Comparison of Bacterial Growth in Single and Mixed Populations of Sewage Origin

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INTRODUCTION

Of all the microscopic forms of life directly or indirectly involved in the removal of organic matter from waste waters, bacteria are recognized as the most important. No one specific genus or strain of bacteria is responsible for waste water purification. Usually, large numbers of different strains of bacteria are expected to be present in biological sludges. No attempt is made to control which types of organisms are present in waste treatment reactors, and the "how" and "when" of bacterial predominance changes are largely unknown.

In dealing with heterogeneous populations, massive changes in bacterial predominance are fairly common and have been observed in chemostats operated in the Bioengineering research laboratories at Oklahoma State University. It was felt that if a better understanding of the mechanisms of bacterial interaction were obtained, this knowledge would be helpful in predicting possible predominance changes in reactors such as activated sludge aeration tanks, and might result in improved design procedures and provide insights into more close control of treatment plant efficiency.

The work reported here represents a portion of long-term studies being conducted by us and pertaining to factors which affect changes in bacterial predominance. The results presented herein include a study of the biochemical response and growth kinetics of selected pure cultures and of mixtures of pure cultures.

LITERATURE REVIEW

One of the first investigators to observe and report changes in bacterial predominance was DeBarry (Waksman, 1937). He observed that if two organisms were grown together one would eventually predominate over the other. Various investigators have also reported on beneficial effects of mixed populations in fermentations. Sherman and Shaw (1921) reported that fermentation of lactose to propionic acid would take place faster by the combination of *Streptococcus lacticus* or *Lactobacillus casei* with *Bacterium acidipropionici* than by the single species acting alone. Sanborn (1926) observed that the decomposition of cellulose by *Cellulomonas folia* was aided by the presence of other organisms which would provide essential components.

Savastano and Fawcett (1929) observed the selective effect of temperature on mixed cultures. Fawcett (1931) observed that investigators using pure cultures in plant pathology research were not obtaining satisfactory results; he recommended that the effects of known mixtures of microorganisms and their relation to plant diseases be more actively investigated.

Waksman (1937) listed the following factors as determining the extent of the development of any one group of organisms in natural substrates:

1) food supply, inorganic materials

2) environmental conditions, such as temperature and oxygen supply

3) the presence of other organisms producing toxic or stimulating substances

4) the presence of predators.

In the waste treatment field, activated sludges have been studied and found to be composed of microorganisms including bacteria, molds and protozoa, and metazoa such as rotifers, insect larvae and worms (Rich, 1963). Wattie (1942) classified all floc-forming organisms as members of the species *Zooglea ramigera.* However, Winogradsky (1935) had previously found that the predominant organism in activated sludge was of the genus *Nitrocystis.* Jasewicz and Porges (1956) observed in batch studies that 74% of the bacteria in the assimilative phase were either *Bacillus* or *Bacterium,* while these genera comprised only 8% of the organisms present in the endogenous phase. Rao and Gaudy (1966), in long-term studies with heterogeneous populations, found that the relationship between initial solids concentration and COD removal rate varied for a single substrate, as did the cell yield. These variations were correlated to observed changes in predominance in the experimental units during the period of the study. Jeris and McCarty (1965) suggested that anaerobic digester failures may occur due to a change in predominance of acid-forming bacteria resulting in the accumulation of different substrates for which the appropriate species of methane bacteria are not present.

It can be seen from this brief review that changes in species predominance in waste water treatment processes are of significant interest to the biological waste treatment field.

Materials and Methods

Synthetic waste—The constituents of the synthetic waste used in this study are given in Table I.

Table I. Composition of Synthetic Waste

Constituent	Concentration mg/l
Glucose	100 - 1000
$(NH_4)_2SO_4$	500
$MgSO_4 \cdot 7\ H_2O$	100
$MnSO_4 \cdot H_2O$	10
$FeCl_3 \cdot 6\ H_2O$	0.5
$CaCl_2$	7.5
KH_2PO_4	526
K_2HPO_4	1070

Equipment used in obtaining growth curves—The shaker flasks used for growth curve experiments were of a special design and were fitted to tubes which permitted light transmittance readings at 540 m_μ by inverting and placing the tubes in a spectrophotometer (Bausch & Lomb Spectronic 20). The flasks were shaken at a constant speed of 90 strokes/min in a water bath shaker apparatus operated at a temperature of 25 C.

Viable cell counts—Viable bacterial counts were obtained by the spread-plate surface-counting technique.

Analytical Techniques—Glucose was measured using the Glucostat technique. Biological solids concentrations were determined by the membrane-filter technique (0.45 μ). Oxygen uptake was determined using a Warburg respirometer operated at a shaker rate of 110 oscillations/min and constant temperature of 25 C. This oscillation rate in the Warburg was found to give comparable kinetics to the shaker speed of 90 strokes/min on the constant temperature shaker apparatus used for the determination of growth curves.

Bacterial cultures—The pure cultures of bacteria used in these studies were either isolated from sewage or known to be present in the sewage. These organisms were selected for study because their growth characteristics when plated on an agar surface were such as to allow rapid and accurate identification. The organisms used were as follows:

Pseudomonas aeruginosa

Serratia marcescens

Escherichia coli, K-12

An unidentified organism, hereafter called *Yellow organism*

An unidentified organism, hereafter called *Blue organism.*

Experimental protocol—Investigations were conducted in three phases:

1) growth studies using pure cultures

2) biochemical behavior of pure cultures

3) studies using mixed pure cultures.

Specific aspects of experimental protocol are presented below.

RESULTS

1. *Growth studies of pure cultures*—A series of growth flasks containing five different glucose concentrations were set up in duplicate for each of the pure cultures studied. Light transmittance readings were made throughout the course of growth, and the readings were converted to optical densities and plotted. Growth curves for an experiment using *Pseudomonas aeruginosa* are shown in Figure 1, and in Figure 2 the same data are plotted on semi-logarithmic paper. The straight-line sections of the curves were used to determine the logarithmic growth rate. Growth-rate values are plotted vs. substrate concentration in Figure 3.

Some time ago, Monod (1949) observed that the value of the logarithmic growth rate, μ, is not constant, but depends upon the concentration of the limiting growth metabolite. Based on his experimental results, he developed the following equation:

$$\mu = \mu_{max} S/(k_s + S)$$

where:

μ_{max} = maximum growth rate

S = concentration of limiting metabolite

k_s = saturation constant, numerically equal to the substrate concentration at which the growth rate is $\mu_{max}/2$. Values of μ_{max} and k_s can be obtained as shown in Figure 3.

