

Doing more with less? Balancing sampling resolution and effort in measurements of protistan growth and grazing-rates

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Abstract

The dilution-method has been key in establishing the role of protistan-grazing in marine foodwebs. Yet its laborious application limits the sampling-resolution achieved. We assessed the reliability of an abbreviated method known as the 2-point by analyzing 77 dilution-experiments performed using 4–5 dilutions in diverse biotic and abiotic conditions. Our aim was to inform practitioners on how experimental design and nonlinear feeding behaviors affect the accuracy of 2-point rate-estimates. We found good agreement between rate-estimates of both phytoplankton growth (μ) and grazer-induced mortality (g) from either method, even though the comparison included experiments with nonlinear feeding-responses. The accuracy of 2-point estimates was similar to the inherent standard deviation of dilution-series estimates ($\pm \sim 0.1 \text{ d}^{-1}$). Nonlinear feeding-responses did not alter overall rate-estimates, negating the need for more than two dilution-levels. Decreasing dilution resulting in an increase in biomass from 10% to 40% resulting in increased biomass increased the median difference between 2-point and dilution-series estimates ~ 3 -fold and increased 2-point estimates' variance ~ 2 -fold, both for μ and g . Recognition of these biomass vs. accuracy tradeoffs enables practitioners to choose whether to procure more biomass at the expense of constraining estimate variance. Using duplicate bottles at each dilution level doubled average accuracy of 2-point estimates. The reduction in effort and water-needs afforded by the 2-point design facilitates acquisition of higher-resolution data of predation-rates across seasons, latitudes, and in response to diverse environmental conditions in the ocean, which is critically needed to decipher abiotic and biotic drivers of protistan-grazing and to parameterize protistan herbivory in global biogeochemical models.

Microzooplankton occupy a pivotal position in pelagic food webs. As herbivores, the dominantly protistan grazers exert significant influence on the species composition, size structure, and abundance of phytoplankton (Verity 1986; Strom et al. 2007; Mariani et al. 2013), thereby affecting primary production and the flow of carbon in the ocean (Legendre and Le Fèvre 1995; Legendre and Rassoulzadegan 1996; Calbet and Landry 2004; Banse 2013). Key agents of geochemical cycles (Buitenhuis et al. 2010) and important constituents of the microbial loop (Azam et al. 1983), phagotrophic protists also are prey to meso- and macrozooplankton (Schmidt et al. 2006; Saiz and Calbet 2011), acting as an important trophic link channeling primary production up the food web. Thus, quantification of these predators' feeding rates is crucial to understanding trophic linkages and biogeochemical cycles.

Although the significance of microzooplankton grazing has been widely recognized, empirical measurements have been challenging. Since its introduction, the dilution method (Landry and Hassett 1982) has become the standard protocol to quantify protistan-grazing rates in mixed plankton assemblages (see Calbet and Landry 2004). Landry and Hassett (1982) developed the dilution method because, contrary to earlier methods used to quantify feeding rates of mesozooplankton (Frost 1972; Heinbokel 1978), a “true” control treatment devoid of predation pressure by protistan predators is not achievable when dealing with natural field samples, since heterotrophic protists occupy the same size spectrum as their prey and thus predator and prey cannot be separated. Thus, dilutions are used. Diluting samples reduces encounter rates between predator and prey, but does not completely eliminate grazing. The dilution method presents several advantages: it is conceptually simple; it requires limited manipulation of the fragile plankton assemblage; and it measures both instantaneous rates of phytoplankton growth (μ) and grazer-induced mortality (g).

Nevertheless, the method still carries limitations associated with the incubation process, such as the difficulty to

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recreate the in situ light field or level of turbulence (McManus 1995; Ross et al. 2011). Furthermore, preparing the dilutions requires manipulation of large volumes of seawater, and pre- and post-incubation analyses are extensive. The logistical effort required by the dilution method to obtain even a single rate measurement precludes acquisition of the large sample sizes needed to fill knowledge gaps with respect to species-specific, or seasonal, environmental, and regional variations in grazing rates (see review by Schmoker et al. 2013). The method's logistics also limit empirical investigations of physical, chemical, and/or biological covariates of grazing rate magnitude, hindering predictions about food web responses to climate-driven changes in ocean ecology (Caron and Hutchins 2013).

In their introduction of the dilution method, Landry and Hassett (1982) suggested that any two dilution-levels could be used to estimate grazing rates, providing a "short cut" alternative to the dilution-series. Easier and faster to use, this "2-point method" (Gallegos 1989) offers the capacity to increase measurement frequency. One significant concern regarding the 2-point approach is that, in contrast to measurements made along a dilution gradient, using two points does not allow detection of deviations from linearity, potentially violating a critical assumption of the dilution method, i.e., that grazing pressure is a linear function of dilution. Deviations from linearity may result from feeding thresholds (Lessard and Murrell 1998), from grazer mortality in a very dilute treatment (Dolan et al. 2000), or from a feeding saturation response at high food levels (Gallegos 1989), all of which can yield misestimates of grazing rates. The effect of nonlinearity in dilution experiments has been repeatedly discussed, and methods have been proposed to extract rate-estimates when feeding behaviors are not strictly a linear function of biomass (i.e., dilution) (e.g., Gallegos 1989; Evans and Paranjape 1992; Elser and Frees 1995; Landry et al. 1995; Moigis 2006; Li et al. 2017). It is however uncertain whether practitioners of the dilution method routinely examine and address occurrences of nonlinearity in their experiments, as to our best knowledge, such reports are uncommon. Thus, if linearity is either not assessed or deviations not reported, the advantages of performing a full series of dilutions may not compensate for the amount of labor associated with its application.

Many studies have used the 2-point method successfully (Landry et al. 1984, 2008, 2009, 2011a, 2011b; Worden and Binder 2003; Strom and Fredrickson 2008; Menden-Deuer and Fredrickson 2010; Lawrence and Menden-Deuer 2012). Rates estimated using the 2-point approach are considered conservative (Worden and Binder 2003; Lawrence and Menden-Deuer 2012) and do not vary significantly from rates obtained using a full dilution-series protocol (Worden and Binder 2003; Strom and Fredrickson 2008). Yet these findings are based on limited analysis, and the sampling design varies widely among studies, including the dilution-

level used, the method of calculating rates, and the number of replicates per dilution, warranting a more thorough evaluation of the 2-point method.

In an effort to promote the use of the 2-point method, Chen (2015) assessed the "accuracy" of 2-point rate-estimates of phytoplankton growth (μ) and heterotrophic-protist grazing (g) by analyzing an extensive global data set. Chen (2015) concluded that greatest accuracy of 2-point estimates was achieved using a highly diluted bottle, and that replication was not absolutely necessary, and that one could use only one bottle per end-point, i.e., two per experiment.

Here we build on the analysis by Chen (2015), by providing practitioners with quantitative information on the consequences on the mean and variance of rate-estimates of making specific choices regarding the number of dilutions, degree of biomass dilution, and replication. To do so, we analyzed a data set not previously subjected to this kind of analysis to compare estimates of phytoplankton growth and grazing mortality as well as the associated error estimates obtained using only two points to those obtained using linear regression of a dilution-series. We addressed the linearity concern by comparing 2-point estimates to the best estimates that can be obtained from nonlinear dilution curves. Finally, we assessed the effect of the level of dilution and replication on the accuracy and the variance of 2-point estimates. We do not aim to be prescriptive in the application of the method. Rather we wish to present the quantitative consequences of specific experimental-design choices on the ultimate quality of the data. We found that the 2-point method provides reliable estimates of growth and grazing rates, that variance of estimates is reduced in a predictable manner when higher dilution levels and replication are used, and that nonlinearity of feeding responses, although a regular occurrence, does not systematically bias the reliability of the rate estimates. Given the need to both increase resolution of grazing-rate measurements and test potential biotic or abiotic covariates (e.g., species composition, light, mixed layer depth, seasonality, location) that can provide predictive and mechanistic insights into the drivers of grazer induced mortality rates, we found the 2-point approach to be a desirable alternative to fully resolved dilution series.

