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Growth Rate Determination of Heterogeneous Microbial Population in Swine Manure

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ABSTRACT

The effect of manure concentration on the growth of the heterogeneous microbial population under batch condition was studied. Four manure concentrations were used in the study. The dehydrogenase activity was used as a measure of the active biomass in the manure. The chemical oxygen demand test was used to measure the change in organic material caused by biological activities. The growth curve of the heterogeneous microbial population in swine manure was essentially similar to that of a pure culture grown batchwise in that it had the four principle phases: lag, exponential growth, stationary, and death. The exponential growth phase followed a diauxic growth pattern. High concentration of manure had an inhibitory effect on the microbial growth. Manure diluted less than 1:3 (manure:water) depressed the specific growth rate of the microbial population.

Index Entries: Growth rate; heterogeneous population; swine manure; batch operation; dilution; diauxic growth.

INTRODUCTION

Continuous culture is a system of cell growth into which nutrients are being continuously added and from which spent culture medium is being continuously removed. At steady state, the viable population of cells

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is kept constant; the removal of dead and viable cells from the system is being balanced by new cell growth. The choice of a design retention time for a continuous culture operation to treat animal manure must be based on the anticipated growth rate of the heterogeneous population of microorganisms.

Growth rates of microorganisms have traditionally been measured in batch culture. However, organisms grown axenically (i.e., in a culture of a single species of microorganisms) on a simple, sterilized medium are unlikely to behave in the same way as they would in a complex environment such as animal manure. In the latter instance, other organisms are present and may further complicate the situation by competing for available nutrients and by producing various toxins. In addition, there is also a basic difficulty associated with the measurement of the microbial growth in animal manure (*1*).

The average concentration of solids in animal manure may range from 5 to 20%, depending on the size and type of animal, the feed ration, and how the production operation is designed and operated (*1,2*). Animal manure with a high solids concentration must be diluted before treatment by aerobic systems. The maximum concentration of manure for satisfactory growth of aerobic microorganisms has not yet been reported in the literature. The knowledge that there are inhibitory levels of metabolic end products for most microbial cultures (*3*) and that there are inhibitory effects of the various end products and heavy metals found in animal waste on the microbial activities (*2,4,5*) leads to the question of whether manure, diluted only two or three times, would inhibit microbial growth. The effect of manure concentration on microbial growth in an animal waste treatment system is, therefore, expected to be profound.

OBJECTIVES

The aim of this investigation was to study the effect of swine manure concentration on the growth of heterogeneous microbial population under batch operation and to determine the maximum growth rate of the microbial population.

MICROBIAL GROWTH

The rate of increase of cellular material under batch conditions is proportional to the quantity of cell present in the system according to the following equation.

$$dX / dt = \mu X \tag{1}$$

where

dX / dt is the rate of increase of cellular material (g cm^{-3} h^{-1})

X is the total mass of cells per unit volume (g cm^{-3})
t is the time (h)
μ is the specific growth rate (h^{-1})

The specific growth rate (μ) depends on the concentration of the limiting substrate in the growth medium. Monod in 1942 proposed the following equation to describe the relationship between the specific growth rate and the limiting substrate concentration (3).

$$\mu = \bar{\mu}\, S / K_s + S \quad (2)$$

where

$\bar{\mu}$ is the maximum specific growth rate (h^{-1})
S is the concentration of the limiting substrate (g cm^{-3})
K_s is a constant, equal to the substrate concentration at 1/2 the maximum specific growth rate (g cm^{-3})

MEASUREMENT OF MICROBIAL GROWTH

The absolute measurement of microbial growth is dry weight increase (3,6), but it is difficult to separate microorganisms from a complex medium like swine manure. Measuring the change in optical density of a suspension of microorganisms can lead to error since the suspension includes both living and dead cells, as well as particulate organic matter (3). Methods based on the determination of cell constituents are very expensive (7). It is, therefore, very important that all these interactions, difficulties, and limitations be taken into account when investigating microbial growth in swine manure.

The rate of electron transfer is affected by the activity of the coenzymes nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD), which act as intermediate electron acceptors. If a suitable electron acceptor dye is present, the activity of NAD and FAD can be measured by the visible color changes of the dye. One such dye is triphenyl tetrazolium chloride (TTC). This dye has been used successfully in dehydrogenase activity studies of activated sludge (5,8–10). In the presence of dehydrogenase enzymes, TTC is reduced to triphenyl formazan, a red dye that can be extracted and determined colorimetrically. Dehydrogenase activity per unit biomass synthesized was observed to be constant for a given substrate–microorganisms system at all specific growth rates (1,11).

MATERIALS AND METHODS

This study was conducted in twelve 500 mL Erlenmeyer flasks, capped with porous plastic stoppers. The flasks were mounted on a controlled environment reciprocating shaker. The temperature in the shaker chamber was maintained at 21°C. The shaker speed was 250 rpm.

Table 1
Characteristics of Swine Manure Used in the Batch Culture Stu∟,

Characteristic	Measured value	Unit
Chemical oxygen demand	132700.00	mg/L
Total Kjeldhal nitrogen	9240.00	mg/L
Ammonia nitrogen	5430.00	mg/L
Total solids	123400.00	mg/L
Volatile solids	83.44	%
Fibrous materials[a]	35.54[c]	%
Fibrous materials moisture content	78.26	%
Filtrate[b]	66.46[c]	%
pH	7.00	

[a]Fibrous mat collected on a number 16 mesh sieve (1.19 mm).
[b]Liquid drawn through number 16 mesh sieve under a 67 kPa vacuum.
[c]Percent by volume from the original volume of raw manure.

Dissolved oxygen in the manure was measured by a polarographic electrode connected to a dissolved oxygen meter. A single electrode pH probe connected to a pH meter was used to measure the pH in the manure. The absorbency of formazan in the TTC test was measured by a spectrophotometer at a wavelength of 484 mμ.

The raw manure (feces and urine) used in this experiment was collected from a finishing swine building. Some characteristics of the raw manure used in the study are presented in Table 1. The raw manure was mixed thoroughly and an equal volume of deionized water was added. The diluted manure was then sieved through a number 16 mesh sieve (1.19 mm opening size) to remove hair and other particulates. The required manure strength was obtained by adding deionized water to the sieved manure. Four dilution ratios of manure to water (1:1, 1:2, 1:3, and 1:4) were used.

For each dilution ratio, 3 flasks were used. A total of 12 flasks were, therefore, required for this experiment. An amount of 1.5 L of manure-water mixture was prepared in a 2-L beaker for each dilution ratio. The mixture was kept well mixed by means of a magnetic stirrer. Three portions of exactly 250 mL each of the mixture was transferred to the three flasks assigned for this particular dilution. The same procedure was carried out with the other dilution ratios. The flasks were fastened to the shaker and the shaker was then turned on. After approximately 20 min of shaking, to saturate the samples with oxygen first, 1 mL inoculum from an aerated swine manure was added to each flask. This moment was taken as "time zero."

