A STUDY OF THE EFFECT OF DILUTION RATE ON BACTERIAL POPULATIONS

by

Hugh Randall Edwards

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INTRODUCTION

Many problems in sanitary engineering require knowledge of the growth dynamics of microbial systems. For example, currently, most research on biological waste treatment is based on highly empirical approaches. Elucidation of the fundamental biology is necessary before a meaningful approach for improving complex biological processes can be formulated. A knowledge of the effects of environmental pressures on mixed culture population is necessary before an understanding of population dynamics in such environments as streams, sewage treatment facilities, and water treatment facilities can be reached. Mixed cultures studied by batch techniques tend to give results which are difficult to interpret because of simultaneous changes in such factors as cell number and age, pH, nutrient concentration, waste product accumulation, metabolic intermediate pools, and the like. Continuous flow procedures tend to establish steady-states which hold constant such environmental factors as nutrient concentration, average physiological age, and physio-chemical conditions.

At fixed nutrient concentration and dilution rate not exceeding the maximum growth rate, a pure

culture will in theory reach a steady-state population. When the dilution rate is greater than the growth rate of the organism, wash-out should occur. In a random mixed culture, dilution rate should have the same effects upon the various organisms if they are not interacting. The organisms with slower growth rates should be washed from the growth vessel before those with faster growth rates. At increased dilution rates, the number of species of organisms should therefore decrease with only the more hardy and faster growing organisms remaining.

In this research the effects of dilution rate on population were studied for a pure culture of Bacillus cereus, a mixed culture of Bacillus cereus and Aerobacter aerogenes, and a completely random mixed culture. The objects of this study were: 1) to determine whether steady-state populations are reached in pure continuous flow cultures of encapsulated organisms such as Bacillus cereus, 2) to compare these results to the continuous flow theory, 3) to determine the effect of dilution rate upon population of the defined mixed culture B. cereus and A. aerogenes, and, 4) to determine the effects of dilution rate upon a random mixed culture.

REVIEW OF LITERATURE

Theory of the Continuous Culture of Bacteria

Herbert, et al (1956) have developed equations based on the analysis of Monod (1942) for growth of bacteria in continuous culture. The essential feature of this classical analysis of the continuous culture is that microbial growth in this system achieves a steady-state condition. To obtain these conditions it is assumed that sterile media is allowed entrance into a completely mixed growth vessel containing a pure culture. Such environmental factors such as pH, concentration of nutrients, metabolic products and oxygen supply also reach steady-states.

Consider bacteria growing in such a continuous culture, the inflowing media containing a single limiting organic substrate at a concentration S^1. It is assumed all other substrates are present in excess, and the oxygen supply is always adequate; the supply of organic substrate is therefore the sole growth-limiting factor. The variables of interest are the substrate concentration and the dilution rate. A complete theory must be developed on how the variation of these environmental factors can affect growth rate and concentration of organisms.

When no organisms are being supplied from external sources, the organisms are growing in the continuous culture growth vessel at a rate described by equation (1) and being simultaneously washed away at a rate determined by equation (2).

$$\frac{dx}{dt} = kx \qquad (1)$$

$$-\frac{dx}{dt} = Dx \qquad (2)$$

x = concentration of organisms in the growth vessel (dry weight of organisms per volume at a time t)

k = the specific growth rate

D = the dilution rate (flow rate entering the system divided by the volume of the growth vessel).

The net increase in concentration of organisms is given by:

Increase = growth - output

$$\frac{dx}{dt} = kx - Dx \qquad (3)$$

therefore if $k > D$ the value $\frac{dx}{dt}$ will be positive and there will be a continuous increase in organism concentration until a shortage in the critical nutrient reduces k. If $D > k$, $\frac{dx}{dt}$ is negative and the organism concentration will decrease, eventually to zero, therefore the culture will

be washed from the growth vessel. When k = D, $\frac{dx}{dt} = 0$ and x is constant; therefore a steady-state is reached. This equation has been experimentally verified for various microbial systems. To understand completely what dilution rate gives a steady-state the effect of dilution rate upon substrate concentration must also be examined.

In the culture vessel, substrate is entering at a concentration S^1, being consumed by the organisms and flowing out at a concentration S. The net rate of change of substrate concentration is obtained by equation (4).

Increase = input - output - consumption (4)

Schulze (1964) shows that Y, the yield factor is not constant. The variance of Y, which is given by the ratio of weight of bacterial cells produced to the weight of substrate consumed per unit time, is conveniently established at a series of varying growth rates k by the continuous flow technique. The amount of substrate consumed is given by (S^1-S) times the feed per hour, and the amount of cells produced is given by the concentration x times the feed volume per hour. The flow rates cancel out and

$$Y = \frac{x}{S^1 - S} \qquad (5)$$

where S^1 = substrate concentration in feed, mg/l

S = substrate concentration in growth vessel or in growth vessel effluent, mg/l

In the Schulze experiment it appears that at high growth rates substrate is more effectively converted to cell material.

The rate of substrate consumption per gram cell weight per hour at various specific growth rates can easily be calculated from continuous flow data:

$$k_s = \frac{f S^1 - f S}{Vx} = \frac{D S^1 - D S}{x} \qquad (6)$$

where k_s = specific rate of glucose uptake in mg per gram cell weight per hour, and

V = volume of growth vessel

A plot of k_s versus D = k as given by Schulze demonstrates that k_s increases in direct proportion to the growth rate following the equation

$$k_s = n + h\,k \qquad (7)$$

where n = y - intercept, representing grams of glucose uptake per gram cell weight per hour, k = 0.

h = constant, representing grams glucose consumed per gram cell weight formed

The constant k is the inverse of the corrected yield constant Y^1. The specific rate of substrate removal

can therefore be expressed as

$$k_s = n + k\,Y^1 \qquad (8)$$

and the growth rate can be related to the rate of substrate removal by

$$k = Y^1\,(k_s - n) \qquad (9)$$

In the development of continuous flow equations the growth rate is usually assumed to be a constant fraction of the substrate removal rate;

$$dx/dt = -\,Y\,ds/dt \qquad (10)$$

and inversely the rate of substrate consumption is given by:

$$\frac{ds}{dt} = -\,\frac{dx}{Ydt} = kx/Y \qquad (11)$$

Now it appears that equation (10) has to be changed because of a small amount of substrate consumption which is evident even if no growth occurs so that

$$\frac{ds}{dt} = -\,(nx + kx/Y^1) \qquad (12)$$

and

$$\frac{ds}{xdt} = k_s = -(n + k_1/Y^1) \qquad (13)$$

Equation (13) makes it possible to compute the net yield Y at various levels of k. By definition

$$Y = \frac{k}{k_s} \qquad (14)$$

and therefore

$$Y = \frac{k}{n + (k/Y^1)} \qquad (15)$$

This demonstrates that the actual yield factor will decrease with decreasing growth rates.

A balanced equation for the net rate of change of substrate concentration in the growth vessel can be written as:

$$\frac{ds}{dt} = D S^1 - D S - x (n + k/Y^1) \quad (16)$$

For steady state conditions when $\frac{dx}{dt} = 0$ we have

$$D = k_m (1 - e^{-c \bar{S}}) \quad (17)$$

where k_m = maximum growth rate constant and

c = constant

and

$$\bar{S} = \ln \left(\frac{k_m}{k_m - D} \right) \Big/ c \quad (18)$$

Where $\bar{S}$ = substrate concentration in growth vessel or effluent at steady state.

