemental nitrogen and carbon source.

C. Phase 3

1. Pure culture batch systems

The growth rate values obtained for the pure cultures in the shaken flask studies seemed well suited for use as a means of predicting the predominant organism in a mixture of the cultures. When glucose minimal medium was employed as the substrate, a relatively wide range of μ_{max} values was found, with each culture possessing a value significantly unlike that of another culture. A wide range of μ_{max} values was also found when glucose minimal medium with Bacto-peptone was employed; however, cultures B and C were found to have nearly similar μ_{max} values. Thus it appeared that if the μ values of these cultures in a given medium could satisfactorily be employed for the prediction of their predominance in mixed culture systems a model set of cultures isolated from sewage had been obtained.

Further investigation of the growth rates of the selected cultures in larger volume reactors with bubble aeration and magnetic stirring revealed that this method of cultivation produced a change in the growth rate of certain cultures. This change was in general an increase in μ values, the degree of which was dependent upon the particular culture and the medium. The growth rate of culture A appeared essentially unaffected (essentially no growth) by the method of cultivation when grown in glucose minimal medium; however, when cultivated in glucose minimal medium with peptone (250 mg/l) by means of bubble aeration the growth rate increased. Therefore it appeared that when some sufficient quantity of peptone was present the growth rate could

become glucose limited under conditions of bubble aeration. The same pattern of growth rate alteration was found to exist for culture B, i.e., essentially no effect upon μ by the different methods of cultivation in glucose minimal medium, but an increase in μ for glucose minimal medium with peptone when bubble aeration was employed.

The cultivation of organism C by the bubble aeration method increased its growth rate in both media. The most significant increase in μ occurred in the medium which contained peptone. The cause for such a notable increase in growth rate with the bubble aeration method was possibly due to an oxygen limitation in the shaken flask experiments. This possibility was investigated with culture C and the use of duplicate incubation conditions except for the shaker oscillation rate. No appreciable difference was found in the growth rates obtained from those flasks shaken at the normal rate and those shaken much more vigorously. The μ values agreed with those previously determined by the shaken flask method for culture C on each medium. Therefore, no definite evidence was found to confirm oxygen limited growth in the shaken flask studies.

Culture D exhibited similar μ values when simultaneously cultivated by each method in glucose minimal medium. A slight beneficial effect was noted with the addition of peptone in the shaken flask experiments. The effect of peptone on μ for culture D when cultivated by the bubble aeration method was investigated and no difference in μ was noted because of the presence of peptone in the medium. However, the values of μ in each reactor were greater than those determined by either the shaken flask method or another bubble aeration experiment which had employed only glucose minimal medium. No valid explanation

can presently be given for this difference in μ values between the separate experiments.

Since such a general difference in μ was found to exist for different methods of cultivation and since the sequences of μ values for the four cultures were found to be dissimilar according to the method of cultivation employed, it would seem that, in order to determine the "proper" or "correct" value of μ for a given purpose, such determinations should be made under conditions similar to those for which the value(s) will actually be employed.

Each value of μ reported in Table XII and in certain other places noted in this work have been validated by at least two, and in many instances three, separate parameters, i.e., optical density, viable count, and biological solids. The values of μ for each culture as found by the separate methods were identical in many instances or agreed within acceptable limits by use of the optical density determination and at least one other analytical growth parameter.

Observations were made of each culture's response when grown in each medium as a pure culture batch system by the bubble aeration method. The results of these studies were later used for comparison with the response of mixed culture continuous flow systems in an attempt to elucidate the role of each culture in the system, or for an observation as to how the response of an individual culture may vary between a pure culture batch system and a mixed culture continuous flow system.

