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Estimating size-dependent growth and grazing rates and their associated errors using the dilution method

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Abstract

Size-dependent properties are pervasive in nature but difficult to measure for natural communities. Here, we develop a technique to estimate size-specific phytoplankton growth and grazing rates based on the two-point dilution method, enhanced by the acquisition of the size spectra of the phytoplankton in the samples. We describe a way to estimate standard deviations associated with the rate estimates, which can be applied either to the size-dependent or total community rates. We tested the accuracy of rates estimated using the size-dependent dilution method by applying it to dilution experiments simulated using a complex size-structured ecosystem model. The strong agreement between model and size-dependent dilution method rates (two-sample Kolmogorov-Smirnov test, $P = 1$) supports the accuracy of this new technique. Because size-dependent rates vary with the size interval over which they are calculated, we display the size-dependent growth and grazing rates and their standard deviations as a function of the size interval. This technique easily allows the assessment of rates for any size class of interest. Finally, we apply the size-dependent dilution method to data collected in the equatorial Pacific. There is a general agreement between size-based and previously published taxonomic-based rates, with differences reflecting the extent to which size classes are mixtures of taxa. The use of the size-dependent dilution method will provide new insights into the structure and dynamics of planktonic communities. Future applications of this method to other natural communities will help in assessing the size-dependencies of phytoplankton growth and grazing rates in their environments.

Organism size plays a major role in structuring planktonic communities (Chisholm 1992; Ichinokawa and Takahashi 2006), in determining pathways of flows in marine food webs (Ryther 1969; Hansen et al. 1994) and in regulating rates of biogeochemical fluxes and carbon sequestration (Legendre and Rassoulzadegan 1996). Size also influences how planktonic organisms relate to their hydrodynamic environment (Koehl and Strickler 1981; Monger and Landry 1990), as well as how they partition nutrients (Eppley et al. 1969; Moloney and Field 1989), growth (Schlesinger et al. 1981; Tang 1995; Nielsen 2006), respiratory losses (Banse 1976; Tang and Peters 1995), and other metabolic processes (Joint and Pomroy 1988; Joint 1991; Gillooly et al. 2001) among the coinhabitants and potential competitors in a given environment. An understanding of size-specific processes is, therefore, important for understanding planktonic ecosystem dynamics.

Measurements of growth and grazing rates are central to understanding plankton community dynamics. Despite their significance, accurate determinations of such rates in general, and size-dependent rates in particular, are difficult. Size-specific estimates of growth and grazing rates have come from laboratory experiments with limited species (Capriulo and Ninivaggi 1982; Monger and Landry 1991; Neuer and Cowles 1995), from syntheses of many such studies (Banse 1976; Hansen et al. 1997), or from theory (Moloney and Field 1989, 1991; Armstrong 1994; Poulin and Franks 2010). However, very few size-resolved rate data for growth or grazing have been acquired for natural assemblages (but see Marañón 2008).

Here, we introduce a method to estimate size-dependent growth and grazing rates that is built upon the two-point dilution technique. This size-dependent dilution method requires counting and sizing of phytoplankton and can be implemented using laboratory or natural samples. Because cell counting and sizing by microscopy is often labor intensive, the numbers of cells enumerated are often low, leading to potentially significant statistical errors in rate estimates.

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Therefore, we introduce a method for calculating the standard deviations of individual size-dependent rate estimates, which can also be applied to community-level rates. We assess the efficacy of the size-dependent dilution method by simulating dilution experiments with a complex, nonlinear size-structured nutrient-phytoplankton-zooplankton (NPZ) model and comparing the known model rates with those estimated by the method. Finally, we apply the size-dependent method to field data collected from the equatorial Pacific.

Materials and procedures

Size-dependent dilution method

Dilution is a commonly used experimental technique to measure bulk, community-averaged rates of phytoplankton growth (μ, d^{-1}) and microzooplankton grazing mortality on phytoplankton (g, d^{-1}) by altering the encounter rates of grazers and prey (Landry and Hassett 1982). Biomass-specific rates of microzooplankton grazing and phytoplankton growth are assumed to be constant and unaffected by dilution. Thus, the logarithm of the measured net rate of phytoplankton growth over an incubation period (t, usually 1 d) should vary linearly with the dilution effect (d, the fraction of unfiltered seawater) on grazer biomass:

$$\text{net growth rate} = = \frac{1}{t}\ln\left(\frac{P_t}{P_0}\right) = \mu - dg \tag{1}$$

where P_0 and P_t are initial and final concentrations of phytoplankton, respectively. The traditional dilution experiment involves several treatments with different fractions of unfiltered seawater (i.e., several values of d), allowing Eq. 1 to be solved by regressing the net growth rate against d and finding μ (y-intercept) and g (negative slope). However, where it can be reasonably assumed or tested that linearity exists (Landry et al. 1995, 2011b), the experimental design can be streamlined to two treatments—the natural, undiluted seawater sample and a single diluted treatment—and solved algebraically for the two unknowns (Landry et al. 1984, 2011b). Adopting subscript designations such that P_0 and P_t are initial and final concentrations of phytoplankton for the undiluted treatment and $P_{0,d}$ and $P_{t,d}$ are corresponding variables for the diluted treatment, growth and grazing rates are computed as follows:

$$\mu = \frac{1}{t(1-d)}\left[\ln\left(\frac{P_{t,d}}{P_{0,d}}\right) - d\ln\left(\frac{P_t}{P_0}\right)\right] \tag{2}$$

$$g = \frac{1}{t(1-d)}\left[\ln\left(\frac{P_{t,d}}{P_{0,d}}\right) - \ln\left(\frac{P_t}{P_0}\right)\right] \tag{3}$$

To apply the dilution approach to generating size-dependent rate data, size measurements of individual phytoplankton cells must be integrated into the experimental analysis. Microscopes, flow cytometers (Ackleson and Spinrad 1988), Coulter counters (Sheldon and Parsons 1967), and FlowCAMs (Sieracki et al. 1998) are all possible instruments for producing such data. Their strengths and weaknesses are discussed below (*see* "Discussion"). Once the cell sizes in each diluted and undiluted treatment at each time point are determined, the range of cell sizes can be split into discrete size classes and the abundances of cells in each size interval determined. The application of Eq. 1 to each of those size intervals can be used to calculate the size-dependent net growth rate for a given size class. That is,

$$\text{net growth rate for size class } i = \frac{1}{t}\ln\left(\frac{P_{t,i}}{P_{0,i}}\right) = \mu_i - dg_i \tag{4}$$

where $i = 1, 2, \ldots, n$ for n size classes, $P_{0,i}$ and $P_{t,i}$ are initial and final phytoplankton concentrations (cells mL^{-1}) for size class i, and μ_i and g_i are the growth and grazing rates, respectively, for size class i. When multiple dilution treatments are used, a linear regression can be used to calculate μ_i and g_i. For two treatments, the size-dependent growth and grazing rates can be estimated by applying Eqs. 2, 3 to each size class, namely

$$\mu_i = \frac{1}{t(1-d)}\left[\ln\left(\frac{P_{t,d,i}}{P_{0,d,i}}\right) - d\ln\left(\frac{P_{t,i}}{P_{0,i}}\right)\right] \tag{5}$$

$$g_i = \frac{1}{t(1-d)}\left[\ln\left(\frac{P_{t,d,i}}{P_{0,d,i}}\right) - \ln\left(\frac{P_{t,i}}{P_{0,i}}\right)\right] \tag{6}$$

where $P_{t,d,i}$ and $P_{0,d,i}$ are, respectively, the initial and final diluted treatments for size class i.