Since k = D at steady-state

$$\bar{x} = \frac{D (S^1 - \bar{S})}{n + \frac{D}{Y^1}} = \frac{Y^1 (S^1 - \bar{S})}{\frac{n Y^1}{D} - 1} \quad (19)$$

where $\bar{x}$ = cell concentration in growth vessel or effluent at steady state. Substituting into equation (19) equation (18) produces

$$\bar{x} = Y^1 \left[S^1 - \ln \left(\frac{k_m}{k_m - D} \right) \right] \frac{c}{\left(\frac{n Y^1}{D} + 1 \right)} \qquad (20)$$

These equations define the substrate concentration and the cell concentration at steady-states for low dilution rates as D = 0.02 up to where D = k_m.

Herbert et al (1956) formulated the critical value of dilution rate, D_c, above which complete "wash-out" occurs. From equation 3 it can be seen that this critical value is reached at the highest possible value of k. This is theoretically attained when $S^1 = S$. The critical value is given by

$$D_c = k_m \left(\frac{S^1}{k_s + S^1} \right) \qquad (21)$$

Van Niel (1955) discusses the situation where a complex mixture of organisms is grown on a medium which has been adjusted to favor certain species. In a race to survive, the desired organisms outgrew the others and predominate and can then be isolated. This enrichment technique demonstrates the importance of nutrition to the relative growth of organisms in a mixed culture.

Jennasah (1964) discussed bacterial enrichment by means of the chemostat. Various ways of directing such enrichments are tested and the control of the enrichment of several species of heterotrophic bacteria from one particular sample of water was discussed.

Parker and Snyder (1961) developed and described a continuous two-stage system for investigation of interactions between two or more species of bacteria grown in mixed culture. The system employed two or more chemostatically limited stages of pure cultures feeding to a non-limited mixed continuous growth cell. Comparisons were thus possible at any point on the growth curve of numbers and products of organisms in pure culture with numbers and metabolic products resulting from interactions between the same organisms growing in mixed culture. Data was expressed in terms of generation time. Results obtained with a mixture of S. salivarius and V. alcalescens indicate an interaction with the generation times of each of these organisms being greatly increased when the organisms were grown with each other.

Shindala, et al (1965) showed the interaction of P. vulgaris and S. cerevisiae is a true commensalism in that one organism benefits but has no effect on the other. The bacterial growth was shown to follow that of the yeast in a relationship that was not linear. Coulter Counter

methods were used to give counts of each organism based on size differences in the same sample.

Gilley et al (1965) have shown that yeast in continuous culture show oscillations in growth rate induced by changes in dilution rate, medium concentration, or temperature. Abrupt changes in dilution rate do not always give smooth transitions from one steady state to another. Instead, decaying oscillations in population seem to occur. The magnitude of the oscillations was shown to be a function of medium concentration and magnitude of dilution rate change. Population counts were done by the electronic Coulter Counter method.

OBJECT

The initial object of this research was to study a random mixed culture in a continuous flow system. The initial plan was to determine the amount of variation of organism in the mixed culture and by forcing environmental conditions to be able to select simple species from the mixture.

METHODS AND MATERIALS

A. Description of Continuous-Flow Vessels

1. In the initial series of experiments two 8 liter media reservoirs were used to supply media by constant head gravity flow (Fig. 1) to growth vessels of 100 or 300 milliliters capacity. Each resevoir was supplied with air kept aseptic by passage through a cotton plugged glass tube.

The growth vessels (B Fig. 1) were plastic bottles cut at various heights. A knotch was cut in the rim of the vessels to give the desired volume by locating the position of overflow. The growth vessels were left open to the atmosphere so maximum exposure to random bacteria could occur. The components of the vessels were continuously stirred magnetically. A cardboard pad was placed between the magnetic stirrers and the growth vessels in order to provide heat insulation. The effluent went to the sink. Samples for staining were removed directly from the growth vessels by use of a loop needle. Samples for Petroff-Hausser counting were removed directly from the growth chamber with sterile pipettes. One milliliter of this sample was added to two milliliters of a 25% solution of Crystal Violet for counting the free swimmers (organisms

not constituents of the slime) in the sample by the Petroff-Hausser method.

When slime became too heavy the vessel walls were scrubbed with a test tube brush. This was usually done about once a day.

The medium composition is listed in Table 1. The medium and hoses were autoclaved at 15 p.s.i. for 15 minutes. The growth process was operated in a $20^{\circ}C \pm 1\ C^{\circ}$ constant temperature room.

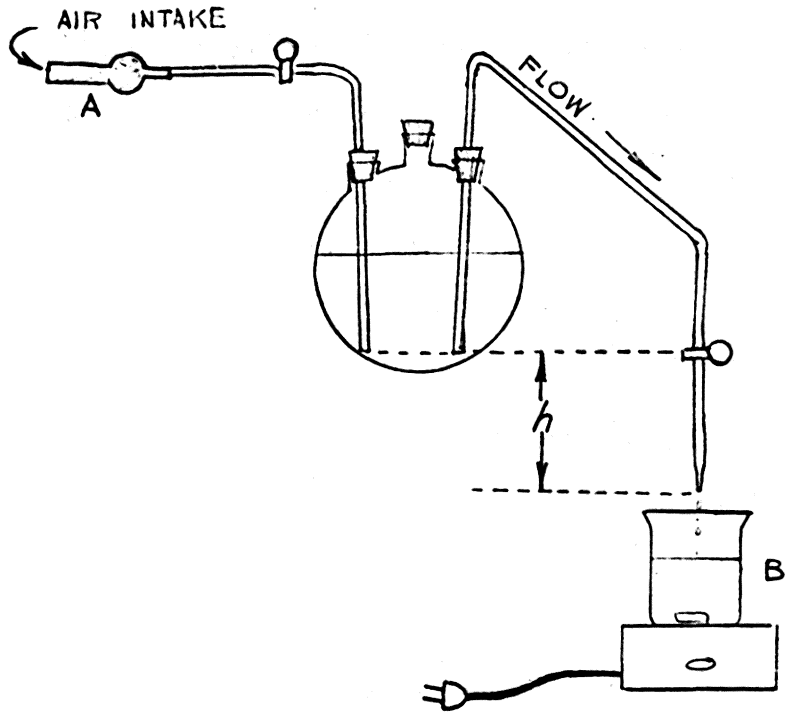

Figure 1.

2. In the aseptic series of the experiments, two 12-liter reservoirs, prepared and connected as previously mentioned except no constant head was required, were connected to a 15-liter pyrex reservoir (B Fig. 2). Air was supplied to this reservoir from the atmosphere through a cotton plugged glass tube. Flow passed from the reservoir to a three-way flow divider (C Fig. 2) and then each hose was fitted into a peristaltic pump (D Fig. 2). Next the flow entered the cotton plugged growth vessels (E Fig. 2) through hypodermic needles.

The growth vessels were 500 mililiter Erlenmeyer flasks with glass side arm effluent tubes located at heights to permit overflow at 200 milliliters. The vessels were attached via steel clamps to a home made shaker (F Fig. 2).

The home made shaker consisted of a plywood board suspended by four coil springs above a plywood base. The suspended plywood top was connected by a steel connecting rod to a steel flywheel to attain a three inch stroke. The flywheel was driven by a 1/8 horsepower Westinghouse Electric motor. The speed of the flywheel was controlled by a zero-max clutch apparatus.

The entire apparatus was connected and autoclaved for 15 minutes at 15 p.s.i. The growth process took place in a 20°C $\pm$ 1 C° constant temperature room.

After the growth vessels were partially filled with medium, each was identically inoculated with the random culture grown in the previous experiment.

