APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1987, p. 2914–2921
0099-2240/87/122914-08$02.00/0

Vol. 53, No. 12

Tests of the Critical Assumptions of the Dilution Method for Estimating Bacterivory by Microeucaryotes

SARAH C. TREMAINE AND AARON L. MILLS*

Department of Environmental Sciences, University of Virginia, Charlottesville, Virginia 22903

Received 28 July 1987/Accepted 22 September 1987

The critical assumptions of the dilution method for estimating grazing rates of microzooplankton were tested by using a community from the sediment-water interface of Lake Anna, Va. Determination of the appropriate computational model was achieved by regression analysis; the exponential model was appropriate for bacterial growth at Lake Anna. The assumption that the change in grazing pressure is linearly proportional to the dilution factor was tested by analysis of variance with a lack-of-fit test. There was a significant ($P < 0.0001$) linear ($P > 0.05$) relationship between the dilution factor and time-dependent change in ln bacterial abundance. The assumption that bacterial growth is not altered by possible substrate enrichment in the dilution treatment was tested by amending diluted water with various amounts of dissolved organic carbon (either yeast extract or extracted carbon from lake sediments). Additions of carbon did not significantly alter bacterial growth rates during the incubation period (24 h). On the basis of these results, the assumptions of the dilution method proved to be valid for the system examined.

Awareness of the role of bacteria in element cycling and energy flow in the detrital food web has resulted in increasing interest in the quantification of protozoan grazing on bacteria (11, 16, 20, 27, 31–33, 37, 44, 47). Direct quantitation of bacterivory is technically difficult, but recent methodological and theoretical advances have been made. Methods currently being developed and evaluated include labeling of food particles and calculation of grazing from the growth of the prey community when grazing pressure is relieved by selective chemical inhibition, filtration, or dilution. Measurement of the grazing rate with radiolabeled bacteria (25, 26) or fluorescence-labeled particles (latex microspheres or natural bacterial assemblages [2, 36, 39]) assumes that there is no ingestion bias by the protozoan grazers. Preliminary evaluations of these methods have been equivocal in their assessment (39; S. C. Tremaine and A. L. Mills, *in* S. S. Rao, ed., *Microbial Interactions in Acid Stressed Aquatic Ecosystems*, in press). Measurement of the grazing rate with selective eucaryote inhibitors assumes that upon inhibition of grazers, observed bacterial growth is quantitatively equal to the grazing rate (4, 14, 26). With use of a procaryote inhibitor, it is assumed that a decrease in the number of bacteria can be used as a measure of grazing (14, 21, 49). Incomplete inhibition of target organisms and inhibition of nontarget organisms precludes use of inhibitors in quantitative grazing studies (36, 41, 42). The filtration method assumes that the observed increase in bacterial number upon removal of grazers by filtration is equal to the grazing rate when grazers are present. Incomplete removal of microprotozoans during filtration (7, 14) and removal of particle-attached bacteria (47, 48; Tremaine and Mills, in press) necessitate careful application of this method to quantitative grazing studies.

The dilution technique assumes that observed bacterial growth in a diluted water sample is quantitatively equivalent to the grazing rate in unaltered samples (8, 24). Early evaluations of the dilution technique have been promising (24); however, questions have been raised as to the validity of the model used to calculate bacterial grazing mortality and of two assumptions implicit in the technique. It is the purpose of this paper to review and test these assumptions.

Theoretical considerations. Bacterial mortality due to protozoan grazing can be derived from bacterial growth equations and a comparison of apparent bacterial growth rates in experiments with or without (or with quantitatively reduced) predators. Bacterial growth in the absence of grazing mortality or substrate limitation is characterized by the exponential growth equation $dN/dt = \mu N$, where μ is the instantaneous bacterial growth rate (hours^{-1}), N is the number of bacteria (cells · milliliter^{-1}), and t is time. Apparent bacterial growth in the presence of bacterivorous protozoans (r_n [hours^{-1}]) (8) can be described by the equation $dN/dt = r_nN$, where r_n is less than μ by an amount equal to the grazing mortality (g [hours^{-1}]) such that

$$d\ln N/dt = r_n = \mu - g \quad (1)$$

The apparent bacterial growth rate, r_n, is evaluated as the slope of a least-squares regression of $\ln N_t$ versus t (from the relationship $\ln N_t = N_0 + r_nt$), when bacterial number has been quantified over time in unaltered water samples. Dilution of whole-water samples has been used to quantitatively reduce bacterial grazing mortality in an experimental unit (24) such that

