

Experimental and Modeling Studies of a Four-Trophic Level Predator-Prey System

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Abstract. Experimental studies of a microbial food chain involving organic carbon substrates, *Enterobacter aerogenes*, and ciliate protozoans *Paramecium primaurelia* and *Didinium nasutum* were conducted in stirred, aerated batch cultures. Quantitative measurements were made of organic carbon levels and of cell numbers, mean cell volumes, and total biovolumes for all three microbial populations. A mathematical model based on Monod kinetics was developed to describe this four-trophic level predator-prey system. The model was formulated in terms of biovolume, which is the product of cell numbers and mean cell size, and includes terms for biovolume decay. Batch culture data were used to derive parameter values, and model simulations were compared to experimental results. Despite the significance of *Paramecium-Didinium* studies in ecological literature, the entire food chain has not been previously studied or modeled.

Introduction

With the development of continuous culture devices for long-term growth of microorganisms under constant conditions and the mathematical equations to describe microbial growth dynamics, a number of investigators have studied predator-prey systems involving heterotrophic protozoa and various prey organisms [12]. These studies have in common the measurement of all trophic levels, which requires use of electronic counting techniques for assaying microbial populations, and the use of mathematical models based on Monod kinetics, which explicitly define growth of one microorganism in terms of its food resource. One of the earliest such studies of heterotrophic protozoan and bacterial populations in continuous culture was that of Drake et al. [10] who studied predator-prey interactions between the myxamoeba *Dictyostelium discoideum* and the bacterium *Escherichia coli* B/r. Oscillations in predator, prey, and substrate were observed [33]. Other continuous culture studies have examined ciliate protozoa as bacterial predators [6, 9, 15]. In the most complex continuous culture study to date, Jost et al. [19] and Tsuchiya et al. [34] examined the effect of predation by *Tetrahymena pyriformis* on competition between *E. coli* and *Azotobacter vinelandii* for glucose substrate. Bader et al. [2] reported on predator-prey interactions between the ciliate *Colpoda steini* and blue-green

alga *Anacystis nidulans*. All of these studies involve only three trophic levels. Studies involving interactions between protozoa have not yet been reported [12].

Interactions between heterotrophic protozoa, however, have been a favorite subject of study among experimental and theoretical ecologists because they exhibit a range of population interactions similar to metazoans and can therefore serve as models of general biological systems [13, 17, 20, 23–25]. Because heterotrophic protozoa reach large population sizes in small volumes and possess rapid growth rates, they are convenient for testing mathematical models of population phenomena, such as competition and predation. Gause [13] was one of the first experimental ecologists to use heterotrophic protozoa as general biological systems to test mathematical models of various population phenomena. In one series of classical experiments, Gause selected a microbial predator-prey system, which consisted of the bacterial grazer *Paramecium caudatum* and its predator *Didinium nasutum*, and tried to determine what factors led to coexistence or extinction in predator-prey systems. On the basis of these experiments, Gause concluded that in a homogeneous environment the predator-prey relation was unstable and led to self-annihilation. More recently, Luckinbill [21, 22] showed that the outcome of the *Paramecium-Didinium* interaction in a homogeneous system could be altered by varying the level of dissolved organic carbon provided to the bacteria. Experimental ecologists, however, have not quantified the levels of dissolved organic carbon or bacteria [13, 21, 22, 25]. When mathematical models have been applied by experimental ecologists to protozoan systems, the models have been of the logistic and Lotka-Volterra type rather than a Monod-type model. Salt [30] pointed out that Gause's work has had a very strong influence on ecological concepts about predation. Clearly, a more dynamic approach to the *Paramecium-Didinium* system in which all trophic levels are measured and modeled would be appropriate.

In the present paper, the growth and interactions in a four-trophic level system consisting of organic carbon substrates, the bacterium *Enterobacter aerogenes*, grazing ciliate *Paramecium primaurelia* and predatory ciliate *Didinium nasutum* are studied in batch cultures. Electronic counting and sizing techniques are examined for all three microbial populations in mixed cultures. A mathematical model based on Monod kinetics is developed, optimal constants are derived from batch culture experiments, and the model is used to simulate the system. These results are an essential first step toward study of the entire system in continuous culture and testing of theories on predation phenomena.

Materials and Methods

Microorganisms

Enterobacter aerogenes (ATCC 13048) was selected as a suitable food organism and was maintained aerobically on trypticase soy agar plates. *Paramecium primaurelia* (stock 540) was obtained in axenic culture from the laboratory of Dr. S. L. Allen, Division of Biological Sciences, University of Michigan. Stock cultures of *P. primaurelia* were grown on *E. aerogenes* in 5 ml of culture

medium. *Didinium nasutum* (ATCC 30399) was maintained in cultures of *P. primaurelia* and transferred to fresh cultures every other day. The general procedures used for sterilization of *Didinium* have been described previously [1].

Culture Medium and Methods

The culture medium contained in g/liter: $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$, 1.34; KH_2PO_4 , 0.68; NaOH, 0.08; KCl, 0.037; NaCl, 0.117; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.123; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.014; $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.002; NH_4Cl , 0.191; Cerophyl, a brand name for dehydrated cereal grass (Cerophyl Corp., Kansas City, Missouri), 2.50. The pH of the medium was about 7.2. Before autoclaving, the medium was mixed, boiled for five min, and filtered through 18.5 cm rapid filter paper (Scientific Products) and twice through pre-washed Type A-E 47 mm glass fiber filters (Gelman Corp.) to remove particles likely to interfere with electronic counting of bacterial cells. A few sprays of antifoam A (Dow Corning) were added to reduce foaming. Sterile culture medium would not support growth of axenic *P. primaurelia*.