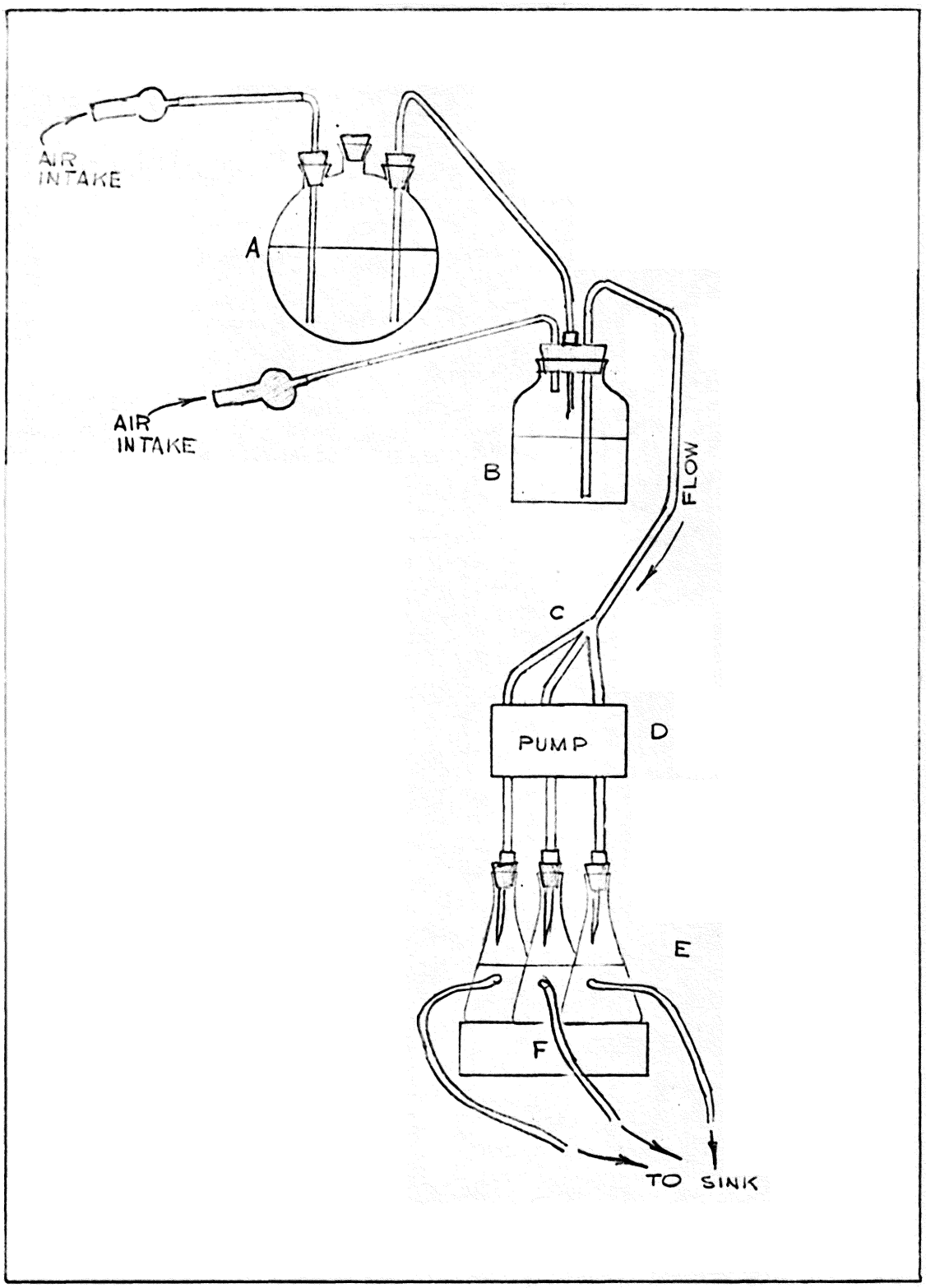

Figure 2.

3. In the defined culture experiments the continuous flow apparatus consisted of a 12-liter reservoir feeding an Erlenmeyer flask containing 186 milliliters with effluent through a glass tube system (Fig. 3). Aeration was accomplished by blowing air from the laboratory compressed air system (Fig. 3) into the culture vessel through a cotton plugged glass tube. The culture vessel was held in a 30°C water bath, and medium was fed with a peristalic pump (Sigmamotor Company, Middleport, New York). Magnetic mixing of the culture vessel was employed. The system is shown in Figure 3.

Samples were taken from the effluent tube for Petroff-Hausser and Coulter counting. One milliliter of the sample was added to 99 milliliters of a solution that contained 0,9 grams of Nacl per 100 milliliters of distilled water. This sample was then counted by the electronic Coulter Counter with a 50 micron aperture. The Coulter Counter had settings of the following: 1) threshold reading of 4, gain selection of 3, and an aperture current setting of 7.

After adding 10 liters of medium (Table 2) to the reservoir the apparatus was connected and autoclaved at 15 p.s.i. for 15 minutes. Following cooling the vitamins were added to the medium and operation of the

system was begun.

After the growth vessel contents had reached 186 milliliters, pumping was stopped so inoculum could be added to the growth vessels. The air intake was also disconnected from the laboratory compressed air system to prevent contamination of the media reservoir, and to prevent immediate washout of the inoculum.

Cultures of B. cereus and A. aerogenes which were grown in test tubes of media (Table 2) at 37°C were used as inoculum. Initially B. cereus was added to the growth vessels of the continuous flow system. The organisms were grown in batch culture for 18 hours before starting continuous flow. After the population of B. cereus alone was determined at several flow rates, A. aerogenes was added to the system without interupting the flow.