Five mL samples were drawn from 2 flasks of each manure dilution at any sampling time in a way that over the entire period of the experiment equal volumes of samples would be removed from each of the 12 flasks. Sampling was frequent (every 2 h) during the first 24 h, since most of the change was expected to take place during the initial period of the experiment. During the period of 24–48 h, sampling was done every 6 h. This was followed by 24-h sampling intervals until the end of the 144-h shake-flask experiment. The TTC test and the COD test were performed on each sample. The TTC test was performed as described by Ghaly (*1*). The COD test was made on the mixed liquor sample (total COD) and on the filtrates passing through 0.45 mμ millipore filter (soluble COD). The COD test was performed as described in APHA (*12*).

RESULTS AND DISCUSSION

Substrate Measurement

The chemical oxygen demand (COD) test was used in this study to determine the variation of organic content of the swine manure with time. The test was utilized to measure the change in COD caused by biological oxidation. The results obtained from the COD test are presented in Figs. 1 and 2. The reductions in both the total and soluble COD were strongly affected by the manure concentration.

The soluble COD results illustrate the ability of the aerobic microorganisms to utilize readily available carbon. The measured soluble COD curves (Fig.1) displayed 3 main stages. During the first stage there was no or little change in the soluble COD. This was followed by a rapid soluble COD removal during the second stage. The soluble COD then remained constant during the third stage. The duration of the period of rapid soluble COD removal was related to the manure concentration; the higher the concentration the longer was the period of rapid soluble COD removal. The total COD (Fig. 2) went through similar stages. Unlike the soluble COD, however, the total COD continued to decline until the end of the experiment.

The initial and final values of the total and soluble COD as well as the amount of reductions in the COD are reported in Table 2. The reductions in both the total and soluble COD were dependent on the initial concentrations of manure. The four manure–water mixtures used in this study (1:1, 1:2, 1:3, and 1:4 manure–water ratio, respectively) resulted in reductions in the total COD of 64, 70, 77, and 80% and reductions in the soluble COD of 85, 89, 90, 95%, respectively. For all manure concentrations, at the end of the experiment the reduction in the total COD was about twice as large as was the reduction in soluble COD. This indicates that organic materials initially present in a solid form had been solubilized as a result of the production of extracellular enzymes by the microorganisms. The soluble material were then metabolized by the microorganisms.

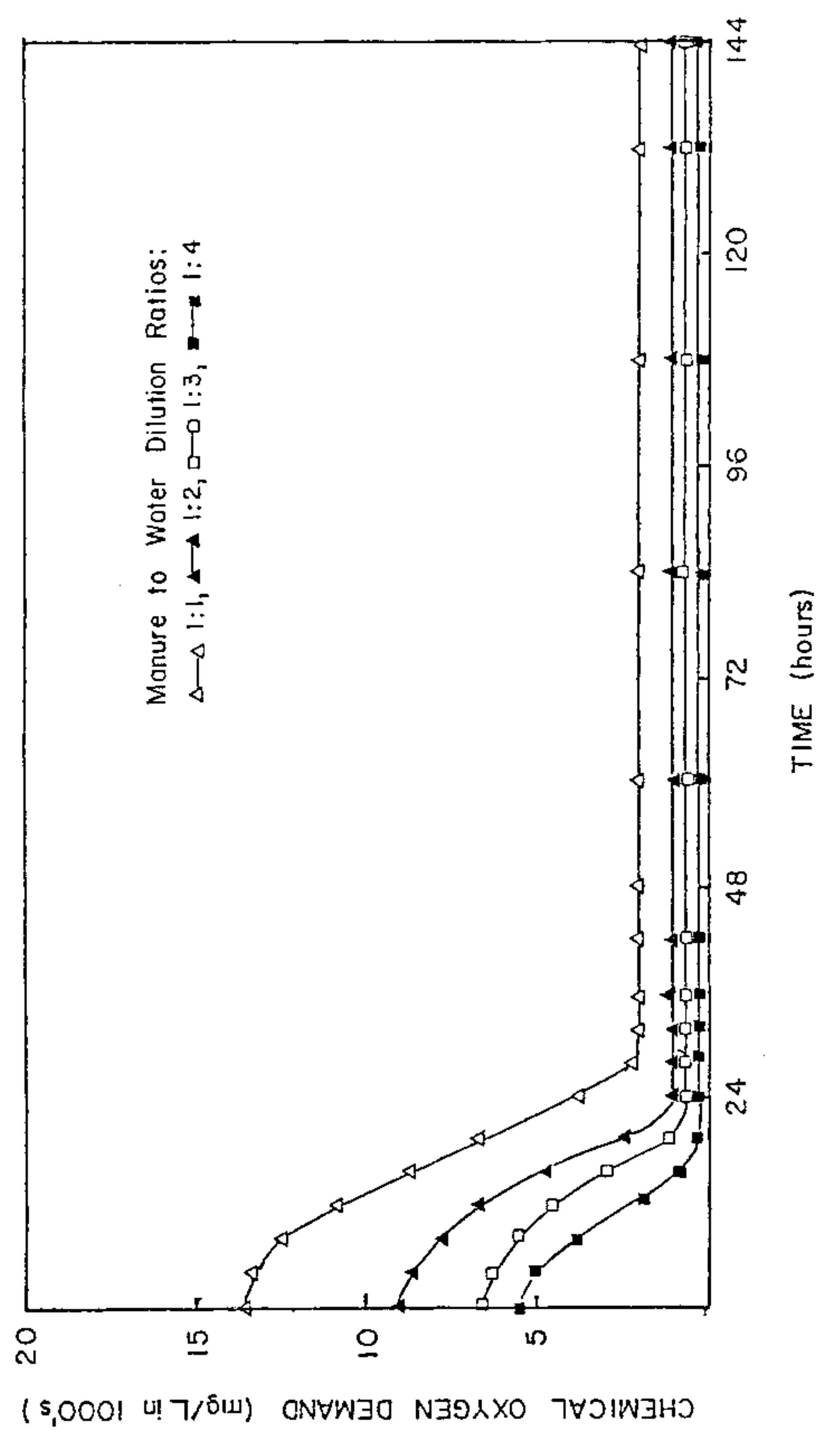

Fig. 1. The soluble chemical oxygen demand.