Batch culture experiments were conducted at $20^\circ \pm 1^\circ\text{C}$ in a constant temperature chamber. The batch culture vessels were assembled from two-liter flat-bottomed pyrex boiling flasks equipped with ports for inoculation, sample removal, aeration with humidified sterile air, and air exhaust. The working volume in all batch culture experiments was 1.5 L. Air flow was maintained at 1 L/min. A magnetic stir bar provided continuous mixing. Culture samples were removed with sterile 25 ml syringes and checked for contaminating microorganisms by streaking and incubating trypticase soy agar plates for two days at 37°C .

Analytical Techniques

Organic Carbon Analysis. Samples for carbon analysis (9–10 ml) were filtered through prewashed $0.45 \mu\text{m}$ filters into sterile test tubes containing two drops of $1:4\text{H}_2\text{SO}_4$ and stored at $2\text{--}3^\circ\text{C}$ until analyzed on a Beckman Model 915 total organic carbon analyzer. At least four replicates were run for each sample and carbon standard analyzed. Growth of *E. aerogenes* in the culture medium left about 54% refractory organic carbon in solution; this refractory carbon was subtracted from total carbon to give an estimate of utilizable organic carbon present during experiments.

Bacterial Population Analysis. Electronic estimates of cell concentration, mean cell volume, and biovolume of *E. aerogenes* were obtained with a Coulter Model ZB equipped with a $30 \mu\text{m}$ aperture tube and connected to a Model P-64 size distribution analyzer, which was calibrated with $1.305 \mu\text{m}$ polystyrene spheres (Coulter Electronics). Preliminary experiments indicated that an amplification switch setting of one-half and aperture current of one-quarter were optimal. Culture samples were diluted with $0.45 \mu\text{m}$ filtered 0.9% NaCl to give cell counts from 5,000 to 50,000/0.05 ml. For each sample, six to eight replicate counts and three size distributions were obtained. Electronic counts were compared with viable counts on trypticase soy agar plates. Matched weight filters (47 mm, $0.45 \mu\text{m}$ filters which are matched to ± 0.1 mg, Millipore Corp.) were used to determine bacterial dry weights (biomasses) for comparison with electronic biovolume estimates.

Paramecium Population Analysis. Electronic estimates of cell concentration, mean cell volume, and biovolume of *P. primaurelia* were obtained with a Coulter Model B equipped with a $560 \mu\text{m}$ aperture tube and connected to a Model P-64 size distribution analyzer and X-Y recorder. Calibration for *P. primaurelia* volume analysis required $40 \mu\text{m}$ and $50 \mu\text{m}$ polystyrene spheres (Duke Standards). An amplification switch setting of two and aperture current of two or one were optimal for *P. primaurelia*. Culture samples of 25 ml or 50 ml were diluted in 0.225% NaCl to a total volume of 225 ml, which gave cell counts of 10–600/2 ml. In 0.9% NaCl, *P. primaurelia* became

swollen, causing size distributions to shift, and eventually burst. In the empirically derived 0.225% NaCl, however, the protozoa remained active, size distributions were stable, and the protozoa grew when provided with bacteria. For each sample, five replicate protozoan counts and two size distributions were measured. A computer program was used to integrate the graphs of cell size distribution to derive values for mean cell size and biovolume. Electronic estimates of cell numbers were compared with optical counts, which were obtained by counting all *P. primaurelia* in five replicate 1 ml subsamples as they were drawn into a micropipette. To obtain optical measurements of cell volume for comparison with electronic estimates, 50 or more *P. primaurelia* were transferred to a drop of 5% NiSO₄ on a microscope slide. The length and width of the first 25 cells were then measured at 200× and their volumes computed based on the formula for an ellipsoid.

Didinium Population Analysis. Electronic estimates of *D. nasutum* numbers, mean cell sizes, and biovolumes were made on the same Coulter counter equipment as *P. primaurelia*. Calibration for *D. nasutum* volume analysis required 80 μm polystyrene spheres (Duke Standards). For *D. nasutum*, an amplification switch setting of four and aperture current of four or two were used. The predators were counted at the same dilution in 0.225% NaCl as were their prey. When both protozoa were present in culture samples, the sample volume permitted only five *D. nasutum* cell counts and one size distribution to be recorded after counts and size distributions of *P. primaurelia* had been collected. After *P. primaurelia* became extinct, however, two size distributions of *Didinium* were made. At Coulter settings of amplification four and aperture current four or two, only *Didinium* were counted, but at settings used for *P. primaurelia*, both protozoa were counted. The number of *P. primaurelia* was determined by subtracting the count of *Didinium* from the total count. *Didinium* were, however, too large to be included in *Paramecium* cell size distributions. A computer program was also used to integrate cell size distributions. Optical counts of *D. nasutum* were made by micropipette as with *P. primaurelia*. Optical measurements of predator cell volume were made by expelling 40 to 50 *D. nasutum* into a drop of Bouin's solution. The length and width of the first 25 *D. nasutum* were measured and volumes computed with the formula for an ellipsoid.