$$r_d = \mu - Xg \quad (2)$$

where r_d is the apparent bacterial growth rate in the diluted water sample (hours^{-1}) in which grazing mortality (g) has been reduced by decreasing the encounter frequency between predator and bacterial prey proportional to the dilution factor (X). The dilution factor is calculated from the ratio of initial prey concentration in the diluted water versus the prey number in the unaltered control water. r_d is evaluated as the slope of the least-squares regression of $\ln N_t = \ln N_0 + r_dt$. Grazing mortality is the product of the predator concentration (P) and the second-order rate constant (g') such that $dN/dt = \mu N - g'PN$ or $d(\ln N)/dt = \mu - g'P$. The dilution technique determines grazing mortality empirically from combining equations 1 and 2 into

$$g = (r_d - r_n)/(1 - X) \quad (3)$$

* Corresponding author.

by using r_d, r_n, and X determined experimentally, while grazing mortality rate, bacterial production, and total grazing mortality can be calculated.

Bacterial growth can also be evaluated under substrate limitation by using Monod kinetics which have been applied to bacterial growth in chemostats (3):

$$\mu = \mu_{max}S/(K_s + S) \quad (4)$$

where μ_{max} is the maximum bacterial growth rate at substrate (S) saturation and K_s is the saturation constant (the concentration at which $\mu = \mu_{max}/2$). To evaluate bacterial growth rates subject to grazing mortality and substrate limitation, equations 2 and 4 can be combined:

$$r_d = [\mu_{max}S \cdot (K_s + S)^{-1}] - Xg \quad (5)$$

This would be difficult to evaluate experimentally. However, if equation 5 is evaluated at only one value of X but several values of S, the effect of substrate concentration alone on the apparent bacterial growth rate can be considered and some conclusions can be drawn about substrate effects on the estimates of grazing mortality.

There are three assumptions implicit in the dilution technique as it is most commonly used. (i) The change in bacterial number over time follows an exponential model as opposed to a linear model (8, 24, 47). (ii) Grazing mortality is a constant and is linearly related to the dilution factor (24). (iii) Bacterial growth rates are constant and unaffected by the dilution treatment (6, 12, 24). Enhanced bacterial growth in experimental flasks could be caused by either carbon enrichment, due to an increase in absolute carbon concentration (increased milligrams of C · liter^{-1}) as a result of lytic release during filtration (12), or decreased competition for the available carbon, due to the dilution of cell number (increased picograms of C · cell^{-1}) (22). This paper addresses each of these assumptions to determine if any are violated. The study revealed that the assumptions are not violated, and therefore estimates of grazing mortality and bacterial production made by using the dilution technique are reasonable and valid.

MATERIALS AND METHODS

Site and general protocol. The research was conducted in the Contrary Creek arm of Lake Anna, an impoundment in east-central Virginia. This arm of the lake receives acid mine drainage (pH 2.5 to 3.5; sulfate, 2 to 20 mM) from an abandoned pyrite mine in the Contrary Creek drainage basin (17). Benthic bacterial metabolism, i.e., sulfate reduction, iron reduction, and fermentation (A. L. Mills, P. E. Bell, and A. T. Herlihy, *in* S. S. Rao, ed., *Microbial Interactions in Acid Stressed Ecosystems*, in press), neutralizes acid from the water column within a centimeter of the sediment-water interface (18), creating a habitat of high bacterial activity (Mills et al., in press) and high concentrations of available carbon (S. C. Tremaine, P. E. Bell, and A. L. Mills, unpublished data).

Three replicate samples from the sediment-water interface were collected for each grazing experiment between July and November 1986. Water samples were collected with a peristaltic pump through autoclaved tubing into an ethanol-washed, thermally insulated container. The experiments were begun within 3 h of sample collection. Treatment and control (CTL) flasks (2 liter) were set up in triplicate with 500 ml of sample in each flask. The CTL flasks contained unaltered samples of water from the sediment-water interface containing both bacteria and bacterivorous protozoans. The specific treatments varied depending on the hypothesis being tested but generally involved dilution of lake sediment-water interface samples with filter-sterilized lake water from the same site.

Sterility was achieved by filtering each sample sequentially through a Whatman no. 4, a Whatman no. 5, and a Nuclepore 0.2-μm-pore-size filter; vacuum never exceeded 100 mm Hg (13.3322 kPa). The flasks were maintained in subdued light at a constant temperature, within 5°C of in situ temperatures. Subsamples (10 ml) were taken from each of the flasks at 6-h intervals for 24 h and put into 4-dram vials (~10 ml). Each vial was rinsed four times with filter-sterilized (0.2 μm) deionized water and held frozen until use to prevent bacterial contamination.