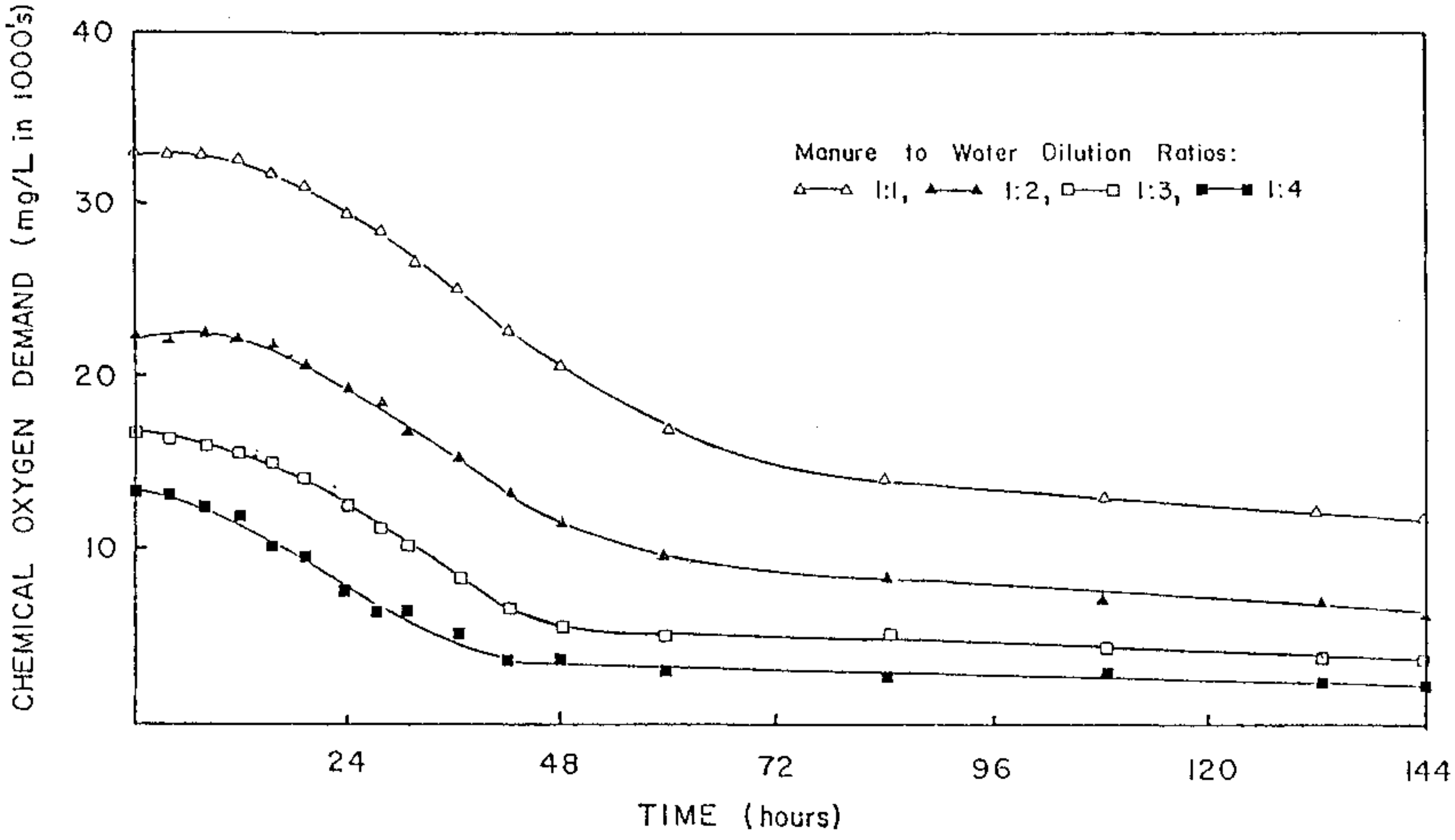

Fig. 2. The total chemical oxygen demand.

Table 2
The Chemical Oxygen Demand Values of the Batch Culture Operation

	Total COD				Soluble COD			
Dilution,	Initial,	Final,	Reduction		Initial,	Final,	Reduction	
manure–water	mg/L	mg/L	mg/L	%	mg/L	mg/L	mg/L	%
1:1	33075	12025	21050	63.64	13564	2000	11564	85.26
1:2	22028	6668	15360	69.73	9047	995	8052	89.00
1:3	16538	3803	12735	77.00	6782	670	6112	90.12
1:4	13230	2646	10584	80.00	5425	271	5154	95.00

Microbial Growth

The dehydrogenase activity was used as a measure of active biomass in preference to other biochemical parameters because of the simple nature of the dehydrogenase test. Also, the dehydrogenase activity per unit biomass synthesized was observed by Ghosh (*11*) to be a constant for a given substrate–organism system at all observed specific growth rates. The measured dehydrogenase activity was, therefore, considered to be related to the concentration of the active microbial population in swine manure, in accordance with the finding of Ghosh (*11*). This relationship was also confirmed by Ghaly (*1*) The formazan yield curve is, thus, considered as the growth curve of the heterogeneous population.

The formazan yield values of the growing heterogeneous population are presented in Fig. 3. The 4 manure concentrations yielded 4 different growth curves, all having a similar shape but with different magnitudes. The shape of these curves is essentially analogous to that obtained from a pure batch culture. The important feature of these curves is that the 4 principal phases encountered in the history of a microbial culture grown in batch operation can be clearly recognized. These were: the lag phase, during which the growth rate was zero; the exponential growth phase, during which the growth rate had a constant, maximum value; the stationary phase, during which the growth rate again was zero; the death phase, during which the growth rate had a negative value. These 4 phases are interconnected by transition periods during which the growth rate changed continuously.

There was also an observed intermediate lag phase during the period of exponential growth. This is an expected phenomenon for a mixed population growing in a complex medium such as swine manure. In a complex medium, microorganisms exhibit a growth patern different from that in a single substrate. The microorganisms initially consume 1 substrate preferentially and subsequently the initial growth rate slows as this substrate is exhausted. After a period of adaptation, during which the cells synthesize new enzymes required to utilize the alternate substrate, the lag period is followed by a new exponential growth phase. This phenomenon was

