THE CHEMOSTAT

A CONTINUOUS FLOW ALGAL ASSAY

by

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INTRODUCTION

During the last few years the use of continuous cultivation of organisms has become very popular. However, continuous cultures are not new; in fact, they were used back before 1900 to produce vinegar and yeast and to treat municipal wastes. One of the first laboratory scale units was set up by Ketchum and Redfield in 1938 (see Malek and Fencl, 1966) in which diatoms were grown in artifically illuminated tanks. The first real understanding of the fundamental relations in continuous cultures became known when the papers of Monod (1949) and Novick and Szilard (1950) were published. Since then many aspects of algal growth and physiology have been studied using continuous culture systems. The effect of high intensity light upon the growth of chlorella was studied (Matthern, et al, 1969). Wassink, et al, (see Burlew, 1953) showed that the efficiency of light-energy conversion in chlorella is 12 to 20 percent when the illumination is not too high, but decreases to 2 to 3 percent in full summer light. Pipes and Koutsoyannis (1962) observed that the population density of chlorella in light limited culture is directly proportional to detention time. Kok studied the effect of flashing light on chlorella growth while Tamiya, et al, observed the effect of diurnally intermittent light on chlorella growth (see Burlew, 1953). Temperature, light, and nutrient interactions during continuous culture were studied by Maddox and Jones (1964). Bacterial cultures have been used to study the influence of growth conditions on cell wall composition (Tempest and Ellwood, 1969). Limiting nutrient studies on bacteria were investigated by Slezak and Sikyta (1967) and Tempest, et al, (1965, 1967). Studies of fungal growth and intermediary carbon metabolism under steady and non-steady state conditions were made by Carter and Bull (1969).

The current interest in continuous-flow steady state systems stems from the need for a laboratory method of evaluating biostimulatory response. Using continuous-flow systems it is possible to relate the growth rate to the limiting substance, determine yield and decay constants for a given substrate, and then estimate the standing crop of algae that will be supported by a given nutrient concentration. With the chemostat it is possible to determine the nutrient concentration which produces the maximum growth rate under any set of environmental conditions. However, the relationships between growth rate, nutrient concentrations, light intensity, photoperiod, temperature, and other factors must be determined. Once these relationships are determined, it should be possible to formulate mathematical models of algal productivity. The coefficients obtained from continuous flow cultures could be used to model a natural aquatic ecosystem using the kinetic equations derived from chemostat theory. However, Porcella (see Middlebrooks, et al. 1969) recommends against this; he believes that chemostat data should be used to gain useful information on productivity, nutrient cycling and successional relationships. Growth, decay, and yield coefficients derived from chemostat data could, however, be used as rate coefficients in sophisticated mathematical models. It should be emphasized that the chemostat is basically a highly simplified simulation of an aquatic ecosystem; thus, direct application of chemostat kinetics to complex natural ecosystems is not feasible.

A continuous flow system can be characterized as a constant volume system, being in a dynamic steady state with constant concentrations of all components, with all reactions proceeding at a constant rate. There are two general types of continuous systems: the turbidostat and the chemostat.

The turbidostat maintains a constant density by using a photocell adjusted to a specific light intensity; when the density of the culture reduces the light intensity, a valve opens allowing fresh medium to enter while an equal volume of the culture overflows into a sampling container. Up to about 1961, turbidostats were used more than chemostats, but due to their high cost and inconvenience, turbidostats are rarely used now.

Unlike the turbidostat, which is actually a semi-continuous system, the chemostat is a continuous flow system which maintains steady state by keeping a constant flow rate and volume. To maintain a constant volume, the flow in must equal the flow out. Furthermore, steady state is maintained only if the chemostat is completely mixed and kept in a constant environment, that is, constant temperature, light, etc. Figure 1 shows a model of a chemostat; the chemostat can be described as a Continuously Stirred Tank Reactor (CSTR) where the concentrations of substrate and organisms in the chemostat are equal to the effluent concentrations. By keeping the volume constant and adjusting the flow rate, any desired residence time can be maintained. Algal cells are added to the chemostat at a concentration X_0. After a certain time, the algal population reaches a steady state concentration X_1. The initial substrate concentration S_0 also reaches a steady state concentration S_1, where S_0-S_1 equals the amount of substrate utilized by the algal population X_1. Steady state is maintained when the cell growth rate equals the rate at which cells are washed out of the system.

