Microbial Ecology

Determining Parameters of the Numerical Response

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Abstract

The numerical response, the change in specific growth rate with food concentration, is a fundamental component of many aquatic microbial studies. Accurately and precisely determining the parameters of this response is essential to obtain useful data for both aut- and synecological studies. In this work we emphasize four points that are often ignored in designing numerical response experiments: (1) the inclusion of subthreshold concentrations (i.e., where growth rate is negative) in the experimental design; (2) an appropriate allocation of effort, i.e., the superiority of choosing more individual prey concentrations rather than replicating fewer; (3) the potential superiority of replicating experiments rather than simply replicating treatment in a single experiment; and (4) the placement of most measurements near the lower end of the concentration gradient, well below the asymptote, possibly following a geometric progression. We illustrate the first point by examining a small subset of published data on planktonic oligotrich ciliates and then, using a Monte Carlo simulation, rigorously evaluate the experimental design, supporting the remaining points.

Introduction

The numerical response, the change in specific growth (μ) rate of a predator with prey concentration, is a fundamental component of many aquatic microbial studies. Typically this relationship is modeled following a rectangular hyperbolic function with a nonzero intercept; this model is known to be a good predictor of the numerical response (e.g., [1, 11, 15]):

$$\mu = [\mu_{max}(p - p')]/[k + (p - p')]$$

where μ is the specific growth rate (d^{-1}); μ_{max} is the maximum specific growth rate; p' is the threshold concentration (prey mL^{-1}, the concentration where $\mu = 0$); k is a constant (prey mL^{-1}); and p is the prey concentration (prey mL^{-1}).

Such numerical responses are often incorporated into microbial food web models [4]. Furthermore, the parameters of the response are often used to compare taxa (e.g., [11]) and are used to assess the autecology of species [5, 16]. Consequently, for these comparisons to be made it is essential to obtain accurate and precise estimates of the parameters for this response.

Effort must thus be directed at optimizing the basic experimental design associated with determining the numerical response. Choosing appropriate prey concentrations is a key component of such experimental design, if a precise and accurate response is to be obtained. Parallel concerns arise in the study of enzyme kinetics, using the Michaelis–Menten function (e.g., [14]) and in algal studies, using photosynthesis vs irradiance functions (e.g., [6]). Here we emphasize four points: (1) the inclusion of subthreshold concentrations in the experimental design; (2) an appropriate allocation of effort, i.e., the superiority of choosing more individual prey concentrations rather than replicating fewer; (3) the potential superiority of replicating experiments rather than replicating treatment in a single experiment; and (4) the placement of most measurements near the lower end of the concentration gradient, well below the asymptote, possibly following a geometric progression. We illustrate the first point by examining a small subset of published data on planktonic oligotrich ciliates and then, using a Monte Carlo simulation, support the remaining points; Monte Carlo analysis is recommended to assess the precision and accuracy of nonlinear curve-fitting methods [8].

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Methods

Using values based on some of our own numerical response experiments on planktonic oligotrich ciliates (unpublished data) and those in the literature [5, 15], we established a "model" numerical response with the following parameters: $\mu_{max} = 1.34\ d^{-1}$, $k = 3.75 \times 10^6$ prey mL^{-1}, and $p' = 1.38 \times 10^6$ prey mL^{-1}. We emphasize that this "model" response does not necessarily reflect any one ciliate species; it is only used to "seed" the Monte Carlo simulation with values that approximate those found in nature. Using this response, we then explored where measurements should be taken to maximize parameter accuracy and precision. Nine experimental cases were explored; all were based on 12 measurements—a minimum number of measurements recommended for such plots [14] and assuming that real experiments might be logistically limited to near this number.

Brief descriptions of each case follow: case 1 (*Unreplicated*), where measurements were made at various prey concentrations, with more at lower prey levels but with no systematic choice of concentrations (Fig. 1a), i.e., this is in practice what many researchers do; case 2 (*Replicated 1*), which was similar to case 1, i.e., no systematic placement, but triplicate measurements were made at each concentration (Fig. 1b); case 3 (*Replicated 2*), where three replicate measurements were made at the threshold (p') and k-concentrations and near μ_{max} (Fig. 1c)—this simulates the unlikely case where measurements are made exactly at k and p' and at levels that approximate μ_{max}; case 4 (*Replicated 3*), where triplicate measurements were made at concentrations that were at some distance from the from k, p', and concentrations giving μ_{max} (Fig. 1d)—if case 3 is a "best-case" for replication, this can be considered a "worst-case"; case 5 (*Even*), where measurements were spread evenly over the range of prey concentrations (Fig. 1e); case 6 (*Bias low*), where many measurements were made at lower concentrations, i.e., near to the parameters, k and p', and few were made near μ_{max} (Fig. 1f); case 7 (*Bias high*), where more measurements were made near μ_{max}, and few were made near k and p' (Fig. 1g); and finally, cases 8 and 9 (*Geometric 1 and 2*), where measurements were spread systematically, following a geometric progression (a low value was chosen and then repeatedly doubled), i.e., placing more emphasis on lower food levels (Fig. 1h, i).