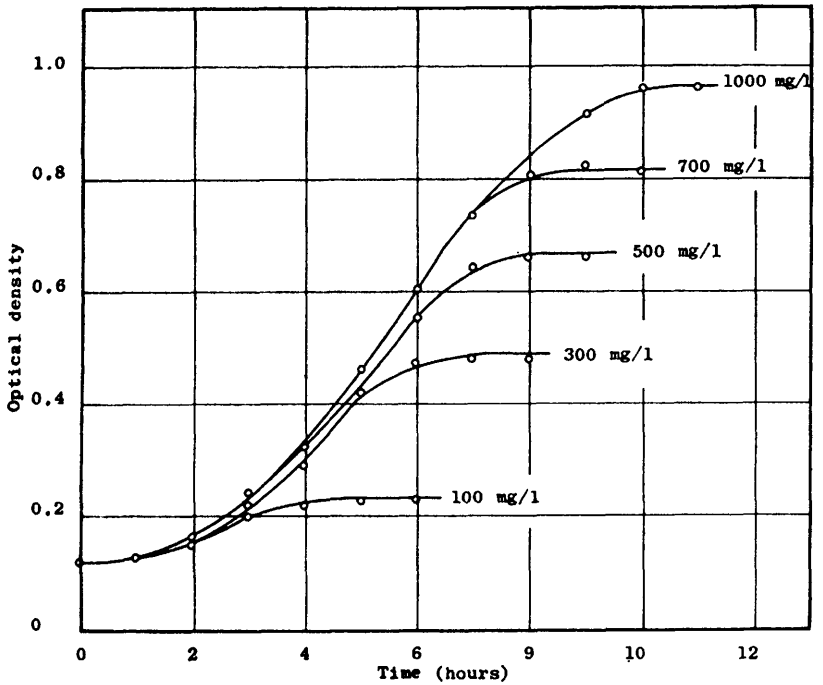

Figure 1. Growth of *Pseudomonas aeruginosa* on various concentrations of glucose

In a similar manner, Figures 4, 5, and 6 show growth curves and a plot of μ vs. S for the *Yellow organism*. This organism is particularly interesting because of the sensitivity of its growth rate to changes in substrate concentration. Values of μ_{max}, k_s, and cell yield for the five organisms studied are shown in Table II.

2. *Biochemical behavior of pure cultures*—In these experiments COD removal, oxygen uptake, glucose removal, and biological solids concentration were used as parameters to assess the biochemical behavior of the cultures during growth. Figures 7 through 11 show the biochemical behavior of the pure cultures under study. From these figures it can be seen that the *Blue organism*, *Pseudomonas aeruginosa*, and *Serratia marcescens* produced considerable amounts of metabolic intermediates and/or end products during the substrate removal period. This may be discerned by comparing the COD removal curve and the glucose COD utilization curve. *Escherichia coli* K-12 also produced intermediates, although at a slower rate than the three previously mentioned organisms. It is seen in Figure 10 that a very limited amount of material was excreted into the medium during metabolism by *Yellow organism*.

3. *Studies using mixtures of pure cultures*—These studies were performed by setting up three different systems at the same substrate concentration; two for each pure culture, and one for the mixed system. COD, glucose, and solids concentration for the two single organism systems were not determined, since the biochemical behavior of the pure cultures already had been shown (Figures 7 through 11).

Escherichia coli K-12 and the *Yellow organism* were found to have relatively low μ_{max} values, and it was desirable to determine the behavior

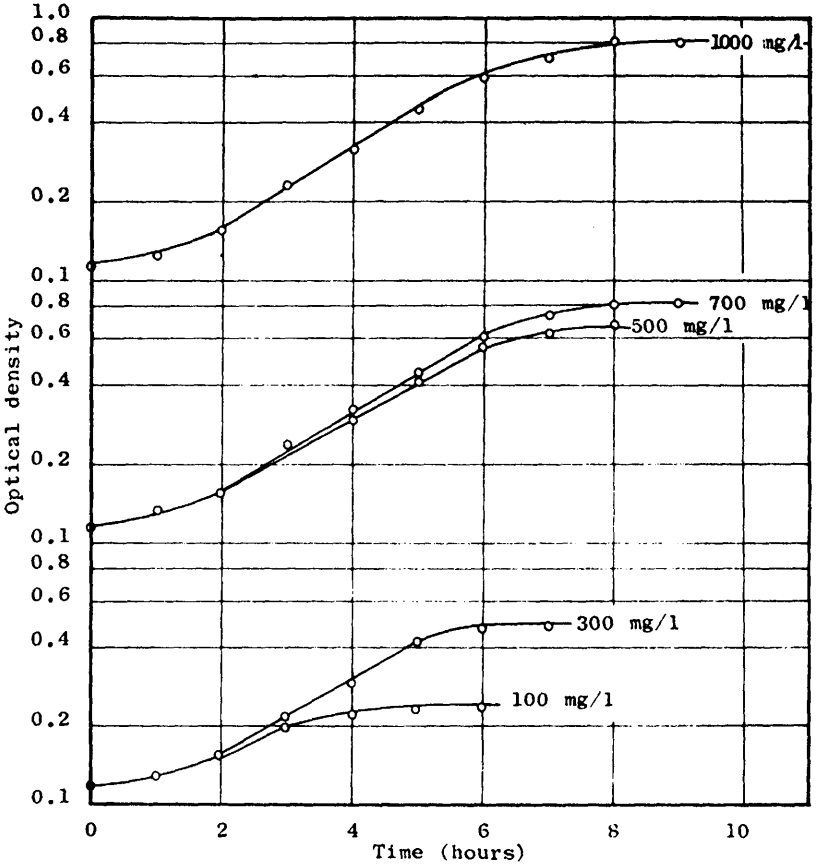

Figure 2. Effects of glucose concentration on growth rate of *Pseudomonas aeruginosa*

TABLE II. KINETIC CONSTANTS AND CELL YIELD FOR SELECTED ORGANISMS

Organism	μ_{max}	k_s	Cell Yield mg Solids/mg Glucose
Blue organism	**0.375**	**22**	**0.697**
Pseudomonas aeruginosa	**0.340**	**40**	**0.482**
Serratia marcescens	**0.290**	**55**	**0.447**
Yellow organism	**0.220**	**230**	**0.473**
***Escherichia coli* K-12**	**0.170**	**20**	**0.424**

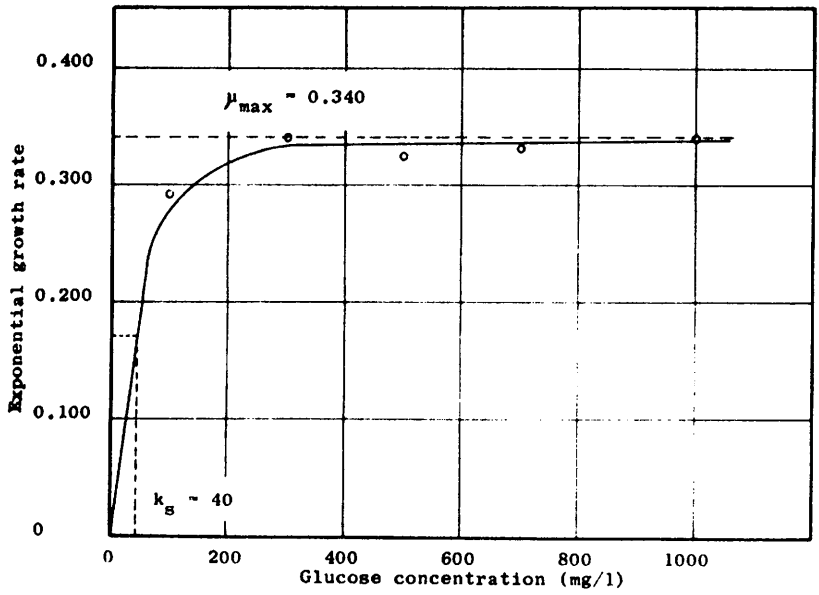

Figure 3. Determination of maximum growth rate (μ_{max}) and saturation constant (k_s) for *Pseudomonas aeruginosa*