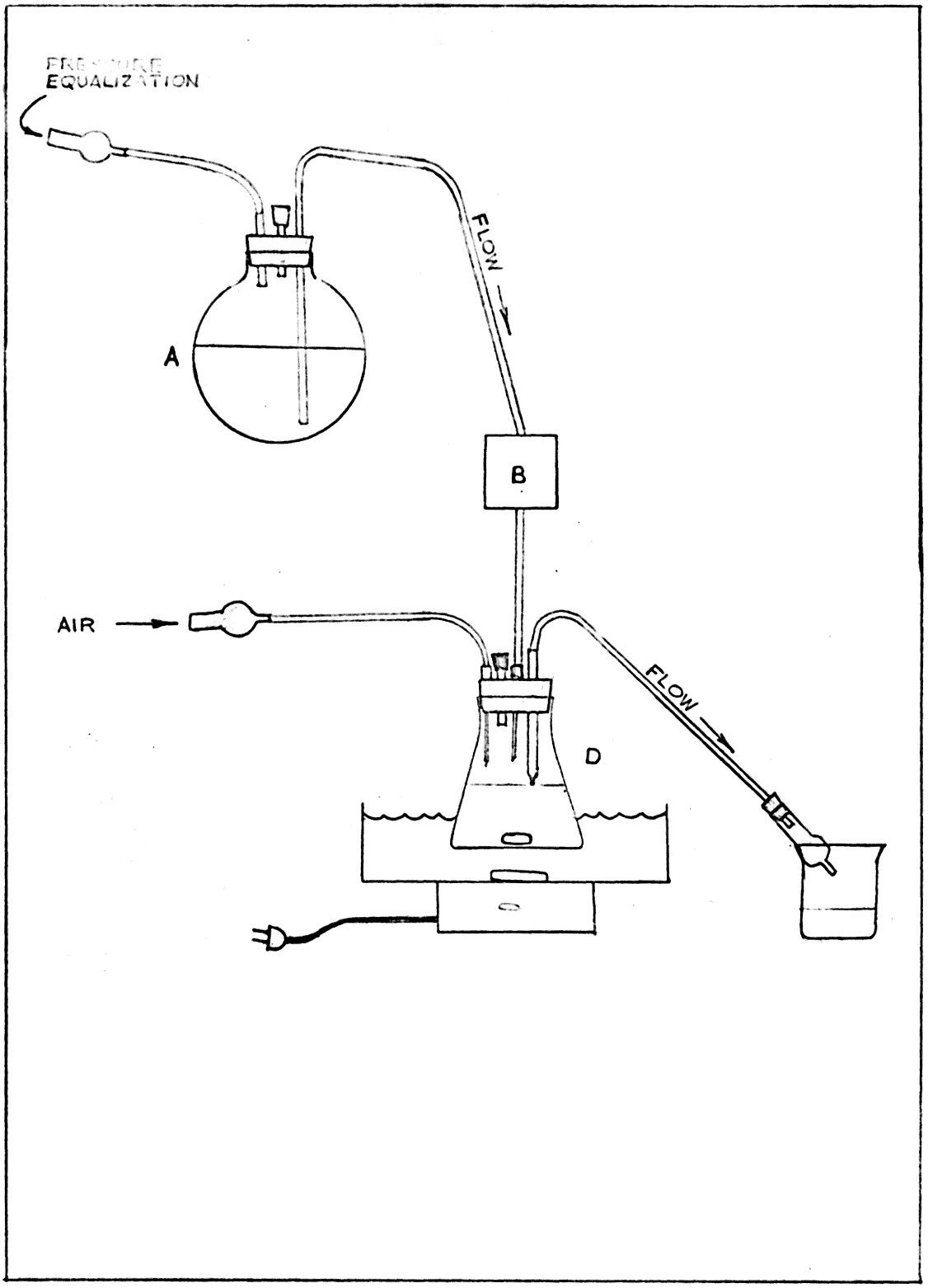

Figure 3.

B. Cultures and their Maintenance

Cultures used in the first two experiments were collected by leaving the growth vessels of the initial experiment open to the atmosphere for maximum exposure to random bacteria. The types of bacteria present in the culture were: small rods with a diameter of roughly one micron, some encapsulated and some not; large rods with diameters as large as 5.0 microns, some encapulated and some not; small and large cocci some free and others trapped in slime. There was also a great number of slime fioccules both large and small.

Bacillus cereus (Strain 1) and Aerobacter aerogenes (Strain 1) used in the defined culture experiments were obtained from the culture collection of the bacteriology department, Virginia Polytechnic Institute. B. cereus is a rod shaped organism, 1.0 to 1.2 by 3.0 to 5.0 microns, with square ends, aerobic, facultative anaerobic, usually occuring in short to long tangled chains. The species dealt with was filamentous, encapsulated and gram variable. Optimum growth occurs at 30°C. A. aerogenes is a rod shaped organism, 0.5 to 0.8 by 1.0 to 2.0 micron, occuring singularly, encapsulated, gram negative, aerobic, gas producer. Optimum growth occurs at 30°C. Both cultures were initially grown on Difco cystine tryptic agar then transferred by use of a loop needle to test tubes of medium (table 2).

These test tube cultures were incubated at 37°C for a minimum of 48 hours. After incubation these cultures were used for inoculum for the continuous flow systems.

C. Composition and Preparation of Media

Medium of the composition shown in Table 1 were used in the random mixed culture experiments. Medium of the composition shown in table 2 was used in experiments on defined cultures.

Vitamins added to media used in the defined culture experiments were dissolved in distilled water and filtered through a milipore membrane filter to sterilize. No particular amount of vitamins were used, but always more than 5 milliliters of the vitamin stock solution were added to the 10 liter reservoir of media.

COMPOSITION OF MEDIA

Table 1

Medium 1

Component	Quantity
Potassium phosphate, monobasic (KH_2PO_4)	6 g
Potassium phosphate, diabasic (K_2HPO_4)	14 g
mg $SO_4 \cdot 7\ H_2O$	0.2 g
Fe SO_4	0.0010 g
$(NH_4)_2\ SO_4$	3.0 g
Dextrose ($C_6H_{12}\ O_6 \cdot H_2O$	2.0 g
Distilled Water	8 liters

Table 2

Medium 2

Component	Quantity
Dextrose ($C_6H_{12}O_6 \cdot H_2O$	4 g
$(NH_4)_2SO_4$	2 g
$K H_2PO_4$	6 g
K_2HPO_4	14 g
Sodium Citrate 2 H_2O	1 g
$Mg SO_4 \cdot 7 H_2O$	0.2 g
Distilled Water	10 liters

Vitamins

for Experiment C and D	Quantity/100 ml of H_2O
Biotin	10.0 mg
Calcium Pantothenate	1.0 mg
i - inositol	5.0 mg
Thiamine	1.0 mg
Pyrid oxine	200 mg

RESULTS

In the experiments with random mixed cultures the dilution rates were kept constant at $D = 0.6\ hr^{-1}$ for the 100 milliliter growth vessel. These vessels were left open to the atmosphere for continuous exposure to random bacteria. Counts were made of the organisms not trapped in the slime. Population as a function of time is given in Fig. 4. The population seems to oscillate randomly. This great oscillation is in line with the results of Cassell et al (1964).

Since random oscillations of population levels occurred in the opened vessel study, it was decided to eliminate the variable of ambient reinoculation from the system and see how soon the growth contents of three aseptic growth vessels became dissimilar. Equal inoculum from the original experiment were added to each of the three growth vessels and continuous flow started. Samples were taken from all three growth vessels at various times. Bacterial counts were high and slime production was great as in the open mixed culture experiments. There was no way to do quantitative work because of clumped organisms, slime on the walls, and inadequacies of Petroff-Hausser counting, thus this approach was discontinued. However, growth floccules similar to activated sludge floccules were noted. Also, from visual observations slime formed in the vessels at different rates and variations were evident from