For each case, datasets were generated with the above "model" parameters. Datasets for each case were error-corrupted [*sensu* 9] by the addition of random error (generated in a normal distribution with a mean of 0 and a standard deviation of 1 using the "NORMAL" function of SPSS 11.0 for Windows, SPSS Inc., Chicago), at a constant 25% of the value of μ at a given prey concentration; i.e., the coefficient of variation of μ was constant regardless of the prey concentration.

For each of the nine cases, 100 simulated datasets were generated. For each simulation (12 data points) data were fit to the numerical response (see equation and parameters above), using a nonlinear regression package (SigmaPlot 5.0 and SigmaStat 2.0, SPSS) that applies the Marquardt–Levenberg algorithm to iteratively fit data to a defined curve and provides asymptotic standard errors for parameters. The algorithm determines the line of best fit by minimizing the sum of squares of differences between the dependent variable and the observations; this method can be more accurate than linear transformation when dealing with rectangular hyperbolas and is needed when a nonzero intercept is included in the formulas [1, 8].

From the 100 replicate simulations, three statistics were determined for each parameter (μ_{max}, k, p'): the mean from the 100 simulations, the standard error of this mean (a statistic used to assess how close the estimated mean is to the model parameter), and the mean of the asymptotic standard errors for parameters, generated for each nonlinear curve fit (this statistic assesses the precision of the estimated parameter for a single fit but does not indicate how well it estimates the model parameter).

Results and Discussion

Our first point is that subthreshold values of growth rate (i.e., mortality rates) may be needed to adequately estimate the threshold concentration. For instance, researchers have determined the numerical responses of *Strombidium inclinatum*, possibly the most rigorously studied oligotrich ciliate (e.g., [5, 15]), previously referred to as *Strombidium sulcatum* [13]. Simply for illustrative purposes, using the computer packages mentioned above, the numerical response was fit to the data for *S. inclinatum* presented in these two studies ([5], values obtained from their Fig. 3; [15], values obtained from their Table 1). The parameters and error estimates for these fits are presented in Table 1. The estimate of the threshold parameter (p'), obtained from these studies was, not significantly different from zero in four of the five experiments (t-tests, $P < 0.05$, Table 1). Although several studies clearly indicate that *S. inclinatum* consumes bacterial-sized particles (e.g., [5, 15]), our analysis of existing data would suggest that the ciliate could theoretically survive at food levels that are not significantly different from zero ($P < 0.05$, Table 1). It is important to bear in mind that the asymptotic error terms from nonlinear fits, that we have used for statistical tests, may be inaccurate by two- to threefold [8], and thus this is not necessarily a rigorous test.

Another reason for the large errors, and consequent unreliable estimates of p', we have determined using previous studies (Table 1) stems, at least in part, from our extrapolation: the threshold parameter is inappro-