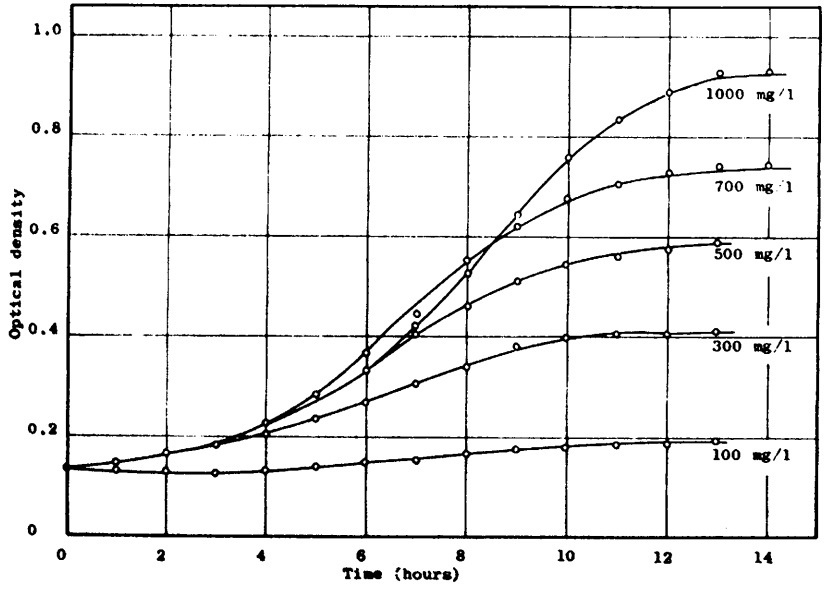

Figure 4. Growth of *Yellow organism* on various concentrations of glucose

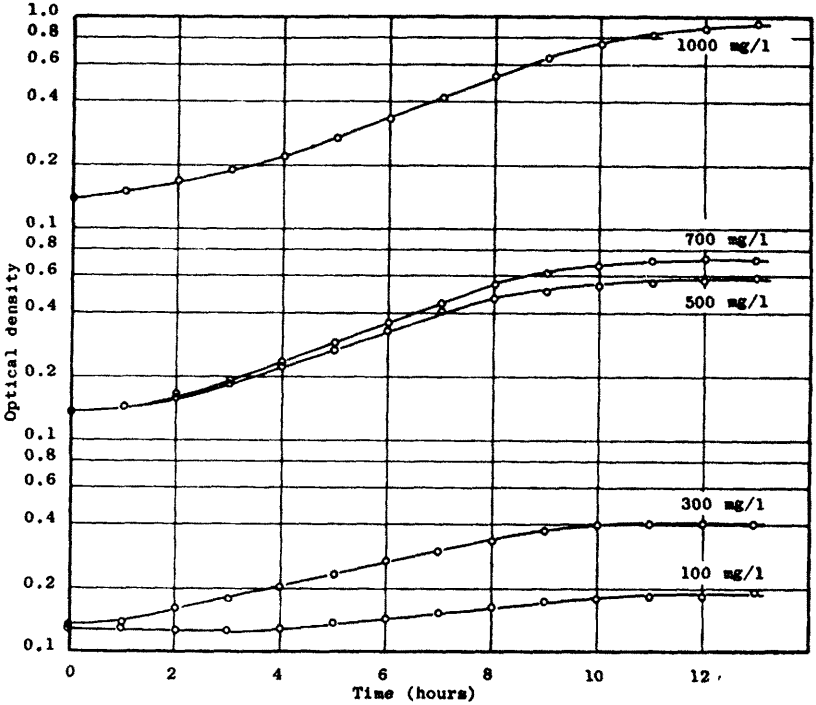

Figure 5. Effects of glucose concentration on growth rate of *yellow organism*

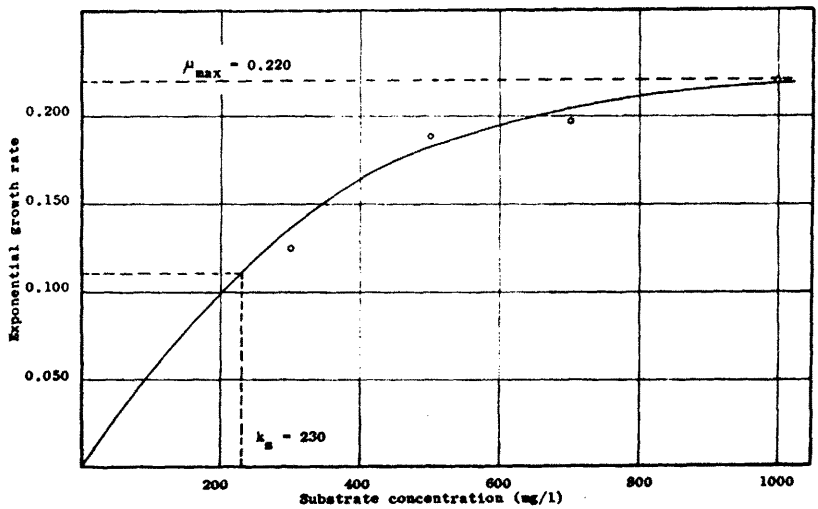

Figure 6. Determination of maximum growth rate (μ_{max}) and saturation constant (k_s) for Yellow organism

of these organisms when grown in combination. Figure 12 shows the biochemical response of the mixed system. A small amount of metabolic intermediates or end products was produced in this experiment, and it is seen that a slightly higher yield was obtained for the mixed system than for the pure cultures alone. For a period of 7½ hr the oxygen uptake of the mixed system was very close to the sum of the oxygen uptake of the pure culture systems. Indeed, the uptake of the mixed system was slightly higher than the sum of the pure cultures (Table III). In Figure 13 the viable growth curves of the mixed cultures are shown. The growth patterns in the mixture were similar to those for each organism alone, and the final viable counts were very close. Additional experiments performed with the same organisms confirmed these results. From the data presented in the figures it may be concluded that there was no antagonistic relationship between these organisms; there was, on the other hand, a slight increase in the system activity resulting from combined growth.