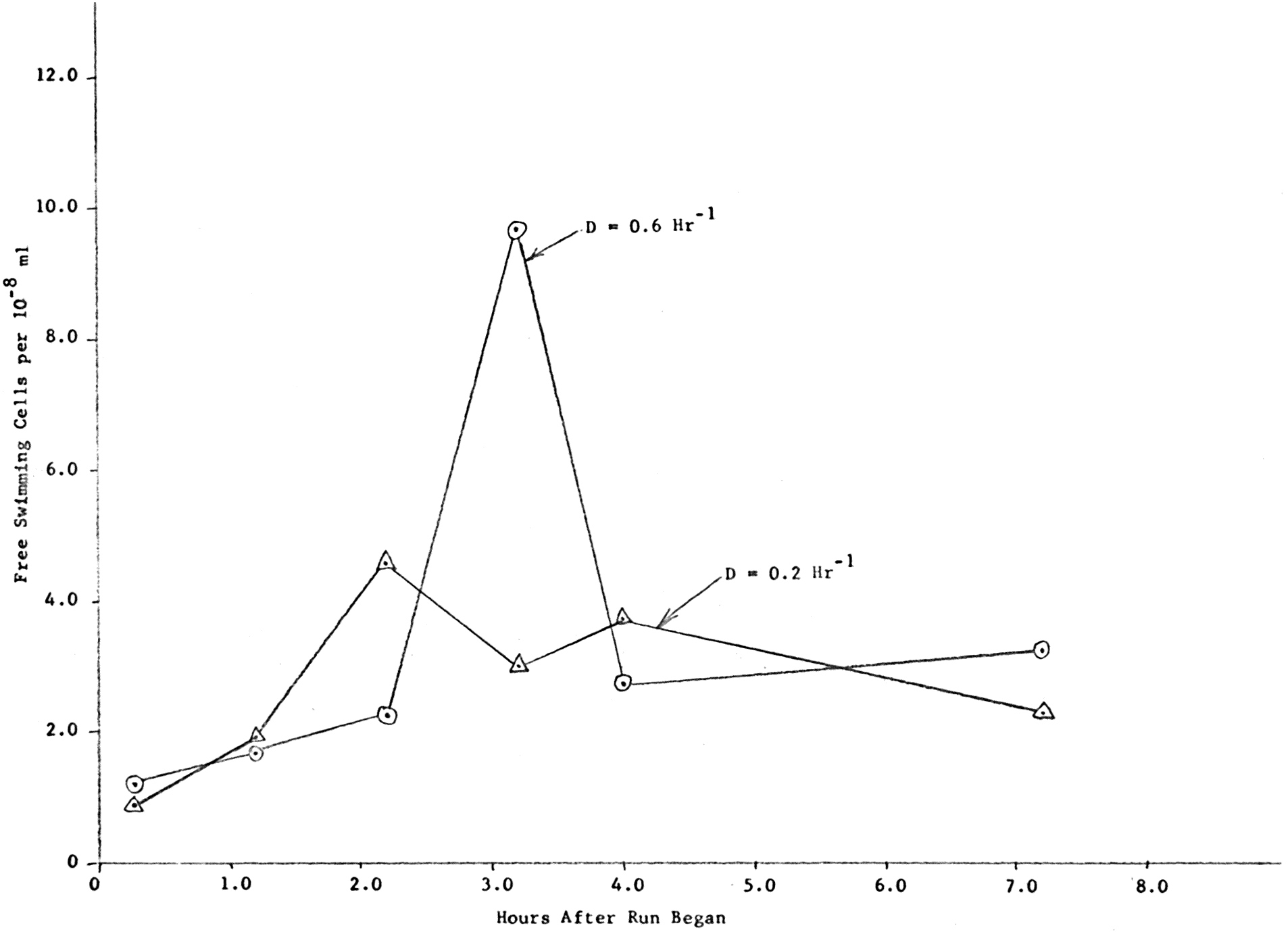

Figure 4 Population Changes in a Random Mixed Culture

the start. After the continuous flow operation was stopped, the slime on the sides of the vessels took from one to four weeks of batch culture to slough off. A greater time was required for the more dense slime to slough off the container walls.

Since results from these random mixed culture growth systems were hard to interpret, it was decided to do population studies on pure cultures of bacteria at various dilution rates.

A different medium (Table 2), more suited to the selected cultures, was used. Sterile test tubes of this medium were inoculated with the various organisms. This batch method of growth provided an index of the ability of the organisms to grow in this media. Results from this test are shown in Table 3.

Table 3

Growth of different test cultures in the chemically defined medium (Table 2)

Organism	Growth
Staphylococcus epidermidis	0
Bacillus cereus	++
Aerobacter aerogenes	+++
Proteus vulgaris	0
Serratia marcescans	0
Eschericha coli	++++
Saccharomyces cerevisiae	++++

Bacillus cereus was chosen as the test pure culture for the continuous flow process because of its satisfactory growth rate. B. cereus would later be studied in mixed culture. It was believed that in a mixed culture of the two species that A. aerogenes would overgrow B. cereus. Population levels of the pure culture of B. cereus at various dilution rates were determined. Measurements were taken after the dilution rate was constant for 12 hours to insure establishment of steady-state conditions. The population levels at various dilution rates are shown in Fig. 5 for Petroff-Hausser and Coulter Counter Counting methods. The Petroff-Hausser methods always gave higher numbers of cells present because doublets, groups, or flocs of cells give only single pulses on the Coulter Counter. The Coulter Counter and Petroff-Hausser methods gave roughly parallel growth trends. Population increased from that at a dilution rate of D 0.065 hr^{-1} to a higher level at D = 0.085. Reductions in population levels then occurred and continued up to the highest dilution rate tested of D = 0.72 hr^{-1}. The reduction in population did not follow continuous flow theory for non-clumping organisms in that the population level should remain fairly constant for dilution rates up to a critical rate beyond which there should be an almost immediate reduction to a zero population level.

With the pure culture behavior of B. cereus somewhat established, it was of interest to add a second organism to the

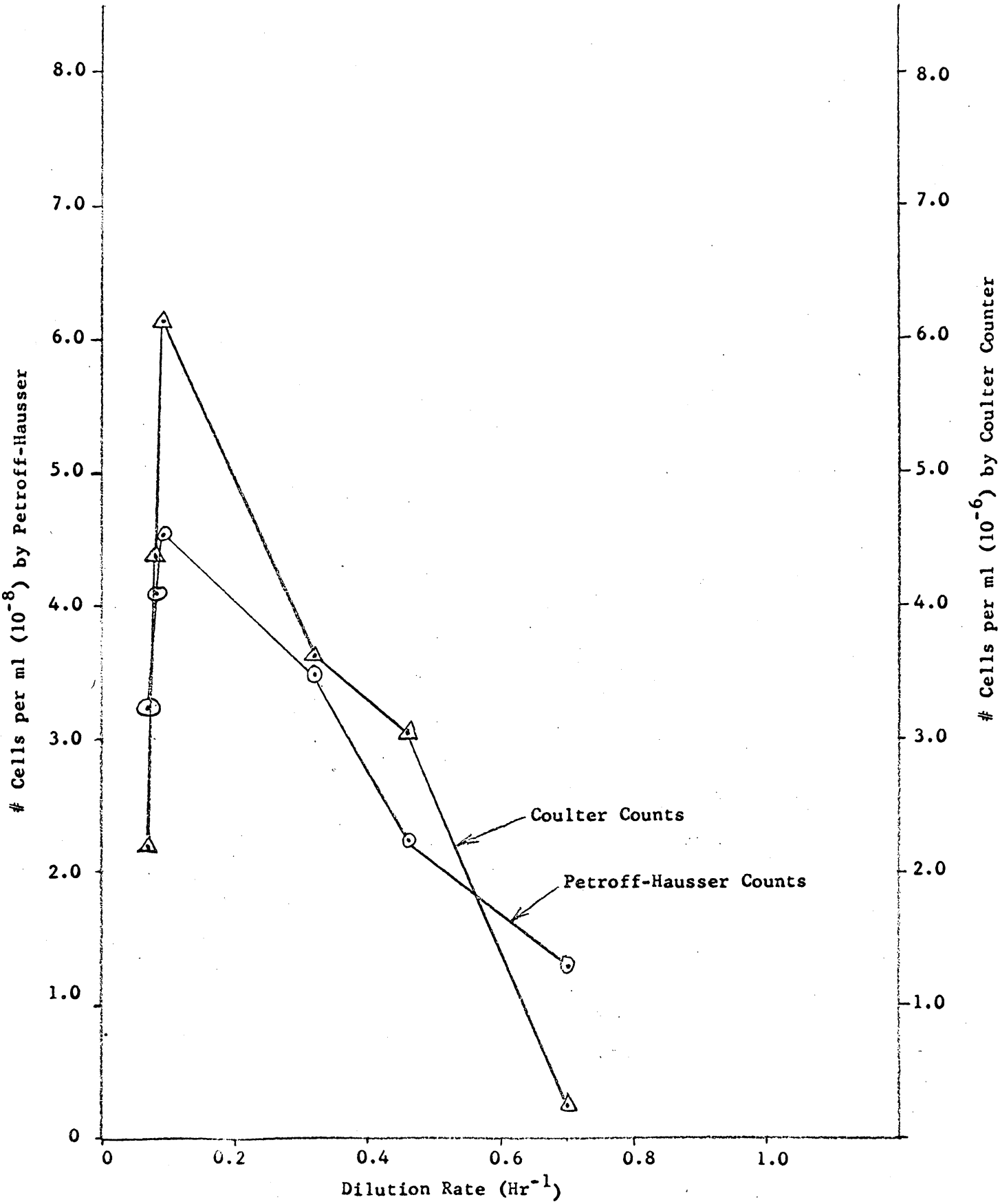

Figure 5 Population of B. cereus as a function of Dilution Rate

continuous flow system and run population counts on this defined mixed culture population. Counts verses time from the time of inoculation of A. aerogenes were made on this culture at a dilution rate of D = 0.065. The results are shown in Fig. 6.