The subsamples were preserved with 10% buffered glutaraldehyde (pH 7.0) to give a final concentration of 1% glutaraldehyde (5). Portions (1 ml) of each subsample were stained, and bacteria were counted by using the acridine orange direct count method (19) within 1 week of the experiment. At least five fields or 200 cells were counted per slide.

Dissolved organic carbon (DOC) concentrations were determined either by infrared spectroscopy with an MSA model 202 infrared-CO_2 analyzer for low-carbon-level samples (45) or by the method of Lu (28) for high-carbon-level samples. Treatments with no carbon added (NC) were achieved by passing filter-sterilized lake water through an activated-charcoal column. To test the effectiveness of the charcoal column in adsorbing DOC, a 5-mg · liter^{-1} yeast extract (YE) standard (6.7 mg of C · liter^{-1}) was passed through the activated charcoal, and the resulting DOC was 1.56 mg of C · liter^{-1} or a 77% decrease in DOC.

Examination of the residuals. To test whether the linear or exponential model was more appropriate, least-squares regressions were performed on bacterial number versus time for data collected from diluted water samples. Residuals were plotted versus time and examined; the procedure was then repeated by using logarithmically transformed bacterial numbers (23, 35).

Varying the dilution factor. To test the assumption of proportionality, three dilution factors were used in the experimental design: 1:2, 1:3, and 1:5 (whole water to filtered water) with three independent replicates per treatment. Regressions were run on ln bacterial number versus time (ln N versus t) for the independent replicates, and apparent bacterial growth rates were taken as the slopes of the regressions (from equation 2). A least-squares regression was performed on apparent bacterial growth rate versus the dilution factor. The critical test of the assumption was to determine whether the dilution factor was linearly related to the grazing rate; this was tested by conducting an analysis of variance (ANOVA) with a lack-of-fit test (23). The lack-of-fit test allows the rejection of the straight-line-model assumption if the data indicate that a more complex model is warranted. The residual sum of squares is partitioned into a component describing pure error (by using replicate observations at one value of X, the dilution factor) and a component describing the extent of lack of fit of the assumed straight-line model. The test statistic is an F statistic (mean square lack of fit/mean square pure error; $\alpha = 0.05$), with the null hypothesis being that the straight-line model is appropriate (23).

Effect of substrate concentration on bacterial growth rates. Two experiments were conducted to examine the effect of organic carbon concentration on bacterial growth rates in diluted samples. The first experiment used a CTL and three

treatments: (i) 1:3 dilution in which the diluent was 0.2-μm-filtered lake water (DIL); (ii) 1:3 dilution, as above, with added YE; and (iii) 1:3 dilution, as above, with no organic carbon in the diluent (NC). In the NC treatment, carbon was removed from 0.2-μm-filter-sterilized lake water by passing it through an activated-charcoal column. YE was added to the YE treatment flasks from a filter-sterilized stock solution to make a final experimental concentration in excess of 100 mg · liter^{-1}. An ANOVA was run to determine the source(s) of variance in the estimate of apparent bacterial growth rate given variations in the initial carbon concentration.

The second experiment used the addition of organic carbon extracted from Lake Anna sediment and plant material. The treatments were (i) CTL, (ii) 1:3 DIL, (iii) unaltered lake water with added carbon (CTL+), and (iv) 1:3 dilution with added carbon (DIL+). The actual DOC concentrations were determined as described above. A two-way ANOVA was conducted to evaluate the sources of variance in the estimate of apparent bacterial growth rate given the two factors, dilution and carbon augmentation.

RESULTS

Validity of the exponential model. The first assumption of the dilution method was that the change in bacterial number over time follows an exponential model. A least-squares regression of bacterial number versus time for a dilution-treated water sample yielded residuals that were heteroscedastic and autocorrelated when plotted versus time (Fig. 1). The regression assumption requiring equal variance for the dependent variable (N) for any value of the independent variable (t) was violated for the linear model. To stabilize the variance, the data were ln transformed and regressed against time. The residuals of the transformed data were homoscedastic when plotted against time (Fig. 1). We performed this analysis for 12 dilution grazing experiments covering 2 years of sampling and always found that transformation was required to stabilize the variance. Therefore, the ln transformation was sufficient to stabilize the variance, i.e., the exponential model is appropriate (23, 40).