The response of culture A in glucose minimal medium was typical of that previously observed, i.e., essentially no growth occurred. In the medium which also contained peptone the initiation of peptone

utilization preceded significant growth and the utilization of peptone appeared to continue through the period of greatest growth. Some acidic intermediates were produced (acetic acid was detected by gas chromatography). However, the pH did not decrease to such a degree as to be a likely cause for the observed decrease in growth. More likely the decrease in growth was in response to the requirement for peptone or some constituent thereof. This organism was found to grow so slowly under the experimental conditions employed that it would seem unlikely that it would be able to compete well with faster growing organisms in a mixed culture system.

Culture B exhibited an increase in growth rate when peptone was present in the medium. Unlike culture A, however, it did not appear to require the assimilation of a readily measurable amount of peptone before glucose utilization commenced. This observation appeared to be in accord with the findings of the earlier studies for the determination of μ_{max} and K_s where it was seen that culture A possessed a much higher K_s value for peptone than did culture B. Organism B was found to remain viable at pH values as low as 4.7. Acetic acid was among the intermediates produced. The results shown in Figure 15 are particularly interesting when the initial and later responses of culture B are noted in the two media. While the inclusion of peptone in the medium caused an increase in the rate of growth and subsequently a more rapid removal of glucose than that observed in the system which lacked peptone, a comparison of the two systems after 24 hours revealed that the system without peptone had produced nearly the same cell mass (but slightly less viable count) as the system with peptone and appeared to contain a lesser concentration of intermediates (by COD calculation).

The response of culture C as shown in Figure 16 indicated that an initial beneficial effect resulted from the addition of peptone to the glucose minimal medium. The growth of culture C in both media was found to be significantly retarded before the complete utilization of the glucose had occurred. It was noted that although both systems used about the same amount of glucose more cells were produced and lesser amounts of intermediate COD accumulated in the GMM + P reactor. The low pH present in both systems and the presence of considerable amounts of intermediates gave an indication that organism C might be sensitive to the decrease in pH. The results of the experiment with culture C shown in Figure 17 indicate that when the buffer concentration was increased three fold the pH depression was decreased and a slightly more rapid initiation of glucose utilization occurred. Complete glucose removal was obtained in each system (normal buffer and triple buffer concentration) in contrast to the results shown in Figure 16. The probable cause for this is that the initial substrate concentration (in Figure 17) was not sufficient to allow for the production and accumulation of intermediates to such a degree as to produce a detrimental decrease in pH. The system with the greater buffer concentration exhibited a shorter lag, but had the same growth rate as the normal buffer system.

It was observed (Figures 17 and 18) that following the removal of glucose a rise in pH and decrease in intermediate COD occurred with a concomitant increase in viable count. The removal of intermediate COD was more complete in the GMM + P system than in the GMM system. It was noted, however, that the biological solids did not increase significantly following the exhaustion of glucose from the medium. Possibly

this response was due to a temporary storage of carbohydrate and its subsequent utilization for growth. This could explain the apparent paradox of increased viable count without an increase in biological solids in terms of "fat" and "lean" cells. The relatively fast growth rate of organism C coupled with an ability to remove carbohydrate from the environment and store it for later use could serve as a means for the establishment and retention of this culture's predominance in a mixed population in which the limiting carbon source is a carbohydrate.

Culture D produced acidic intermediates to such an extent that the resultant decrease in pH interfered with the organism's ability to continue the utilization of glucose (Figure 19). The inclusion of peptone in the glucose minimal medium resulted in a shortened lag period and a slightly higher glucose removal than for the non-peptone systen before the systems apparently became pH limited. While this culture was found to be capable of a rapid rate of growth and glucose removal, barring pH limitation, the overall efficiency of COD removal was poor because of the resultant high degree of production and accumulation of intermediate COD. Culture D produced nearly equal concentrations of intermediate COD from each medium and within the period of investigation (Figure 19) neither system appeared to utilize the intermediates produced. However, when the glucose concentration in the medium was decreased (Figure 20), thereby decreasing the concentration of accumulated intermediates and lessening the resultant decrease in pH, complete removal of glucose was achieved with a sequential utilization of the intermediate products after a brief lag period.