The biochemical response of a mixed system consisting of a fast-growing organism, *Serratia marcescens*, and a slow grower, the *Yellow organism*, is shown in Figure 14. The mixed-culture yield was slightly higher than that for the pure cultures. The oxygen-uptake curve of the mixed system closely resembles that of *Serratia marcescens* alone, and during the initial stages of the experiment the oxygen uptake of the mixed system was very close to the summation of oxygen uptakes for the individual organisms (see Table IV). Viable-count data during these experiments are shown in Figure 15. It is clearly seen that in the mixed system *Serratia marcescens* predominated over the slow-growing organism. It seems apparent that the response is dependent upon the relative growth rates.

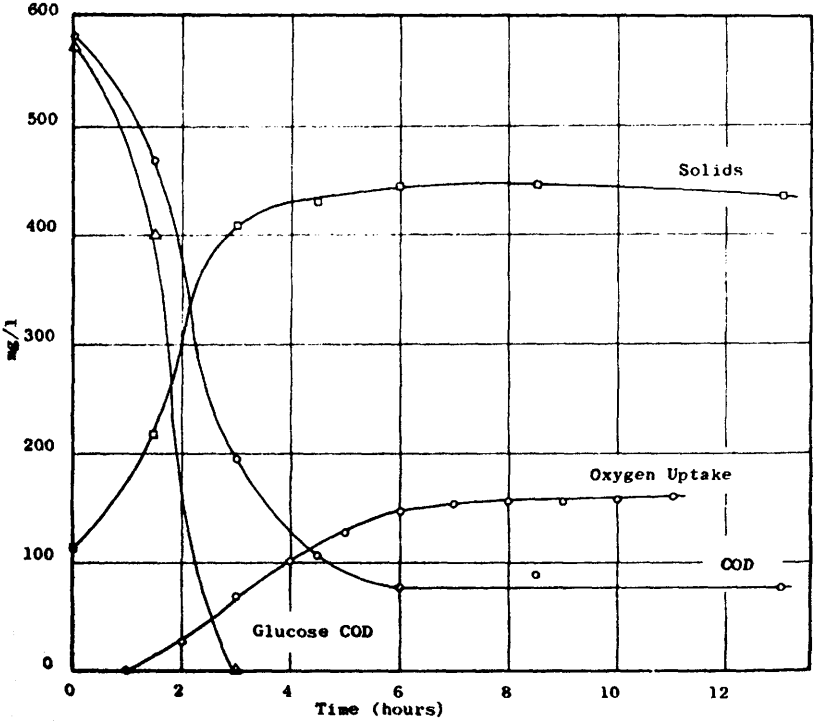

Figure 7. Substrate removal and growth, *Blue organism*

TABLE III. OXYGEN UPTAKE AND VIABLE COUNT FOR *E. coli* AND *Yellow organism* IN PURE CULTURES AND IN MIXED CULTURES

Time	E. coli K-12		Yellow organism		Sum	Mixed System		
	O_2 Uptake	Viable Count	O_2 Uptake	Viable Count	O_2 Uptake	O_2 Uptake	Viable Count	
							E. coli	Yellow organism
0 hr		7.80×10^7		2.23×10^8			5.00×10^7	2.39×10^8
1½	6		6		12	13		
2¼		6.50×10^7		2.33×10^8			1.05×10^8	2.35×10^8
2½	13		14		27	29		
3½	22		26		48	51		
4¼		7.90×10^7		1.75×10^8			9.50×10^7	5.80×10^8
4¾	35		46		81	87		
5½	44		62		106	115		
6¼		1.56×10^8		4.15×10^8			1.80×10^8	6.05×10^8
6½	58		87		145	156		
7½	74		113		187	192		
8½		2.75×10^8		8.85×10^8			2.60×10^8	7.70×10^8
8½	93		140		233	216		
10¼		5.45×10^8		1.04×10^9			5.80×10^8	9.90×10^8

Oxygen Uptake, mg/l
Viable Count, cells/ml

TABLE IV. OXYGEN UPTAKE AND VIABLE COUNT FOR *Serratia marcescens* AND *Yellow organism* IN PURE CULTURES AND IN MIXED CULTURES

Time	Serratia marcescens		Yellow organism		Sum	Mixed System		
	O_2 Uptake	Viable Count	O_2 Uptake	Viable Count	O_2 Uptake	O_2 Uptake	Viable Count S. marcescens	Viable Count Yellow organism
0 hr		2.40×10^{8}		1.09×10^{8}			1.85×10^{8}	9.50×10^{7}
1½	28		8		36	38		
2		2.10×10^{8}		1.73×10^{8}			1.55×10^{8}	1.35×10^{8}
2½	68		17		80	76		
3½	94	5.75×10^{8}	29	1.50×10^{8}	123	107	6.55×10^{8}	4.00×10^{7}
4½	127		45		172	145		
5		1.07×10^{9}		2.35×10^{8}			7.75×10^{8}	4.80×10^{7}
7		8.15×10^{8}		8.35×10^{8}			1.02×10^{9}	1.00×10^{7}
8½	190	1.17×10^{9}	144	9.95×10^{8}	334	203	1.02×10^{9}	6.00×10^{7}