Materials and procedures

Data source

We used a data set consisting of results from 77 dilution experiments performed by our laboratory at different times of the year in a wide range of geographic regions, chlorophyll concentrations, temperatures, and plankton species composition. Some experiments were conducted at sea aboard research vessels: in the North Atlantic (NAt) during early spring ($N = 15$), and along the West Antarctic Peninsula (WAP) during austral late fall ($N = 11$) and austral late spring ($N = 13$). The remaining experiments ($N = 38$) were

conducted onshore at the University of Rhode Island Graduate School of Oceanography during summer, with water collected from Narragansett Bay (NB) at the site of the NB long-term phytoplankton time series ($N = 36$; <http://www.gso.uri.edu/phytoplankton/>), or from a dock in Jamestown, Rhode Island ($N = 2$).

Details of the methods used for setting up the experiments and for chlorophyll a (chl a) measurements have been previously described (e.g., Morison and Menden-Deuer 2015). Water was collected either from the surface by bucket grabs, or from the subsurface, often at the apparent chl a maximum, using Niskin bottles mounted on the ships' CTD rosette. To exclude larger grazers, the collected water was gently screened using a 200 μm mesh, except for 8 experiments in NB when the mesh used was 150 μm . We refer to this screened fraction as "whole seawater" (WSW). A portion of the collected water was gravity-filtered through a 0.2- μm membrane capsule (Pall) to obtain filtered seawater (FSW). FSW and WSW were mixed in various proportions to achieve a series of dilutions. The number of dilutions varied from 4 to 5, except for a subset of experiments (NAt; $N = 6$), in which only three dilution levels were used. Once prepared the various dilutions were dispensed by siphoning into clear 1 or 2 L polycarbonate bottles. The number of replicates per dilution level varied between 2 and 3. In most cases, all bottles were amended with macronutrients (N, P, and Si) except when in situ nutrients were assumed to be in excess. To serve as nutrient controls, additional 100% replicates were incubated without added nutrients, or with nutrients when none had been added to the dilution series. In a few cases, these additional nutrient controls were also included at the 10% WSW dilution level. At sea, incubations took place in on-deck tanks at depth-adjusted irradiance simulated by using either screen mesh or blue light filter. Onshore, incubation bottles were strapped to rotating plankton wheels. Incubation vessels were kept at ambient temperature by flow-through water. Initial chl a concentration in the 77 experiments ranged from 0.17 to 18.5 $\mu\text{g L}^{-1}$, and incubation water temperature that varied from -1°C to 26°C .

Phytoplankton growth and grazing rates

For each experiment, the apparent phytoplankton growth rate (k) in each replicate at each dilution level was calculated from 24-h changes in chl a:

$$k = 1/t * \ln (P_t/P_0) \quad (1)$$

where t was the incubation duration (in units of day), and P_0 and P_t represent the chl a concentration at the beginning and end of the experiment respectively. From the replicates of each dilution level, an average k value was calculated \pm one standard deviation of the mean (SD).

The potential for nutrient limitation was assessed by comparing k in nutrient amended and nonamended replicates using a paired t -test. If a significant difference was found

between apparent phytoplankton growth rates (k) in nutrient amended and nonamended WSW treatments, only k values from nutrient amended treatments were used in rate calculations, as nutrient limitation would affect k in the diluted and undiluted treatments differently and violate the dilution method's assumption that k is independent of phytoplankton cell density.

Estimation of rates from dilution series

When based on the full series of dilutions, rates of instantaneous phytoplankton growth (μ) and grazing mortality (g) were determined following the Landry and Hassett method (1982), in which the coefficients of a linear regression of k vs. the dilution factor yield μ and g from the y -intercept and negative slope respectively. For all experiments, the hypothesis that there was no significant relationship between dilution factor and apparent growth rate (i.e., regression slope = 0) was tested. When a regression slope was not significantly different from zero ($p > 0.05$), g was set to 0, and the average k of all dilution levels was used as an estimate of μ (Murrell et al. 2002; Chen et al. 2009). Dilution series for which the linear regression yielded a positive slope, i.e., negative g ($N = 7$) were not further included in the comparisons, reducing the initial number of experiments used in the present analysis to 70.

Estimation of rates for experiments with nonlinear feeding responses

We tested whether the dilutions-series data deviated from the dilution method's linearity assumption using ANOVA on the residuals of the regression (Zar 2010). Out of the 70 dilution experiments available for analysis, 28 (40%) exhibited statistically significant deviations from linearity. To determine the linear portion of the data for these experiments, we applied piecewise regression models using the R package "stats," and identified the breakpoint in the original linear regression as the dilution at which the model has the minimum residual standard error (Crawley, 2013). Results of the R analysis indicated a feeding saturation response in 17 out of the 28 experiments (24% total). Other deviations from linearity were associated with low apparent growth rates in the 10% WSW dilution ($N = 7$). A set of rates adjusted for nonlinearity was obtained by estimating μ from the y -intercept of a new regression of only the linear portion of the data, following Moigis (2006). For cases of feeding saturation, which represent the majority of nonlinear dilution experiments, the method we used yields similar estimates as the stepwise regression approach (Rivkin et al. 1999; Gutiérrez-Rodríguez et al. 2010, 2011), which is based on the same rationale as the 3-pt approach suggested by Gallegos (1989). Elser and Frees (1995) addressed nonlinearity by a similar, though nonlinear piecewise fitting approach assuming an upper feeding threshold (i.e., their incipient limiting concentration).

Once μ was determined from the linear portion of the data, grazing rate g was estimated using the equation

$$g = \mu - k_1 \quad (2)$$

where k_1 refers to the average k in the undiluted treatment.

Estimation of 2-point rates

To determine 2-point estimates, we used as one endpoint the average apparent growth rate in the nondiluted treatment (k_1), and as the other endpoint the average k in either one of three dilution levels corresponding to a fraction of 10 (± 2) %, 20 (Mean 22 ± 4 %), and 40 (Mean 44 ± 7 %) WSW. In what follows, we refer to 2-point rate estimates as μ^* and g^* followed by a subscript indicating the dilution level based on which the rate estimate was calculated (e.g., μ^*_{10} and μ^*_{20} for instantaneous growth rates estimated based on apparent growth rate k taken from the 10% and 20% WSW dilutions respectively).

When using the most diluted treatment ($\sim 10\%$ WSW), we assumed that k for that dilution ($k_{0.1}$) served as a reasonable estimate of phytoplankton instantaneous growth rate unaffected by grazing, i.e. $\mu^*_{10} = k_{0.1}$ (Worden and Binder 2003; Strom and Fredrickson 2008; Menden-Deuer and Fredrickson 2010). To calculate the corresponding 2-point grazing rates, we followed procedures by Landry et al. (1984) and the following equations:

$$g^*_{10} = \mu^*_{10} - k_1 \quad (3)$$

For estimating grazing rates using either the 20% or 40% WSW dilution level, we used the equation:

$$g^*_{20(\text{or } g^*_{40})} = (k_d - k_1) / (1 - x) \quad (4)$$

where k_d represents the average k in the diluted treatment, and x is the corresponding fraction of WSW (Landry et al. 1984). Once g^*_{20} or g^*_{40} were obtained, corresponding 2-point phytoplankton growth rates were then determined from the equation:

$$\mu^*_{20(\text{or } \mu^*_{40})} = g^*_{20(\text{or } g^*_{40})} + k_1 \quad (5)$$

Comparison of 2-point estimates and dilution-series rates

Rate estimates based on the 2-point approach fail to identify nonlinearity in the feeding response. To quantify the importance on 2-point estimates of not being able to detect nonlinear feeding responses, we compared 2-point and dilution-series estimates in two ways. First, we used adjusted dilution-series rates, i.e., those obtained after testing for deviations from linearity and adjusted as described above. Excluded from this comparison were dilution-series experiments exhibiting such deviations from linearity that no adjustment was possible ($N = 8$). Second, we also performed the comparison using “nonadjusted” dilution-series rates, i.e., those obtained without testing the regressions for

deviations from linearity but rather taken at face value. For dilution-series that met the linearity assumption, there is no difference between nonadjusted and adjusted rates.