Proportionality of the dilution factor. The second assumption of the dilution method is that the reduction in grazing mortality is directly proportional to the dilution factor. This assumption was tested by varying the dilution factor. The results show that as the volume of the diluent increased, the apparent bacterial growth rate (slope of the least-squares regression of ln N versus t) increased (Fig. 2 and Table 1). To determine whether the increase in apparent bacterial growth rate was directly proportional to the dilution factor, apparent bacterial growth rates were plotted against the dilution factor (Fig. 3). The relationship appeared to be linear with a correlation coefficient (r) of 0.90 and a slope of 0.120 (standard error of the estimate of the slope, ±0.014). The slope of this plot was the grazing rate, g, which was not significantly different from the grazing rate calculated from the individual bacterial growth rates minus the control and corrected for the dilution factor: 0.098 ± 0.012 (equation 2 and Table 1).

An ANOVA was performed with a lack-of-fit test to assess the appropriateness of the straight-line relationship between bacterial growth rate and dilution factor (Table 2). The ANOVA indicated a significant ($P < 0.0001$) relationship between the variables, and the variance due to lack of fit was not significantly different from the pure-error variance ($P > 0.05$). These analyses indicate that the straight-line assumption was reasonable and the assumption of proportionality between the dilution factor and the apparent bacterial growth rate was not violated in the application of the dilution method.

Bacterial growth rates. The third assumption of the dilution technique is that bacterial growth rates are unaltered by the dilution treatment. Shear stress on cells during filtration causing protozoan cell lysis and carbon enrichment of the diluent would be the most likely cause of enhanced bacterial growth in dilution-treated samples. Two experiments were

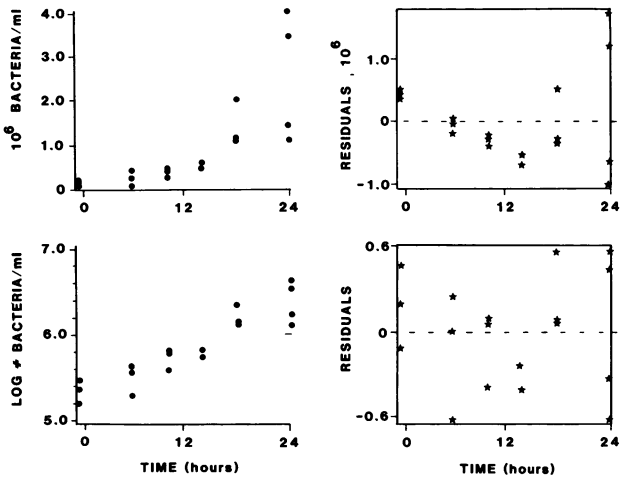

FIG. 1. Bacterial growth in a diluted water sample and evaluation of linear and exponential models by the examination of the residuals from least-squares regressions plotted versus time. Each datum point in the plots of bacterial number versus time is the bacterial concentration for each of three independent replicate water samples; counting error associated with each point is <10%.

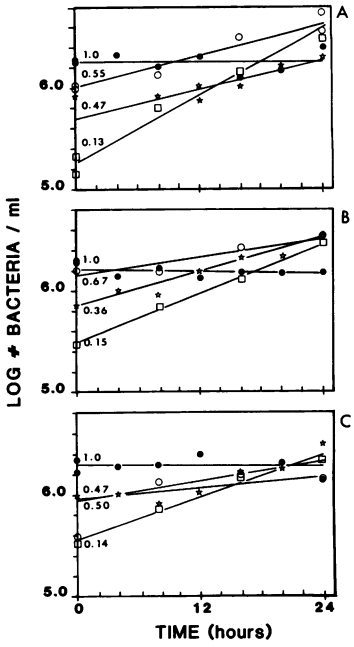

FIG. 2. Changes in numbers of bacteria in a sediment-water interface microbial community over time with four dilution factor treatments: an undiluted control (1.0; ●) and dilutions of 1:2 (○), 1:3 (☆), and 1:5 (□). Actual dilution factors are given on the least-squares regression lines (Table 1). The three panels represent the results from three independent replicate water samples.