Oxygen Uptake, mg/l
Viable Count, cells/ml

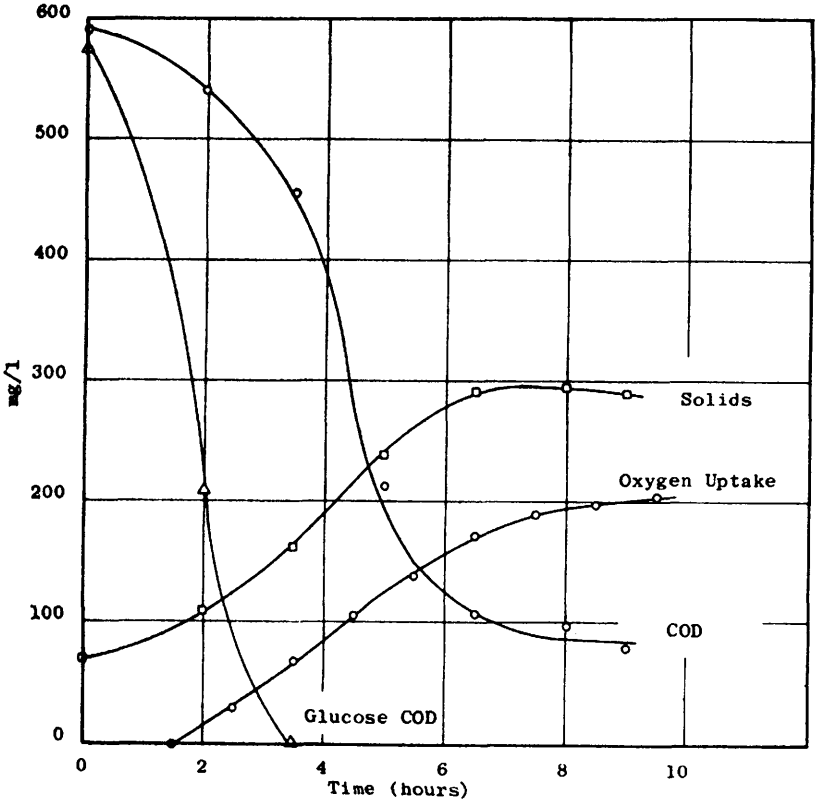

Figure 8. Substrate removal and growth, *Pseudomonas aeruginosa*

Experiments were also conducted using a fast-growing organism *(Serratia marcescens)* in combination with *Escherichia coli* K-12, which exhibited the lowest μ_{max} value of the five microorganisms studied. A small inoculum of *Serratia marcescens* and a fairly large inoculum of *Escherichia coli* were incubated together. Figure 16 shows that *Serratia marcescens* rapidly outgrew *Escherichia coli*. This experiment demonstrates how rapidly changes in predominating species can be brought about. It is also interesting that a marked change in the color of the mixed liquor, i.e., from whitish to red, took place during the experiment. In this case, using pure cultures as in the work of Rao and Gaudy (1966) using heterogeneous populations, the change in the color of the aerating mixed liquor provided definite indication of change in predominance.

Two fast-growing organisms, *Pseudomonas aeruginosa* and *Blue organism*, were combined for experimentation. The biochemical behavior of the system is shown in Figure 17. As expected, substrate removal was extremely rapid. The oxygen uptake of the mixed system is very close to the sum of the uptakes of the pure-culture systems (see Table V). The viable-count data for this experiment are shown in Figure 18. It may be surmised from this experiment that these organisms participated in direct competition for substrate without any apparent antagonistic effects.

TABLE V. OXYGEN UPTAKE AND VIABLE COUNT FOR *Blue organism* AND *Pseudomonas aeruginosa* IN PURE CULTURES AND IN MIXED CULTURES

Time	Blue organism		Pseudomonas aeruginosa		Sum	Mixed System		
	O_2 Uptake	Viable Count	O_2 Uptake	Viable Count	O_2 Uptake	O_2 Uptake	Viable Count	
							Blue organism	Ps. aeruginosa
0 hr		5.05×10^7		7.50×10^7			6.85×10^7	7.60×10^7
1¼	11		10		21	23		
1½		1.52×10^8		1.31×10^8			1.35×10^8	1.50×10^8
2¼	33		28		61	61		
3		2.50×10^8		4.55×10^8			3.60×10^8	4.20×10^8
3¼	70		52		122	113		
4½		5.05×10^8		2.65×10^8			5.25×10^8	7.85×10^8
5¼	128		127		155	195		
6		7.20×10^8		1.23×10^9			5.80×10^8	8.20×10^8
7¼	165		212		377	216		
7½		7.85×10^8		1.74×10^9			7.00×10^8	1.09×10^9

Oxygen Uptake, mg/l
Viable Count, cells/ml

Discussion and Conclusions

The data obtained from the growth experiments with pure cultures were used in the determination of the kinetic growth-constants of each culture by the graphical method shown in Figures 3 and 6. Using μ_{max} as the principal growth parameter, the results of mixed culture experiments show that it is possible to predict the predominating organism when two strains are placed together, assuming that there is an appreciable difference in their respective growth rates. Relating these observations to the biochemical behavior of the pure cultures, it can be seen that the fast-growing organisms metabolize glucose at a high rate, while at the same time introducing large amounts of metabolic intermediates and/or end products into the medium. These intermediates appear to be sequentially metabolized immediately after the glucose is exhausted, indicating the presence of constitutive enzymes in the organisms which are appropriate for the assimilation of the intermediate products. These findings suggest the possibility that these fast-growing species may affect other species through the production of the elaborated products. However, no evidence of antagonistic relationships was found; indeed, no antagonistic relationships of any type were noted. The oxygen uptake data point, however, to a small but noticeable beneficial relationship in the mixed cultures.

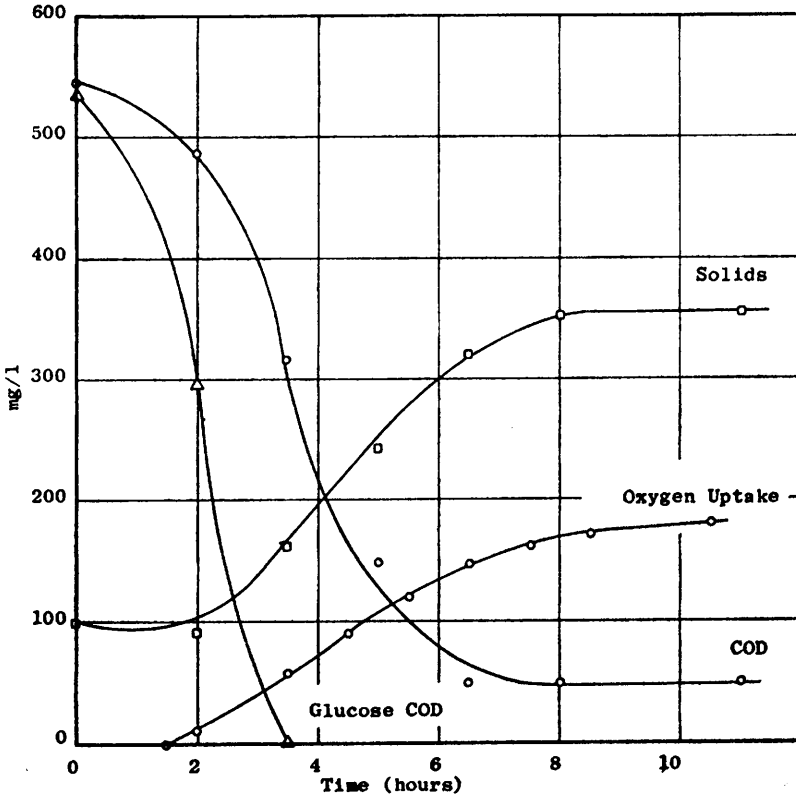

Figure 9. Substrate removal and growth, *Serratia marcescens*

If, as shown in these experiments, the most important factor governing the predominance of one strain over another is simple competition for substrate, then it may be possible to predict predominance in more complicated systems. The present line of investigation is being expanded to include more complicated environments, e.g., more than two species, multi-substrate media, continuous flow conditions, etc. It should be emphasized that changes in predominance in continuous-flow reactors do occur in an, as yet, unpredictable manner; therefore the problem would appear to be more complex than that presented by simple substrate competition. Furthermore, the presence of other types of organisms such as protozoa in activated sludge and their effects on predominance contribute to the general complexity of predicting species predominance and changes in specific population density. Much more work is needed on the mechanisms of species predominance before attempts can be made to control plant operations to select and maintain the most desirable organisms. Also, while much more experimentation is needed in order to draw sound conclusions for less complicated systems, tentative patterns for predicting predominance may be drawn from these simple model systems. For example, it would appear that in these studies, in which there was no evi-