Standard deviation for rate estimates

For typical dilution experiments (multi-treatment or two-point), growth and grazing rates are usually estimated from chlorophyll or high-performance liquid chromatography (HPLC) pigment concentrations, which are relatively precise stock measurements. Where individual cell counts are required, as in this method, the counting errors can be large relative to the rate estimates, depending on the counting technique used and the phytoplankton cell size spectrum. Here, we present a method for quantifying error—the standard deviation—associated with size-dependent estimates of μ and g derived from initial and final cell counts for any two dilution treatments (Eqs. 5 and 6). We used only two treatments because it is analytically tractable; however, the error can be extended to multiple treatment experiments using a variation of the error of a linear regression. For clarity in the description below, we leave out the subscript i.

As seen in Eqs. 2, 3, 5, 6, the growth and grazing rates are calculated from the ratio of two cell counts: initial and final. If we assume that the cell counts per unit volume can be modeled by Poisson distributions, the standard deviations for μ and g can then be estimated from the approximate standard deviations for the ratios of Poisson-distributed variables.

As described by Gu et al. (2008), given two Poisson rates, ρ_0 and ρ_1, with associated sampling frames (space or time interval) κ_0 and κ_1, respectively, testing whether their ratio is 1.0 is equivalent to testing if $\ln\left(\frac{\rho_0}{\rho_1}\right)=0$. Using the delta method, the standard deviation of $\ln\left(\frac{\rho_0}{\rho_1}\right)$ is approximately

$$\sigma = \sqrt{\frac{1}{\kappa_0\rho_0}+\frac{1}{\kappa_1\rho_1}} \quad (7)$$

For a dilution experiment, let $P=\frac{N}{v}$ where N is the number of cells counted and v is the volume sampled. Subscripts indicate the treatment to which the samples relate, such that P_0 and P_t are initial and final cell concentrations for the undiluted treatment, and $P_{0,d}$ and $P_{t,d}$ are similar counts for the diluted treatment. The same subscript designations apply to N and v. Assuming that P can be modeled as a Poisson distribution, v is the sampling volume, and incubation time t and fraction of unfiltered seawater d are constants, the standard deviation for the net growth rate (*see* Eq. 1) in the undiluted sample is

$$\sigma_{undiluted} = \frac{1}{t}\sqrt{\frac{1}{P_t v_t}+\frac{1}{P_0 v_0}} = \frac{1}{t}\sqrt{\frac{1}{N_t}+\frac{1}{N_0}} \quad (8)$$

Likewise, the standard deviation for the diluted sample is

$$\sigma_{diluted} = \frac{1}{t}\sqrt{\frac{1}{N_{t,d}}+\frac{1}{N_{0,d}}} \quad (9)$$

Using the rules for arithmetic operations on standard deviations and Eqs. 2, 3 for calculating estimates of μ and g, the standard deviation for the growth rate σ_μ can be approximated as

$$\sigma_\mu = \frac{1}{t(1-d)}\sqrt{\frac{1}{N_{t,d}}+\frac{1}{N_{0,d}}+d^2\left(\frac{1}{N_t}+\frac{1}{N_0}\right)} \quad (10)$$

and the standard deviation for the grazing rate σ_g as

$$\sigma_g = \frac{1}{t(1-d)}\sqrt{\frac{1}{N_{t,d}}+\frac{1}{N_{0,d}}+\frac{1}{N_t}+\frac{1}{N_0}} \quad (11)$$

Eqs. 10, 11 can be used to calculate standard deviations for both the growth and grazing rates based on the number of cells counted in each treatment sample. For size-based analyses, the equations can be applied to each size class separately to determine the standard deviation for the size-specific rates. Standard deviations for total community growth and grazing rates can also be determined from Eqs. 10, 11 by grouping all cells together.

Varying the upper and lower bin edges

If growth and grazing rates vary with organism size, the size-dependent rates are a function of the size interval i, which is itself defined by a lower bound p and an upper bound q, where $p < q$. The calculations described above can thus be extended to account for all choices of p and q, binning the data into varying size intervals. Using this new notation, the size-dependent growth and grazing rates can be denoted $\mu_{p,q}$ and $g_{p,q}$, respectively. Interpolation between the size bins gives size-dependent growth and grazing rates for any size interval of interest.

Plotting growth and grazing rate estimates as a function of size-interval edges p and q is a compact way to represent size-dependent rates that highlights interesting size-dependent patterns (Fig. 1). For instance, diagonal lines along these graphs represent specific binnings of the data (shown in the two-dimensional plot on the right of Fig. 1). Contour lines can be overlain to show values of the standard deviations of the rate estimates for any size interval. Moving from the main diagonal toward the upper left corner corresponds to the data binned into increasingly larger size intervals until the whole community rate is reached in the upper left corner. Because the lower bin edge p must be strictly less than the upper bin edge q, there are no values for the lower right portion of the graph.

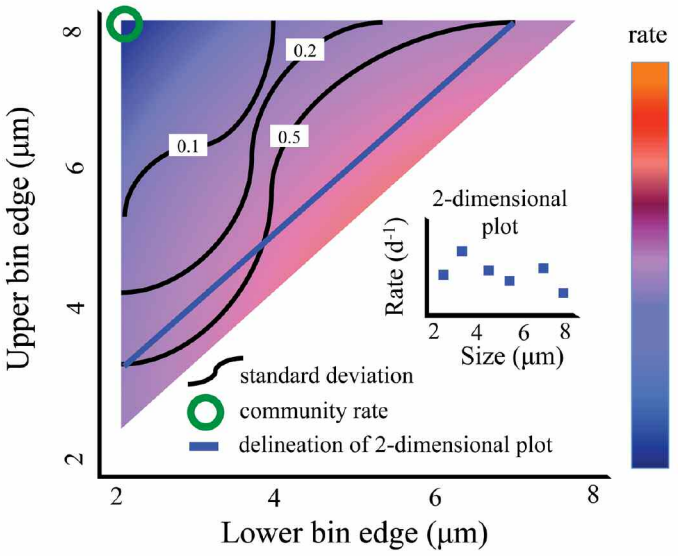

Fig. 1. Schematic diagram of rate estimates for a variety of size intervals. The rate estimates are calculated as a function of the x-axis (the lower edge of the size interval) and the y-axis (the upper edge of the size interval). Two-dimensional plots, standard deviations, and the rate for the entire community can also be plotted on the same graph.

This graphing technique avoids the need to recalculate the rates for each choice of bin width. All potential bins are accommodated, including a single bin giving the bulk community rates.