TABLE 1. Protozoan bacterivory calculated from the change in ln bacterial number with time in a dilution experiment in which the dilution factor was varied

Sample	X	r_d (cells · h^{-1})	Grazing mortality of bacteria[a]		Bacterial production[b]	
			h^{-1}	10^5 cells · ml^{-1} · h^{-1}	h^{-1}	10^5 cells · ml^{-1} · h^{-1}
A	1.00	0.0005				
	0.55	0.0570	0.0906	1.63	0.0912	1.64
	0.47	0.0409	0.0762	1.37	0.0760	1.37
	0.13	0.1144	0.1308	0.55	0.1313	2.36
B	1.00	−0.0074				
	0.67	0.0416	0.1480	2.87	0.1405	2.53
	0.36	0.0521	0.0935	1.81	0.0861	1.55
	0.15	0.0991	0.1251	2.43	0.1177	2.12
C	1.00	−0.0073				
	0.47	0.0289	0.0686	1.32	0.0599	1.08
	0.50	0.0536	0.1223	2.35	0.1152	2.07
	0.14	0.1002	0.1241	2.38	0.1168	2.10
Mean (SEM)			0.1088 (±0.0091)	1.86 (±0.240)	0.1040 (±0.009)	1.87 (±0.161)

[a] For calculation of grazing mortality, see equation 3.
[b] Bacterial production, $\mu = r_n - g$.

conducted to examine the possible effect of carbon enrichment on this assumption. The first experiment had four treatments with increasing DOC concentrations. These concentrations were estimated to be 2.9 mg of C · $liter^{-1}$ for the NC treatment, 5.7 mg of C · $liter^{-1}$ for the CTL and DIL treatments, and >100 mg of C · $liter^{-1}$ for the YE treatment.

Mean apparent bacterial growth rates increased with increasing carbon concentration (Fig. 4). The ANOVA (Table 3) assessed the variance contributions of replication and carbon concentration, and the latter was broken down into three a posteriori contrasts. There was no significant difference in the estimates of bacterial growth rate among the replicates ($P > 0.05$). The results of the individual contrasts of bacterial growth rates at different carbon levels were as follows. The rate in DIL was not significantly different from that in YE ($P > 0.05$), and that in CTL was not significantly different from that in NC ($P > 0.05$). However, the rate in DIL was significantly different from that in NC ($P < 0.05$). The latter contrast explained 41.2% of the variance in the estimates of apparent bacterial growth rate. These results are understandable in light of the bacterial growth response to substrate concentration (Fig. 4). Here we see that bacterial growth reached a maximum of approximately 0.05 h^{-1}. Bacterial growth was not significantly different in the DIL and YE treatments, despite a 1-order-of-magnitude difference in DOC concentration. Bacterial growth rates in DIL and NC treatments were significantly different, and DOC concentration in the latter was 30% of that in the DIL treatment.

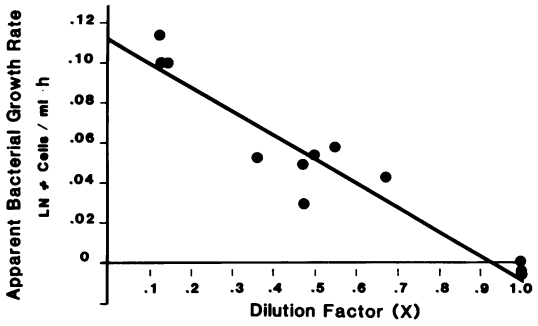

FIG. 3. Effect of dilution factor (X) on apparent bacterial growth rate (slope of the least-squares regression of ln N versus t). A dilution factor of 1.0 was the undiluted control. The least-squares regression line is plotted ($y = 0.112 + 0.120\ X$; $r^2 = 0.90$; standard error of the slope = 0.014). The slope of the regression line is an estimate of bacterial mortality due to protozoan grazing.

The second experiment to test for substrate enrichment effects on bacterial growth rate had controls with or without added carbon (CTL+ and CTL, respectively) and diluted with or without added carbon (DIL+ and DIL, respectively) treatments. The carbon addition was calculated to have been equal to that amount released if 50% of the protozoans present in the water sample had lysed. These values were calculated for protozoans by using the following conversion factors: concentration of protozoa, 10^3 cells · ml^{-1} (unpublished data); weight-to-volume factor, 0.52 pg (dry weight) μm^{-3} (15); and average protozoan volume, 20,000 μm^3 (unpublished data) (13). Cells are assumed to be 40% carbon on a dry-weight basis (34). Given the number of milliliters filtered, 2.8 mg of C · $liter^{-1}$ could have been added to the filtrate owing to protozoan lysis during filtration. For bacteria, the conversion factors were as follows: concentration of bacteria, 10^6 cells · ml^{-1}; weight-to-volume conversion (dry weight), 0.162 · $volume^{0.91}$ (30); and average volume, 0.08 μm^3. Cells are 50% carbon on a dry weight basis (43). Given the volume actually filtered, 0.0042 mg of C · $liter^{-1}$ could have been added if all the bacteria lysed upon filtration. A