Mathematical Model

The mathematical model presented in this section is based on simple material balance and saturation kinetics to describe growth as a function of resource utilization. Monod [27, 28] developed a series of equations to describe saturation of a bacterial population specifically in terms of utilization of some essential substrate. A detailed theoretical development and experimental testing of Monod's equations has been presented by Herbert et al. [16], and therefore only a brief summary of Monod's equations is presented here. When all essential substrates are present in excess, bacterial growth in batch culture can be described by the familiar exponential equation

$$\frac{1}{B} \frac{dB}{dt} = \mu \quad (1)$$

where B is concentration of bacteria at time t in mg/L, dB/dt is the rate of change of the population, and μ, the rate of increase per unit organism concentration, is the specific growth rate in h⁻¹. Monod [27] first showed that the relationship between the specific growth rate and the substrate concentration could be described by

$$\mu = \mu_{\max} \frac{S}{K_s + S} \quad (2)$$

where S is substrate concentration in mg/L, μ_{max} the maximum growth rate constant in h⁻¹, and K_s the saturation constant in mg/L, which is that substrate concentration at which μ = ½ μ_{max}. The form of equation 2 is hyperbolic with μ being proportional to S at low concentrations but approaching a limit of μ_{max} at high concentrations. Hyperbolic functions occur commonly in predator-prey

models [26]. Finally, Monod [28] also showed that bacterial growth rate was a constant fraction of the substrate utilization rate

$$\frac{dB}{dt} = -Y \frac{dS}{dt} \quad (3)$$

where Y is a dimensionless yield constant which relates substrate utilization to bacterial growth. Equations one to three can be combined to give a quantitative description of bacterial growth and substrate utilization in batch culture and, with minor changes, in continuous culture.

Mathematical equations similar to those in equations one to three were subsequently used to describe growth of ciliate protozoa on bacterial prey in both batch and continuous culture. Proper and Garver [29] first showed that the specific growth rate of the ciliate *Colpoda steini* on *Escherichia coli* could be described by an expression identical to equation two. At low bacterial concentrations, protozoan growth is limited by frequency of encountering bacteria but at high bacterial concentration growth can be limited by rate of ingestion or digestion. The mathematical model for changes in substrate, bacteria, and protozoa in batch and continuous culture has been described previously [6, 9, 10] and subjected to detailed analysis of the behavior of its solutions [5, 18, 33]. In experimental studies, this model has been used extensively to describe three trophic level systems involving the ciliate *Tetrahymena pyriformis* feeding on bacteria [6, 9, 19], the myxamoeba *Dictyostelium discoideum* on *E. coli* [10, 33], and the ciliate *Colpoda steini* on the blue-green alga *Anacystis nidulans* [2].

The mathematical model for a four trophic level system involving *Paramecium* and *Didinium* was obtained by adding an equation for the second predator to the previously described three trophic level model. As in equation two, the specific growth rate of *Didinium* was described as a hyperbolic function of *Paramecium* concentration. Experiments by Salt [30] and Hewett [17] support such a formulation for *Didinium*. The equations for the four trophic level predator-prey system in batch culture are

$$\frac{dS}{dt} = -\frac{\mu_{\max}}{Y} \frac{S}{K_s + S} B \quad (4)$$

$$\frac{dB}{dt} = \mu_{\max} \frac{S}{K_s + S} B - d_b B - \frac{\eta_{\max}}{W} \frac{B}{K_b + B} G \quad (5)$$

$$\frac{dG}{dt} = \eta_{\max} \frac{B}{K_b + B} G - d_g G - \frac{\sigma_{\max}}{Z} \frac{G}{K_g + G} P \quad (6)$$

$$\frac{dP}{dt} = \sigma_{\max} \frac{G}{K_g + G} P - d_p P \quad (7)$$

S is the concentration of utilizable organic carbon substrates in the culture vessel in mg/L. B , H , and P are the concentrations of *E. aerogenes*, *P. primaurelia*, and *D. nasutum*, respectively, in $\mu\text{m}^3/\text{mL}$, and μ_{\max} , η_{\max} , and σ_{\max} are the corresponding maximum specific growth rates in h^{-1} . Y , W , and Z are yield constants and K_s , K_b , and K_g saturation constants. Y is expressed in $\mu\text{m}^3/\text{mg}$, and W and Z are dimensionless. K_s is in mg/L, and K_b and K_g are expressed in $\mu\text{m}^3/\text{mL}$. The terms d_b , d_g , and d_p are biovolume decay constants expressed in h^{-1} .

Monod [27] originally considered dry weight to be the appropriate measure of bacterial concentration, but with the exception of Canale et al. [6] and Canale and Cheng [7], who used biovolume as the concentration unit of the ciliate predator, most batch and continuous culture studies have used cell numbers. It is not appropriate to use cell numbers as a measure of concentration in equations 4–7 because cell numbers may represent different biomasses if cell sizes are variable. Therefore, the four trophic level model was formulated in terms of biovolume, which is the product of cell numbers and mean cell size. Because biovolume declines due to both cell lysis and reduction in cell volume when food resources are depleted, first-order biovolume decay terms were included in the model [6, 7]. Incorporation of these decay terms is equivalent to assuming a maintenance metabolism for the organisms. When food resources are depleted, metabolism proceeds at the expense of accumulated biovolume.