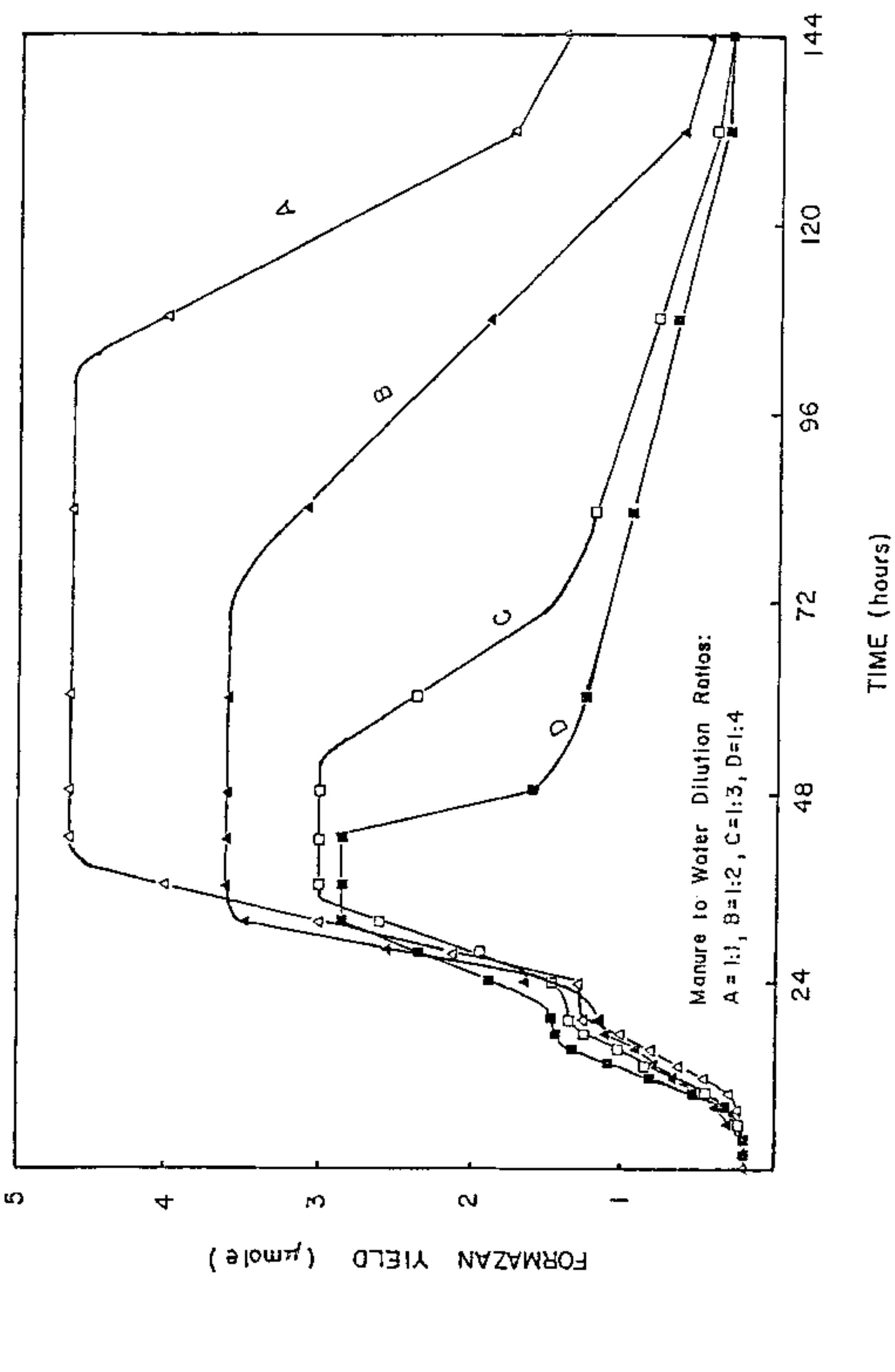

Fig. 3. The formazan yield of the growing heterogeneous population in swine manure.

discovered by Monod in 1942 and is called "diauxic growth." Its occurrence has also been reported by several other workers (*1,6,13,14*).

The formazan yield values obtained from each dilution of manure were plotted on a semilogarithmic coordinate paper, as shown in Fig. 4. This was done to linearize the exponential portions of the growth curve. The specific growth rate (μ) of the heterogeneous population was then calculated from the slope of the linear portion of the growth curve according to the following equation.

$$\mu = 2.303 \times \text{slope} \tag{3}$$

The generation time (g) or mean doubling time is defined as the time required for all components of the culture to increase by a factor of 2. The relation between μ and g is as follows (3).

$$\mu = (\ln 2) / g = (0.693) / g \tag{4}$$

Equation (4) was then used to estimate the generation time (g). The graphical method described by Stainer et al. (3) was used to calculate the duration of the lag phase, as illustrated in Fig. 5. The minimum required retention time was taken as the reciprocal of the maximum growth rate. All these calculated values are presented in Table 3. The duration of the stationary growth phase was also determined from Fig. 3. It was found to be 62, 34, 18, and 10 h for manure–water mixture rations of 1:1, 1:2, 1:3, and 1:4, respectively.

The microbial growth in swine manure was strongly affected by the manure concentration. The observed variances in the initial lag phases were probably caused by the differences in concentrations of manure used in this experiment compared to that used for inoculum preparation. The inoculum was prepared in a manure–water mixture having 1 part manure to 2 parts water. Thus, change of manure concentration resulted in a longer lag phase, since an acclimatization period was necessary. The second lag phase was constant (2 h), regardless of the manure concentration, since the microorganisms had been growing in their media and were, therefore, acclimatized to them.

A plot of specific growth rate of the first growth phase vs initial soluble COD is shown in Fig. 6. A similar plot of the specific growth rate of the second growth phases is also shown on the same graph. During the first growth phase, microorganisms were observed to grow faster in more diluted manures than in concentrated ones. However, there was no difference between growth rates obtained in manures of 1:1 and 1:2 dilution ratios. The apparent inhibitory effect of high manure concentration on microbial growth might be a result of the presence of high levels of heavy metals and other toxic materials in concentrated swine manure, as reported by Robinson and Loehr (4) and Coackley and O'Neill (5). It might also be a result of the nutrient concentrations occurring at inhibitory levels. This phenomenon was reported by Stainer et al. (3), who stated that at low