Assessment

Comparison of analytical and empirical rate standard deviations

To test our analytical estimates of the standard deviation for the size-dependent growth and grazing rates, the results of Eqs. 10, 11 were compared with bootstrapped standard deviations. To perform this comparison, a size-dependent dilution experiment was simulated (without the use of the size-structured NPZ model) with $\mu = g = 0$. That is, the mean number of cells in each size class was the same for both the initial and final samples. The number of cells in each size class was chosen randomly with equal probability from a phytoplankton size spectrum with a slope of –1.5 and an intercept of 10^4 cells mL^{-1} μm^{-1}, with the initial and final cell counts chosen separately and independently. The diluted initial and final samples had 1/3 the cell concentration of the undiluted samples. The initial and final diluted and undiluted treatments were randomly resampled 20,000 times each. These bootstrapped samples were then used to calculate the standard deviations for the size-dependent growth and grazing rates (both zero) and compared with the empirical standard deviations estimated using Eqs. 10, 11 (Fig. 2). The strong agreement between the analytical and empirical estimates of the standard deviations supports the use of Eqs. 10, 11 for providing accurate standard deviation values. The small differences between the empirical and theoretical error bounds were expected, given the random sampling that was used to calculate the bootstrap results.

The increasing spread in both the theoretical and empirical standard deviations with increasing cell size (Fig. 2) arises because the underlying size spectrum has a negative slope: there are fewer large cells compared with small cells, as is typical for most natural planktonic communities (Platt and Denman 1977; Sprules and Munawar 1986; Chisholm 1992). The standard deviations given by Eqs. 10, 11 decrease rapidly with increasing N, the number of cells in a size class (Fig. 3). Thus, the binning of the cells into size classes affects the accuracy of the estimated growth and grazing rates; fewer cells in a bin give larger standard deviations and less accurate rate estimates.

Given these relationships, it is worth exploring a variety of methods for choosing the size intervals. Possibilities for dividing cells into different size classes include a linear (e.g., Sheldon et al. 1972) or logarithmic scale (e.g., Platt and Denman 1977) or taxonomic groupings (Laurion and Vincent 1998); each of these methods trades off resolution and accuracy. Resolution is lost for larger size intervals, but rate estimates are more accurate (Fig. 4). Size-dependent bin widths of data are one way to vary the cell counts used for rate estimates. In addition, cell counts can be increased by pooling data from replicate experiments, bootstrapping samples, or collecting more data. Different combinations of these strategies can be used to lower the rate estimate errors to desirable levels.

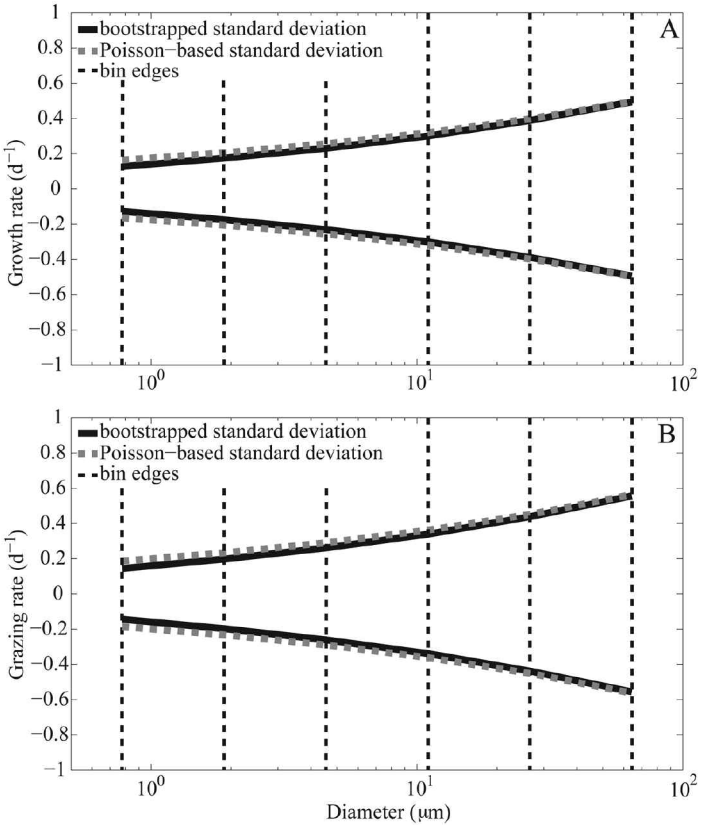

Fig. 2. A comparison of the bootstrapped standard deviations with the Poisson-based standard deviations calculated from Eqs. 10 and 11 for size-specific growth rates (A) and size-specific grazing rates (B).

Experiments with different dilution factors

As described above, we only compute standard deviations from Eqs. 10, 11 using the results of two dilution treatments. To apply the equations to a full dilution experiment with multiple treatments, therefore, a choice must be made as to which diluted treatment to use as a contrast to the natural (undiluted) sample. The closer the diluted treatment is to the concentration in the natural sample (i.e., the closer d is to 1), the higher the standard deviation of the rate estimates (Fig. 5). More accurate rate estimates may, therefore, come from using the most diluted treatment. Unfortunately, this is also the treatment for which the cell density is sparse and dilution artifacts (e.g., Dolan et al. 2000) are most likely. The best choice of the diluted treatment is thus a compromise between getting the largest dilution effect and other practical concerns, such as cell counts.

Model assessment of the size-dependent dilution method

To test the accuracy of the size-dependent dilution method, we used a size-structured plankton ecosystem model (Poulin and Franks 2010) to simulate a dilution experiment. The

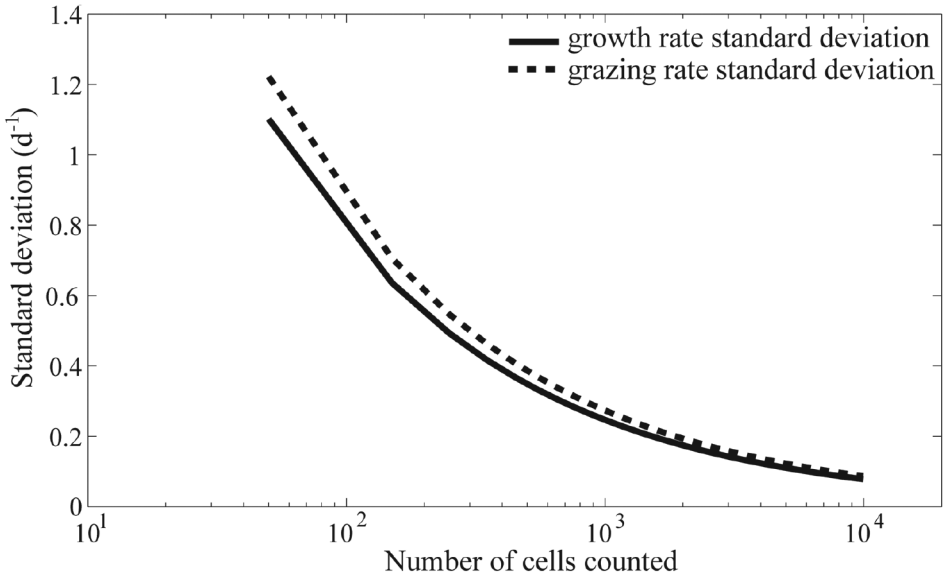

Fig. 3. Plot of Eqs. 10 and 11 of the change in the standard deviation with cell count.