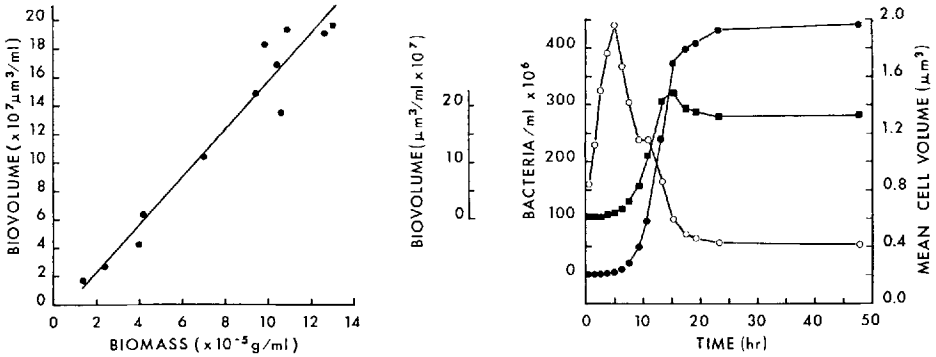


Fig. 1. Linear relationship between bacterial biovolume estimates and the corresponding determinations of bacterial biomass (dry weight).

Fig. 2. Changes in cell numbers, mean cell volume, and biovolume during batch culture growth of *Enterobacter aerogenes*. (●) cell numbers, (○) mean cell volume, (■) biovolume.

Results

The growth and decline of each microbial population was examined in batch cultures to determine patterns of changes in cell numbers, mean cell volumes, and biovolumes and to correlate measurements of these parameters made by electronic particle counters and size distribution analyzers with measurements made by more conventional methods. Total electronic counts of *E. aerogenes* exceeded plate counts during lag and stationary phases when intact but non-viable bacterial cells were present. During exponential phase, the correspondence was quite close. The total number of particles is probably a better indication of available protozoan food than viable cells alone. Comparison between electronic counts and plate counts of bacterial cell numbers indicated that the presence of the protozoa did not adversely affect the accuracy of the electronic estimates (Tables 1 and 2). In Table 1, *P. primaurelia* reduced its prey to an average concentration of 3.2×10^5 viable cells/ml. The data in Table 2 were obtained during the experiments shown in Fig. 9, but the data in Table 1 were from an experiment separate from those in Fig. 8. The linear regression for electronically determined bacterial biovolume vs biomass, determined by measurement of dry weight (Fig. 1), was highly significant ($P < 0.001$). Changes in cell numbers, mean cell sizes, and biovolumes of *E. aerogenes* during batch culture growth are shown in Figure 2. Bacterial numbers remained constant during lag phase, but average cell size increased rapidly and reached a maximum shortly after the culture entered exponential phase. Mean cell size declined during exponential phase and reached a constant minimum size of $0.42 \mu\text{m}^3$ in stationary phase. Maximum biovolume did not correspond to maximum cell numbers but occurred at about 15 h when mean cell volume was still greater than minimum cell size.

Electronic and optical estimates of cell numbers and mean cell volumes of the grazing ciliate *P. primaurelia* each closely corresponded, and consequently biovolume estimates were highly correlated (Fig. 3). The pattern of changes in cell numbers, mean cell volumes, and biovolumes of *P. primaurelia* during

Table 1. Comparison between electronic counts and plate counts of *Enterobacter aerogenes* in a stirred, aerated batch reactor with *Paramecium primaurelia*.

Time (h)	Coulter counts ^a × 10 ⁶ cells/ml	Plate counts ^b × 10 ⁶ cells/ml
49.67	706.18	600
73.14	642.25	437
97.08	491.00	380
122.47	119.70	70.7
145.67	3.013	0.190
169.92	0.850	0.156
193.25	1.170	0.297
218.67	0.387	0.223
239.17	0.346	0.226
263.58	0.119	0.264
288.58	0.326	0.345
311.95	0.488	0.527
336.41	0.600	0.660

^a Based on 6–8 replicate counts

^b Based on 3 replicate platings

Table 2. Comparison between electronic counts and plate counts of *Enterobacter aerogenes* in a stirred, aerated batch reactor with *Paramecium primaurelia* and *Didinium nasutum*.

Time (h)	Coulter counts ^a × 10 ⁶ cells/ml	Plate counts ^b × 10 ⁶ cells/ml
48.10	693.05	520
69.30	455.67	340
91.92	4.23	0.6
140.77	28.60	24.5
168.77	33.99	23.0
187.21	29.04	16.0

^a Based on 6–8 replicate counts

^b Based on 3 replicate platings

batch culture growth is shown in Figure 4. Again mean cell volume initially increased and reached a maximum as the population entered exponential phase. Mean cell size then declined rapidly during exponential phase and continued to decline slowly during the remainder of the experiment. Cell numbers also showed a slow decline from the maximum level. Maximum *Paramecium* biovolume occurred about 70 h after inoculation of the batch culture with protozoa, but cell numbers did not reach the maximum level until more than 90 h had elapsed (Fig. 4). Biovolume decline was the product of declining cell numbers and mean cell volume.

The correspondence between electronic and optical measurements of cell

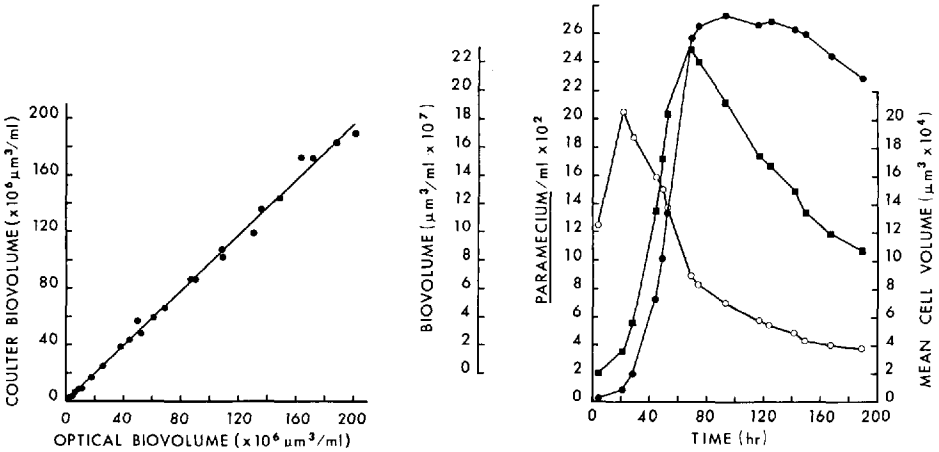


Fig. 3. Linear relation between electronic biovolume estimates and optically measured biovolumes of *Paramecium primaurelia*.