As can be seen in Fig. 6, the total population was reduced from that of the initial pure culture to a new lower steady-state condition. The total population count by the Coulter Counter may have been reduced below that of the original because of a greater number of floccules present or from some inhibition that A. aerogenes has on B. cereus. After the steady-state was accomplished, a Petroff-Hausser count was made to determine relative proportions of each organism. It was determined that 70% of the population consisted of A. aerogenes and 30% of the population was B. cereus.

Since a steady-state condition had been reached at a dilution rate of D = 0.065, it was decided to test other dilution rates for steady-states. Fig. 7 gives the results of this effort. Although there seemed to be some deviation from a steady-state at these dilution rates, it seemed that these deviations could have resulted from experimental inconsistances rather than random oscillations of growth. The steady-state population obtained when D = 0.3079 hr^{-1} was lower than when D = 0.354 hr^{-1} or when D = 0.065 hr^{-1}. Also the population count at a steady-state for D = 0.354 hr^{-1} and D = 0.065 hr^{-1}

are almost the same.

These results did not follow the steady-state continuous flow theory for a pure culture in that at the highest dilution rate used the population level was greater than at an intermediate dilution rate.

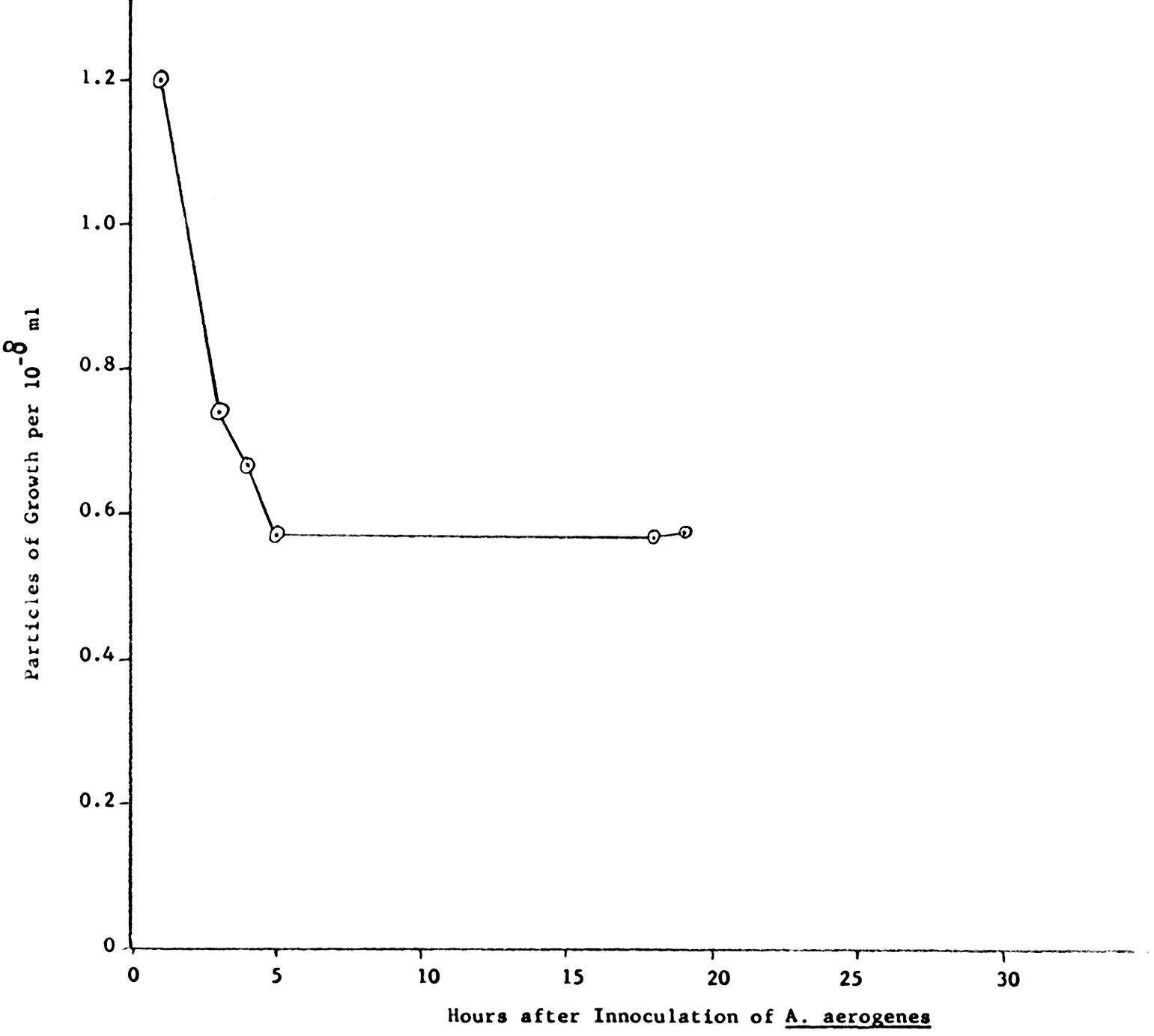

Figure 6 Trends of Total Counts of Bacillus cereus and Aerobacter aerogenes vs Time after Innoculation of A. aerogenes

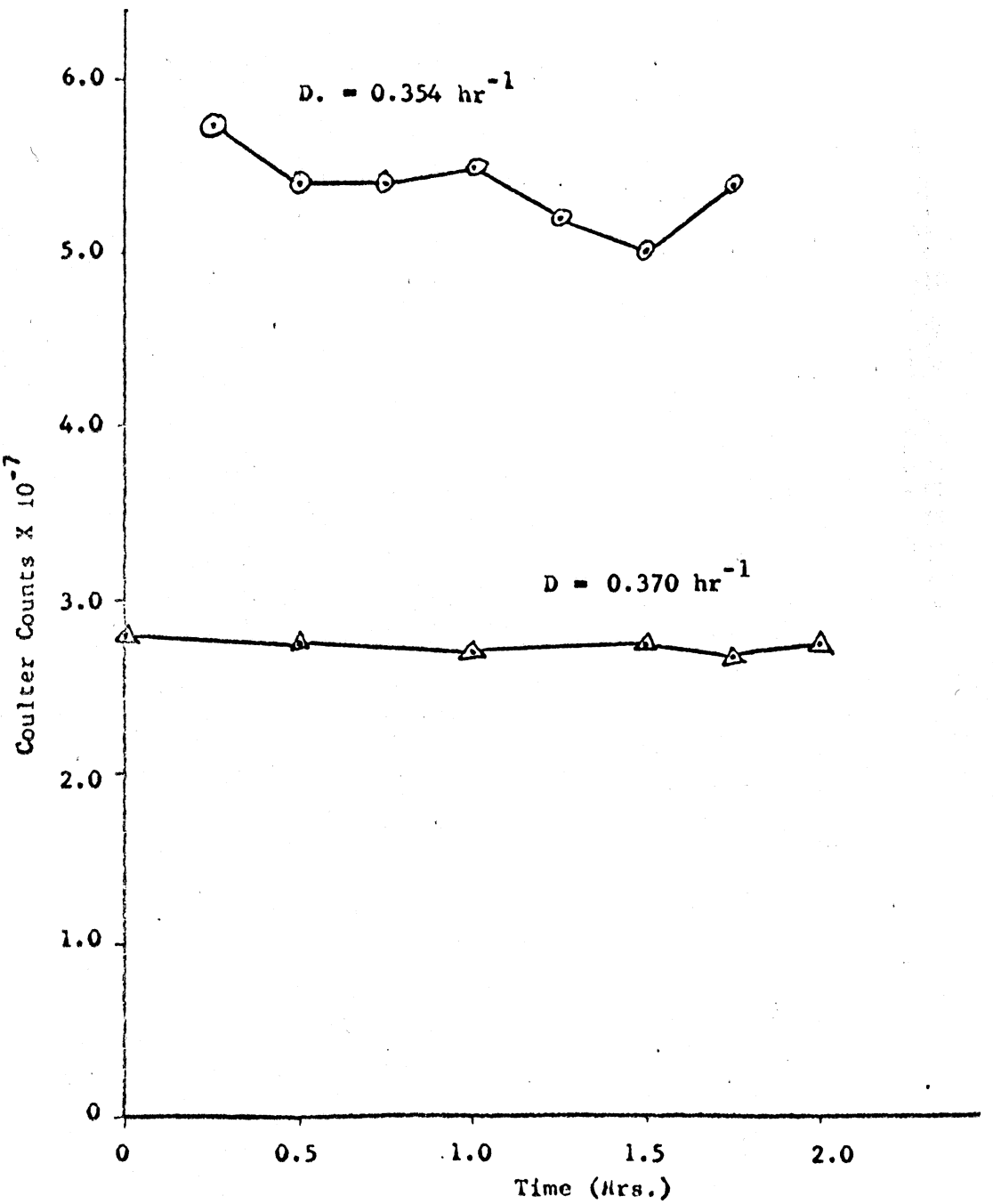

Figure 7 Population Counts of a Culture of Bacillus cereus and Aerobacter aerogenes by the Coulter Counter Method