The mathematical theory of continuous flow systems was derived from the typical microbiological growth curve obtained in batch cultures. Chemostats are usually run so that the algal population are in the

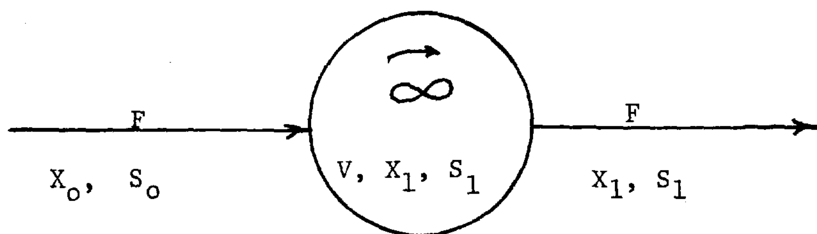


FIGURE 1. SINGLE STAGE STIRRED REACTOR (CSTR)(From: Provisional Algal Assay Prod.)

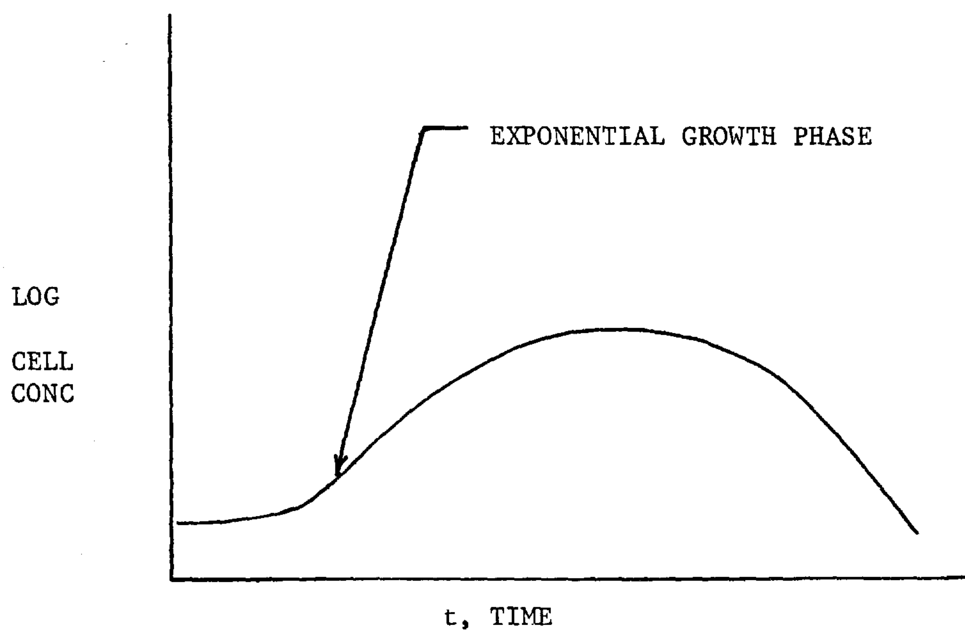


FIGURE 2. GROWTH CURVE FOR A BATCH REACTOR (From: Provisional Algal Assay Proc.)

exponential growth phase (see Figure 2). In this phase, the algal growth rate is a maximum and balanced growth is achieved. With balanced growth, all nutrients are present in excess and development is not inhibited by products of metabolism. Continuous culture allows the algae to grow in the exponential growth phase where they are permanently "physiologically young" (Malek and Fencl, 1966). The main advantage of continuous cultivation is the steady state condition that evolves. Cultivation can proceed for as long as desired or can be changed simply by regulating the nutrient flow rate.