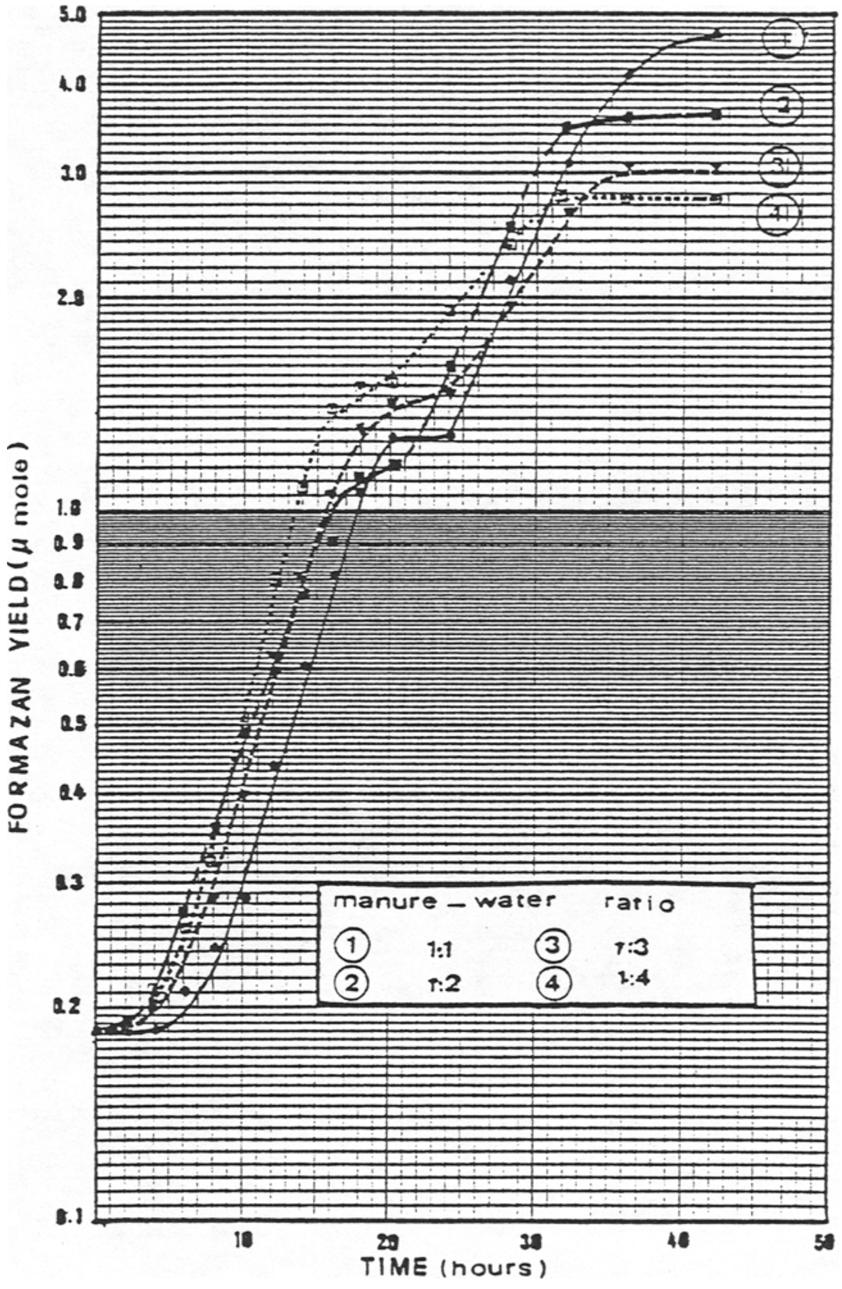

Fig. 4. The exponential growth phase of the heterogeneous microbial culture as measured by formazan yield.

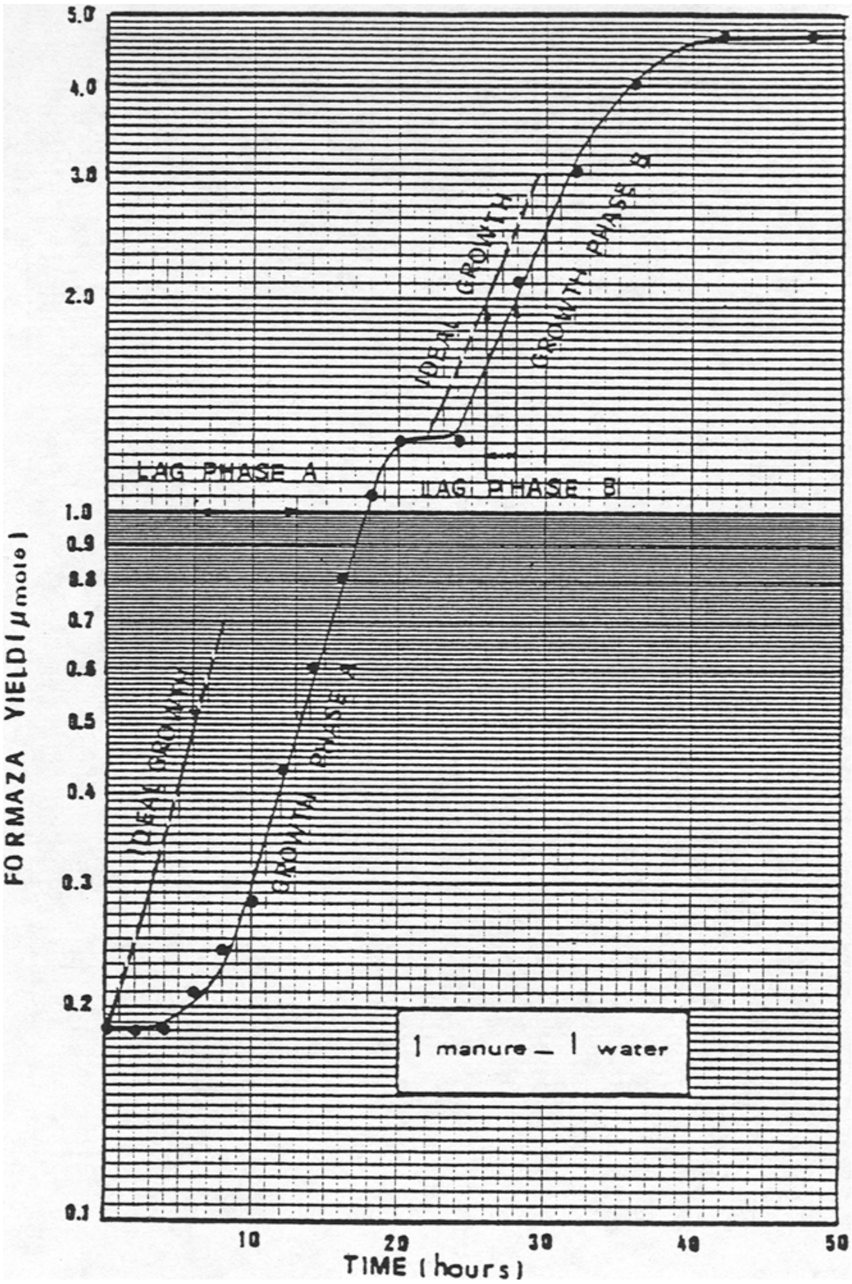

Fig. 5. Graphical determination of the lag phase.

Table 3
Measured Values of Some Kinetic Parameters of the Batch Culture Operation

	First growth phase, A				Second growth phase, B			
	Dilution, manure-water				Dilution, manure-water			
Parameters	1:1	1:2	1:3	1:4	1:1	1:2	1:3	1:4
Soluble COD, mg/L	13564	9047	6782	5425	5500	3750	1000	450
Lag phase, h	7.00	3.75	5.00	6.00	2.00	2.00	2.00	2.00
Doubling time, h	4.62	4.62	4.07	3.01	6.30	6.93	13.76	11.55
Maximum specific growth rate, h^{-1}	0.15	0.15	0.17	0.23	0.11	0.10	0.07	0.06
Minimum retention time, h	6.66	6.66	5.88	4.35	9.09	10.00	14.28	16.66

nutrient concentrations, the growth rate of microorganisms is closely proportional to nutrient concentration, but as the concentration increases, the growth rate rises rapidly to a maximum value that is maintained until the nutrient concentration reaches an inhibitory level, at which point the growth rate begins to fall again. This phenomenon was also confirmed by Ghaly (*1*). In the second growth phase, the growth of the heterogeneous population was also affected by the manure concentration. In contrast to the first growth phase, during the second growth phase higher manure concentration resulted in higher specific growth rates. As evident from Fig. 6, cultures that had grown the slowest during the first growth phase grew the fastest during the second growth phase. The shape of the specific growth rate–manure concentration curve of the second growth phase (Fig. 6, lower curve) seemed to follow Michaelis-Menten kinetics. These results were interpreted to mean that during the second growth phase, the microorganisms were growing under nutrient limited conditions.

The maximum specific growth rate ($\bar{\mu}$) and the substrate constant (K_s) were determined by the method described by Loehr (*2*). The results are illustrated in Fig. 7. The values of μ and K_s were 0.103 h^{-1} and 3300 mg/L of soluble COD, respectively.