Fig. 4. Changes in cell numbers, mean cell volume, and biovolume during batch culture growth of *Paramecium primaurelia*. (●) cell numbers, (○) mean cell volume, (■) biovolume.

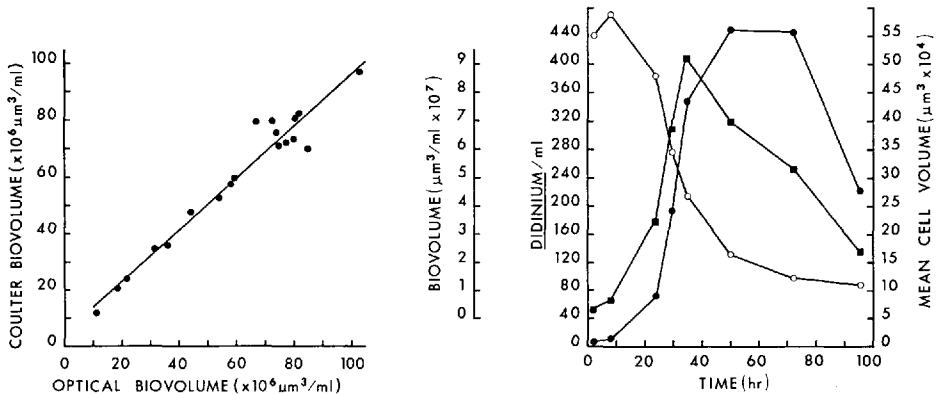


Fig. 5. Linear relation between electronic biovolume estimates and optically measured biovolumes of *Didinium nasutum*.

Fig. 6. Changes in cell numbers, mean cell volume, and biovolume of *Didinium nasutum* during batch culture growth. (●) cell numbers, (○) mean cell volume, (■) biovolume.

numbers and mean cell volumes of the predatory ciliate *Didinium nasutum* also proved quite satisfactory, although initial counts were somewhat scattered because they were based on very small numbers of cells. The resulting biovolume estimates were highly correlated (Fig. 5). Changes in cell numbers, mean cell volumes, and biovolumes of *D. nasutum* during growth in batch culture are shown in Figure 6. The initial increase in mean cell volume was slight, presumably because the predators were near their maximum cell size in the inoculum. Mean cell size declined rapidly as the predators grew exponentially,

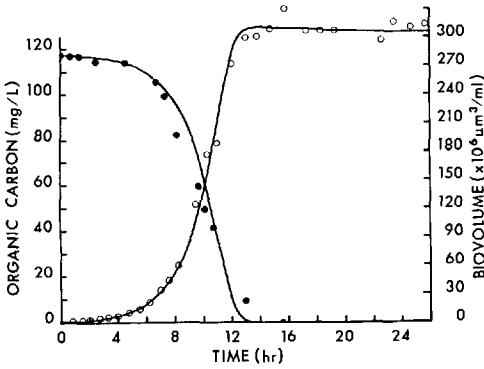


Fig. 7. Removal of utilizable organic carbon (●) and production of *Enterobacter aerogenes* biovolume (○) in three replicate batch cultures. The curves are fitted model calculations.

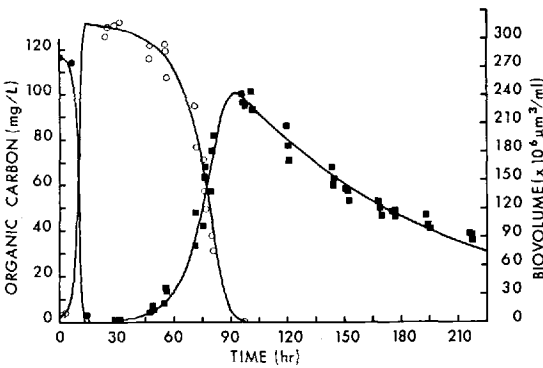


Fig. 8. Removal of organic carbon (●) and changes in biovolume of *Enterobacter aerogenes* (○) and *Paramecium primaurelia* (■) in three replicate batch cultures. All curves are fitted model calculations. Cell numbers for one of the replicates shown in this Figure are included also in Figure 4.

and cell numbers also dropped sharply after 80 h. Maximum *Didinium* biovolume occurred at about 35 h when the number of predators (347/ml) was below the maximum number (450/ml) reached at 50 h. The predators therefore continued to divide at the expense of their mean cell volume.

After the electronic techniques for counting and sizing the microbial populations were verified, a series of replicated batch culture experiments were conducted. The interaction between dissolved organic carbon substrates and *Enterobacter* was first examined, then grazing on bacteria by *P. primaurelia*, and finally predation on *Paramecium* by *Didinium nasutum*. From these replicated batch experiments the values of growth constants, expressed in terms of biovolume, were derived for use in the mathematical model represented by equations 4–7. Batch culture interactions were simulated by the model and compared to the observed data.