DISCUSSION

In a random mixed culture continuously reinoculated ambiently from exposure to random bacteria, population levels seemed to fluctuate. These random oscillations in free swimmer population are of great interest and importance because some factors which may cause fluctuations are the variable inoculation of random bacteria and the possible interaction of bacteria. While the free swimmer population level oscillated randomly, slime continually increased until the vessel held mostly slime. The slime production was possibly a product of some interaction of a few species of the cultures in the mixed culture system.

The results from the use of an aseptic continuous flow random mixed culture system suggested that quantities of slime and activated sludge type of floccules did not establish steady-state growth patterns. Once slime became attached to the sides of the growth vessels, the growth rate of the slime seemed to increase. Furthermore, the slime continually changed the environment in the growth vessel. Organisms became entrapped in this slime and therefore it was impossible to control these growth factors.

In the pure continuous culture of B. cereus population

levels decreased as dilution rates increased. Population levels did not follow continuous culture theory in that population levels decreased gradually over a series of dilution rates instead of decreasing sharply at the critical dilution rate. Since B. cereus formed capsules and some became attached to the growth vessel walls, it was impossible to provide the constant control over growth factors that normally would be expected in a continuous flow system. Slimes which entrapped these organisms could possibly have decreased medium detention and increased oxygen supply per unit volume by decreasing the volume of container. Also the time interval required for washout of bacteria and metabolic waste products could have been increased from these items being held firm in the slime connected to the vessel wall.

When A. aerogenes was added to the system already containing B. cereus, it was believed that the more rapid growing A. aerogenes would completely take over the system, Total population levels decreased after the addition of A. aerogenes. Petroff-Hausser counts showed after several days that the population was composed of 70% of A. aerogenes and 30% of B. cereus. It is believed that the only reason that B. cereus was not washed completely from the system was because of the continuous reinoculation of this organism from the slime on the container walls.

Population levels of the mixed culture did not follow the continuous flow theory for a pure culture in that counts at the higher dilution rates some times were greater than those at lower dilution rates. These discrepencies from continuous flow theory might have been the result of the unpredictable inoculation pattern of organism coming from the slime floccules. Also, it was quite possible that some unknown interaction existed between these two organisms.

This study has shown some general trends in population levels at several dilution rates, but more research needs to be done in this field. Some studies should be made on factors that could possibly control slime production. If slime production could be held to a minimum, quick and accurate results of population levels could then be determined with the use of the Coulter Counter. Some suggested studies on the possible control of slime production are:

1. The effect of pH upon slime production in mixed cultures.
2. The effect of oxygen supply upon slime production in mixed culture.
3. The effect of medium composition and concentration upon slime production in mixed cultures.

By finding the condition of minimum slime production a greater number of mixed cultures can then be studied in the future.

Other research suggestions are:

1. The affect of sinusoidal application of flow to a defined mixed culture of B. cereus and A. aerogenes to determine whether there is an immediate response in population levels.
2. The use of sinusoidal application of medium to a defined mixed culture of B. cereus and A. aerogenes to determine the growth response of these organisms to food supply.
3. The use of sinusoidal pH variance on mixed cultures in general to determine the growth responses of these organisms to pH.

These are only a few suggestions for future research projects on mixed cultures, but much research is still needed.

CONCLUSIONS

1. At various detention times population levels of free swimmer organism in a random mixed culture fluctuated continuously. Therefore, a random mixed culture must be considered a dynamic system.
2. Slime production increased steadily throughout the random mixed culture runs and therefore was controlled by factors such as being offered longer contact with the medium by being attached to the wall surfaces.
3. Population levels of encapsulated B. cereus did not have a sharp reduction in magnitude at one dilution rate but decreased gradually over a series of dilution rates. Therefore, the production of these capsules must offer a more protected environment than that of a bacteria that is not encapsulated.
4. The culture of B. cereus was not completely overgrown after the addition of A. aerogenes. Therefore, reinoculation of the system with B. cereus from the wall slime probably occurred.
5. Population levels of the defined mixed culture at higher dilution rates were not always lower than those at lower dilution rates and therefore did not follow continuous flow theory for a pure culture.

SUMMARY

Population levels were studied at various constant dilution rates for a random mixed culture that was constantly exposed to bacteria from the atmosphere. A continuous flow constant head apparatus was described. The Petroff-Hausser method of counting was used to determine the population levels. Random oscillations in free swimmer population of the random culture were noted, but slime built up until there was little free liquid.

An aseptic continuous flow system for the study of a random mixed culture was described. The quick production of slime and large numbers of bacteria proved this system of no great advantage over the system with constant exposure to the atmosphere. Activated sludge like floccules and slime were noted to grow at three different rates in the three identical growth vessels.

Bacillus cereus population levels were noted at various flow rates in a continuous flow system. Slime floccules were formed and some slime was noted connected to the growth vessel walls. Population levels taken by Coulter Counter method did not conform to the continuous flow theory for a pure culture in that there was not an immediate reduction in population level at the critical dilution rate but a series of reductions over a range of several dilution rates.

Population levels of a defined mixed culture of B. cereus and Aerobacter aerogenes were studied. A reduction in total population of the mixed culture from that of a pure culture of B. cereus was noted. Petroff-Hausser counting showed that after several days the total population of the mixed culture was approximately 70% of the original. A. aerogenes population counts were taken at several dilution rates. These levels did not follow continuous flow theory for a pure culture in that at a high dilution rate a larger count was noted than the count at a lower dilution rate. It was decided that slime on the walls of the growth vessel continually changed the environment of the culture and made it difficult to control all the environmental factors even in a continuous flow process.

Also this method was too laborious and it was almost impossible to get quantitative data by Petroff-Hausser counting because there was too wide a spectra of organism.

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APPENDIX I

Theory of the Coulter Counter

In the Coulter Counter a suspension is made to flow through a small aperture in a glass wall that separates the suspension into two electrically isolated portions. Electrodes placed in the suspension on either side of the wall are connected to a direct current supply. The total current passing through the suspension between the electrodes must pass through a small aperture. Because the resistance of any portion of a fluid varies inversely with its area, the resistance of the fluid in the small aperture will be very much higher than that of the rest of the suspension, and the current between the electrodes will depend almost entirely on the resistance of particles this resistance will be constant, but when a particle enters the tunnel a reduction in the volume of the fluid in the aperture occurs, the resistance rises, and the current between the electrodes drops. As a particle passes through the aperture the current will fall and then rise again to its original level; the magnitude of this current pulse will indicate the volume of the particle. By use of a suitable conversion factor the particle volume can be related to the diameter of a sphere of the same volume. Adjustment of the threshold level above which pulses are counted enables the determination of the particle size distribution.