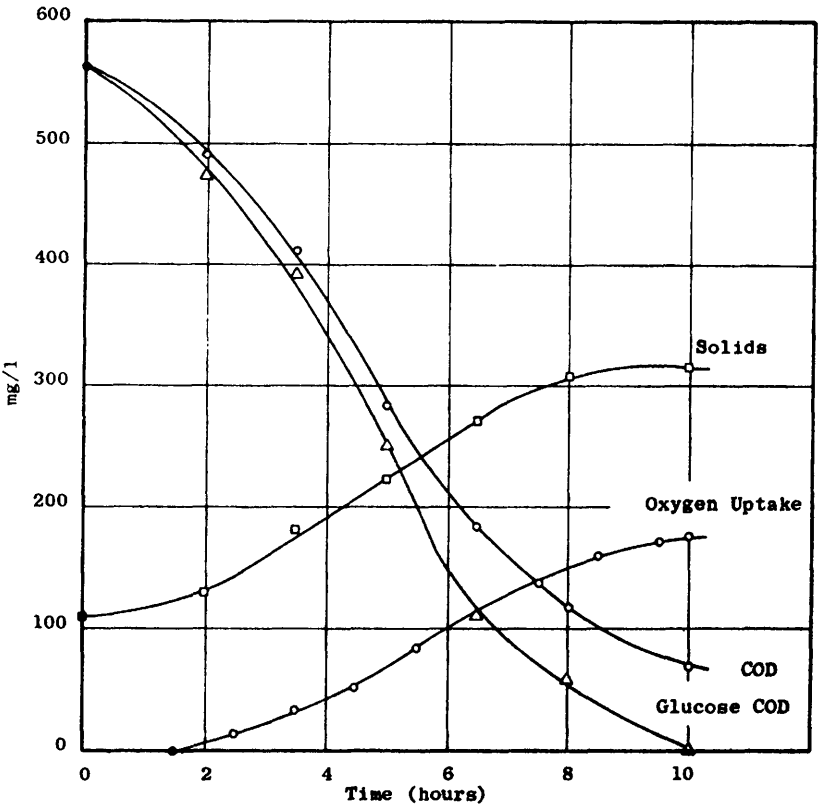

Figure 10. Substrate removal and growth, *Yellow organism*

dence for antagonism and where the competitive situation involved one of simple competition for available substrate, the organism which exhibited the highest value of μ_{max} may be expected to become predominant. Considerably more work is needed in discontinuous systems to determine the effect that k_s may have in establishing predominance for organisms with approximately the same μ_{max} value.

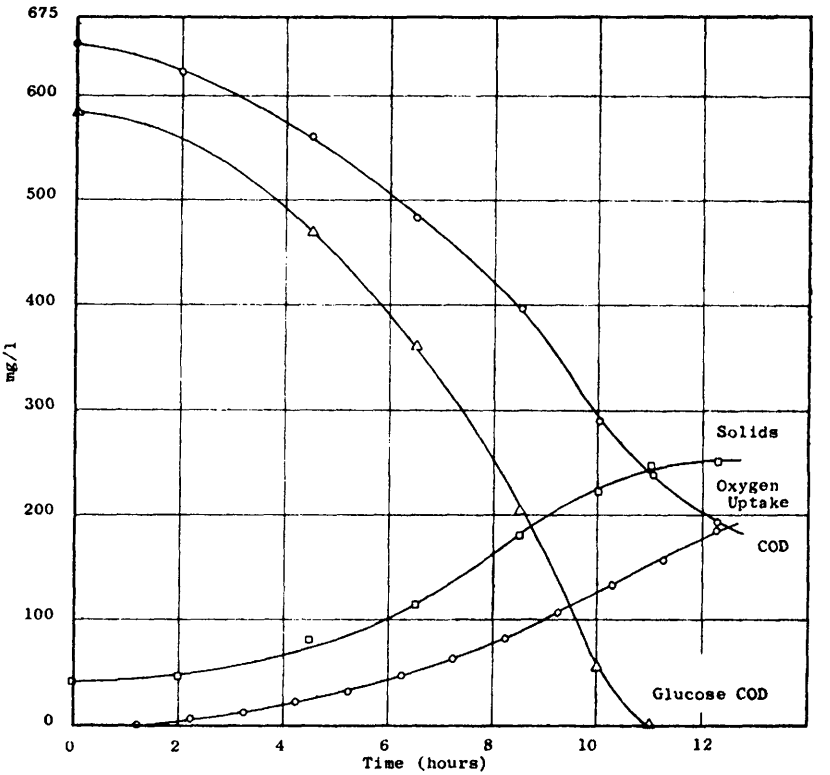

Figure 11. Substrate removal and growth, *Escherichia coli*, K-12

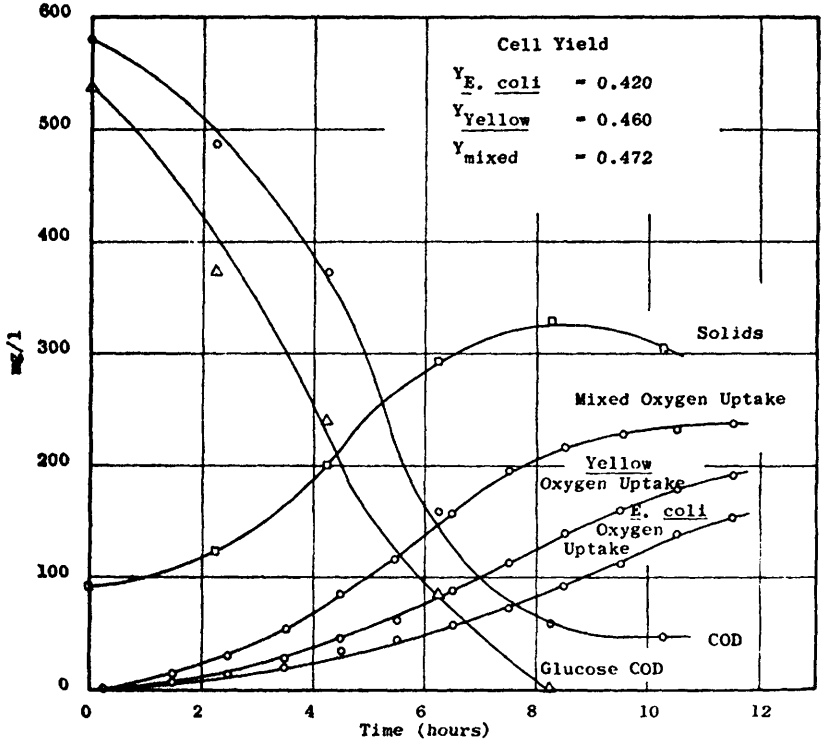

Figure 12. Growth response of a mixture of *Yellow organism* and *Escherichia coli*, K-12