Three replicate stirred, aerated batch cultures were run simultaneously starting with the same initial organic carbon substrate concentration and inoculum of *Enterobacter aerogenes*. The standard bacterial inoculum used in all batch culture experiments was 25 ml of 24-h old loop-inoculated medium. The initial bacterial cell concentration averaged about 2.4×10^6 cells/ml, and the initial mean cell size was about $0.6 \mu\text{m}^3$. Organic carbon and bacterial biovolume were followed for more than 300 h. The results were so similar that the data were pooled for graphing and analysis. During the first 12 hours of the exper-

Table 3. Optimized growth parameters for Monod model with biovolume decay constants.

Organic carbon—<i>Enterbacter aerogenes</i>	
μ_{\max}	$= 0.621 \text{ h}^{-1} (14.9 \text{ da}^{-1})$
Y	$= 2.66 \times 10^6 \mu\text{m}^3/\text{ml}/\text{mg/L}$
K_s	$= 24.3 \text{ mg/L}$
d_b	$= 0.001 \text{ h}^{-1} (0.024 \text{ da}^{-1})$
T_b (doubling time)	$= \ln 2/\mu_{\max} = 1.12 \text{ h}$
<i>Enterobacter aerogenes-Paramecium primaurelia</i>	
η_{\max}	$= 0.132 \text{ h}^{-1} (3.17 \text{ da}^{-1})$
W	$= 0.968 \mu\text{m}^3/\text{ml}/\mu\text{m}^3/\text{ml}$
K_b	$= 90.4 \times 10^6 \mu\text{m}^3/\text{ml}$
d_g	$= 0.0090 \text{ h}^{-1} (0.212 \text{ da}^{-1})$
T_g (doubling time)	$= \ln 2/\eta_{\max} = 5.25 \text{ h}$
<i>Paramecium primaurelia-Didinium nasutum</i>	
σ_{\max}	$= 0.160 \text{ h}^{-1} (3.84 \text{ da}^{-1})$
Z	$= 0.608 \mu\text{m}^3/\text{ml}/\mu\text{m}^3/\text{ml}$
K_g	$= 29.67 \times 10^6 \mu\text{m}^3/\text{ml}$
d_p	$= 0.0234 \text{ h}^{-1} (0.56 \text{ da}^{-1})$
T_p (doubling time)	$= \ln 2/\sigma_{\max} = 4.33 \text{ h}$

iment, organic carbon was depleted and the maximum bacterial biovolume was produced (Fig. 7). During the remainder of the experiment, bacterial biovolume declined slowly due to cell lysis. Optimal values of growth constants were determined by a computer program which minimized the sum of squared deviations between observed data and model predictions (Table 3). The curves in Figure 7 were produced by numerical integration of equations 4 and 5, without the term for *Paramecium* grazing.

Three replicate batch cultures were then monitored for 225 hours for organic carbon, bacteria, and *P. primaurelia*. Each batch culture received 15 ml of 72-h old *P. primaurelia* culture 27 h after inoculation with bacteria. The initial concentration of *P. primaurelia* averaged about 16 cells/ml with a mean cell size of about 146,000 μm^3 . The resulting data were again grouped for analysis (Fig. 8). After 100 h, the bacterial population remained at $0.1\text{--}1.6 \times 10^6 \mu\text{m}^3/\text{ml}$ as biovolume of *Paramecium* steadily declined due to the combined effects of declining cell numbers and mean cell size. Optimal parameter values were calculated by computer program (Table 3), and the smooth curves in Figure 8 were obtained by numerical integration of equations 4, 5, and 6, without the term for *Didinium* predation.

A final three replicate batch cultures were followed for 195 h, and changes in all four trophic levels were measured. Each batch culture received 20 ml of 48-h old *Paramecium* culture 24 h after bacterial inoculation. The *Paramecium* inoculum resulted in an initial concentration of about 14 cells/ml with a mean cell size of about 159,000 μm^3 . After *Paramecium* reached maximum biovolume, 20 ml of 48-h old cultures of *Didinium nasutum* were added to each batch culture at 92.5 hours. The initial concentration of *Didinium* was about

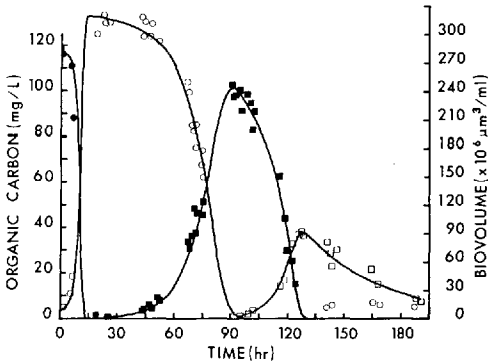


Fig. 9. Removal of organic carbon (●) and changes in biovolume of *Enterobacter aerogenes* (○), *Paramecium primaurelia* (■), and *Didinium nasutum* (□) in three replicate batch cultures. All curves are fitted model calculations. Cell numbers for one of the replicates shown in this Figure are included also in Figure 6.