In summary of the pure culture studies, it was found that although the selected organisms were isolated from a common source their

individual metabolic requirements and responses were widely variable. They possessed a variable requirement for some substance other than the constituents of glucose minimal medium. The constituents, or some particular constituent, of Bacto-peptone were found to satisfy this requirement, at least in part. Possibly the inclusion of some organic nitrogen source (as in peptone) other than the ammonium sulfate nitrogen source in the minimal medium was beneficial for the growth of these organisms and could produce an increase in growth rate and a decrease in intermediate product accumulation. All organisms produced considerable quantities of metabolic intermediates which commonly included acetic acid. When sufficient glucose was present to permit the production and accumulation of these acidic intermediates, the resulting pH decrease could result in a slowing or cessation of glucose utilization. The ability to utilize the intermediates varied with the culture, but was found to be dependent upon the pH of the environment.

2. Mixed culture shaken flask studies

The results of the mixed culture batch studies illustrated the importance of the interactions that may occur among bacteria which may cause their response to differ from that observed in pure cultures. One of the more notable effects which resulted from the combination of cultures was the apparent growth stimulation exhibited by various cultures, especially those which grew most slowly in the pure culture systems. Of special interest was the growth of culture A when combined with other cultures in glucose minimal medium since it was unable to grow in this medium as a pure culture.

The repeated apparent inhibition of culture D by culture C is of particular importance in view of efforts to predict culture predominance

from values of μ in pure cultures; these were the two fastest growing organisms studied. Based on the individual μ values (shaken flask) one would likely have predicted that organism D would predominate; however, the results of the mixed culture studies indicated that because of some interaction in the mixed populations such a prediction would have been erroneous. In fact, later evidence from the continuous flow studies further demonstrated the capability of organism C to predominate over organism D. While the means by which organism C was able to predominate over organism D were not wholly clarified by this study, the evidence found here and in connection with the pure culture experiments tend to support the fact that the mechanism for organism C's predomination can begin while both cultures are in the logarithmic growth phase and may be related to an ability of culture C to assimilate carbohydrate, in a storage form, for later growth. The effect of culture C in those systems containing both cultures C and D was apparent in that the μ of the mixed population system was nearly that for a pure culture of C, i.e., much less than μ for culture D.

It should be understood that the viable count data upon which the discussion of relative population densities is based was taken at the time of initial inoculation and near the end of the logarithmic growth phase for the pure and mixed culture systems. Thus it does not necessarily apply to the prediction of the relative population composition in the stationary or declining growth phase.

The organisms selected for this study did not form flocculent particles or adhere to the reactor walls when grown as pure cultures in shaken flasks. However, when certain of these organisms were combined, such growth characteristics occurred after 24 to 36 hours.

Flocculent or adherent growth was especially prevalent in the mixed cultures which contained organism B. Such growth characteristics apparently occurred only after a period of several hours following the exhaustion or severe depletion of the substrate and were not observed at other times nor during the continuous flow operations.

3. Mixed culture continuous flow studies

The results of the experiments which employed the four mixed culture systems will be discussed on the basis of the findings for each system and then discussed together in view of the common findings that are disclosed.

The continuous flow studies on mixed population AB gave further insight into the interactions that occurred in this system and confirmed certain findings from the mixed population shaken flask studies. Organism B predominated over organism A in each experiment even when A was given an initial numerical advantage. Therefore, it would appear that the respective growth rates determined the predominant organism. As previously observed both organisms were found to have a greater growth response when peptone was included in the glucose minimal medium. Culture A, which was unable to grow as a pure culture in glucose minimal medium (GMM), exhibited an ability to grow nearly as well in association with culture B in GMM as with B in GMM + P. Such a beneficial response from the association could hardly have been predicted from the individual pure culture studies.

The beneficial effects of peptone in the medium for the mixed population AB may be especially noted by the disruption of the systems when the reservoir feed lines were exchanged so as to change the feed to the reactors from one which contained peptone to a no-peptone feed

and vice versa (Figure 21). Also when the units were allowed to "endogenate" after the feed was shut off a greater quantity of intermediate COD was removed from the unit which contained peptone (Figures 22 and 23).