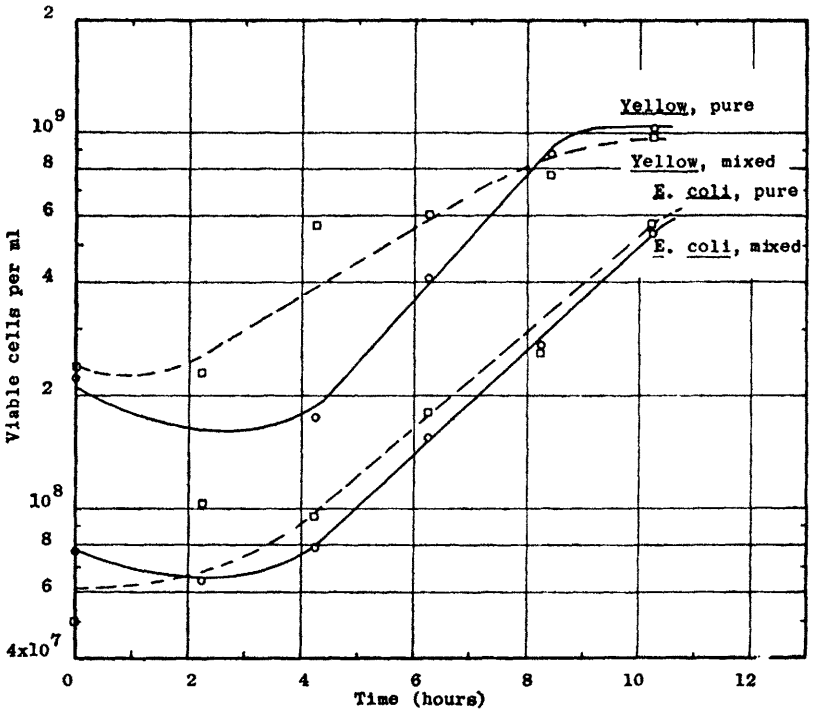

Figure 13. Viable counts for *Yellow organism* and *Escherichia coli*, K-12 in a mixed system

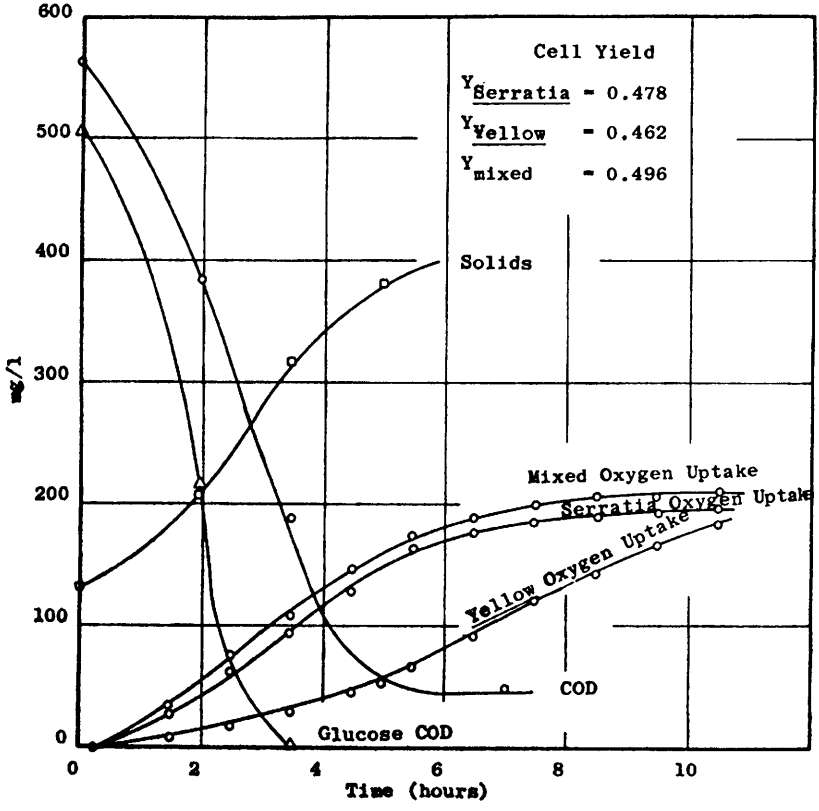

Figure 14. Growth response of a mixture of *Serratia marcescens* and *Yellow organism*

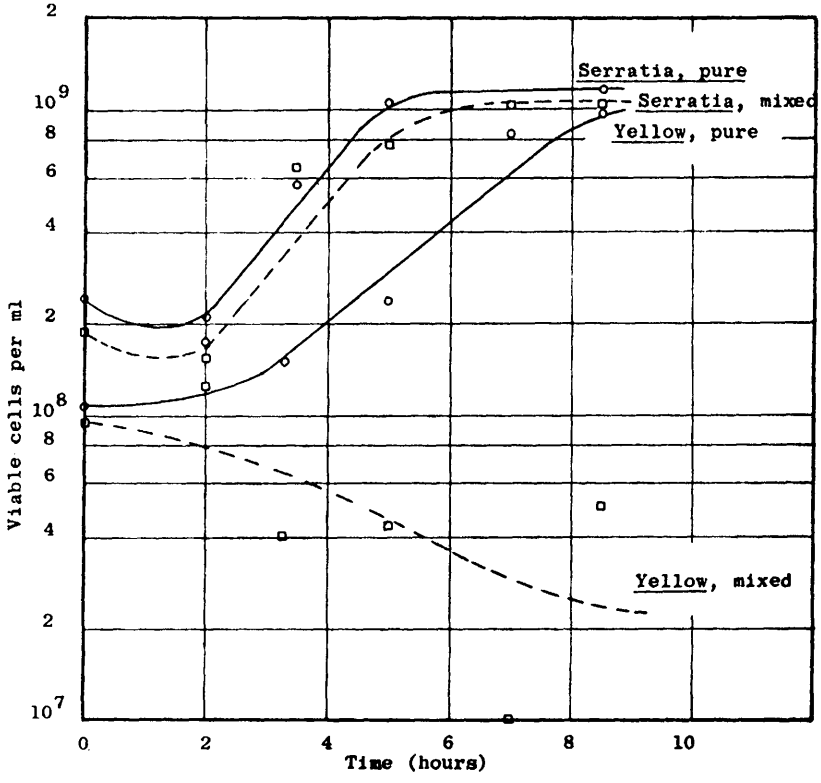

Figure 15. Viable count for *Serratia marcescens* and *Yellow organism* in a mixed system