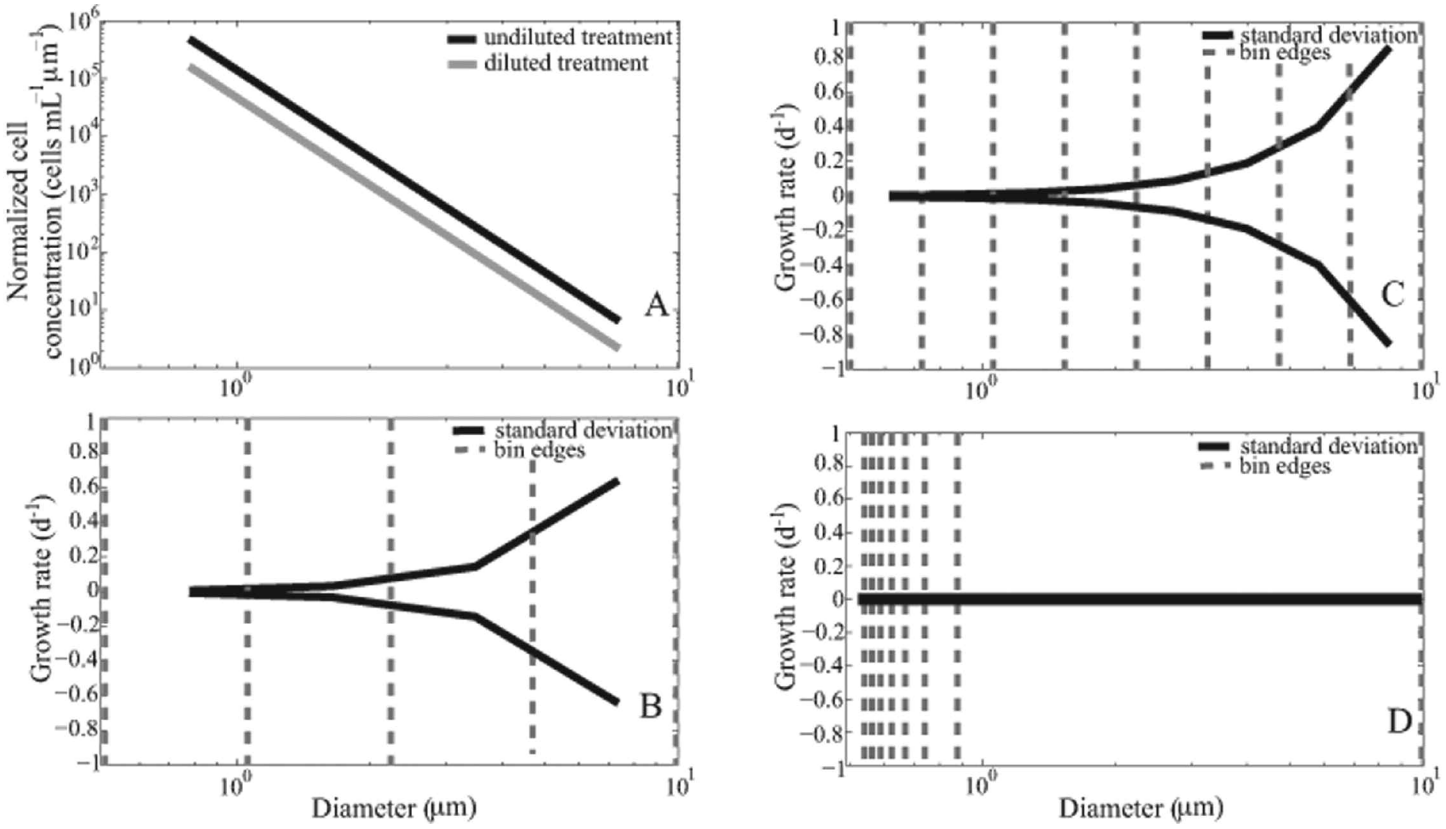

Fig. 4. Examples of standard deviations for the growth rate calculated using Eq. 10. For each plot, the same size distributions shown in A were used. The undiluted spectrum has a slope of –5 and an intercept of 105 cells mL^{-1} µm^{-1}. The sample volume was 1 mL. The fraction of unfiltered seawater was 1/3. For B, the size distribution was divided into 4 size classes that are equal in logarithmic space. For C, the size distribution was divided into 8 logarithmically equal size classes. For D, the size distribution was again divided into 8 size classes, but the divisions were made so each size class had the same number of cells.

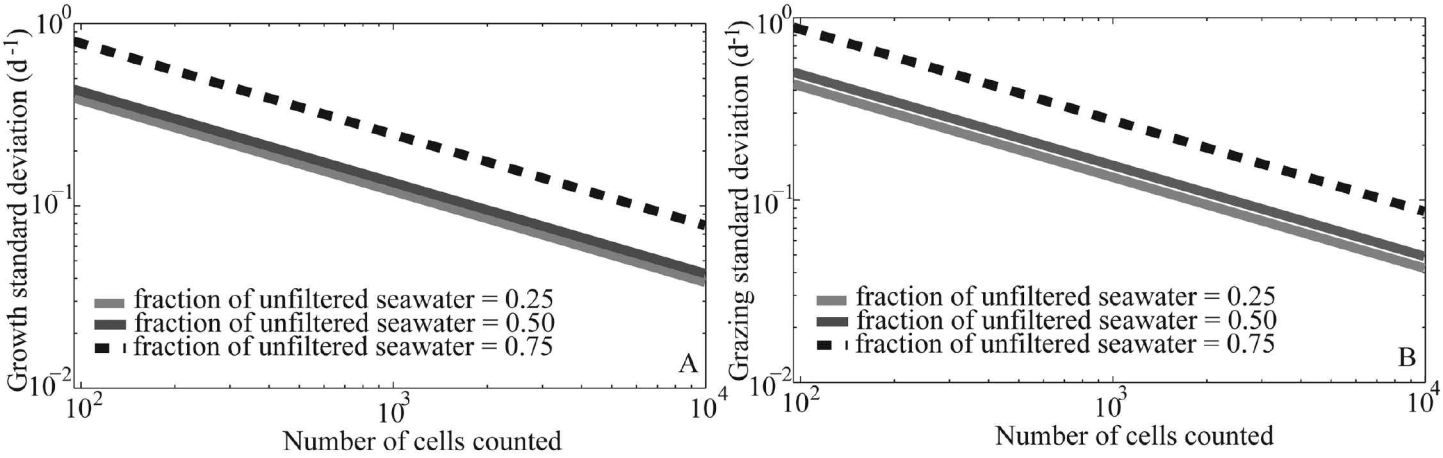

Fig. 5. The standard deviations for different fractions of unfiltered seawater, d. A. The standard deviations for the growth rate, calculated using Eq. 10. B. The standard deviations for the grazing rate, calculated using Eq. 11.

Table 1. Parameter values for the size-structured NPZ model used to evaluate the size-dependent dilution method.

Parameter	Coefficient	Exponent	Units
Total nutrients, N_{tot}	10	NA	μmol N L^{-1}
Zooplankton half saturation constant, K	0.08	–0.55	μmol N L^{-1}
Zooplankton grazing rate, ζ	2.468	–0.58	d^{-1}
Zooplankton assimilation efficiency, γ	0.4	0	dimensionless
Zooplankton loss rate, δ	0.1	0	d^{-1}
Phytoplankton half saturation constant, k	0.08	0.88	μmol N L^{-1}
Phytoplankton growth rate, η	0.7	–0.75	d^{-1}
Phytoplankton loss rate, λ	0.1	0	d^{-1}

known model rates were then compared with rates calculated by applying the dilution equations (Eqs. 5, 6) to the model output (initial and final phytoplankton concentrations binned into size intervals). We chose this approach for testing the method because it provides a controlled and unambiguous comparison of accurately known growth and grazing rates.