When all of the measured parameters were taken into account, it appeared that a "steady-state" condition was not established during the course of operations except for relatively brief periods and possibly near the end of the continuous flow operation (Figure 23).

Some initial dilute-out of organism A was found to occur in both reactors at the start of flow. Culture B was not initially diluted from the reactor to an extent that its population density in the reactor decreased. The decrease in the population of B which occurred during the experiments appeared to coincide with the decrease in pH (to about 6.6 to 6.8) and the increase in residual glucose COD, particularly in the GMM reactor. Therefore, the pH sensitivity of organism B may be a controlling factor in the determination of the relative populations of A and B and the overall efficiency of COD removal.

The growth response of mixed population BC in the continuous flow studies (Figures 24 and 25) served to illustrate the fallacy of the conclusions which might be made if the results of short term batch studies (shaken flask) were applied to the prediction of results for longer term continuous flow operations. Culture B was unable, initially, to compete for substrate on an equal basis with culture C, especially when the available glucose supply became minimal. On the basis of shaken flask μ values it might have been anticipated that while culture C would decisively predominate in the GMM reactor a nearly equal population of each culture, B and C, would occur in the GMM + P reactor.

If the population predictions were made using the μ values obtained from the bubble aeration studies, results more nearly like those observed in continuous flow might have been made in which organism C predominated even when culture B was continuously inoculated into the reactor along with the substrate (Figure 25).

In the reactor which received peptone with the GMM, less intermediate COD accumulated. Such intermediates as were elaborated in the GMM reactor represent a loss of potential growth and are the cause of a resultant pH less favorable for the organisms. The pH of the mixed liquor does not wholly account for the decrease in the population of B because B was seen to be able to maintain a relatively stable higher population level at even lower pH in the mixed population experiments with culture A.

While culture C was capable of producing a red-brown colored soluble pigment, such pigment production was observed only after colony development for about four days incubation (25^{o} C) on nutrient agar or prolonged growth in liquid glucose minimal media supplemented with more than 1000 mg/l peptone. No pigment coloration from culture C was observed in the continuous flow reactors to suggest that the elaboration of this pigment into the mixed liquor might be related to the decrease in population of organism B or in the following experiment with mixed population CD.

The response of the mixed population CD is another example which illustrates the importance of the method used for the determination of μ if respective μ values are to be used for the attempted prediction of the predominant population. The shaken flask method yielded a greater μ value for culture D in each medium, whereas the bubble aeration

method gave the greater μ for D in GMM and for C in GMM + P. The short term shaken flask study with the CD mixtures had indicated that organism C was able to retard the growth of organism D. An explanation involving an ability of culture C to rapidly assimilate glucose into a storage form for latent growth was proposed. Such a mechanism may have also existed in the system shown in Figure 26 where organism D was able to compete favorably with organism C for glucose only so long as excess glucose was present. When the glucose concentration became minimal, a shift in the predominant population from D to C occurred. The pH of the mixed liquor in the reactors is not a likely cause for the decrease in the population density of organism D in light of D's ability to grow at even lower pH values in the pure culture studies (Figures 19 and 20).

The population density of organism C in each reactor compared favorably with that produced in batch studies on similar substrate concentrations (Figures 17 and 18). The population of organism D became nearly stable at about one thirtieth of its comparative pure culture density (Figure 20). This leads to the speculation that organism C is nearly the sole utilizer of the glucose and that organism D may be able to maintain a viable population in the continuous flow reactors in part due to its ability to utilize certain metabolic intermediates better than organism C.

Of all the systems examined, the mixed population CD most nearly attained "steady-state" operation. All measured parameters except total COD, or more specifically calculated intermediate COD (assuming minimal peptone utilization), became nearly constant after 8 to 10 detention periods. The increase in total COD in the mixed liquor was due to the elaboration of metabolic intermediates which were not completely

utilized by either of the cultures, at least not within the allowed period of acclimation.