The duration of the stationary growth phase and the size of microbial population (as measured by formazan yield) were also affected by manure concentration. Increasing manure concentration increased both the duration of the stationary growth phase and the maximum population size.

The allowable manure concentration and the retention time (the reciprocal of the specific growth rate) are considered to be of prime importance for the design of continuous aerobic treatment. High manure concentration and short retention time are desirable features for such treatment systems. A high manure concentration allows a small size treatment system to be used, short retention time results in a high manure throughput.

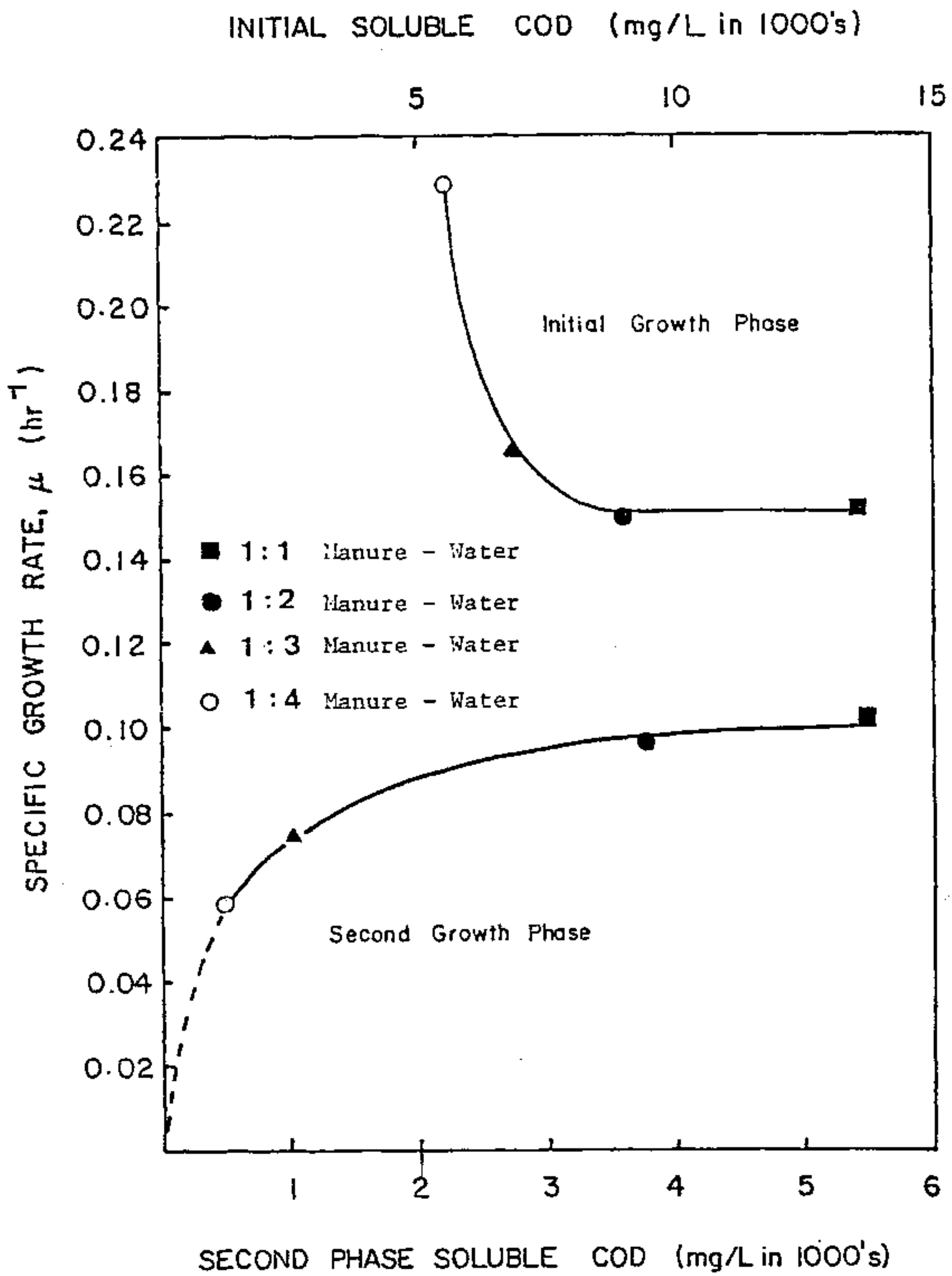

Fig. 6. Effect of manure concentration as measured by soluble COD on the maximum growth rate of heterogeneous culture.