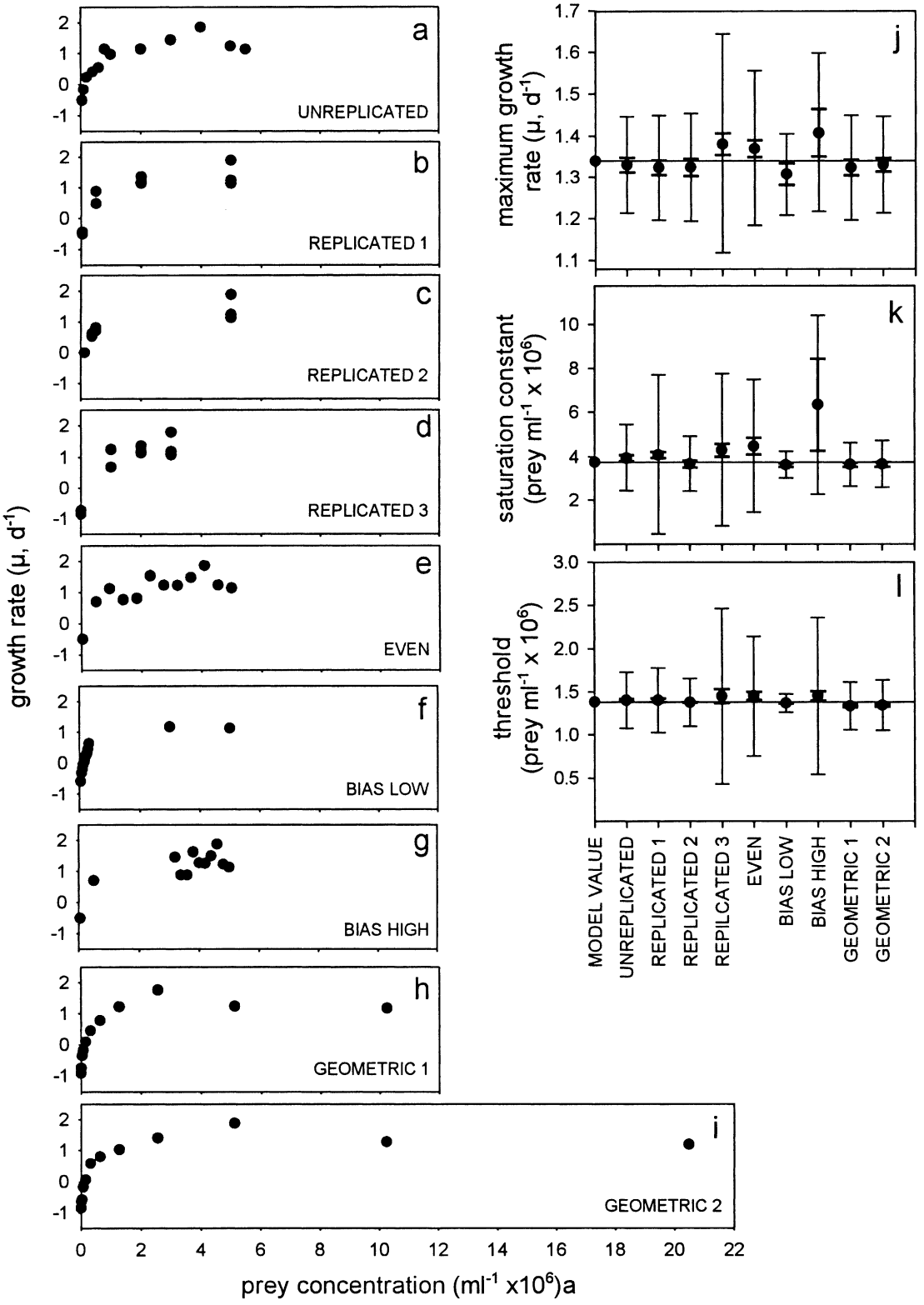

Figure 1. Monte Carlo simulated data of the numerical response, investigating where measurements are best made. (a–i) Examples of the nine cases of where measurements might be placed (see Methods for details). (j–l) Estimates of precision and accuracy of the three parameters of the numerical response (see text for equation; μ_{max}: the maximum growth rate; k: a constant; p': the threshold concentration). Solid points represent mean estimates (n = 100) of the parameters; the vertical line and the first data point are the model parameter value (i.e., the "true" value that was being estimated); thick error bars represent the standard error associated with 100 estimates of the parameters made by the Monte Carlo simulations; thin error bars represent the mean (n = 100) of the asymptotic standard errors for parameters, generated by the nonlinear curve-fitting package (see Methods for details).

Table 1. Nonlinear curve fits of the numerical response (specific growth rate vs food concentration) for the ciliate *Strombidium inclinatum* growing on bacteria[a]

Study	*Parameter*	*Parameter estimate*	*SE*	*P*
[15] exp. 1	μ_{max}	3.35	0.322	
	k	4.92×10^6	2.05×10^6	
	p'	7.93×10^5	3.66×10^5	0.08
[15] exp. 2	μ_{max}	1.12	0.059	
	k	3.19×10^6	7.94×10^5	
	p'	9.36×10^5	1.98×10^5	<0.01*
[15] exp. 3	μ_{max}	0.860	0.087	
	k	3.25×10^6	2.06×10^6	
	p'	1.71×10^5	6.91×10^5	0.81
[15] exp. 4	μ_{max}	1.96	0.401	
	k	5.95×10^6	1.10×10^7	
	p'	-4.99×10^6	1.03×10^7	0.65
[5]	μ_{max}	3.04	0.392	
	k	2.94×10^6	1.46×10^6	
	p'	-7.61×10^4	5.06×10^5	0.89