The Poulin and Franks (2010) model includes nonlinear nutrient uptake by phytoplankton and nonlinear grazing of phytoplankton by zooplankton. The model contains seven parameters that can be size dependent: maximum phytoplankton growth (η) and zooplankton grazing rates (ζ), half-saturation constants for phytoplankton nutrient uptake (k) and zooplankton grazing (K), phytoplankton (λ) and zooplankton loss rates (δ), and zooplankton assimilation efficiency (γ). Adding size-dependencies to the parameters doubled the number of parameters to estimate, as both coefficients and exponents are required for allometric scaling. Total nitrogen concentration for the system, N_{tot}, is the only parameter that does not have the potential to be size-dependent.

The model was parameterized based on 1) previously published allometric scalings, 2) a literature review of size-dependent relationships, 3) the generation of a realistic planktonic size spectral slope at steady state, and 4) the maintenance of a zooplankton:phytoplankton biomass ratio < 1 (Table 1). Generally, the maximal growth rates of phytoplankton and grazing rates of zooplankton and the half-saturation constant for grazing decreased as functions of size, whereas the phytoplankton half-saturation constant for nutrient uptake increased with size. All other variables were not size dependent (i.e., their exponents were zero).

Using the analytical solutions for the Poulin and Franks (2010) discrete model, we found steady-state values for the state variables (nitrogen, phytoplankton, and zooplankton) and used them to initialize a 24 h, two-point dilution experiment (Fig. 6) with d = 0.33.

The growth and grazing rates estimated by applying the size-dependent dilution method to the size spectra and the actual rates from the model were not statistically different (two-sample Komolgorov-Smirnov test, P = 1) (Fig. 7), and a strong positive correlation was seen across all size classes for both rates. This assessment shows that the size-dependent dilution method accurately estimates size-specific growth and grazing rates, despite the complexity and nonlinearities of the simulated community. Further modeling work (Taniguchi et

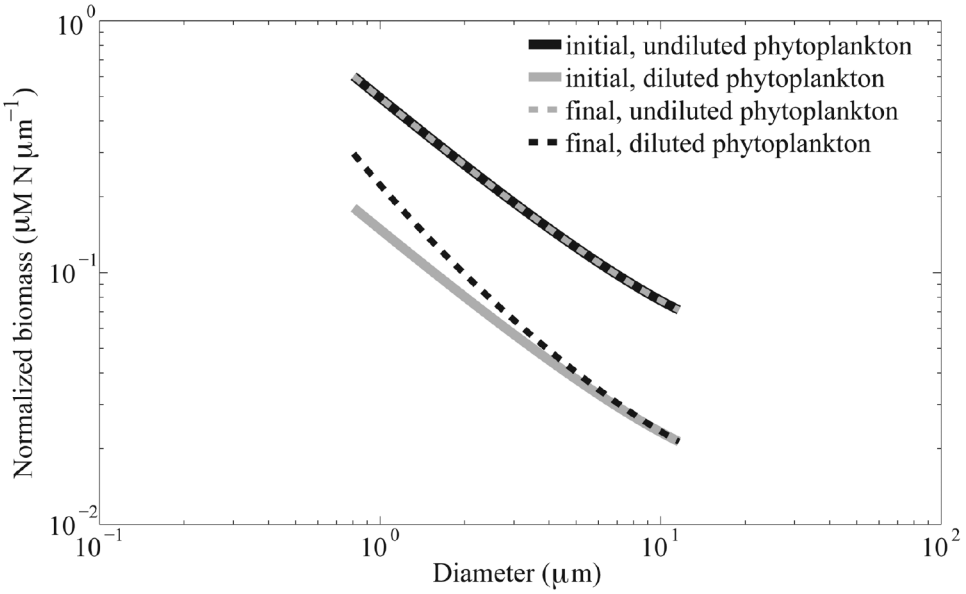

Fig. 6. The phytoplankton size spectra generated from the size-structured NPZ modeled dilution experiment. These distributions were used to calculate size-dependent growth and grazing rates based on Eqs. 5 & 6, respectively.

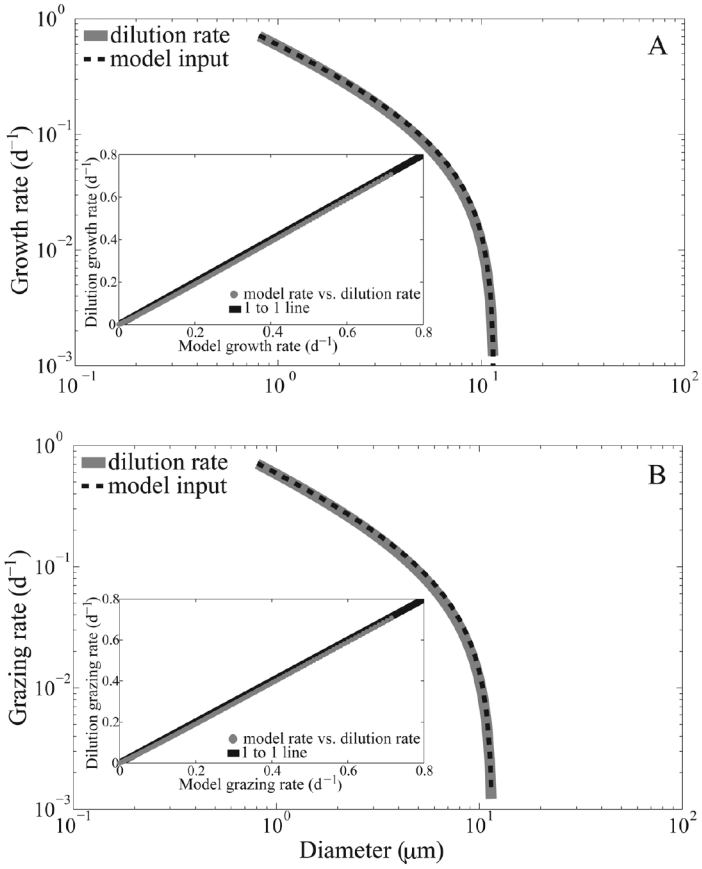

Fig. 7. Comparison of the growth (A) and grazing (B) rate values calculated from the size-dependent dilution method and those from the size-structured NPZ model.

al. in prep.) shows that this method will also accurately estimate rates under nonsteady conditions, with grazers consuming multiple size classes of producers, and with omnivory.