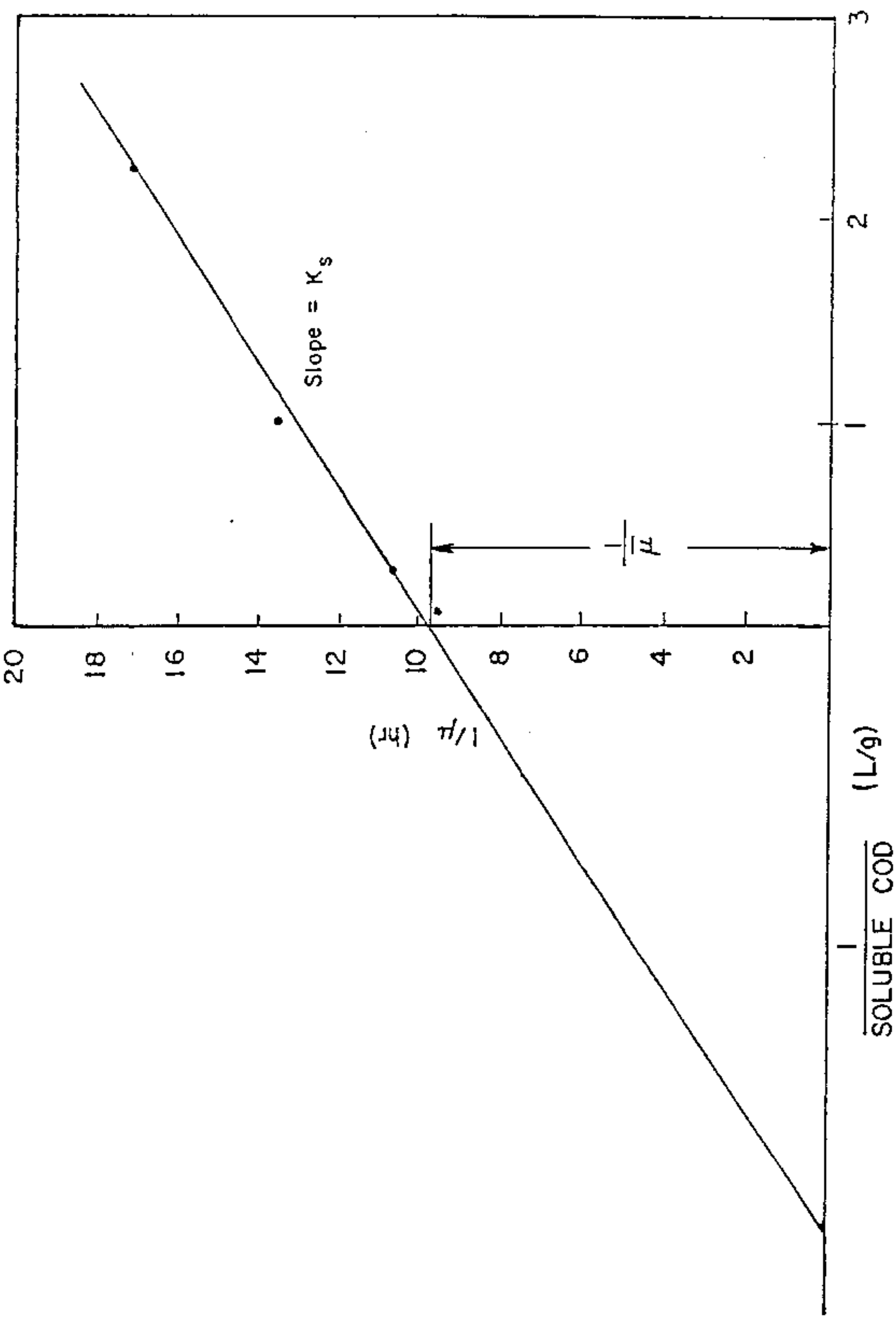

Fig. 7. Graphical determination of K_s and $\bar{\mu}$.

From the results obtained from batch culture operation, it is evident that these two factors are interrelated; the retention time increases (i.e., the specific growth decreases) as the manure concentration is increased. Thus, in the design of a continuous aerobic system, the two factors may be traded off against each other to find the lowest total cost.

Dissolved Oxygen

The dissolved oxygen results are shown in Fig. 8. Initially, the mixed liquor was saturated with oxygen before the inoculum of microorganisms was added. The observed oxygen saturation value was slightly different for different manure concentrations; the higher the manure concentration the lower was the oxygen saturation value. Changes in the concentration of dissolved salts present in animal manure were probably the principal cause of the variation.

The dissolved oxygen concentration in animal manure was strongly affected by manure concentration. For all manure concentrations, the dissolved oxygen dropped sharply during the first 42 h of the shake-flask operation, remained at its minimum value for a period of time, and then increased again. The duration of the period of minimum dissolved oxygen values was related to the manure concentration; the higher the manure concentration the longer was the period of low DO. The final concentrations of dissolved oxygen at the end of the experiment were, however, lower than the initial values for all concentrations of manure.

The observed DO minimum of 1.5 mg/L is higher than the range 0.2–0.6 mg/L, suggested by Porges et al. (*15*) to maintain an active aerobic animal waste treatment, which indicated that the oxygen uptake rate of the heterogeneous population was independent of the oxygen concentration; oxygen was, therefore, presumably not a growth limiting factor during this experiment.

pH

The pH results are shown in Fig. 9. The initial pH value of the swine manure used in this experiment was very close to 7.0. Stevens and Cornforth (*16*) suggested that the near neutral pH may be a result of the presence of dissolved ammonium bicarbonate. According to their interpretation, dissolved carbon monoxide (CO) in animal manure may combine with the ammonia present to form ammonium bicarbonate, thus keeping the pH value near neutral. However, manures of different concentrations had different initial pH values (6.65, 6.75, 6.85, and 6.95 for 1:1, 1:2, 1:3, and 1:4 dilutions, respectively). The greater the amount of water added the higher was the pH value.

For all manure concentrations, 3 distinct stages of pH change were observed during the first 36 h of the shake-flask operation. There was a rapid initial pH increase during the first 12 h, followed by a rapid pH decrease during the second 12 h, and then a rapid increase again during the

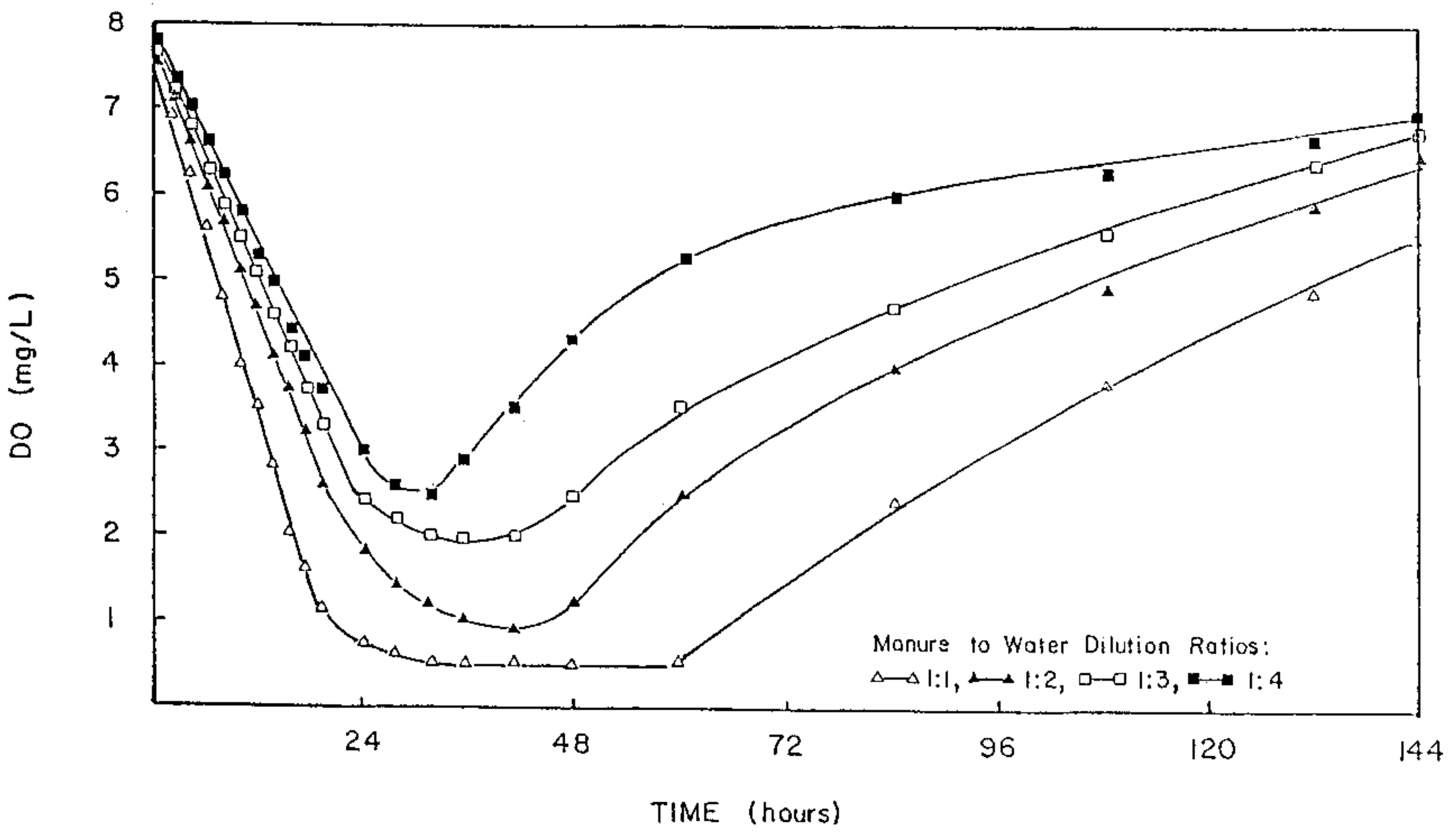

Fig. 8. The dissolved oxygen.