TABLE 2. ANOVA with a lack-of-fit test for the assumption of a straight-line model for regression of change in ln bacterial number with time in dilution experiments[a]

Source of variance	df	F	P
Dilution factor	1		
Total error	10	91.74	<0.00001
Lack of fit	7		
Pure error	3	6.53	>0.10

[a] The correlation coefficient (r) for the model was 0.950.

total of 2.8 mg of C · liter^{-1} could have been added if all bacteria and protozoans lysed during filtration. Thus, 1.4 mg of DOC · liter^{-1} were added to the CTL+ flasks. That amount represents 25% of the total available DOC, which was measured and found to be 5.7 mg · liter^{-1}.

An ANOVA was run on the bacterial growth rates (the slopes of the regressions of the change in ln N versus t; Table 4). The ANOVA indicated the following. (i) The difference in bacterial growth rates among the replicates did not contribute significantly to the variance in bacterial growth rate estimates. (ii) The carbon addition had a marginal effect on the bacterial growth rate estimates ($P > 0.05$). (iii) Dilution was the only significant factor affecting bacterial growth rate estimates ($P < 0.005$). (iv) The carbon by dilution interaction was not a significant contributor to the variance in the estimates of bacterial growth rates. Carbon addition accounted for only 16% of the variance in the estimates of bacterial growth rates, while dilution accounted for 64%. The carbon by dilution interaction term indicates that the difference in bacterial growth rates between DIL and DIL+ treatments versus CTL and CTL+ treatments was not significant.

DISCUSSION

Bacterial growth in a closed system, such as a flask, follows a sigmoidal growth curve described by the logistic model (46, 47). However, observed bacterial growth in water taken from an open system, diluted, and incubated can be described by exponential, linear, or logistic regression models depending on the initial number of active bacteria and the substrate concentration. Landry and Hassett (24) derived equations for the dilution method by using an exponential growth model. Exponential growth is observed in closed systems when $N \ll N_{max}$ (or the substrate is effectively not limiting). Wright and Coffin (47) have consistently found a linear relationship between bacterial number and time in dilution experiments and have applied this linear relationship to the logistic model for grazing calculations. Linear growth is observed in closed systems when $N \simeq N_{max}/2$. Ducklow and Hill (8) described dilution experiments in which no growth was observed as predicted as N approaches N_{max}. Analyses of regression residuals provide the data required to evaluate the appropriate model for calculation of grazing parameters. We tested the statistical appropriateness of the linear and exponential models and found that the exponential model was appropriate to model bacterial growth in dilution treatments from the Lake Anna sediment-water interface.

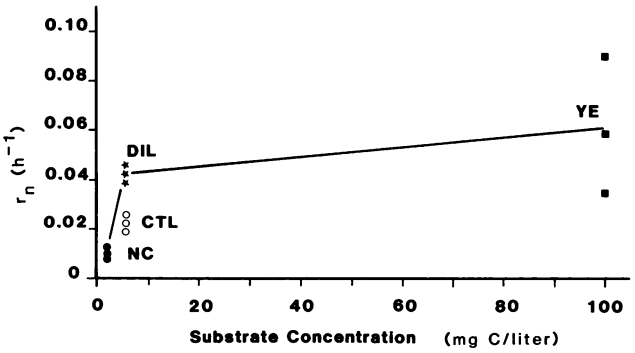

FIG. 4. Effect of substrate concentration on apparent bacterial growth rate (r_n) in a carbon addition and removal dilution experiment. The four treatments are as follows: CTL, undiluted control; DIL, 1:3 dilution; YE, 1:3 dilution with YE added to a final concentration in excess of 100 mg · liter^{-1}; and NC, 1:3 dilution in which the filter-sterilized diluent was passed through an activated-charcoal column to remove DOC. Three independent, replicate sediment-water interface samples from one station at Lake Anna, Va., were used for each treatment.