[a]Parameters and their associated standard errors (SE) are presented for the numerical response (see text for equation). *P* values apply to *t*-tests, assessing whether the threshold concentration (p') is significantly different from zero; * represent estimates that are significantly different at $\alpha = 0.05$. [15] exp. 1–4 refer to four separate experiments presented in that study. Parameters are described in the text.

priately predicted from beyond the range of the observations, as no negative growth rates were determined in these studies. Thus, the lower estimates of threshold concentration (Table 1) are clearly imprecise and possibly inaccurate and may not be appropriate to determine the role of this ciliate as a bacterivore at low food concentrations. Admittedly, this was not the intent of the previous studies; we simply use these works to illustrate the point, and by no means do we ridicule these fine works. We simply point out that studies should, and more recently often do, include subthreshold concentrations to adequately parameterize the numerical response (e.g., [16]).

In fact, determining mortality rates may be important for other reasons. For instance, planktonic ciliates suffer high mortality rates below threshold levels of their phytoplankton prey, and these threshold levels are likely to be encountered *in situ* [11]. Furthermore, the threshold parameter (p') may be used to compare different planktonic species, indicating that similar taxa have distinctly different responses [11], and to compare single species under different ecological conditions, indicating, for instance, the response to temperature (e.g., [16]). Accurate estimates of these values could thus substantially alter food web models that include protozoa (see [4]), and if the threshold level is used as a key measurement to compare taxa, it needs to be precisely and accurately estimated.

Our second conclusion from this study is that there are certain misconceptions regarding experimental design of growth and feeding experiments. Many studies of functional and numerical responses seem to be based on an assumption that failing to replicate measurements at any single food concentration represents a flawed experimental design. There seems to be some confusion here, between tests that compare discrete treatment effects, which require replication (e.g. ANOVA), and regression analysis that does not require replication of discrete treatments (see [17]). Experimental design for regression can, of course, include replication, and replication provides data to test assumptions regarding homoscedasticity [17], but is it the best allocation of effort for nonlinear regression? The older literature on enzyme kinetics (Michaelis–Menten functions) recommended that replicate measurements be made at the various substrate concentrations, primarily for exploratory purposes (e.g., [2]). However, following the introduction of nonlinear curve-fitting methods, recent assessments of such Michaelis–Menten responses have recommended against replication, in favor of spreading measurements along the concentration gradient [8, 14]. Similarly, for nonlinear curve fitting of photosynthesis vs irradiance studies, which follow an analogous relationship to the numerical response, it has been recommended that measurements be spread out along the irradiance gradient rather than replicating individual irradiances [6]. What is gained by choosing more points is the superior ability to evaluate the goodness of fit of the predicted model and to specify the error terms in the parameter estimates [8].

However, quantitatively assessing the benefits of placing measurements at various concentrations along a gradient is intractable or difficult using standard statistical methods, and Monte Carlo simulations have been recommended as a means to assess such error in nonlinear curve fitting [8]. Thus, using a Monte Carlo simulation, we examined whether, for the numerical response, replication is needed and then assessed where it is best to place measurements to improve accuracy and precision of estimates.

Our analysis suggests that if measurements are replicated ($n = 3$) at or very close to the parameters (i.e., μ_{max}, k, p') to be estimated (Fig. 1c), then the estimates are both relatively accurate and precise (Fig. 1j–1). However, if measurements are replicated and spread across the range (Fig. 1b), the estimates of the parameters are no better, and possibly worse, than if the same number of total measurements (unreplicated) were spread across the same range (Fig. 1a, j–1). Furthermore, if the measurements are replicated at poorly chosen concentrations (Fig. 1d), then resulting parameter estimates will be relatively imprecise and potentially inaccurate. Although replicated measurements at ideally chosen concentrations have the potential to provide better estimates of parameters, such ideal choices are, paradoxically, dependent on knowledge that does not exist at the outset. Indeed, there is a greater potential for poor choices of replicated concentrations to result in poor estimates of parameters than is the case in unreplicated studies. We therefore recommend, where there are constraints in the number of samples that can be examined, against replication and in favor of spreading measurements along the concentration gradient (c.f. [6, 14]).