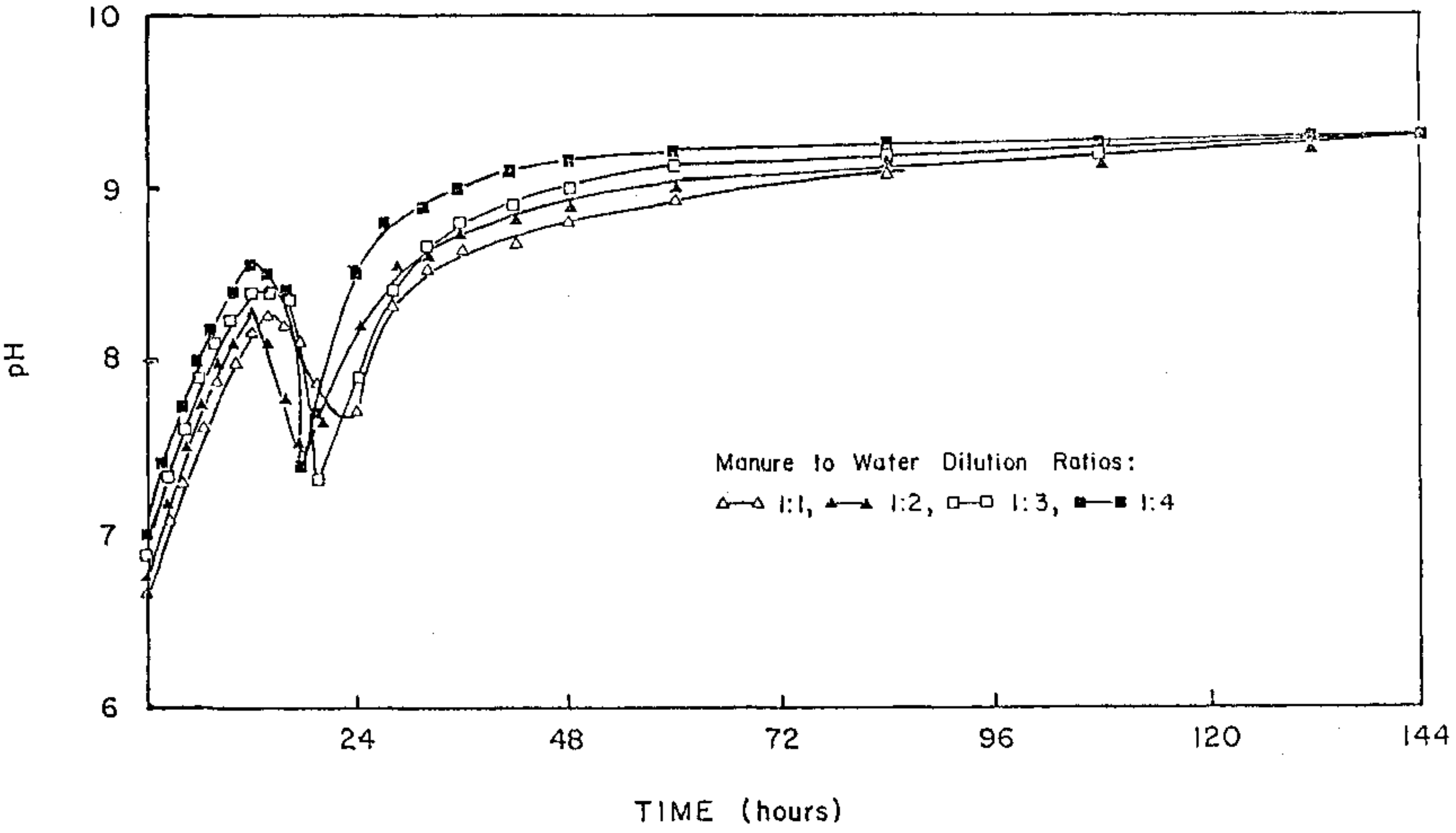
Manure to Water Dilution Ratios:
1:1, 1:2, 1:3, 1:4
TIME (hours)
pH
10
9
8
7
6
24
48
72
96
120
144

Fig. 9. The pH.

third 12 h. After 36 h, the pH remained high and slowly increased until it eventually reached 9.3 at the end of the experiment. According to Stevens and Cornforth (*16*), degradation of organic matter and urea hydrolysis may result in an increased rate of ammonia production, causing the pH to rise. Another explanation for the rise in pH could be the utilization of acidic substrates by microorganisms, as readily available nutrients, which may increase the relative buffering capacity of ammonia present in the treatment system. Phillips (*17*), Robinson, and Loehr (*4*) all reported similar final pH values.

For all manure concentrations, the decrease in pH took place during the period of the first lag phase. During this period, the microorganisms may have produced acidic substrate or utilized ammonium thereby reducing the pH. Phillips (*17*) reported a similar shaped pH curve but did not, however, discuss the cause of the intermediate drop in the pH.

CONCLUSIONS

The following conclusions were drawn from this study.

1. Dehydrogenase activity measurement, expressed as formazan yield from TTC, can be used to obtain the growth curve of a heterogeneous microbial population grown in swine manure.
2. The growth curve of the heterogeneous population was essentially similar to that of a pure culture grown batchwise in that it had four principal phases; lag, exponential growth, stationary, and death. The exponential growth phase followed the diauxic growth pattern.
3. An inoculum transferred to a different medium required a period of adaptation (lag phase), during which time new enzymes had to be synthesized and the control mechanism regulating the synthesis of various cell components had to be brought into step with the new situation. The duration of the lag phase depended on the magnitude of the difference between the two media.
4. High manure concentrations had an inhibitory effect on the microbial growth. Manure diluted less than 1:3 (manure:water) depressed the specific growth rate of the heterogeneous microbial population.
5. During the second growth phase, the microorganisms seemed to grow under nutrient limiting condition. The specific growth rate of the microbial population appeared to be related to the manure concentration and exhibited Michaelis-Menten kinetics.
6. The smallest specific growth rate of the heterogeneous population grown in swine manure (of 1:1 manure-water dilution ratio) was 0.15 h^{-1}. A retention time of longer than 7 h would

be necessary in order to not wash out the microbial population from a continuous treatment unit.

7. The reduction in both the total and soluble COD is dependent on the initial manure concentration, decreases with increasing manure concentration.
8. The dissolved oxygen decreased very rapidly during the period of exponential growth and increased again when the culture entered the stationary growth phase.
9. The pH of untreated swine manure was near neutral (6.65–6.95), but it increased during the aeration process and eventually reached 9.3.

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