The chemostat apparatus need not be complicated; Figure 3 shows a simple chemostat proposed by The Joint Government-Industry Eutrophication Committee on Methods (Pearson, *et al.*, 1968). Operation consists of filling the chemostat with the test substrate, innoculating it with algal seed, and adjusting the flow rate so as to give the desired residence time. Environmental conditions such as temperature, light, intensity, and relative humidity must be kept constant. Daily observations of effluent volume, cell count in the reacter, pH, and conductivity should be made. At least twice each week the limiting substrate concentration and cell mass should be determined. For proper operation, the feed rate and the degree of mixing should be checked regularly. Using the data collected, the kinetic characteristics of the system can be modeled.

KINETICS

Kinetic equations for continuous algal cultures are possible because of the relationships between growth rate, the limiting nutrient, and growth yield. When these three relationships are combined using material balances, steady state kinetic equations can be formulated.

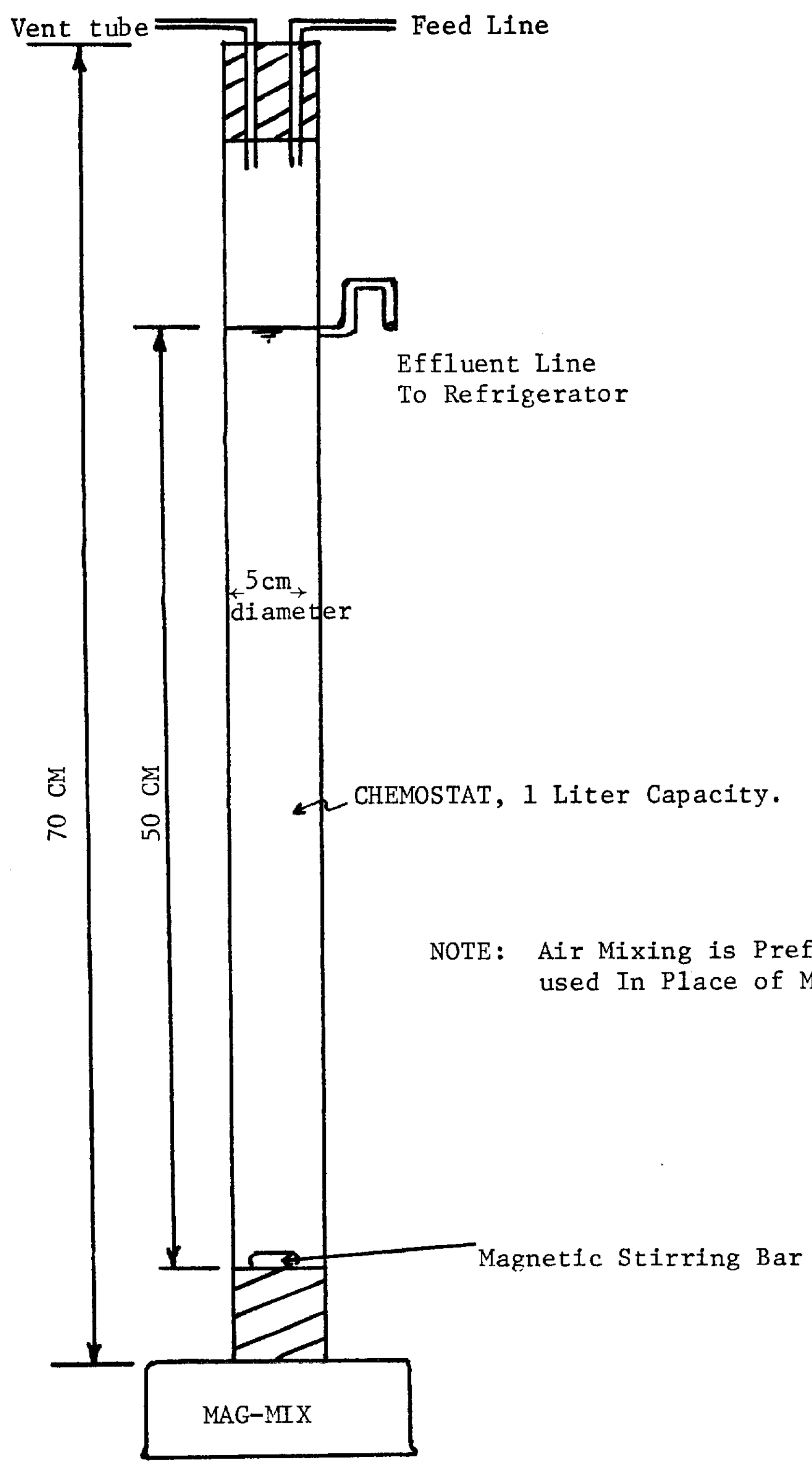


FIG. 3 DETAILS OF PROPOSED CONTINUOUS CULTURE APPARATUS (CHEMOSTAT)
(From: Provisional Algal Assay Procedure)

The specific growth rate is a function of the limiting nutrient in the reactor. According to Monod (1950) and others, the relation between the specific growth rate and the limiting nutrient is expressed by the Michaelis-Menten equation:

$$(1) \qquad \mu = \hat{\mu} \left[\frac{S}{K_s + S} \right]$$

where

μ = specific growth rate, T^{-1}

S = growth limiting substrate concentration, ML^{-3}

K_s = half-saturation constant, ML^{-3}

$\hat{\mu}$ = maximum specific growth rate, T^{-1}

In 1913, Michaelis and Menten formulated their equation using the method of equilibrium rather than steady state. Today, however, enzyme kineticists use a Michaelis-Menten equation developed from steady state considerations. Both equations are identical except for the interpretation of the K_s values; the steady state K_s value includes dissociation of the enzyme-substrate complex while the equilibrium K_s value does not.