7 cells/ml with a mean cell size of $607,000 \mu\text{m}^3$. The resulting data were again pooled for analysis (Fig. 9). Up to 90 h, the pattern of changes in *Enterobacter* and *Paramecium* were virtually identical to that in Figure 8. After inoculation, *Didinium* rapidly consumed all their prey and then declined. A secondary regrowth of bacteria occurred after *Paramecium* were eliminated. *Didinium* did not appear to eat bacteria, since in these cultures the number of bacteria increased in the presence of *Didinium* after *Paramecium* were eliminated. Apparently, as *Didinium* removed *Paramecium*, a certain amount of organic carbon substrate was released for bacterial growth. The amount of recycled organic carbon could be estimated at 6.4 mg/L, about 6% of initial levels, because the coefficient for conversion of organic carbon to bacterial biovolume and the level of secondary bacterial biovolume produced ($17.0 \times 10^6 \mu\text{m}^3/\text{ml}$) were known. Plate counts indicated that this regrowth represented viable bacteria and not just a buildup of debris (Table 2). Optimal parameters for *Didinium* growth were determined by computer program (Table 3), and the fitted curves in Figure 9 were obtained by numerical integration of equations 4–7.

Discussion

The microbial predator-prey system represented by *Paramecium* and *Didinium* has long been a favorite subject for study by many investigators [13, 17, 21–23, 25], and it has had a major influence on general theories of predation [12, 30]. Despite this interest in the system, no one previously has attempted to measure or to model all four trophic levels involved simultaneously. Before the development of electronic techniques for counting and sizing mixed cultures of microorganisms, the labor involved in measuring organic carbon substrate levels and cell numbers, mean cell sizes, and biovolumes of three microbial populations simultaneously would have made any quantitative investigation of the entire *Paramecium-Didinium* system exceedingly difficult. In the present report, electronic estimates of bacterial biovolume were found to be highly correlated with bacterial biomass (Fig. 1). A number of authors have shown similar high correlations between biovolume and biomass for the algae *Euglena* [8], *Selenastrum* and *Chlorella* [37], and protozoa *Tetrahymena* [4, 32] and *Colpidium* [31]. Drake and Tsuchiya [11] established the accuracy of electronic

techniques for counting mixed populations of microorganisms and applied their procedures to differential counting of mixed cultures of bacteria and protozoa, bacteria and yeast, and large and small bacteria. In the present report, it was found possible to count and size two distinct populations of protozoa in mixed culture. Electronic estimates of protozoan biovolumes were highly correlated with optical measurements of biovolume (Figs. 3 and 5). Differential counting and sizing were possible because the cell size distributions of *Paramecium* and *Didinium* did not overlap appreciably. When batch cultures were inoculated with *Didinium*, the mean cell size of *P. primaurelia* was about $60,000 \mu\text{m}^3$ or less, but the mean cell size of *Didinium* ranged from $440,000$ to $830,000 \mu\text{m}^3$. Thus, the predators were 6–13 times larger than their prey when experiments began. The nearly spherical *Didinium* were also found to have a narrow, symmetrical cell size distribution which was more uniform than some commercial calibration spheres. *Didinium* cells became as small as the largest *Paramecium* only long after all prey had been eaten. Optical methods were preferable only when *Didinium* numbers were very low at the beginning of batch culture growth.

Biovolume was selected as the unit of population measurement because cell numbers may represent different biomasses when cell sizes are variable. Mean cell volumes varied from $0.4 \mu\text{m}^3$ to about $2 \mu\text{m}^3$ for *Enterobacter aerogenes*, from $38,000 \mu\text{m}^3$ – $210,000 \mu\text{m}^3$ for *Paramecium primaurelia* and from $110,000 \mu\text{m}^3$ – $580,000 \mu\text{m}^3$ for *Didinium nasutum* (Figs. 2, 4, and 6). Mean cell size was therefore quite variable in all three microbial populations. The use of biovolume as a measurement of microorganism concentration incorporated this variation into the mathematical model. Maximum biovolume of the resource-utilizing organisms occurred just as that resource was depleted in all three trophic interactions (Figs. 2, 4, and 6). This close coupling between resource consumption and biovolume production did not occur between resource consumption and cell numbers. During lag phase of batch culture growth, cell numbers did not change but mean cell volumes increased (Figs. 2, 4, and 6). Maximum cell numbers occurred after maximum biovolume had been reached and resources were depleted. Thus, the microorganisms continued to divide at the expense of accumulated biovolume, a process Gause [13] described for *Didinium* as producing an increase in the effective "seizing surface." The use of biovolume in the mathematical model avoided the complications this behavior would entail.

The optimal values of all the growth constants in the mathematical model are given in Table 3. The maximum specific growth rate of *Didinium* (σ_{max}) is somewhat larger than that of *Paramecium* (η_{max}), a finding consistent with other reports [13, 21]. The yield coefficients represent gross yields rather than net yields because biovolume decay is included in the model. Because the half-saturation constant for *Paramecium* grazing on bacteria (K_b) was larger than that for *Didinium* capturing its prey (K_p), *Paramecium* were evidently less effective at picking up prey at low concentrations than were *Didinium*. At least in part, this difference in effectiveness may be due to the great difference in the sizes of prey. *Enterobacter* cells average around $0.4 \mu\text{m}^3$ at stationary phase while *Paramecium* cells range from $38,000 \mu\text{m}^3$ –over $200,000 \mu\text{m}^3$. The biovolume decay coefficient for bacteria (d_b) was only about 2% per day compared

with 21% per day for *P. primaurelia* and 56% per day for *D. nasutum*. *Didinium* perhaps expends considerably more energy in pursuit of prey than does *Paramecium*.

The mathematical model presented in this paper shows a reasonable fit to the measured results at high population concentrations. The addition of biovolume decay terms to the basic Monod formulation was essential, particularly for the protozoans which have high rates of biovolume loss. The decline in biovolume after the maximum level was reached was due to the combined effects of fewer cells and smaller average cell sizes. The importance of these biovolume decay terms in the equations for *Paramecium* and *Didinium* growth is evident in Figures 8 and 9. Without a decay term to represent maintenance metabolism, the predicted *Paramecium* and *Didinium* biovolumes would remain at a constant maximum level.