For both phytoplankton growth and grazing-mortality rates, we evaluated the agreement between the 2-point and the dilution-series estimates of μ and g , by performing a model II linear regression analysis for each of the 2-point μ^* estimates (i.e., μ^*_{10} , μ^*_{20} and μ^*_{40}) vs. each adjusted and non-adjusted corresponding dilution-series estimates of μ , and similarly of 2-point g^* estimates vs. each corresponding adjusted and nonadjusted dilution-series estimates of g , which resulted in a total of 12 comparison regressions. Regression slopes < 1 indicate tendency for the 2-point method to underestimate rates, whereas slopes > 1 indicate rate overestimates, and a perfect agreement results in a regression slope of 1. A y -intercept significantly different from zero indicates a consistent bias. We used a one-sample t -test to compare the major axis slope and y -intercept of each comparison regression to theoretical values of 1 and 0, respectively, (Zar 2010).

In several cases, g estimates based on dilution series were ≥ 0 whereas their counterpart 2-point estimates were < 0 ($N = 8, 7,$ and 12 for $g^*_{10}, g^*_{20},$ and $g^*_{40},$ respectively). Although < 0 g estimates are anomalous, these negative 2-point estimates were retained in the comparisons since neglecting them would bias the comparisons' outcome towards better agreement. These < 0 g estimates were however not included in the calculation of 2-point g averages.

Assessing the accuracy of 2-point estimates

To assess the accuracy of the 2-point method, i.e., by how much 2-point estimates deviated from the dilution-series estimates, and to investigate how the magnitude of these deviations was affected by the choice of the diluted treatment, we calculated the difference between each 2-point rate-estimate and the corresponding adjusted dilution-series rate. We further refer to these differences as “2-point errors.” We used the mean standard deviation associated with adjusted dilution-series rates as a criterion against which mean 2-point errors could be evaluated. To assess whether 2-point errors were acceptable, their range was measured against the range within which 95% of the standard deviations of full adjusted dilution-series rates were predicted to occur, which we calculated as $\pm \text{mean error} + t_{(95\%, \text{DF})} \times \text{error SD}$. We considered whether linearity (or deviations thereof) affected the magnitude of the 2-point errors by repeating the analysis using nonadjusted dilution-series estimates. We also examined the potential effects of initial chlorophyll a and temperature on the outcome of the comparison between the two methods.

Assessing the variability of 2-point estimates

To assess the variability of 2-point estimates, for each experiment we calculated all the 2-point estimates that could be obtained from all the possible pairings of each k_d and k_1

Table 1. Summary of location, season, and experimental design of dilution experiments used to compare 2-point and dilution-series rates.

Location	Season	No. of experiments	No. of dilution levels	No. of replicates			Total No. of bottles
				10%	100%	Other dilution levels	
NAt	Sp	6	3	2	2	2	7
NAt	Sp	1	4	0	2	2	10
NAt	Sp	8	5	2	2	2	12
NB	S	1	4	2	2	2	8
NB	S	16	5	2	2	2	10
NB	S	4	5	3	2	2	11
NB	S	9	5	3	3	2	12
NB	S	8	4	3	3	3	12
WAP	AF	1	5	2	2	2	12
WAP	AF	8	5	3	3	2	14
WAP	AF	2	5	3	3	2	16
WAP	Asp	13	5	2	2	2	12
Total		77					

AF, Austral fall; Asp, Austral spring; Nat, North Atlantic; NB, Narragansett Bay; S, summer; Sp, spring; WAP, West Antarctic Peninsula

Table 2. Summary of phytoplankton growth (μ) and grazing mortality (g) rates obtained using either regression analysis of dilution-series data, or the 2-point method with three different diluted treatments containing an average of either 10%, 20%, or 40% undiluted seawater. "Nonadjusted" dilution-series rates are those obtained from the regression coefficients without testing the regression for deviations from linearity. For experiments that exhibited deviations from linearity, dilution-series rates were "adjusted," i.e., obtained from the linear portion of the data only. Averages for g are based on > 0 g estimates only.

	Nonadjusted Dilution-series	Adjusted Dilution-series	2-point estimates per % of WSW in diluted treatment (SD)		
			10%	20%	40%
Growth rates (μ)			μ^*_{10}	μ^*_{20}	μ^*_{40}
Range	-0.16–2.34	-0.16–2.43	-0.17–2.29	-0.35–2.40	-0.35–2.49
Average	0.78 (0.71)	0.82 (0.73)	0.77 (0.68)	0.81 (0.78)	0.72 (0.75)
N	70	62	69	56	67
Grazing rates (g)			g^*_{10}	g^*_{20}	g^*_{40}
Range	0–1.22	0–1.19	0–1.09	0–1.26	0–1.40
Average	0.28 (0.30)	0.30 (0.30)	0.31 (0.26)	0.31 (0.32)	0.30 (0.32)
N	70	62	58	48	50

values, and generated coefficients of variation (CV, as %) from the average and SD of these estimates. This was repeated for each of the three diluted treatments used, and the three groups of CVs were then compared in a Kruskal–Wallis one-way analysis of variance on ranks followed by Dunn's multiple-comparisons test. The latter is a conservative post-hoc test that applies a Bonferroni correction required because of the many comparisons made. Prior to analysis, CVs outliers were removed from the data using the Median Absolute Deviation (MAD) method, which is more

robust than assessing outliers based on the number of standard deviations (Leys et al. 2013).

Assessing the effect of replication on the accuracy of 2-point estimates

We assessed the effect of replication by comparing 2-point errors associated with μ^*_{10} and g^*_{10} estimated based on a different number of replicates. Instead of using a random approach for selecting replicates (*see* Chen 2015), we used a "worst-case" scenario approach, as we wanted to quantify