The maximum specific growth rate, $\hat{\mu}$, is the velocity of the reaction when the substrate concentration approaches infinity, that is, when all of the enzyme is combined with substrate, thus it is the maximum velocity obtainable for a specific enzyme at a given enzyme concentration. The half-saturation constant, K_s is characteristic of a particular enzyme and is equal to the substrate concentration at which the enzyme activity is half-maximum; K_s is also independent of the enzyme concentration.

Since it is assumed that growth rate is a function of the limiting substrate concentration, it seems reasonable to assume that the growth

rate is also a function of the limiting substrate removal rate. However, yield coefficients (mass of cells produced per mass of substrate removed) for both algal and bacterial cultures have been shown to vary (Malek and Fencl, 1961).

A mathematical model for the chemostat is developed by taking material balances of cell mass and substrate. It is assumed that the Michaelis-Menten Equation adequately relates the growth rate to the limiting nutrient concentration, that a constant proportion of the cells are viable, and that the yield coefficient is constant. The following mathematical derivations are a condensation of those presented in the First Progress Report, Eutrophication of Surface Water - Lake Tahoe (McGauhey, *et al.*, 1968).

Material Balance of Cells

$$\frac{dX_1}{dt} V = FX_0 - FX_1 + \mu X_1 V - K_d X_1 V$$

where

X_0 = influent cell mass, ML^{-3}

X_1 = effluent cell mass, ML^{-3}

F = flow rate L^3T^{-1}

V = reactor volume, L^3

k_d = decay coefficient, T^{-1}

At steady state,

$$\frac{dX_1}{dt} = 0$$

Therefore, assuming $X_0 = 0$,

$$(2) \quad \mu - K_d = \frac{F}{V} = \frac{1}{\Theta} = \frac{1}{\Theta c}$$

where

Θ = hydraulic residence time, T

Θc = mean cell age in reactor, T

Note that $(\mu - K_d)$ is the net growth rate. Substituting the Michaelis-Menten Equation into the above and solving for S_1, we get

$$(3) \quad S_1 = \frac{K_s(K_d + 1/\Theta)}{\mu - (K_d + 1/\Theta)}$$

For a particular microbial condition (μ, K_s, K_d), the effluent substrate concentration is a function of hydraulic residence time; that is, as the residence time increases, the effluent substrate decreases.