When all four cultures were mixed the response was rather similar to that found in the two-culture mixed systems which contained culture C (BC and CD). Culture C was the predominant culture with D in secondary predominance. The populations of cultures A and B appeared to be of minor importance in the total system ecology.

As with mixed population CD, which appeared to approach "steady-state" operation with the exception of the total COD, or more specifically the intermediate COD, the four-culture system appeared to approach "steady-state" operation until the systems were disrupted by the exchange of substrate.

The results of the mixed culture short term batch studies conducted by the shaken flask method could not be used for reliable predictions of the viable population response observed in the continuous flow operation although there was an indication that culture C was apparently the cause of a diminished growth of organism D and might therefore gain predominance.

In summary, certain general findings should be noted. The use of the growth rate value, μ, of individual cultures to predict predominance in mixed populations is subject to certain limitations which may be inherent in the cultivation method employed during the determination of μ as well as the possibility of microbial interactions which may affect the growth rate in some manner not revealed by pure culture observations. Generally, μ values may serve to rank predominance, but not necessarily relative sizes of populations of several cultures in mixed populations.

The use of short term batch studies of mixed cultures conducted in shaken flasks may reveal some indications of the initial response that may occur in longer term continuous flow operations. However, when microbial interactions occur, such batch studies may reveal little concerning the events which transpire as the populations seek to become "established" or approach a "steady-state" operation.

The achievement of a "steady-state" continuous flow operation in which all the measured parameters remained constant for more than a relatively short period was not achieved with any of the mixed populations. Nearly steady conditions were, however, attained in some instances with the exception of the intermediate COD. The time required to approach such "equilibrium" conditions was found to be about 8 to 10 detention periods or longer (8 hour detention time).

Several cases were observed in which apparently complete glucose removal occurred and a later increase in intermediate COD occurred. There were also cases in which an incomplete utilization of glucose occurred and a steady rise in the free glucose concentration was observed in the mixed liquor. Such occurrences seem to bear a relationship to the accumulation of metabolic intermediates and the corresponding decrease in pH. It may be proposed that these conditions may be controlled by a decrease in the detention time as long as the cultures are not excessively diluted from the system. The reasoning for this is that if the detention time is too long an incomplete utilization of glucose may occur because of an accumulation of acidic intermediates which may result in a selective population or limit the growth of the population that might be anticipated to develop from a given quantity of substrate. An attempt at pH control through the use of

increased buffer concentration with culture C in GMM + P (Figure 17) indicated some success at overcoming pH effects.

In review of the optical density data (or percent transmittance as originally determined) which were not included whenever viable count and biological solids data were given, it may be said that the measure of percent transmittance was found to be a rapid and rather sensitive measure of the stability of all systems.

The inclusion of peptone in the glucose minimal medium was beneficial in increasing the growth rate of most organisms and was also found to result in a lesser concentration of metabolic intermediates (especially if a minimal utilization of peptone is assumed).

CHAPTER VI

CONCLUSIONS

1. Sewage which has been concentrated by flash evaporation may be used as a substrate in an agar base bacterial counting medium if supplemental salts and buffer are also incorporated into the medium and toxic materials which may be present in the sewage are not concentrated to a critical level. A 30:1 concentration (approximately 3000 mg/l COD) was satisfactory for both enumeration and differentiation. Enumeration alone could be accomplished on unconcentrated sewage agar (if the sewage was strong enough) after about 5 days incubation at 25^{o} C.
2. Nutrient agar (Difco) may serve as a satisfactory medium for the enumeration, differentiation, and isolation of many types of sewage bacteria.
3. The incubation of plates inoculated with sewage at 25^{o} C yielded higher colony counts than incubation at 37^{o} C.
4. Agar surface inoculation by the spot-plate technique produced higher bacterial population densities than inoculation by the spread-plate technique.
5. The addition of Bacto-peptone as a supplemental nitrogen source (or carbon source) in addition to ammonium sulfate in a minimal medium may increase the growth rate or even be necessary for the growth of many bacteria isolated from sewage. The inclusion of

peptone may also result in a decrease in the elaboration of metabolic intermediates or assist in their utilization.