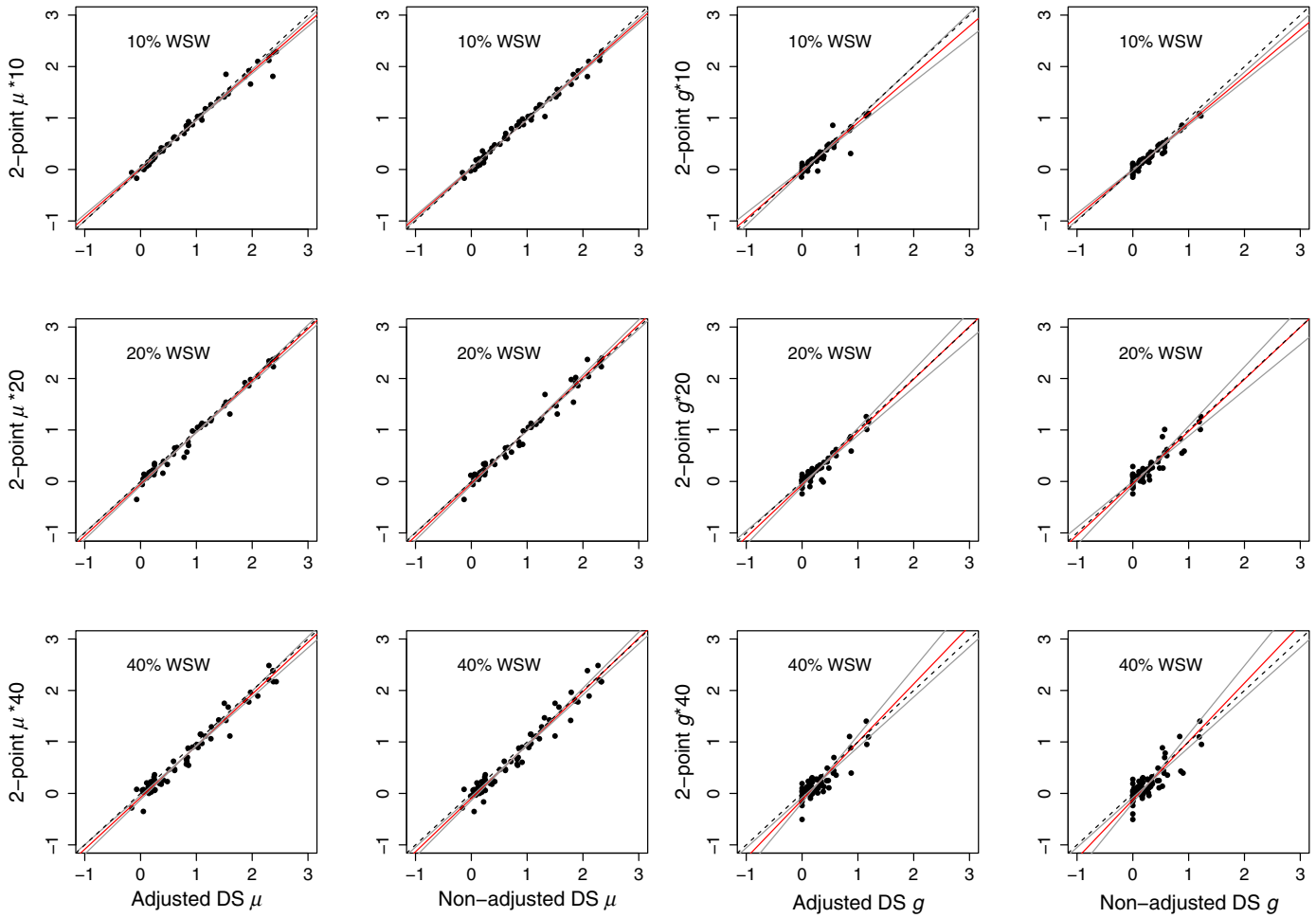


Fig. 1. Two-point vs. dilution-series rate estimates (d^{-1}): 2-point phytoplankton growth rates (μ^*) vs. corresponding adjusted (1st column) or nonadjusted (2nd column) dilution-series rates, and 2-point grazing mortality rates (g^*) vs. corresponding adjusted (3rd column) or nonadjusted (4th column) dilution-series rates. Each row of plots corresponds to 2-point estimates obtained using a diluted treatment with a 10%, 20%, or 40% fraction of seawater (WSW). Diagonal red solid lines represent linear model II regression major axis (MA), gray lines represent 95% CI limits of MA, dashed lines represent 1 : 1 ratio line.

and compare the maximum 2-point errors as a function of number of replicates. Thus, for the analysis we selected replicates that yielded 2-point rate estimates that were most different from the ones obtained through regression of the dilution series. This approach is conservative in that it favors higher degree of replication. The number of comparisons possible depended on the different replication schemes used in the different experiments (Table 1). We compared 2-point errors based on a single bottle to those based on duplicate bottles using a Wilcoxon matched-pairs signed rank test ($N = 69$). Where available, we compared 2-point errors based on up to three replicates for a subset of 24 and 28 experiments for g^*_{10} and μ^*_{10} respectively using a one-way ANOVA with repeated measures, followed by either a Holm Sidak's multiple comparisons test or the nonparametric alternative Friedman test when data were normally distributed (μ^*_{10}) or not (g^*_{10}) respectively.

All statistical analyses were performed at an alpha value ≤ 0.05 . Average values are presented \pm one standard deviation of the mean.

Assessment

Comparison of dilution-series and 2-point estimates

Estimating μ and g based on a series of dilutions or using only two points yielded similar average estimates ($\mu = \sim 0.8 \text{ d}^{-1}$ and $g = \sim 0.3 \text{ d}^{-1}$; Table 2). Increasing the fraction of plankton biomass in the diluted treatment increased the range of 2-point μ estimates from -0.17 d^{-1} to 2.29 d^{-1} for μ^*_{10} , to -0.35 d^{-1} to 2.40 d^{-1} for μ^*_{20} , and -0.35 d^{-1} to 2.49 d^{-1} for μ^*_{40} . The range of 2-point g estimates (not including negative estimates) increased from 0 d^{-1} to 1.1 d^{-1} for g^*_{10} ($N = 58$), to 0 d^{-1} to 1.3 d^{-1} for g^*_{20} ($N = 48$) and 0 d^{-1} to 1.4 d^{-1} for g^*_{40} ($N = 50$).

Table 3. Regression coefficients and goodness of fit comparing 2-point and dilution-series rate estimates of phytoplankton growth, and grazer-induced mortality (shaded columns). Comparisons are made for dilution series estimates that were adjusted for deviations from linearity and before they were adjusted (nonadjusted). The number of experiments available for analysis (N) varied according to the diluted treatment used in the 2-point method, due to differences among experiments in the dilution-series set-up.

	μ^*_{10} vs. μ	g^*_{10} vs. g	μ^*_{20} vs. μ	g^*_{20} vs. g	μ^*_{40} vs. μ	g^*_{40} vs. g
2-point vs. adjusted regression						
Regression slope	0.95	0.94	1.01	1.03	1.01	1.13
Slope standard error	0.017	0.05	0.018	0.050	0.025	0.006
R^2	0.98	0.88	0.99	0.90	0.97	0.77
Testing H_0 : Slope = 1	S	NS	NS	NS	NS	NS
p value	0.002	0.166	0.76	0.63	0.84	0.90
N	61	61	50	50	59	59
2-point vs. nonadjusted regression						
Regression slope	0.95	0.91	1.03	1.01	1.04	1.14
Slope standard error	0.011	0.025	0.021	0.067	0.026	0.09
R^2	0.99	0.95	0.98	0.81	0.96	0.71
Testing H_0 : Slope = 1	S	S	NS	NS	NS	NS
p value	0.000	0.000	0.19	0.87	0.15	0.14
N	69	69	56	56	67	67

Regression analyses comparing estimates of μ based on dilutions series and those based on only two dilution levels showed good agreement between the two methods (Fig. 1). Slopes from these comparison regressions were consistently close to 1, indicating good agreement between the two methods, and varied from 0.95 to 1.01 depending on the dilution level used in the 2-point (Table 3). Comparison slopes were not significantly different from 1 ($p \geq 0.76$), except for estimates of μ^*_{10} (slope = 0.95, $p = 0.002$), indicating an underestimation of 5% from adjusted dilution-series rates (Table 3). In several cases, the y -intercepts of the comparison regression were significantly different from 0, but their magnitude was negligible (data not shown) indicating no systematic over or underestimate of μ .

Results of the comparison regressions were more variable for g than for μ (Fig. 1), indicated by lower values of r^2 (Table 3). Slopes from comparison regressions between 2-point and adjusted dilution-series estimates of g varied from 0.94 to 1.13 (Table 3), and were not significantly different from 1 ($p \geq 0.17$). As with the comparison for μ reported above, y -intercepts were sometimes different from 0 but had negligible magnitudes except for g^*_{40} (-0.13).

Dilution-series estimates had comparable ranges and averages, irrespective of whether adjustments were made for deviations from linearity or not (Table 2). Estimates of μ obtained with or without adjustment adjustment for deviations from linearity had identical ranges from -0.16 d^{-1} to 2.43 d^{-1} but different averages of 0.78 (± 0.71) d^{-1} and of 0.82 (± 0.73) d^{-1} with and without adjustment respectively. Adjusted estimates of g ranged from (-0.01 d^{-1} to 1.19 d^{-1}) and averaged (0.30 ± 0.30 d^{-1}), compared to a range of 0 to 1.22 d^{-1} and an average of 0.28 (± 0.30) d^{-1} without

adjustment (Table 2). Notably, variance was nearly identical for both nonadjusted and adjusted estimates. There was no significant difference between adjusted and nonadjusted dilution-series estimates of g (Wilcoxon, $p = 0.83$, $N = 62$). For μ , the difference was significant (Wilcoxon, $p = 0.003$, $N = 62$), yet its magnitude was negligible (Table 2).