Material Balance of Substrate

$$\frac{dS_1}{dt} V = FS_0 - FS_1 - \left[\frac{VX_1 \mu}{Y}\right]$$

At steady state,

$$\frac{dS_1}{dt} = 0$$

Therefore

$$(4) \quad X_1 = \frac{Y(S_0 - S_1)}{\mu\Theta}$$

Since we assumed a constant yield, we can relate growth rate to substrate removal velocity.

$$(5) \quad \mu = Yq$$

where

Y = yeild coefficient = $\frac{\text{mass of cells produced}}{\text{mass of limiting substrate removed}}$

q = substrate removal velocity, T^{-1}

Substituting Equation (5) into Equation (2), we get

$$(6)\quad \frac{1}{\Theta} = Yq - K_d$$

To evaluate the kinetic growth characteristics, $\hat{\mu}$, K_s, Y, and K_d, equations (1) and (6) are solved graphically as shown in figures 4 and 5.

Data for the above analysis can be obtained by running a series of three steady state experiments for each nutrient and organism. For a given nutrient and organism, three different residence times or feed concentrations should be used, resulting in a different effluent concentration, S_1. Using the system constants ($\hat{\mu}$, K_s, Y, and K_d), the specific growth rate for any steady state nutrient level can be predicted. Thus,

$$\mu = \frac{\hat{\mu}\ S_1}{K_s + S_1} = \text{specific growth rate}$$

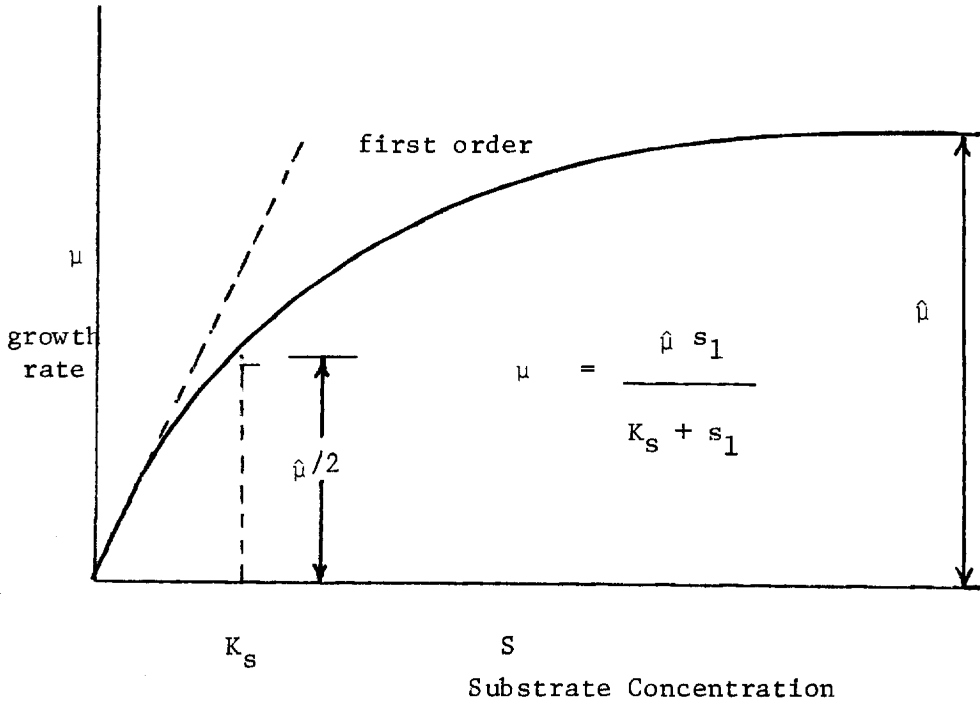
The net growth rate of the system can then be determined using Equation (2),