Where then should measurements be made? Both spreading measurements across the range evenly (Fig. 1e) and placing most measurements near the asymptote of μ_{max} (Fig. 1g) provide relatively inaccurate and imprecise measurements (Fig. 1j–1). In contrast, making most of the measurements at lower concentrations (near k and p', Fig. 1f,h,i), provides both accurate and precise estimates, although limited measurements near the asymptote (Fig. 1f), not surprisingly, yield estimates of μ_{max} that are not accurate (Fig. 1j). Thus, while adding measurements at very high concentrations does little to improve estimates (cf. Fig. 1i), biasing the measurements toward the lower concentrations does improve estimates (Fig. 1f). This last observation has been previously noted [1]; i.e., where there are no points on the asymptote, curves are poorly fit, but if some points occur at concentrations that yield values near μ_{max}, biasing measurements to the lower concentrations improves parameter estimates.

Here, our conclusions are twofold: (1) replication of food concentrations for a single experiment is not essential for this statistical analysis [17]; in any case, such replication is virtually impossible as experimental containers that start with similar prey numbers will inevitably change at different rates during an incubation, and thus the average concentrations will inevitably differ [10]; and (2) effort directed at attempting replication of food concentrations would be much better spent on increasing the number of measurements distributed along the nonlinear response.

One of our initial assumptions was that numerical response experiments are often logistically limited, in terms of the number of food levels applied (the 12 measurements in the Monte Carlo simulation). Therefore, assuming all other factors can be kept constant, repeating the entire experiment would be one method to better estimate parameters. Experiments could then be combined or examined separately as replicates or separate treatments (with time). For instance, Rivier et al. [15] were rigorous and conducted four replicate experiments on the growth rate of *S. inclinatum* (Table 1), and these provide four independent estimates of the model parameters that can be used as true independent measurements. Similarly, Montagnes et al. [12] conducted several discrete numerical response experiments on a clonal culture of the planktonic ciliate *Strombidinopsis cheshiri* but found that there were distinct differences between experiments separated by months (i.e., they were not replicates); they subsequently attributed differences to clonal decline and selfing-conjugation within the clone. To some extent, experiments where food concentrations are independent but are all run at the same time could be considered to lack one level of replication. If "demonic intervention" [7] directionally biased the conditions during a single run of an experiment (e.g., clonal decline [12] or an unnoticed temperature shift altered the response [16]), it could provide precise but inaccurate data. Repeating the experiment would then provide more accurate parameter estimates; thus, we support Rivier et al.'s [15] approach of running multiple experiments.

Numerical response experiments have also been evaluated on criteria other than those in this work. Studies have discussed (1) the analysis of data that follow a rectangular hyperbolic (numerical or functional) response, including the problems associated with food concentration decreasing during incubations, potentially leading to non-steady-state dynamics (e.g., [10]); (2) problems associated with inadequate acclimation periods and ciliates dividing but not growing at subthreshold concentrations (thus numbers may increase but total biomass may not) (e.g., [5, 11]); and (3) the complicated issue of which model should be used to fit the data and how it should be fit (e.g., [1, 3]). These points are essential to recognize when designing and evaluating experiments, but they are not directly relevant to the issues we raise. There is one consideration, however, that is germane: although growth rate ($+\mu$) will be constant at a defined food level, this may not be so for mortality rate ($-\mu$). As ciliates (or other predators) are not in a steady state, mortality rate will depend on the incubation period at a single food level; over extended periods mortality rate might increase or decrease, depending on the adaptive response of the organism. However, predictive numerical response curves that include mortality are needed by ecologists (e.g., [11]), and over relatively short periods we can make the assumption that mortality rate is intrinsic; i.e., it follows the same function as growth rate. Therefore, we add a cautionary note: experimentalists should

apply incubation (starvation) periods similar to those which the predator might experience in nature [11], and modelers must recognize these incubation periods when incorporating a numerical responses into ecosystem models or determining a threshold level.

In conclusion, we emphasize: (1) to estimate the threshold concentration, subthreshold levels should be considered; (2) replicating food levels in a single experiment is unnecessary, virtually impossible, and not an optimal allocation of effort; (3) numerical response measurements should emphasize concentrations below the asymptote, using a geometric progression; and (4) repeating experiments in time/space may provide more accurate parameter estimates than replicating treatment in a single experiment.

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