Failing to adjust estimates from nonlinear dilution experiments did not substantially alter the agreement between the 2-point and dilution-series methods (Fig. 1). Comparison regression slopes using nonadjusted dilution-series estimates were similar to those obtained using adjusted rates, both in their magnitude, and in the fact that they were generally not significantly different from 1 (Table 3). There was an exception to the latter when comparing 2-point rates obtained with a 10% WSW dilution level ($p < 0.001$), which indicated 2-point underestimates of 5% and 9% for μ and g , respectively.

Effect of biomass dilution on accuracy of 2-point estimates

To assess the accuracy of the 2-point estimates, we quantified how much they deviated from adjusted dilution-series rates as a function of the proportion of biomass in the diluted treatment. The average 2-point errors for μ were similar in magnitude to the average standard deviation of 0.04 (± 0.03) d^{-1} of adjusted dilution-series estimates of μ . Median 2-point errors values were -0.02 d^{-1} for μ^*_{10} and μ^*_{20} and were larger (-0.07 d^{-1}) for μ^*_{40} (Fig. 2A). For μ^*_{10} , most errors (87%) fell within the reference limits representing 95% range of standard deviations (± 0.09 d^{-1}) associated with rates from dilution-series (Fig. 2A). Increasing plankton biomass in the diluted treatment decreased that proportion to 80% and 47% for μ^*_{20} and μ^*_{40} , respectively (Fig. 2A).

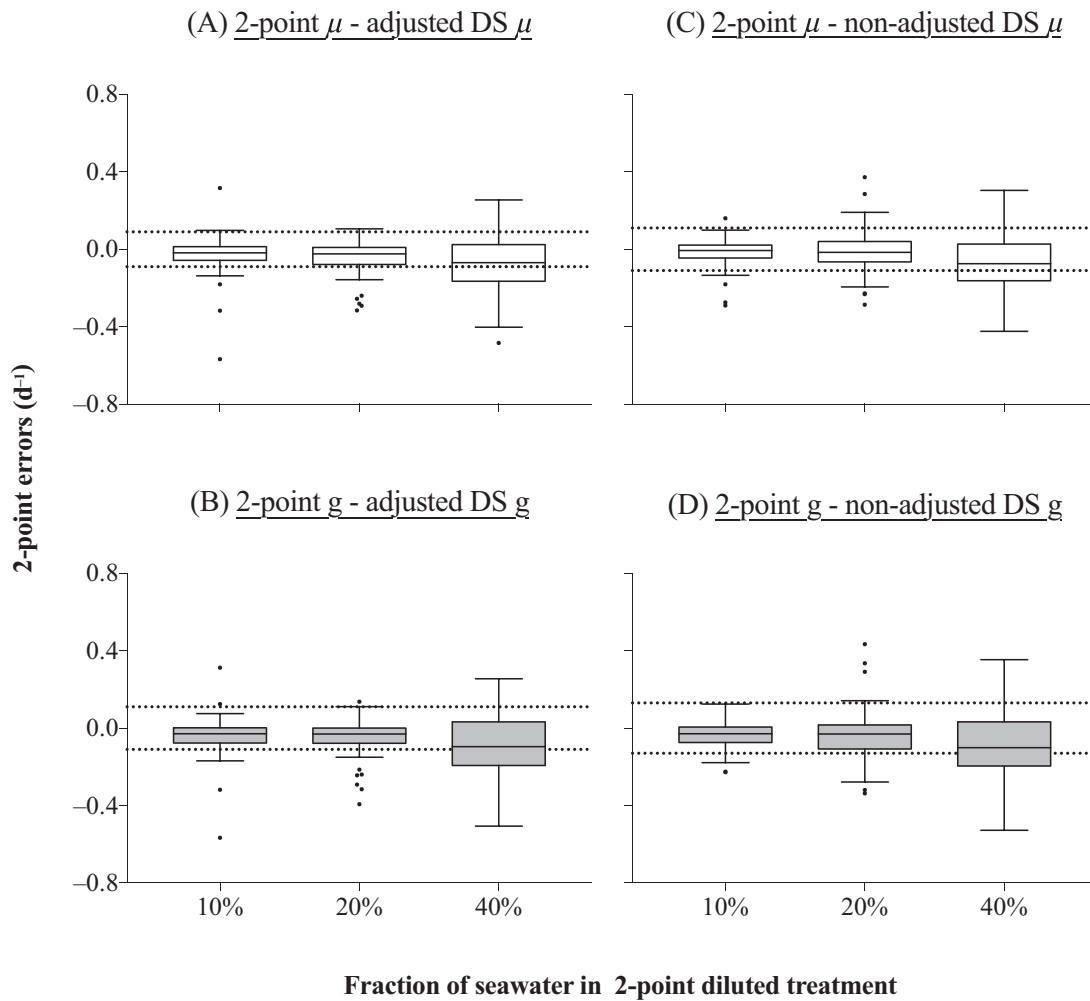


Fig. 2. Two-point errors relative to adjusted (**A** and **B**) and nonadjusted (**C** and **D**) dilution-series (DS) rate-estimates of phytoplankton growth (μ , **A** and **C**) and grazing (g , **B** and **D**) as a function of fraction of plankton biomass in the 2-point diluted treatment (10%, 20%, or 40%). Dotted horizontal lines represent upper and lower limits of interval containing 95% of the standard deviations associated with dilution-series rates. Lines in the middle of the boxes represent median values and whisker extend to 25th (lower) and 75th (upper) percentiles $\pm 1.5 \cdot$ interquartile range. Values higher/lower than upper/lower limits of whiskers are plotted as individual points. Percentiles (P) are computed as $P = a/(n + 1)$, where a is the rank starting at 1 and n is the sample size.

Thus, increasing concentrations of biomass decreased the accuracy of the rate estimates, likely due to the decreasing signal to noise ratio.

Two-point errors for g had median values of -0.03 d^{-1} for both g_{10}^* and g_{20}^* , and increased to -0.1 d^{-1} for g_{40}^* (Fig. 2B). In comparison, dilution-series estimates of g had an average standard deviation of $0.05 (\pm 0.03) \text{ d}^{-1}$. The proportion of 2-point errors falling within the 95% range of standard deviations associated with rates from dilution-series ($\pm 0.11 \text{ d}^{-1}$) was 85% and 80% for g_{10}^* and g_{20}^* , respectively, but this proportion decreased to 46% when increasing to 40% the fraction of plankton biomass in the diluted treatment (Fig. 2B).

Two-point errors tended to be greater for nonlinear than for linear data and varied over a range that was on average 2.7-fold and up to 3.2-fold (for μ) and 2.3-fold and up to

4.5-fold (for g) to greater (Table 4). Nevertheless, as both linear and nonlinear data were treated together in the analysis, for each dilution level used, the median 2-point error was similar, irrespective of whether the dilution-series rates from nonlinear experiments were adjusted (Fig. 2A,B) or not (Fig. 2C,D). The adjustment, however, had an effect on the spread of the 2-point errors (Fig. 2). Adjustment increased the proportion of errors exceeding the limits of the 95% interval used as a reference (Fig. 2), up to 8% (μ_{10}^*) for 2-point rate-estimates of both μ and g based on 10% and 40% WSW treatments, while it decreased it by $\leq 5\%$ for μ_{20}^* and g_{20}^* . This effect was likely due to excluding abnormally low apparent growth rates in the most diluted treatments ($k_{0.1}$) observed in some experiments when estimating adjusted dilution-series rates (*see* method section).

Variability of 2-point estimates

The range of CVs for 2-point estimates increased with increasing plankton biomass in the diluted treatment (Fig. 3), and CVs were substantially (3- to 4-fold) lower for μ than for g . CVs for μ ranged from 0.3% to 30.5%, 0.2% to 30%, and 0.2% to 54% for μ^*_{10} , μ^*_{20} , and μ^*_{40} , respectively, and their corresponding medians of 6.4%, 5%, and 10% differed significantly from each other ($p=0.038$; Fig. 3). CVs for g ranged from 2.7% to 81%, 1.4–123%, and 0.5% to 205% for g^*_{10} , g^*_{20} , and g^*_{40} respectively. Median CV for g^*_{10} (19.8%) was significantly smaller than for both g^*_{20} (34%) and g^*_{40} (42%).