Application to field data

We applied the size-dependent dilution method to field data collected in the equatorial Pacific on the 2005 cruise (EB05) of the Equatorial Biocomplexity project (Nelson and Landry 2011). Given that the size-dependent dilution method worked well under the steady-state conditions of the complex modeled ecosystem, the application of the method to the relatively stable conditions of the equatorial Pacific seemed appropriate. As described in detail elsewhere (Selph et al. 2011), two-treatment dilution experiments were conducted at 8 depths at each of 14 stations along a transect at 0.5°N, with one station at 140°W and the rest approximately 2° apart between 132.5 and 123.5°W. For the present analysis, we used only experiments conducted with surface mixed-layer water (collected in early morning CTD casts, ~0300-0400 hour local time) and incubated for 24 h in calibrated seawater-cooled deck incubators at a 31% of surface irradiance, the conditions for maximum phytoplankton growth (Landry et al. 2011b). Linearity of the grazing response is well documented for this ocean region from a variety of studies, including the present experiments (Landry et al. 1995; Verity et al. 1996; Landry et al. 2000; Landry et al. 2011b).

Dilution treatments (d = 1 and d = 0.37, the latter diluted with water filtered through a 0.1 μm Suporcap filter) were prepared in 2.8-L polycarbonate bottles. Flow cytometry samples (2 mL, 0.5% v/v paraformaldehyde preserved, frozen in liquid nitrogen) were taken from both treatments initially and at the end of the incubation. The samples were later thawed, stained

with 1 µg mL^{-1} of Hoechst 33342 (Monger and Landry 1993), and analyzed with a Beckman-Coulter EPICS Altra cytometer, distinguishing three populations: *Prochlorococcus*, *Synechococcus*, and picoeukaryotes. These taxa were combined for size-specific analysis, and the light-scattering values were normalized relative to an internal standard (0.5 µm yellow fluorescent beads). To convert list mode files from normalized light scatter values to cell diameters for the picoplankton assemblage, the median forward light-scattering values for *Prochlorococcus* and for *Synechococcus* were regressed against literature-based cell diameters for these taxa: 0.55 µm for *Prochlorococcus* (Partensky et al. 1999) and 0.95 µm for *Synechococcus* (Morel et al. 1993). The resulting relationship was used to assign cell diameter estimates to individual cells.

To increase the number of cells in each size class for the rate calculations, the data from individual sampling stations were pooled. Given the observed relative constancy of growth and grazing rates and community composition among stations (Landry et al. 2011a; Selph et al. 2011), combining data sets is expected to highlight rather than mask the underlying size dependencies. Once the data were pooled, the cells were divided into bins with edges of 0.45, 0.65, 1.25, 2.75, and 4.00 µm. These divisions were chosen to correspond approximately to the taxonomic groups *Prochlorococcus*, *Synechococcus*, and pico-eukaryotes. For this data set, highly resolved rate estimates, especially for relatively large (>4 µm) cells, were not possible as those cells were too rare. Therefore, larger cells were not considered. After dividing the cells into size intervals, Eqs. 5, 6 were applied to each size bin to estimate the size-dependent growth and grazing rates. Eqs. (10, 11) were then used to calculate the standard deviations for growth and grazing rates, respectively.

The mean size-class rates of growth and grazing generally decreased with size (Fig. 8), though the standard deviations suggest that there is some overlap between size intervals, particularly between the second and third size intervals in both the growth and grazing rates. However, the rates of the smallest size class were significantly higher than those of all other size intervals, and the largest size class rates were significantly lower than those of the smaller size intervals. These data support the conclusion that the larger cells in this analysis, which have variously been called pico-eukaryotes or eukaryotic ultraplankton (Simon et al. 1994; Zubkov and Quartly 2003) in flow cytometric analyses of oceanic plankton, displayed lower growth and grazing rates than the smaller cells (photosynthetic bacteria).

When plotting rate estimates as a function of the upper and lower bounds of the size intervals, both growth and grazing rates decrease with increasing size (Fig. 9). The highest rates correspond to the size class between 0.48 and 0.52 µm (μ_i = 0.68 ± 0.04 d^{-1} and g_i = 0.75 ± 0.05 d^{-1}), whereas the lowest rates are for cells between 3.13 and 3.36 µm (μ_i = 0.27 ± 0.07 d^{-1} and g_i = 0.31 ± 0.09 d^{-1}). As the size intervals get wider, both growth and grazing rates increase because the higher growth and grazing rates of the more numerous smaller cells contribute disproportionately to the size-interval averages.

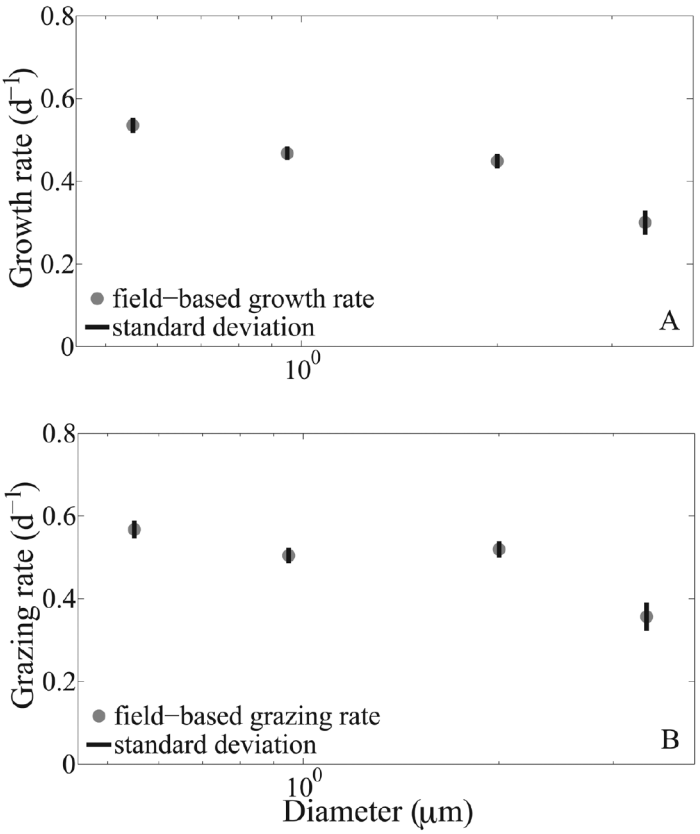

Fig. 8. Application of the size-dependent dilution equations to pooled field samples collected in the equatorial Pacific in 2005. A. The size-based growth rates, calculated using Eq. 5, and the standard deviations, calculated using Eq. 10. B. The grazing rates, calculated using Eq. 6, and the standard deviations, calculated using Eq. 11. Rate values and standard deviations in both panels are plotted at the mid-points of each size bin.

The general agreement between the rates found here and those in other studies in the equatorial Pacific and other oligotrophic areas (Latasa et al. 1997; Worden and Binder 2003; Landry et al. 2011a; Selph et al. 2011) gives some confidence in the size-resolved rates. Of particular relevance is the work of Landry et al. (2011a) and Selph et al. (2011), which include experiments from the same cruise as used here as well as a 2004 cruise to the same region. Those studies focused on taxonomic-based rates generated from HPLC pigment concentrations and flow cytometry data instead of size-based rates. Landry et al. (2011a) found a close coupling between growth and grazing for *Prochlorococcus*, *Synechococcus*, and small eukaryotes, integrated over the full euphotic zone. Selph et al. (2011) did a more detailed study of individual taxonomic groups at discrete depths, including the 31% light level. A comparison of the size-dependent rates from the present study and the taxonomic group rates based on pigment concentrations from Selph et al. (2011) are shown in Table 2. There is generally a good agreement among the rates for *Prochlorococ-*

cus and *Synechococcus* (based on the pigments divinyl Chlorophyll *a* and zeaxanthin) and our rates estimated for the smallest size classes, although the growth rates based on zeaxanthin are slightly higher. Pelagophytes and prymnesiophytes correspond to the larger size categories of the present study; both of those taxonomic groups have higher mean growth rates than we estimated using Eq. 5. These discrepancies may be a consequence of the size classes containing a mixture of taxonomic groups—particularly for the larger size classes—which would not appear in the taxon-based estimates of Selph et al. (2011). It is also of note that pigment-based rates, particularly growth rates, frequently have corrections to account for pigment changes not associated with biomass growth. The accuracy of such corrections can also affect the relationship between pigment-based and size-specific rates.