6. The use of optical density as a means for the determination of the growth rate value, μ, has been validated by the use of simultaneous data for viable count and biological solids to calculate μ values in agreement with those obtained from optical density calculations.

7. For the organisms studied herein it can be concluded that if sufficient carbohydrate resources are present in the medium to support the growth of the selected bacteria, the resultant production and accumulation of acidic intermediates may cause diminished growth of the bacteria. If the pH depression is not severe, certain bacteria may utilize at least a portion of the elaborated intermediates. Generally, the overall COD removal efficiency of the selected organisms was not high because of the production of intermediate COD. The efficiency of COD removal was dependent upon the organism(s), the time of determination (during continuous flow) and the effect of pH upon the organism(s).

8. Pure culture studies do not necessarily serve to predict adequately the behavior of an organism in mixed cultures. Both stimulation and repression of the growth of individual cultures were observed to occur as a result of their association in mixed cultures.

9. The inhibition of the growth of one type of organism by another type may occur while both are in the early phases of growth by means of the suppressor organism limiting the available carbohydrate possibly by accelerated assimilation and storage.

10. The use of the growth rate value, μ, of individual cultures to predict predominance in mixed populations is subject to certain limitations which may be inherent in the method of cultivation employed to determine μ as well as the possibility of microbial interactions which may influence the growth rate in some manner not revealed by pure culture observations. The "proper" or "correct" value of μ should be determined under conditions similar to those for which the value is to be employed. Generally μ values may serve to rank predominance, but not necessarily the relative sizes of populations in mixed culture systems.
11. Short term batch studies of mixed cultures conducted in shaken flasks may provide some indications of the initial response that may occur in longer term continuous flow operations. However, when microbial interactions occur very little insight may be gained into the nature of the system as it approaches stability.
12. The attainment and maintenance of an equilibrium or "steady-state" for all measured parameters in the mixed culture continuous flow systems was not readily developed. Rather slow changes in certain parameters (such as an increase in COD concentration) occurred in most systems. Equilibrium was approached only after about 8 to 10 detention periods or longer.
13. The selection of a proper detention time or buffering system for the bacterial system employed may be beneficial to the minimization of intermediate COD accumulation and the resultant acidic pH which may interfere with the bacterial utilization of substrate and subsequent growth. A three-fold increase in buffer concentration was found to be of some benefit to culture C in a glucose

minimal medium plus peptone batch system, although the initial concentration of substrate did not allow much intermediate production.

CHAPTER VII

SUGGESTIONS FOR FUTURE WORK

1. A study of the response of the pure cultures and mixed cultures (those used in this study and others) under conditions of controlled pH might give further insight into the importance of the pH as a factor in the determination of bacterial predominance.
2. The means by which culture C becomes the dominant population in mixed cultures (especially with other bacteria with rapid growth rates) might be further investigated.

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APPENDIX

CALCULATION OF THEORETICAL DILUTE-OUT CURVE

Procedure for comparison of actual dilute-out with theoretical dilute-out:

1. Fill reactor with 1.0 liter of phenol solution (~1000 mg/l).
2. Dilute phenol out by pumping water into the reactor at a constant rate for a predetermined detention time (e.g., 8 hours) while removing the reactor fluid at the same rate.
3. Periodically collect a sample of the reactor fluid (or effluent) and determine the COD for comparison with the initial COD of the phenol solution.
4. Calculate the theoretical COD remaining for a given elapsed time using the equation:

$$\ln \frac{S}{S_o} = -Dt$$

where: S = the substrate concentration (or COD) at time t

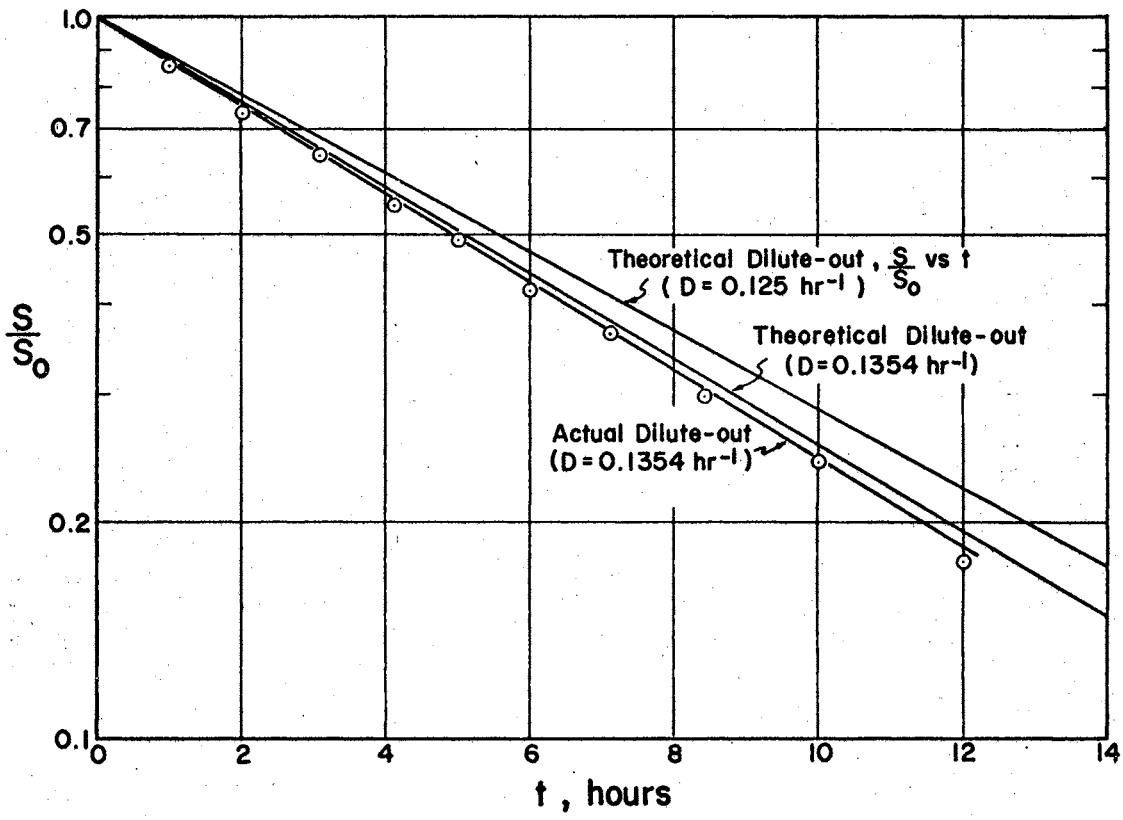
S_o = the initial substrate concentration (or COD), $t = o$

D = the dilution rate, hr^{-1}; $D = \dfrac{1}{\text{detention time (hr)}}$

t = the elapsed time, hours

5. On semi-logarithmic paper plot theoretical $\frac{S}{S_o}$ vs t for the theoretical dilute-out.
6. Determine the actual $\frac{S}{S_o}$ from COD data and plot actual $\frac{S}{S_o}$ vs t on semi-logarithmic paper to compare to the theoretical curve.

Plot of the fraction of original concentration in the reactor vs. time.



Values for theoretical dilute-out using D = .125 hr^{-1} (8 hr. detention)

t	$\ln \frac{S}{S_0}$	$\frac{S}{S_0}$
1	- .125	.8825
2	- .250	.779
4	- .500	.606
6	- .750	.472
8	-1.000	.368
10	-1.250	.2865
12	-1.500	.2235
14	-1.750	.174
16	-2.000	.1355

VITA

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