Effects of replication on the accuracy of 2-point estimates

We investigated the effect of the number of bottles included in the calculation of 2-point rates μ^*_{10} and g^*_{10} on

Table 4. Summary of 2-point errors estimated as the differences between 2-point and adjusted dilution-series rates of phytoplankton growth (μ) and grazer-induced mortality (g), as a function of % biomass in the 2-point diluted treatment, for linear (L) and nonlinear (NL) data.

	10%		20%		40%	
	L	NL	L	NL	L	NL
Phytoplankton growth rate (μ)						
N	41	20	30	20	41	18
Min	-0.1	-0.57	-0.16	-0.32	-0.4	-0.48
Max	0.1	0.32	0.11	0.04	0.11	0.26
Mean	-0.01	-0.08	-0.01	-0.1	-0.07	-0.1
SD	0.04	0.16	0.06	0.12	0.12	0.18
Grazer-induced mortality rate (g)						
N	41	20	30	20	41	18
Min	-0.14	-0.57	-0.21	-0.39	-0.51	-0.48
Max	0.13	0.31	0.14	0.11	0.13	0.26
Mean	-0.02	-0.08	-0.02	-0.1	-0.08	-0.1
SD	0.05	0.16	0.07	0.13	0.14	0.2

their maximal difference from dilution-series rates. For both estimates, increasing the number of bottles per dilution level from 1 to 2 significantly decreased the range of differences (Fig. 4) and reduced the absolute magnitude of the median difference (Table 5) by 1/3 (μ^*_{10}) to 1/2 (g^*_{10}). Increasing the number of bottles per dilution level from 1 to 2 to 3 in the subset of data available with a complete set of triplicates showed the same effect of a reduction in differences (Table 5). The effect of replication on reducing differences in rate estimates based on higher levels of replication was not significant for μ^*_{10} , but the effect was significant for g^*_{10} (Fig. 4).

Effects of initial chlorophyll-a and temperature

There was no correlation between the magnitude of 2-point errors and incubation temperature or initial chl a concentration (Fig. 5). Thus, there was no effect of either incubation temperature or levels of chl a on the quality of the rate estimates from the 2-point approach. This result provides the important insight that the accuracy of 2-point estimates does not inherently decrease due to nonlinear feeding responses that may be expected at either high phytoplankton concentration (feeding saturation) or low phytoplankton concentration (feeding thresholds). Lack of temperature effects on the quality of rate estimates also indicates that the 2-point method delivers results free from seasonal or latitudinal biases.

Discussion

Predation by herbivorous protists is generally accepted as the major loss factor of phytoplankton production (Banse 2013; Steinberg and Landry 2017) and has been hypothesized as a contributing factor in driving large-scale phenomena, such as the North Atlantic Spring Bloom (Behrenfeld 2010; Behrenfeld and Boss 2014). To evaluate predation effects on marine production and test proposed hypotheses, we need to gain a deeper understanding of the critical

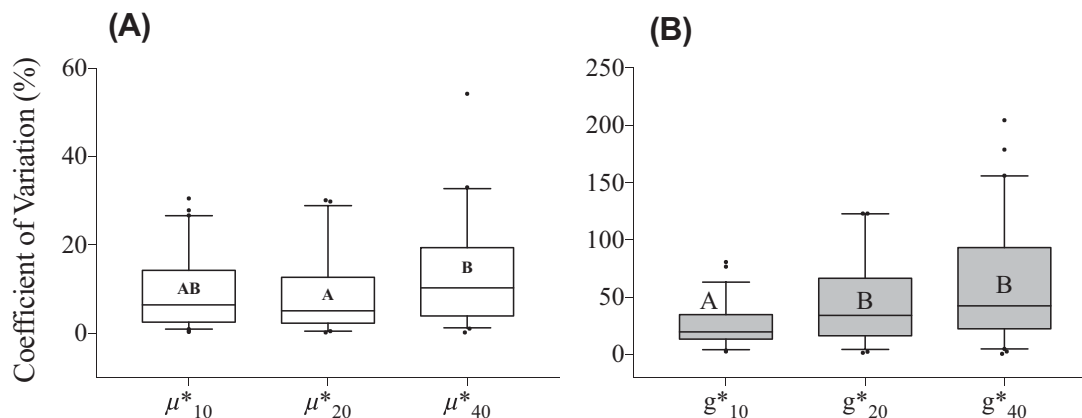


Fig. 3. Coefficient of variation (%) of 2-point estimates of phytoplankton growth (μ , panel **A**) and grazer-induced mortality (g , panel **B**). Note different ranges in y-axes. Box plot specification as in Fig. 2.

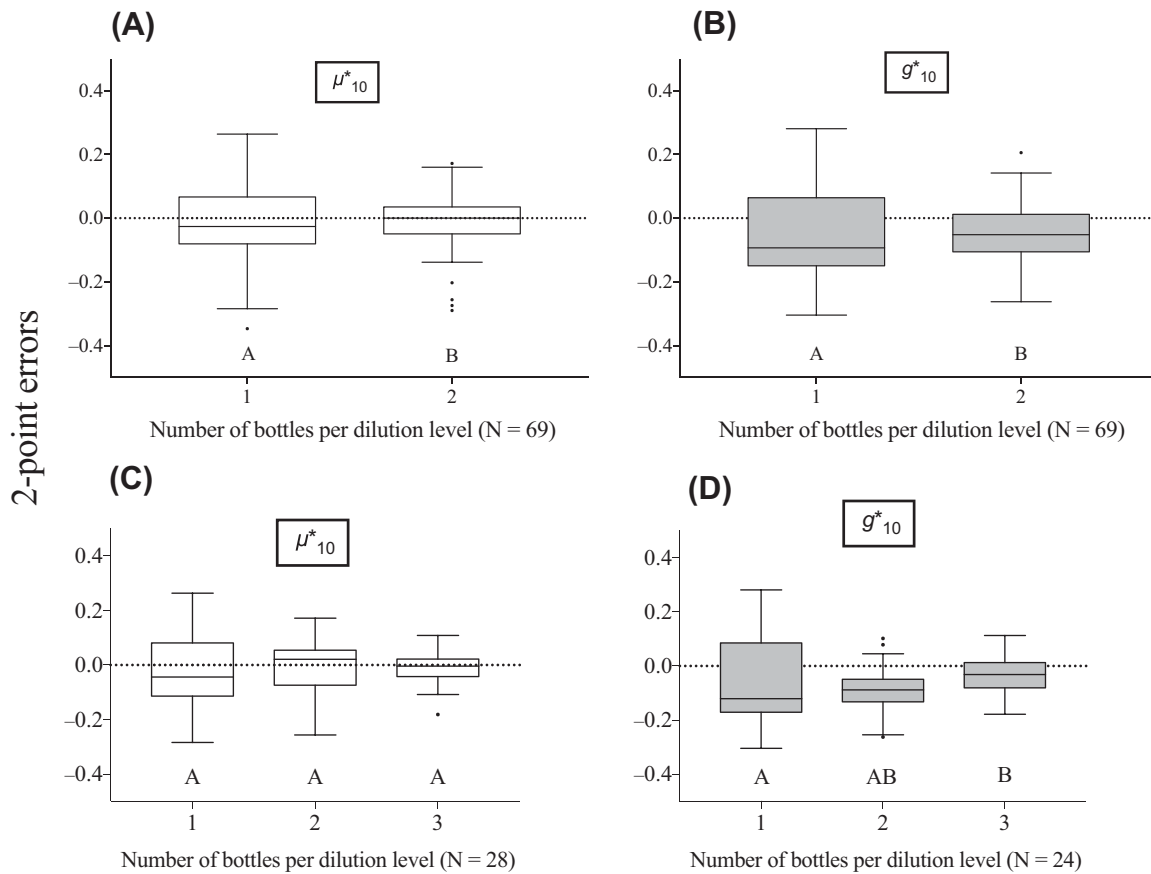


Fig. 4. Two-point errors (d^{-1}) for rates of phytoplankton growth (μ , panels **A** and **C**) and grazing mortality (g , panels **B** and **D**), as a function of number of bottles per dilution level used in the 2-point estimation. Effect of duplication on 2-pt errors is shown in (**A** and **B**), effect of triplicate measurements in (**C** and **D**). Treatments marked with a different letter were significantly different from each other. Box plots specification as in Fig. 2. For clarity, one outlier (-2.97) was omitted from left panels.