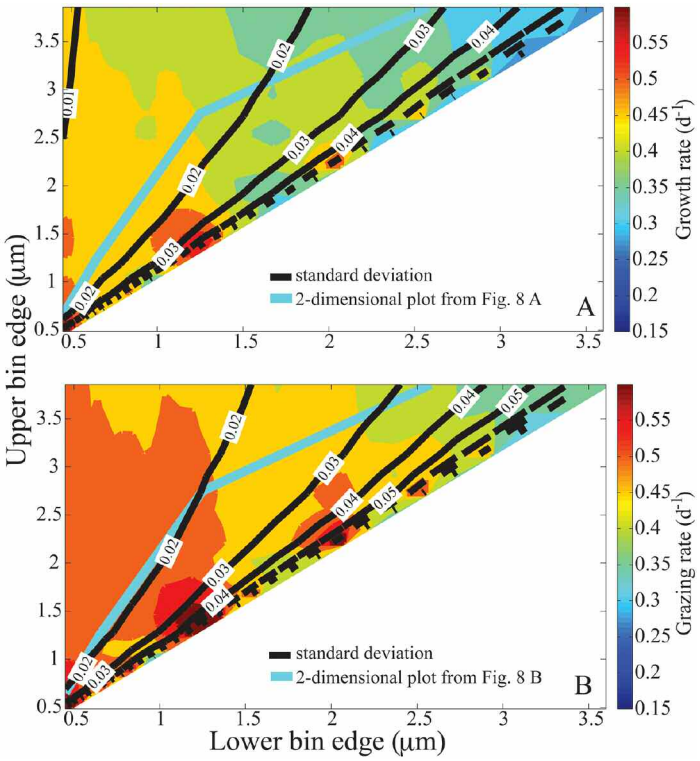

Fig. 9. Application of the size-dependent dilution equations (Eqs. 5,6) to pooled data collected from the equatorial Pacific in 2005. The data are the same as in Fig. 8. However, here they are split into various size intervals within the complete size range. Therefore, the rate estimates are a function of both the lower bin edge (the x-axis) and the upper bin edge (y-axis). Also shown are the two-dimensional data from Fig. 8 as well as the standard deviations, calculated using Eqs. 10,11. A. Growth rates. B. Grazing rates.

A decrease in growth and grazing rates with increasing size has been documented before, both for the general spectrum of organisms (e.g., Fenchel 1974), and for unicellular marine life in particular (e.g., Banse 1976; Hansen et al. 1997). In contrast, other studies have noted that small size may provide a refuge from predation (e.g., Banse 1982; Monger and Landry 1990), which may allow lower growth rates for smaller cells relative to larger ones. The general trend in our results of decreasing growth and grazing rates with increasing size (Fig. 8) tend to support previous observations about an inverse relationship between rates and size. Further studies are needed to assess the generalities of size-dependent rate patterns for a broader size range of phytoplankton and for diverse ecosystems.

Discussion

Size-dependent dilution method

The size-dependent dilution method is a relatively simple approach that can substantially increase the amount of information gathered about the natural planktonic community. With little alteration to the commonly used dilution method, it can provide better parameterizations for size-structured models and enhance our understanding of size-related plankton dynamics.

The execution and the concept of the size-dependent dilution method are not entirely new. Previous studies have used the dilution method to estimate rates of size classes based on size-fractionated chlorophyll (Menden-Deuer and Fredrickson

Table 2. A comparison of growth and grazing rates (d^{-1}) from this study and those based on HPLC pigments taken from Fig. 6 of Selph et al. (2011). The terms in parentheses indicate the pigment representing the taxonomic group(s).

Method	Size class/cell type	Growth rate ± SD	Grazing rate ± SD
Size-dependent dilution method			
	0.45-0.65 µm	0.54 ± 0.02	0.57 ± 0.02
	0.65-1.25 µm	0.47 ± 0.02	0.50 ± 0.02
	1.25-2.75 µm	0.45 ± 0.02	0.52 ± 0.02
	2.75-4.0 µm	0.30 ± 0.03	0.36 ± 0.03
HPLC			
	Prochlorococcus (divinyl Chl *a*), ~0.4-0.8 µm	0.65 ± 0.19	0.54 ± 0.27
	Prochlorococcus and *Synechococcus* (zeaxanthin), ~0.4-1.1 µm	0.78 ± 0.20	0.60 ± 0.23
	Pelagophytes (19-butanoyloxyfucoxanthin), ~1.5-20 µm	1.12 ± 0.16	0.56 ± 0.15
	Prymnesiophytes (19′-hexanoyloxyfucoxanthin), 2-10 µm	0.66 ± 0.32	0.32 ± 0.25

2010), microscopic measurements (First et al. 2007), and flow cytometry (Kuipers and Witte 1999). The major advancement here is the statistical analysis, which allows computation of error estimates, and flexibility in the designation and interpretation of trends among size classes. Whereas size-fractionated chlorophyll could be used for the method put forth here, the size classes would be fixed, and using the error equations (Eqs. 10, 11) and plotting rates as a function of size interval would not be possible. Similarly, previous studies that have used actual cell measurements only reported mean rates for fixed size classes that were sometimes chosen opportunistically (Kuipers and Witte 1999).

Another method to determine size-specific growth and grazing rates is by examining population dynamics of different size classes in predator removal experiments (Glasser 1983). Many of the assumptions and concerns of that method are also applicable for the size-dependent dilution method. For instance, each size class is considered in isolation. Accounting for plankton growing or dividing out of, or remaining within, each size interval is an issue that can only be thoroughly examined using a single species. Addressing the issue of growth and reproduction into and out of a size class for a multi-species assemblage may require some modifications to Eq. 4. Most of the organisms we are considering are bacteria or protists, which would tend to vary in size by only a factor of 2 over their division cycles. If the size classes are large enough (i.e., at least double the size of the previous size class), transfer of organisms between size classes is diminished.

Another potential issue, as proposed by Glasser (1983), involves processes such as aggregation that could be misinterpreted as growth in certain size classes and grazing in others, depending on which size classes gained or lost cells. If such particle size alterations occurred from physical agitation, this artificial growth or grazing effect would at least be common to all treatments. On the other hand, if size changes were mediated by grazers, these artifacts would be more prominent in the less-dilute treatments. Consumers may also add nutrients through excretion, disproportionally stimulating growth in the less-dilute treatments and potentially encouraging the growth of particular size classes. Nutrient-amended dilution experiments (Landry et al. 1995) can help alleviate both this effect and nutrient limitation during the incubation period.