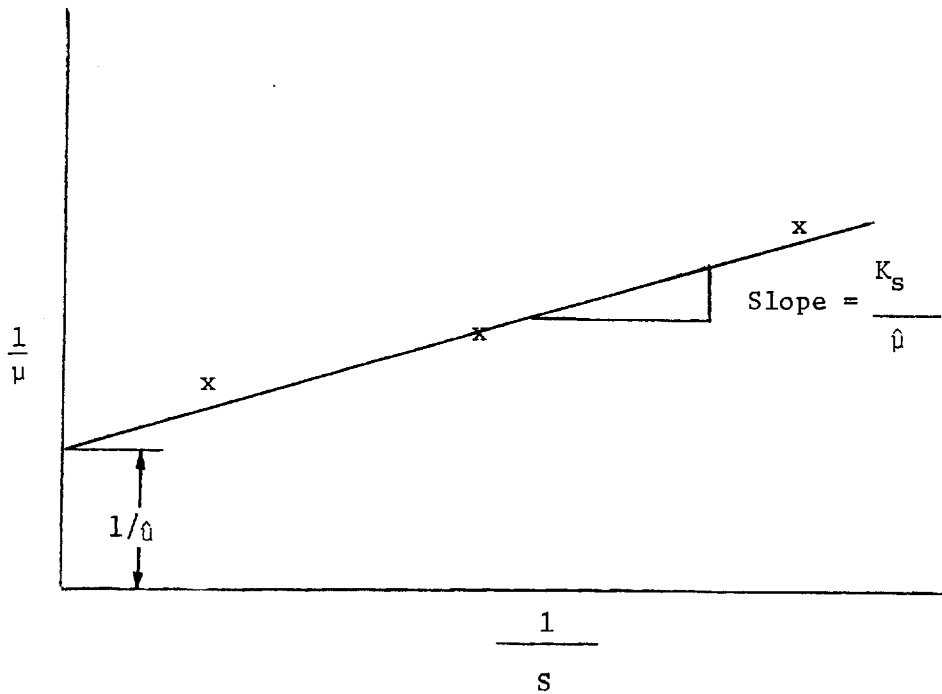
$$\mu - K_d = \frac{1}{\Theta c} = \text{net growth rate}$$

Other kinetic equations have been derived to calculate cell concentrations in continuous cultures. For example,Kono (1968, 1969) notes that predicted cell concentrations using the Monod equation do not fully correspond to observed values in continuous cultures. He derived a rate equation based on critical cell concentration and found good agreement between predicted and observed cell concentrations.

CHEMOSTAT VERSUS BATCH CULTURE

Today, much research is being done on determination of growth rates and nutrient uptake rates. Most of this work is analyzed according to





Straight line form.
$$\frac{1}{\mu} = \frac{K_s}{\hat{\mu}} \cdot \frac{1}{s} + \frac{1}{\hat{\mu}}$$

FIGURE 4 MICHAELIS-MENTEN KINETIC MODEL

(From: Provisional Algal Assay Procedure)