The Monod model predicts that *Paramecium* biovolume should decline continuously to levels below that which would indicate even one cell in the entire batch culture. *P. primaurelia* is actually eliminated entirely, reflecting the fact that protozoan biovolume comes in large discrete units. The Monod model also predicts that *Paramecium* should reduce the population of its bacterial prey to very low densities. Bacterial population levels actually remain steady at $0.2\text{--}1.5 \times 10^6$ cells/ml in batch cultures with *Paramecium* only. Plate counts indicate that these persistent high counts represent considerable numbers of viable bacteria rather than just debris accumulation (Tables 1 and 2). There appears to be a slight increase in viable bacteria in batch cultures containing *Paramecium* after the population of grazing ciliates has declined (Table 1). This regrowth of bacteria may be due to release of carbon substrates by the lysing of protozoan cells combined with a reduction in grazing pressure. In the batch cultures containing the entire food chain, carbon recycling evidently led to a significant regrowth of bacteria after *Paramecium* were eliminated (Fig. 9 and Table 2).

The occurrence of persistent high levels of bacteria in cultures subject to intense grazing by ciliate predators has drawn considerable attention [14, 19, 34–36] and has led to new mathematical formulations to account for it [3, 18]. Habte and Alexander [14] showed that *Tetrahymena pyriformis* failed to consume all bacteria in liquid culture and ceased to take up bacteria when levels fell to $10^5\text{--}10^7$ viable cells/ml. Watson et al. [36] confirmed these observations on *Tetrahymena* and noted that when the feeding rate fell to immeasurably low values, the ciliates tended to swim faster than actively feeding ciliates. These “nonfeeding” ciliates remained viable after 55 h. Two basic proposals have been suggested to account for the persistent levels of bacteria in cultures subject to ciliate grazing. van den Ende [35] proposed that bacteria growing on the vessel walls, where they were presumably free of predation, could produce a slough of bacterial cells that would maintain levels in the medium. Bonomi and Fredrickson [3] examined a mathematical formulation that would add the effects of wall growth to a basic Monod model. Jost et al. [18], however, proposed a generalized Monod model, which he called a multiple saturation model, to include changes in ciliate grazing at low prey density. This multiple saturation model changes equations 5 and 6 to the form

Table 4. Optimized growth parameters for Multiple Saturation model with biovolume decay constants.

<i>Enterobacter aerogenes-Paramecium primaurelia</i>	
η_{\max}	$= 0.0986 \text{ h}^{-1} (2.37 \text{ da}^{-1})$
W	$= 0.965 \mu\text{m}^3/\text{ml}/\mu\text{m}^3/\text{ml}$
K_i	$= 1.108 \times 10^6 \mu\text{m}^3/\text{ml}$
K_{ii}	$= 0.344 \times 10^6 \mu\text{m}^3/\text{ml}$
d_g	$= 0.0089 \text{ h}^{-1} (0.214 \text{ da}^{-1})$
T_g (doubling time)	$= \ln 2/\eta_{\max} = 7.03 \text{ h}$

$$\frac{dB}{dt} = \mu_{\max} \frac{S}{K_s + S} B - d_b B - \frac{\eta_{\max}}{W} \frac{B^2}{(K_i + B)(K_{ii} + B)} G \quad (8)$$

$$\frac{dG}{dt} = \eta_{\max} \frac{B^2}{(K_i + B)(K_{ii} + B)} G - d_g G - \frac{\sigma_{\max}}{Z} \frac{G}{K_g + G} P \quad (9)$$

The multiple saturation model contains two saturation constants— K_i and K_{ii} ; together these give a rough measure of threshold prey density. The multiple saturation model replaces the hyperbolic growth response (equation 2) with a sigmoidal response. The multiple saturation model fit data from a *Tetrahymena-Azotobacter* system better than the Monod model [18].

Because grazing ciliates appear to change their feeding behavior [14, 36], the multiple saturation model was examined with the data for the *Paramecium-Enterobacter* system. Optimal parameter values were determined by a nonlinear regression program (Table 4). Values of the maximum specific growth rate (η_{\max}), yield constant (W) and biovolume decay constant (d_g), are essentially as in Table 3. The two saturation constants, however, are substantially smaller than K_b . The Monod model predicted levels of bacteria less than $10^{-4} \mu\text{m}^3/\text{ml}$, but the multiple saturation model indicates a level of about $1.7 \times 10^2 \mu\text{m}^3/\text{ml}$. Although this predicted level is still less than the observed level of about $1 \times 10^5 \mu\text{m}^3/\text{ml}$, the multiple saturation model is much closer than the Monod model.

In the present study, the Monod model with biovolume decay constants appears to be appropriate for the organic carbon substrates-bacteria system and the *Paramecium-Didinium* system, particularly since *Didinium* can eliminate all of its prey. The multiple saturation model [18] appears to be more appropriate for the *Enterobacter-Paramecium* system, especially since it can partially account for persistent levels of bacteria in batch cultures. Possibly both bacterial wall growth and changes in ciliate grazing behavior are involved in determining the final steady levels of bacteria in batch culture. Continuous culture studies would help decide which model is appropriate for each trophic level. The techniques developed in this study and the mathematical models can be applied to the testing of theories on predator-prey systems in continuous culture and suggest the possibility of studying more complex systems involving competition as well as predation.

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