Other factors involving the predator community that may bias size-dependent rate estimates include the effects of dilution on grazer population growth and mortality, and non-linear grazing. Diluting the predator community can cause unnatural growth and grazing that varies depending on the fraction of unfiltered seawater in the sample. Furthermore, grazers may change grazing behaviors due to the dilution of food concentration. These factors have been brought to light (Gallegos 1989; Dolan et al. 2000; Dolan and McKeon 2005; First et al. 2007) and addressed (Landry et al. 1995; Landry and Calbet 2005), at least partially, for community-level rates from traditional dilution experiments. However, such biases may also differ in their effect on the bulk community rates versus size-dependent rates. For example, First et al. (2007) found that grazer behaviors depended on their size and on the dilution treatment. Some herbivores may grow slowly or die off quickly with decreased food in diluted treatments compared with undiluted treatments. Landry and Calbet (2005) pointed out that the predators of the herbivorous grazers will also be diluted, often leading to a limited net change in grazing activity. Still, if the size-specific grazing impact of the herbivores is altered, the measured size-dependent rates may be biased as a result of the experimental design (i.e., diluting the community). We intend to explore this effect using complex size-structured ecosystem models that include herbivory, carnivory, and omnivory.

Standard deviation for growth and grazing rates

The standard deviations for both growth (Eq. 10) and grazing (Eq. 11) rates provide quantitative measures of the error of our estimated rates. The standard deviation equations can be applied even if time, resources, or experimental difficulties prevent the acquisition of replicates. The equations can be applied to both community data and size-specific data. They can also be used to calculate error estimates even when grazing rates are low [a perceived problem of the dilution method – see Dolan and McKeon (2005) and Landry and Calbet (2005)].

When planning experiments, the standard deviation Eqs. 10, 11 can be useful for estimating the number of cells that should be counted or sample volume that must be analyzed to obtain a specified level of error. If we assume that a system is at steady state (i.e., $N_t = N_0$ and $N_{t,d} = N_{0,d}$), then the number of cells that need to be counted, N, to obtain an error of magnitude σ_μ for growth would be

$$N = \frac{2\left(1+d^3\right)}{d\left[\sigma_\mu t\left(1-d\right)\right]^2} \tag{12}$$

For grazing, the number of cells that need to be counted to obtain an error of σ_g for grazing would be

$$N = \frac{2\left(1+d\right)}{d\left[\sigma_g t\left(1-d\right)\right]^2} \tag{13}$$

For a dilution factor of 0.3 and $\sigma_\mu = 0.1$ d^{-1}, for example, 1400 cells need to be enumerated in each size bin for each sampling time step (initial and final). For a σ_g of 0.1 d^{-1}, about 1800 cells should be counted in each size interval at each time step for each treatment. Knowing cell concentrations, the volumes to be processed are easily calculated from these cell numbers.

Large numbers of cells must be measured in each size class

in the above example, so automated methodologies for cell counting and analysis are recommended. Of the technologies currently available, flow cytometry and FlowCAM are better suited for use in these size-specific analyses than microscopy. However, neither of these methodologies covers the entire phytoplanktonic size spectrum. While Coulter counters are also a viable, automated option, their use is best restricted to cultures due to the machines' inability to distinguish cells from noncells. Therefore, future technologies that could identify phytoplankton cells from nonliving material over a large size range, estimate the sizes of cells, and do so automatically would greatly benefit this type of experimental analysis.

Modeling the size-dependent dilution method

Testing the size-dependent dilution method with the size-structured NPZ model showed that, in a complex modeled ecosystem, the technique outlined in this work can accurately retrieve size-specific growth and grazing rates. The size-dependent dilution method assumes that grazing changes linearly with the fraction of diluted seawater. In the size-structured ecosystem model, however, phytoplankton are directly or indirectly controlled by seven parameters, four of which are size dependent. In particular, growth and grazing in the model are nonlinear. Despite the differences between assumed linearity for the size-specific dilution method and the nonlinear construct of the model, the size-dependent dilution method gave accurate estimates of both phytoplankton growth and grazing rates for all size classes. Not only does the model give us confidence in the method, but it can also be used as a tool for understanding the environmental conditions under which the size-dependent dilution method may or may not work. The model and parameters used here are only one example; future modeling efforts will explore more complex and realistic ecosystem structures and/or different parameterizations to gain a better understanding of the conditions under which the size-dependent dilution method can most appropriately be applied in the field.

Application to field data

Results from the present experiments and statistical calculations yielded four pieces of information: growth and grazing rate estimates, a metric of the error (the standard deviation) of those estimates, the size-dependencies of the rates, and the dependency of those rates on the size interval. The first two properties provide fundamental information about the planktonic community as well as a measure of the accuracy of that information. This knowledge can be used to detail how the base of the food web is changing and provide insight into the processes driving community dynamics (e.g., response to nutrient inputs, grazing pressure from specific consumers). The results can also be compared with species-specific studies, particularly if the size intervals are chosen to correspond directly to taxonomic groups of interest, to contrast and/or complement other measures of community dynamics.

Plotting rate estimates as a function of bin size edges provides a new perspective on size-dependent growth and grazing rates. Such a display of information allows researchers to determine not only how rates change with organism size but also with size resolution.

Our data analyses showed both growth and grazing rates to decrease with increasing cell size. These results support some studies (Fenchel 1974; Banse 1976; Hansen et al. 1997) and tend to refute others (Banse 1982; Monger and Landry 1990, 1991; Marañón 2008). Note, however, that these are realized rates, not the maximum rates that tend to scale allometrically (Hansen et al. 1997; Nielsen 2006). Furthermore, the field population studied here resolves only a limited size range of pico- and small nanophytoplankton. The high cell counts necessary for accurate rate measurements (~10^3 per size class) made it impractical to use counts for larger cells obtained via microscopy. Size-specific rates for cells larger than those considered here may reveal different size-dependent patterns (e.g., Selph et al. 2011). More extensive application of the size-dependent dilution method to natural samples will provide a more quantitative understanding of the size-dependent dynamics of planktonic ecosystems. Quantifying the size-dependent patterns of growth and grazing rates will lead to improved size-dependent models by allowing more accurate parameterizations and stronger tests of the underlying dynamics.

Comments and recommendations

The size-dependent dilution method gives estimates of growth and grazing rates for phytoplankton in different size classes. The quality of these estimates depends on the selection of size classes that will minimize the error bounds on the rate estimates while still allowing some size resolution. Our plots displaying size-dependent rates as a function of size interval provide a concise means of representing all size classes of interest while maintaining the needed balance of minimizing error and maximizing size resolution. Whereas the size-dependent dilution method can be applied with tools presently available, advances in technology should improve the method by allowing larger numbers of cells—particularly the larger cells—to be counted and sized more efficiently. Testing the method with size-structured models of varying complexities will help to determine the ecosystem properties that most strongly affect our ability to extract accurate growth and grazing rate estimates. As it stands, the present method appears to be particularly useful for estimating size-dependent growth and grazing rates for the smallest (and most numerous) size classes of phytoplankton.

The application of the size-dependent dilution method to natural samples will provide important and otherwise difficult-to-acquire information on size-dependencies of growth and grazing rates of actual phytoplankton assemblages. This knowledge will contribute to our understanding of size relationships in ecosystem dynamics and biogeochemical cycling in the oceans.

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