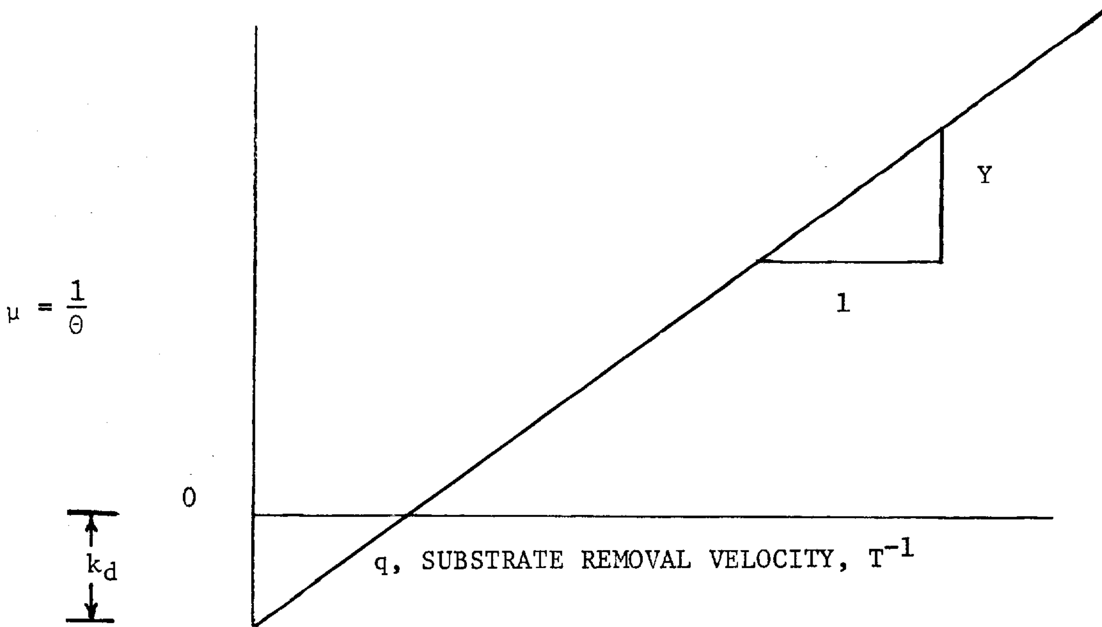


FIGURE 5 PLOT OF CELL CONTINUITY EQUATION,

$$1/\Theta = Y q - k_d$$

(From:Provisional Algal Assay Procedure)

Michaelis-Menten kinetics or some form thereof. However, some researchers work with batch culture methods while others use continuous flow cultures. And herein lies the problem: which method to use: Vaccaro (1969) used batch methods to study the response of natural populations from coastal areas to organic enrichment; Eppley, et al. (1969) used batch cultures to determine half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. Batch methods were used for bioassays for nitrogen and phosphorus with algae and aquatic weeds (Fitzgerald, 1969). The use of batch culture methods for kinetic studies is very popular and many other examples of batch culture research could be cited. We are faced with the situation of having two scientific camps, using two different methods to measure the same parameters.

As mentioned earlier, the chemostat is a dynamic, open system operating at steady state with the algal cells approximately in the same physiological state. The batch unit, however, is a static, closed system, with the physiological state varying with time as shown in Figure 2. Some might object to labeling the batch culture as a "static system" noting that the greatest changes in properties and number of organisms occur in batch culture. However, these changes are due to inhibition of the real dynamics of microbial growth by static conditions (Malek and Fencl, 1966). Fogg (1965) noted that it is difficult to study the effects of mineral nutrient limitation on exponential growth in batch systems since the effect of adding a low concentration of a particular nutrient is to shorten the duration of the exponential phase rather than reduce the relative growth rate. According to Fogg, exponential growth eventually stops in a batch culture because of the following factors: exhaustion of nutrients, decrease in carbon dioxide diffusion, pH change of the culture, light reduction by self-shading, and autoinhibition. The sensitivity and applicability of batch

cultures in quantitating the growth characteristics of limiting nutrients have been questioned (McGauhey, et al., 1968).

Much more information is obtainable from the continuous culture method than from the batch method. One great advantage of the continuous system is that the standing crop level of organisms supported by a given nutrient concentration and residence time can be determined. The batch method merely relates the maximum growth rate to the initial substrate concentration; the substrate concentration at which the maximum growth rate occurs is not known. For continuous culture methods, once the kinetic constants are determined for a given organism and nutrient, the net or gross cellular gross rate attributed to different nutrient concentrations can be estimated. This is not true for batch culture methods.