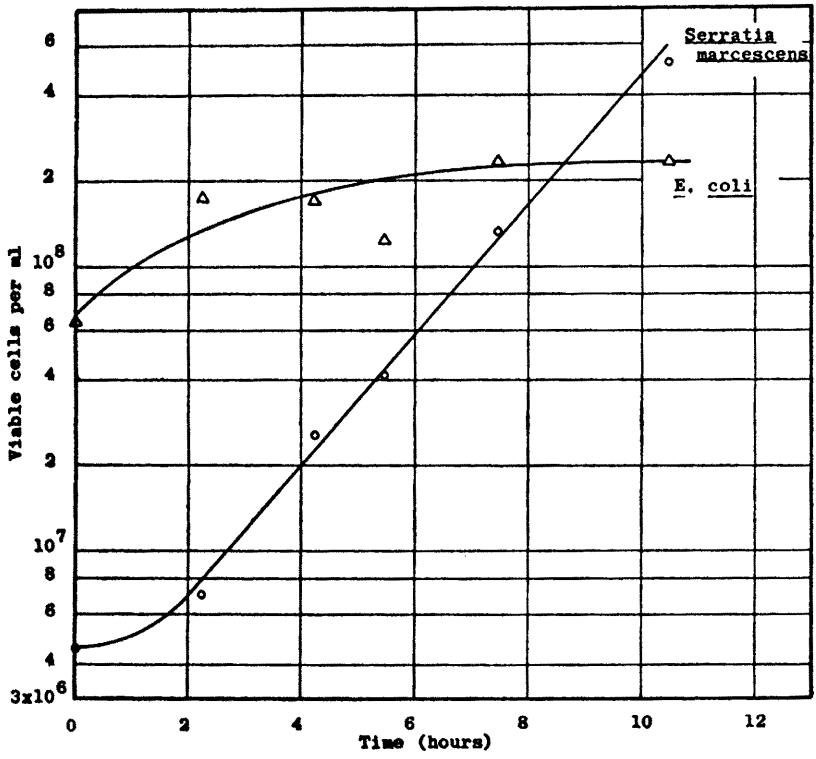

Figure 16. Viable count for *Serratia marcescens* and *Escherichia coli*, K-12 in a mixed system

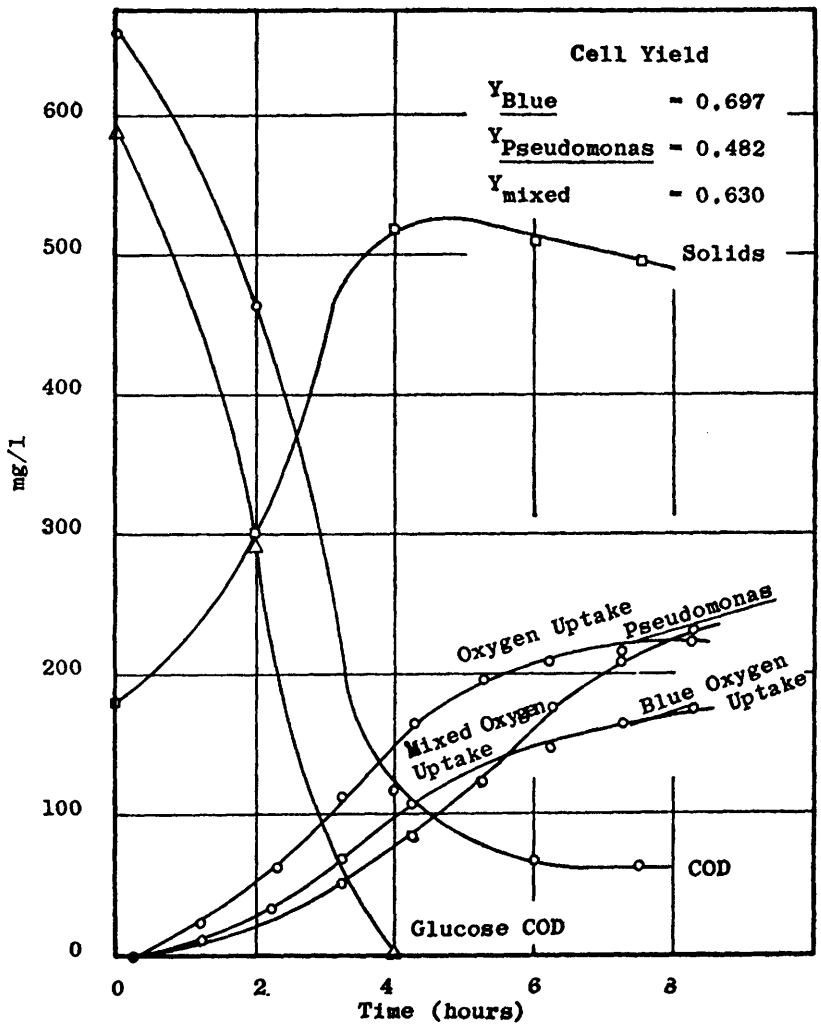

Figure 17. Growth response of a mixture of *Blue organism* and *Pseudomonas aeruginosa*

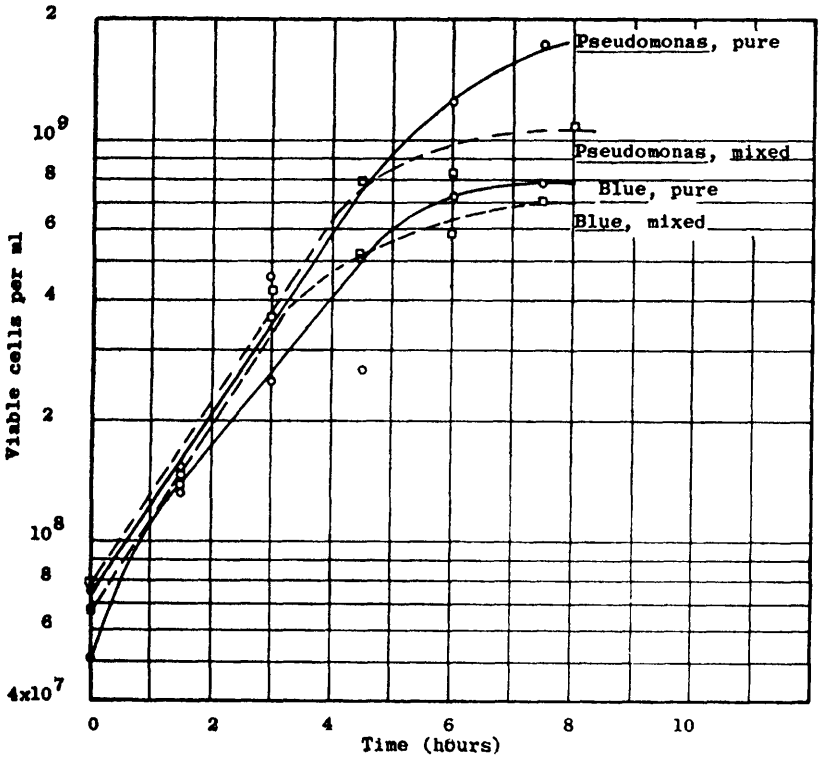

Figure 18. Viable count for *Blue organism* and *Pseudomonas aeruginosa* in a mixed system

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