TABLE 3. Effect of YE and NC treatments on the apparent bacterial growth rate in 1:3 diluted water samples as compared with rates in DIL and CTL treatments[a]

Source of variance	*df*	*F*	*P*	% Variance explained
Replicates	2	1.73	0.255	
DIL vs. YE	1	2.53	0.163	
CTL vs. NC	1	1.19	0.318	
DIL vs. NC	1	9.64	0.021	41.2
Error	6			

[a] At $\alpha = 0.05$, only one a posteriori contrast was significant.

We have shown that apparent bacterial growth rates are a linear function of the dilution factor. Ducklow and Hill (8) suggested that there may be a threshold dilution factor (0.41) above which bacterial growth is not observed. This could be owing either to the N-to-N_{max} ratio having been close to 1 or to substrate limitation. Our results indicate that there is no threshold of this type in the Lake Anna sediment-water system (Fig. 2). This may be owing either to high grazing pressure keeping $N \ll N_{max}$ or to adequate substrate flux or to both.

Landry and Hassett (24) have suggested that protozoan grazing rate may be a step function of bacterial concentration, whereby at low bacterial concentrations the protozoans cannot obtain sufficient nutrition at maximum clearance rates and grazing ceases ($g = 0$). Conversely, at higher bacterial concentrations, protozoans graze at a constant maximal rate. Other researchers have reported that protozoans require a minimal bacterial concentration to sustain protozoan growth of at least 10^6 to 10^7 cells · ml^{-1} on the basis of laboratory feeding experiments (1) and 0.5×10^6 to 2×10^6 cells · ml^{-1} on the basis of energy budgets (9). We did not see a threshold effect of the bacterial concentration on grazing rate (Fig. 5) using the data from the multiple-dilution-factor experiment (Fig. 2 and Table 1) which covered a 1-order-of-magnitude range in the bacterial concentration: 2×10^6 diluted to 0.5×10^6.

Carbon enrichment due to protozoan cell lysis during filtration may confound estimates of grazing mortality and bacterial production (12). The present experiments indicate that the impact of carbon enrichment in the diluent has minimal impact on bacterial growth in comparison to dilution; dilution accounted for 64% of the variance in the estimate of apparent bacterial growth rates, whereas carbon addition (equivalent to 50% of total protozoan carbon) only accounted for 16% of the variance. The effect of the sub-

TABLE 4. Effect of carbon addition on apparent bacterial growth rate in CTL and DIL treatments in a 1:3 dilution grazing experiment

Source of variance	*df*	*F*	*P*	% Variance explained
Replicates	2	0.22	0.807	
Carbon factor	1	5.58	0.056	16.3
Dilution factor	1	21.82	0.003	64.1
Carbon by dilution	1	0.140	0.717	
Error	6			

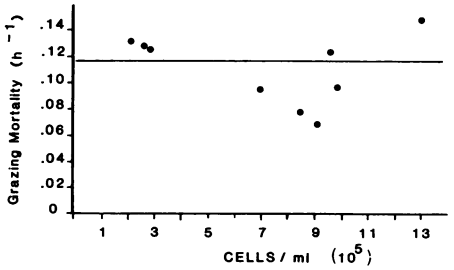

FIG. 5. Effect of bacterial concentration on calculated bacterial grazing mortality due to protozoan grazing by using the dilution technique. The least-squares regression line is plotted ($y = 0.118 + (1.2 \times 10^{-8}\ x)$; $r^2 = 0.03$).

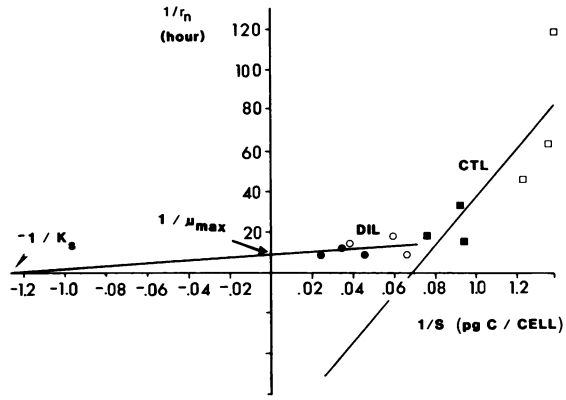

FIG. 6. Double-reciprocal plot of substrate concentration versus apparent bacterial growth rate in dilution experiments with carbon added in three independent samples from the sediment-water interface from Lake Anna, Va. Treatments are as follows: undiluted control with (■) or without (□) added carbon (CTL) and 1:3 diluted water sample with (●) or without (○) added carbon (DIL). The least-squares regression lines are plotted; the x intercept is the negative reciprocal of the half-saturation constant (K_s = 7.99 pg of C · cell^{-1}); the y intercept is the reciprocal of the maximum growth rate (μ_{max} = 0.115 h^{-1}).

strate on estimates of bacterial production in the presence of grazing mortality may be negligible when the substrate is not limiting. Evidence of adequate substrate in the Lake Anna sediment-water system is given in the saturation relationship between bacterial growth rate and substrate concentration (Fig. 4).