The chemostat, however, is not a panacea; it has its disadvantages. Chemostat equipment is, in general, very complicated and expensive; it is often subject to breakdown resulting in the loss of steady-state conditions. Operation of the chemostat is complex and time consuming; it is not recommended for routine bioassays. Excessive growths of some algae on the walls of the chemostat is a common problem, especially with natural algal populations. Porcella (1969) reported that wall growths of diatoms and blue-green algae in a natural algal population were 3-30 times greater than measured in the effluent samples. He recommended that adequate mixing might be achieved through wall scraping or inhibition of those algae which become attached.

In the chemostat, as well as in the batch culture, the limiting nutrient may be added in excess of that needed for growth, resulting in a

data point that has no relation to the growth rate. It has also been established that when the dilution rate varies, the limiting factor can be transferred from one nutrient to another (Malek and Fencl, 1962). When there are several limiting factors present, interpretation of results is very difficult (Porcella, 1969).

It has also been shown that the half-saturation value (K_s) usually decreases as the age of a continuous culture increases. This decrease is probably due to mutation and selection since the lower K_s will give the organism a strong competitive advantage (Malek and Fencl, 1962). However, mutation would only occur if the residence time was exceptionally long.

LAKE TAHOE STUDY

A comprehensive program of chemostat research is being carried on at Lake Tahoe (McGauhey, *et al.*, 1968, 1969). In this study, both batch and continuous cultures were investigated to determine whether wastewater effluents contain materials which stimulate algal growth. Comparisons were made between the batch culture and chemostat to determine which technique to use for the best assessment of the biostimulatory properties of wastewater. It was concluded that the batch assay method is an economical tool for the assessment of the relative enrichment characteristics; that is, the batch assay is a useful but qualitative technique. Quantitative data required for ultimate control of eutrophication must relate algal growth rates to specific nutrient concentrations. Since only the chemostat gives this relationship, evaluation of chemostat data should allow quantitative estimation of the effect of specific nutrients on algal growth.

A comparison of batch and continuous flow assays was made with a nutrient concentration S_o (batch) equal to S_1 (steady state). From kinetic theory, the batch maximum growth rate should be much less than the steady state growth rate in the continuous flow culture. However, this was not observed, in fact, the batch growth rate was about four times greater than the steady state growth rate. Thus, it seems that the initial growth response of the batch culture is much greater than the growth rate maintained under steady state conditions. Measurement of the transient chemostat rate, that is, the growth rate before steady state was reached, showed it to be even greater than the batch rate. The credibility of the batch assay was questioned because of the unusual results obtained. It was concluded that further research was required to study the questionable characteristics of the batch assay and to verify the applicability of the chemostat in determining kinetic constants.

CONCLUSIONS

Like all laboratory techniques, the chemostat is an artificial system that does not consider variations in light intensity, temperature, and other environmental factors. It does not even consider photoperiod, although it has been shown that many algae respond differently to continuous light. The complex relationships between algae and substances in natural waters and associated bacteria are now known well if at all. However, batch cultures are also subject to these limitations.

Porcella (see Middlebrooks, _et al_., 1969) pointed out that environmental factors such as light, temperature, CO_2, and mixing are usually kept constant in order to eliminate their effect on the growth of algae.

Although these environmental factors may control productivity in nature, the growth rate can be related only to the limiting factors in the sample.

More research is needed to determine the effects of light, temperature, CO_2, and other environmental factors on algal growth characteristics. Additional work should be done on chemostats using natural algae populations. As we determine the effects of more environmental factors on growth characteristics, and as we switch to chemostats with natural algae populations, we come closer to simulating the natural aquatic ecosystem.

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NOTE: The presentation at St. Petersburg was made by Dr. Donald O'Connor, Department of Civil Engineering, Manhattan College. Dr. O'Connor's manuscript was not available for publication.