Table 5. Comparison of maximum range and magnitude of differences obtained between 2-point and dilution-series estimates as a function of the number of replicate bottles used in the 2-point experiments.

Replicates	Duplicates				Triplicates					
	μ^*_{10}		g^*_{10}		μ^*_{10}			g^*_{10}		
	1	2	1	2	1	2	3	1	2	3
<i>N</i>	69	69	69	69	28	28	28	24	24	24
Minimum	-0.35	-0.29	-2.97	-0.26	-0.28	-0.26	-0.18	-2.97	-0.26	-0.18
Median	-0.03	0.00	-0.09	-0.05	-0.04	0.02	-0.004	-0.12	-0.09	-0.03
Maximum	0.26	0.17	0.28	0.21	0.26	0.17	0.11	0.28	0.10	0.11
Mean	-0.01	-0.01	-0.09	-0.05	-0.01	-0.006	-0.01	-0.19	-0.09	-0.03
SD	0.11	0.09	0.38	0.10	0.12	0.10	0.06	0.61	0.09	0.08

processes driving phytoplankton grazing mortality in the global ocean. This goal necessitates acquiring higher resolution data of grazer-induced mortality rates from diverse geographic locations, seasons, and environmental and biological conditions. Rate estimates are also needed at higher than daily resolution in order to match observations from

autonomous in situ assets. Acquisition of more frequent grazing-rate estimates could be more easily achieved if experimental logistics can be reduced without sacrificing measurements' accuracy. Using a large and diverse dataset of dilution experiments, we compared estimates of both phytoplankton growth and grazing-mortality rates obtained using

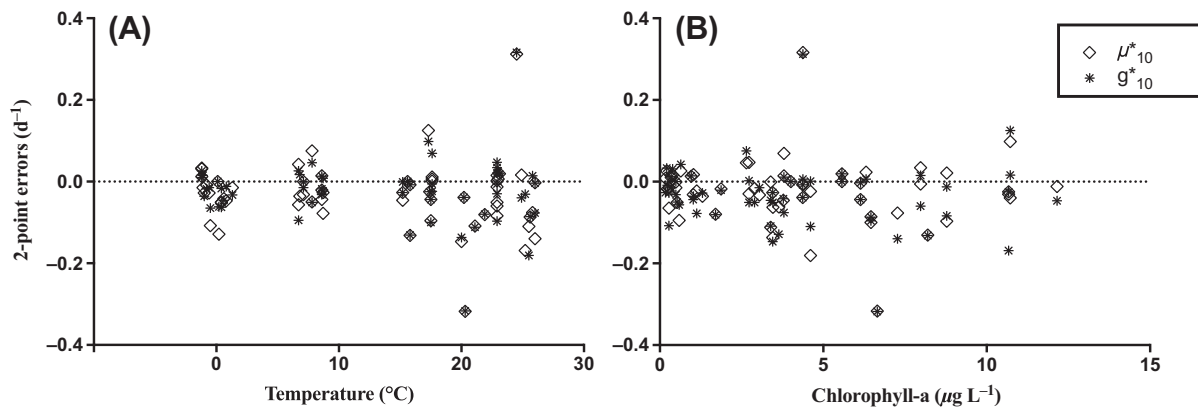


Fig. 5. Errors of rate estimates (d^{-1}) from the 2-point approach, as a function of (A) temperature, and (B) initial chlorophyll a concentration. Phytoplankton growth and grazer-induced mortality rates are based on the 10% WSW diluted treatment (μ^*_{10} and g^*_{10} , respectively).

a series of dilutions with those obtained using only two, and are now able to provide a detailed quantified evaluation of not only the reliability of the 2-point method but also of the trade-offs involved when choosing a sampling design.

Overall, we found that rate estimates for either phytoplankton growth or grazer-induced mortality obtained using only two dilution levels did not substantially deviate from those obtained when using multiple dilutions. Phytoplankton growth rate (μ) estimates based on either two or a series of dilutions were essentially equivalent. Similarly, grazer-induced mortality rates (g) based on two points were overall equivalent to estimates based on a series of dilutions. The accuracy of 2-point estimates was satisfactory, 2-point errors being of a magnitude similar to the inherent error associated with the dilution-series estimates, supporting findings by Chen (2015). Even when deviations from a perfect agreement between methods were statistically significant, which was the case when using a 10% WSW dilution in the 2-point method, 2-point estimates were conservative, meaning the abbreviated method provided underestimated rates of phytoplankton growth and/or grazer-induced mortality. These underestimates were of minimal magnitude, when compared to the dilution series inherent measurement error of 0.1 d^{-1} . At $\mu = 0.69 \text{ d}^{-1}$, the latter would represent a variability of 15%, i.e., three times greater than the deviation between the two methods' estimates of phytoplankton growth ($\leq 5\%$). Furthermore, errors were not compounding systematically (e.g., increasing with increasing rate values), thus the statistically significant deviations of the 2-point rate estimates based on 10% dilution level was truly minor.

Moreover, based on the mean and variance of 2-point errors, it is when the two dilution levels used in the 2-point approach were most dissimilar (i.e., when μ was obtained as the value of k in the treatment containing the smallest fraction of plankton biomass $\sim 10\%$ WSW), that the agreement between the two methods was most robust, confirming

results from previous investigations (Strom et al. 2006; Strom and Fredrickson 2008; Chen 2015).

In choosing the dilution level, there appears to be a biomass vs. accuracy tradeoff: when doubling plankton biomass in the diluted treatment from 10% to 20%, the average accuracy of 2-point estimates was maintained. Further doubling of biomass (to 40%) however increased the proportion of estimates with deviations outside the range of errors from dilution-series two to threefold, i.e., for estimates based on higher proportions of biomass in the diluted treatment, error increased significantly compared to dilution-series errors. An increase in plankton biomass in the dilute treatment may be desirable for post-incubation flow-cytometry or DNA analysis (e.g., Landry et al. 2008, Taniguchi et al. 2012), or a dilute treatment containing $>10\%$ WSW may be necessary to achieve a detectable signal in very low plankton biomass conditions, such as wintertime or the oligotrophic ocean.

Nonlinearity is considered the chief weakness of the 2-point version of dilution experiments and is the fundamental reason to conduct a full regression analysis on at least four dilution levels. Previous comparisons of estimates obtained by dilution-series and 2-point approaches performed in the field have indeed attributed their good agreement to the dependably linear response of phytoplankton apparent growth rate to dilution (Landry et al. 2011b). We found no support for the widespread concern associated with nonlinearity. The inclusion in our analysis of nonlinear data, which represented a substantial fraction of our total data set (1/3), showed that both methods yielded indistinguishable rate estimates, and including nonlinear data in the analysis did not lead to an inherent bias to either under- or overestimate growth or grazing rates. Certainly, as has been noted before (Chen 2015), the good agreement between the two methods does not always hold for individual 2-point estimates, a proportion of which showed departures from the corresponding dilution-series estimates outside acceptable bounds. When obtained

from nonlinear data, individual 2-point estimates did carry two to threefold larger errors than when obtained from linear data. Yet the theoretical significance of losing the ability to assess nonlinearity with the 2-point method does not appear to consistently bias rate estimates, as long as groups of experiments are considered.