To examine further the relationship between the substrate concentration and the system-dependent observed bacterial growth rate, we plotted the reciprocal of the substrate concentration on a per-cell basis versus the reciprocal of the apparent bacterial growth rate determined in the second carbon addition experiment (equation 4; Fig. 6) in a manner similar to that used to linearize saturation (Monod) kinetics. A comparison of the slopes of the double-reciprocal plot of bacterial growth with grazing (CTL and CTL+ treatments) and with reduced grazing (DIL and DIL+ treatments) demonstrates the effect of grazing on the apparent bacterial growth rates and on the calculation of bacterial growth parameters. Diluted treatments yielded acceptable double-reciprocal plots with positive y and negative x intercepts, whereas in undiluted treatments (grazing present), the plots were nonsensical. This observation may be peculiar to this system; however, the dilution method may legitimately provide usable values of μ_{max} and K_s for bacterial growth. For this study, we determined μ_{max} to be 0.115 h^{-1} and the half-saturation constant to be 7.99 pg of C · cell^{-1} (at a mean initial cell concentration of 6.7×10^5 cells · ml^{-1}, K_s was 5.4 mg of C · liter^{-1}).

Reported values for grazing mortality of bacteria arrived at by using inhibition, dilution, filtration, and labeled food particles span only 1 order of magnitude (0.02 to 0.2 h^{-1}; Table 5). This constancy exists despite the difficulties associated with each of the techniques (as described in the introduction) and the additional variability contributed by latitude, salinity, temperature, and substrate availability.

Estimates of bacterial productivity using the dilution technique have been compared with more direct methods, such as incorporation of tritiated adenine (6) or tritiated thymidine (8, 22). The dilution method closely correlated with the uptake methods when compared with use of inhibitors and the frequency of dividing-cell methods of estimating bacterial productivity.

TABLE 5. Comparison of methods for the determination of protozoan grazing mortality, feeding rate, and clearance rate for different ecosystems[a]

Method	Site	Organism	Grazing mortality		Feeding rate (cells · ml^{-1} · h^{-1})	Clearance rate (nl · grazer^{-1} · h^{-1})	Reference(s)
			h^{-1}	10^5 cells · ml^{-1} · h^{-1}			
Dilution	Coastal marine	Flagellates	0.021–0.045	0.27	26.9	20	24
	Warm core rings	Protozoans	0.071 ± 0.01	0.54[b]	ND	ND	8
	Acid lake	Protozoans	0.109–0.009	1.86 ± 0.24	37.2[c]	20[c]	This study
Filtration	West Atlantic Ocean	Protozoans	0.016–0.116	0.4–2.9	ND	ND	48
Inhibition	Coastal marine	Flagellates	0.035	0.41–2.08	ND	ND	14
	Lake	Protozoans	0.011–0.019	0.21–0.35	ND	ND	36
	Estuary	Protozoans	0.022–0.18	0.22–1.8	20–80	2–10	38
Fluorescent microspheres	Lake	*Dinobryon* spp.	ND	ND	30	6	2
	Lake	Ciliates	ND	ND	210	42	2
	Coastal marine	Flagellates	ND	ND	3.3	0.6	29
Cultures	Danish fjord	Flagellates	ND	ND	60	2–80	9, 10

[a] Values are given as previously reported, i.e., as single values, ranges, or with standard deviations. ND, Not determined.
[b] Using the mean initial bacterial concentration from Table 2 (7.66×10^6).
[c] Using 5×10^3 grazers · ml^{-1} determined at the sediment-water interface.

The dilution method was shown not to violate the assumptions implicit in its application to the quantification of bacterial mortality due to protozoan grazing. The dilution method compares well with other indirect (inhibitor, inert particles, and filtration) and direct (tritiated-adenine and -thymidine) methods.

ACKNOWLEDGMENTS

We thank P. H. Rich of the University of Connecticut for analysis of the DOC samples. We are grateful for the invaluable assistance of P. E. McIntire and J. Porter in statistical analyses, and we thank A. J. Bulger for helpful comments on the manuscript.

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