A sample dilution of 1:100 was selected to provide a rate of flow of particles through the aperture without exceeding the safe coincidence correction values. The conductivity of the diluent can be adjusted to provide the optimum electric current flow through the aperture, but 0.9 per cent saline was used throughout this research. The main variables affecting particle concentration analysis are the particle size, the conductivity of the diluent, the counting time, and the aperture diameter.

The basic construction of the Coulter Counter is shown in Fig. 8. One electrode is placed in the beaker containing the sample. Into the same beaker is placed a vertical glass tube, sealed from the contents of the beaker except at the point where the aperture is located. The second electrode is contained in this tube, immersed in the suspension. To the top of the tube is connected a mercury manometer and, through a stop cock, to a vacuum pump. The vacuum pump runs continuously during the counting. When the cock is opened, mercury is drawn from the manometer towards the tube containing the orifice. When the stop cock is closed mercury flows back to its original balanced position, at the same time drawing the sample back into the tube by way of the aperture. Two contacts inserted in the wall of the manometer provide a means of insuring that the number of particles in an

exact volume (0.05 cubic centimeters) of the sample is counted as it passes through the aperture. Counting is started when the mercury contacts the first electrode and stopped when it contacts the second electrode. Thus, the number of particles is always counted in equal volumes of continuous smoothly flowing fluids. A homemade timer calibrated to give a selected time period of about 10 seconds was used throughout this research. This timer assisted a great deal in reducing the frequent plugging of the 50 micron orifice by reducing the time necessary for uniform volumes for counting. This timer also eliminated the time consuming technique of resetting the mercury in the manometer after each count. Calibration and checking of this timer was done previously by Shindala (1965).

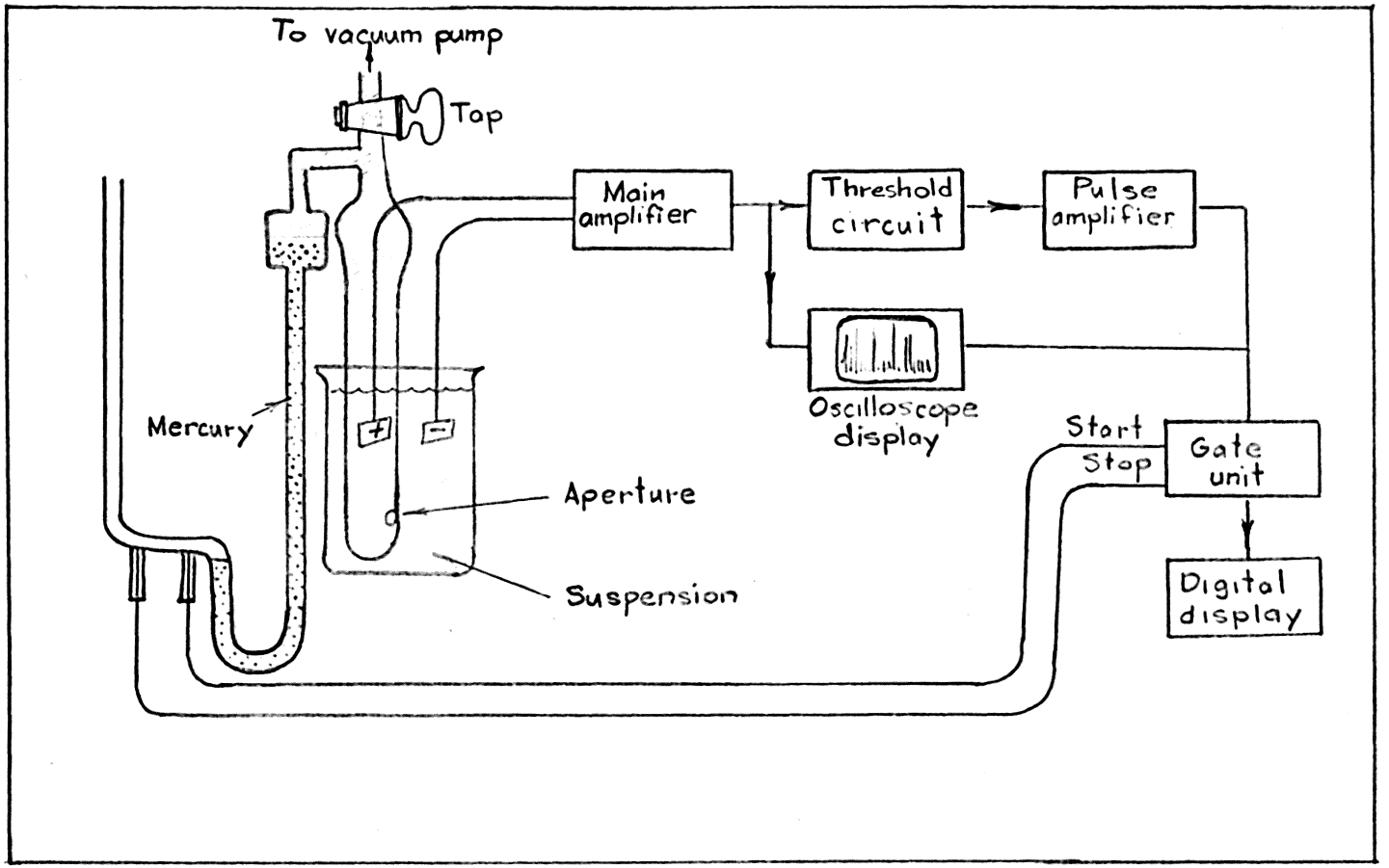

Figure 8. Basic construction of the coulter counter.

APPENDIX II

Cleaning of Glassware

Because minute quantities of vitamins or inhibitors might cause gross errors in the results of the nutritional part of this work, and large numbers of dust particles might effect the Coulter Counter counts or cause the 50 micron orifice to plug, it was necessary to take great care in the cleaning of all glassware used. The glassware was first soaked in 1:100 solution 7X detergent, (Limbro Chemical Company, New Haven, Connecticut) for several hours and then scrubbed and rinsed 10 times in tap water and 8 times in distilled water. The glassware was then shaken by hand and inverted upon paper towels and left in the air to dry. All beakers used that contained samples for the Coulter Counter counts were rinsed 5 times with distilled water before being used. Test tubes used in the preparation of the sample for the Petroff-Hausser method of counting and also used to collect samples were washed and rinsed as previously mentioned except the tubes were dried in an oven for over 2 hours at 200°C for sterilization.

The pipettes were soaked in 1:100 7X solution for more than 24 hours and then rinsed with an automatic continuous device for at least one hour in cold tap water. These pipettes were then placed in aluminum containers and placed in an oven at 200°C for sterilization.

ABSTRACT

Population levels of a mixed culture that was ambiently exposed to bacteria in the atmosphere was studied at various constant dilution rates. Random fluctuations in the free swimmer population were noted. An aseptic continuous flow system proved of no great advantage over that which had ambient innoculation, however, slime production did not follow identical growth patterns in three idential growth vessels.

Coulter counts were made on a pure culture of B. cereus, grown in a continuous flow system at various dilution rates. These counts did not follow continuous flow theory for a pure culture in that there was no sharp drop in count at the critical dilution rate but gradual reductions over a range of several dilution rates.

Total population counts by the Coulter Counter were made on a defined mixed culture of B. cereus and A. aerogenes. After a several days Petroff-Hausser counts showed that 70% of the population was A. aerogenes. Total population was reduced from that of a pure culture of B. cereus. Steady-state populations were noted at various dilution rates. These steady-states, however, did not follow continuous flow theory for a pure culture in that larger counts were noted at a higher dilution rate than some counts at a lower dilution rate. It seemed that slime on the growth vessels' walls continually changed the environment had a greater effect and made it impossible to control all the environmental factors even in a continuous flow process.