As a principal source of nonlinearity, feeding saturation has been the focus of much of the discussion regarding rate estimation from nonlinear dilution-series data and several approaches with various levels of complexity have been proposed (e.g., Gallegos 1989, Evans and Paranjape 1992; Elser and Frees 1995; Rivkin et al. 1999; Redden et al. 2002; Moigis 2006; Teixeira and Figueiras 2009; Li et al. 2017). Like the majority of these prior efforts, in our study we chose to maximize the number of data points included in estimating dilution-series rates, so that we could compare 2-point estimates to the strongest estimates one can secure from a nonlinear dilution series. Following our approach, we find that in the case of feeding saturation, the largest underestimation of either μ or g obtained from a 2-point based on 10% WSW will be equal to the difference between the “true” value of μ in the complete absence of grazers (the y -intercept of the regression line through the linear portion of the data) and the apparent growth rate in the 10% WSW (Supporting Information Fig. S1). This is irrespective of the dilution level at which saturation occurs (Supporting Information Fig. S1). In fact the 2-point method has been found to be a good solution to estimate rates when feeding saturation occurs (Worden and Binder 2003; Chen 2015). Interestingly, if the best approach to deal with nonlinearity in dilution series is to estimate rates using only two dilutions, then the question of nonlinearity being undetectable in the 2-point becomes irrelevant.

Chl *a* is often considered a major driver of nonlinearity: functional responses of feeding saturation are often associated with coastal or estuarine conditions of high chl *a* (e.g., Gallegos 1989, Strom et al. 2001, Teixeira and Figueiras 2009), and nonlinearity could also occur when low chl *a* concentration renders detection of signal difficult for some dilution levels. We found no relationship between chl *a* concentration and deviations from linearity, and neither did chl *a* concentration affect the magnitude of 2-point errors. Nonlinearity occurred across the entire range of chl *a* concentrations, suggesting that neither feeding saturation, nor presence of feeding thresholds or lack of signal at low concentrations, are reliable predictors of predation. Several factors may contribute to obscure the relationship between functional responses and ambient chl *a*. Chen et al. (2014) found microzooplankton grazing half-saturation constants to be highly variable and suggested that this variability may reflect the ability of grazers to acclimate to and fully exploit the ambient prey abundance by minimizing feeding thresholds/food satiation in low/high prey abundance. How fast grazers in situ acclimate to a patchy and fluctuating prey

environment, including in terms of phytoplankton species, is largely unknown. However, we do know that starving predators can rapidly, within minutes, resume grazing even after prey deprivation for days to weeks (Calbet et al. 2013; Anderson and Menden-Deuer 2016). Moreover, heterotrophic protists are highly selective grazers. Species-specific feeding preferences rather than absolute concentration of bulk measurements of prey abundance (chl *a* or carbon) may be stronger determinants of feeding saturation and thresholds (Menden-Deuer and Kjørboe 2016), as well as stronger drivers of realized grazing (Lawrence and Menden-Deuer 2012). Furthermore, predators may rely on prey sources other than those measured by chl *a*, such as bacteria (Strom et al. 2007), and often are mixotrophic (Flynn et al. 2013). Nonlinear responses have also been attributed to changes in the composition and abundance of the predator community during incubations (Dolan et al. 2000; Dolan and McKeon 2004; Agis et al. 2007), or to trophic cascades within the grazing community (Olson et al. 2006; Calbet and Saiz 2013). Our results do not provide a strong indicator for a particular driver of nonlinear feeding responses.

Given the host of theoretical drivers, the occurrence of nonlinearity is impossible to predict and we found no evidence that nonlinearity introduces a systematic bias in rate estimates. Nonetheless, it is greatly advisable to make multiple 2-point measurements in order to secure the most reliable estimates of phytoplankton growth and grazing-mortality rates and best characterize the plankton population dynamics of a system. This emphasis on the importance of conducting multiple 2-point experiments may seem to defeat the initial purpose of the 2-point approach (i.e., that of minimizing sampling logistics). Nevertheless, in a heterogeneous environment one would want to make multiple measurements across gradients of depth, light, or temperature, even when using a full dilution-series. Making multiple measurements would thus be more practical with the 2-point method, since using only two dilutions considerably reduces sampling logistics and strain on water budget, including time needed to prepare the dilutions, and subsequent measurements of chl *a*. Furthermore, when performed at sea, the 2-point method can save a minimum of 25% of seawater needed to be collected when reducing the number of dilutions to two and the number of bottles from 12 to 9 bottles total, and up to 60%, just by reducing the number of bottles from 12–15 to 6 (4 without nutrient control), allowing a greater number of dilution experiments be done in parallel.

Part of our analysis dealt with comparing the magnitude of 2-point errors when using only one hypothetical bottle vs. two or three per dilution levels. The effect of replication on the potential magnitude of the errors from 2-point estimates indicates that inclusion of at least duplicate bottles per dilution level is strongly advisable. This is in contrast with previous recommendations that only one bottle can be

used to obtain reliable results (Chen 2015). Not only does replication increase statistical confidence in the rate estimates, using two instead of one bottle per dilution level reduced the median error of 2-point estimates by $\sim 1/2$ and also decreased the variance of the mean value of that error by a factor of two for μ and four for g . Although the dataset including triplicates was limited, its analysis showed that adding a third bottle further reduced both the magnitude and variance of 2-point mean errors.

While we found robust consistency between phytoplankton growth-rate estimates from either method, such estimates do not necessarily reflect in situ rates. Whether performed using only two or multiple dilutions, grazing experiments carry limitations associated with incubations, one of the most challenging being failure to replicate the in situ light environment in a mixed layer (Ross et al. 2011). This can result in pigment change artifacts, which makes it difficult to interpret the chl *a* signal (*see* Behrenfeld et al. 2016). Light-mediated changes in μ alter the ratio of primary production consumed (i.e., $g : \mu$, Morison and Menden-Deuer 2015). Poorly constrained estimates of μ also jeopardize estimation of potential phytoplankton accumulation rate (i.e., $\mu - g$), an important metric for understanding the annual dynamics of phytoplankton biomass (Behrenfeld 2014). Combining chl *a* based measures of phytoplankton growth with alternate methods, including cell counts performed using flow-cytometry and/or microscopy (Landry et al. 2008, Taniguchi et al. 2012), does not completely overcome the inability of fixed-depth incubations to mimic phytoplankton cells' light history in a vertically mixing water column. Thanks to its simpler execution, the 2-point approach could be used to address the challenge of replicating in situ conditions by making it possible to simultaneously conduct incubations exposed to different simulated light levels.

Comments and recommendations

When using the 2-point approach, statistically indistinguishable and most-constrained 2-point estimates relative to those from a full dilution-series are obtained using a diluted endpoint with low plankton biomass ($\sim 10\%$). If a larger fraction of plankton biomass is needed for additional sample analyses, or if there is a concern that low in situ chl *a* may influence the sensitivity of the method, the experimenter can now, based on our analysis, weigh the trade-offs in choosing the appropriate diluted treatment knowing the consequences on the variance of rate estimates. A most dilute treatment will minimize rate variability relative to a full dilution experiment, whereas increasing biomass in the dilution will maintain a statistically equivalent estimate of the mean rate, but increase the variance. Using duplicate instead of a single replicate bottle at each end-point should

significantly reduce variability of 2-point estimates and still afford considerable logistical advantages.

Extensive characterization of the impact of grazing on phytoplankton dynamics exist for the Equatorial Pacific and has been achieved with ample use of the 2-point approach (i.e., Landry et al. 2003, 2011a, 2011b). The results presented here provide strong support to extend this approach to other regions of the global ocean and to seasons outside annual productivity peaks.

The application of the 2-point sampling design can facilitate acquisition of higher-resolution data of predation rates across seasons, latitudes, and in response to multiple environmental conditions in the ocean—all critical factors necessary to parameterize protistan herbivory in global biogeochemical models and to decipher the biotic and environmental drivers of predation impact on marine production.

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Conflict of Interest

None declared.

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