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# A revised dilution methodology and implications for estimates of rates of plankton mortality

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## Author contribution statement:

SJB and JSW designed the research questions and approach. SJB wrote the code and performed the experiments. SJB and JSW wrote the paper.

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## Scientific significance statement:

Zooplankton grazing is an important driver of plankton mortality and is a core component of the microbial loop. The dilution method is the prevailing tool used to measure the impact of zooplankton in marine microbial communities. However, the theoretical model underlying the interpretation of experimental measurements using this method does not account for niche competition between plankton. As a consequence, we show that the dilution method may conflate the effects of grazing with those of niche competition, implying that previously reported grazing rates could be overestimated. We propose modifying the classical dilution method by diluting only the zooplankton and then measuring the response of plankton. This "Z-dilution" method explicitly accounts for the effects of niche competition in limiting net plankton growth. We find the Z-dilution method provides robust grazing rate estimates in theory and suggest ways in which the relative strength of niche competition to zooplankton grazing could be measured by combining the Z-dilution and classical dilution methods.

## Data availability statement:

Code is available from http://github.com/WeitzGroup/DilutionMethod-NicheCompetition and is archived on Zenodo at http://dx.doi.org/10.5281/zenodo.61196 (Beckett and Weitz, 2016).

## Abstract:

The dilution method is the principal tool used to infer *in situ* microzooplankton grazing rates. However, grazing is the only mortality process considered by the theoretical model underlying the interpretation of dilution method experiments. We show an alternative interpretation arises when there is concurrent niche competition within the plankton community. We find that grazing rates may be overestimated – the degree of overestimation is related to the importance of niche competition relative to zooplankton grazing. Thus, we propose a modification to the dilution method to disentangle the effects of niche competition and zooplankton grazing. Our theoretical results suggest the revised “Z-dilution” method can robustly infer grazing mortality, regardless of the dominant plankton mortality driver. Further, we show it is possible to independently estimate both grazing mortality and niche competition when the classical and Z-dilution methods are used in tandem. We discuss the significance of these results for quantifying plankton mortality rates.

## Key words:

dilution method, mortality rates, zooplankton, plankton, niche competition, Z-dilution

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# 1 Introduction

Plankton are responsible for primary productivity in the oceans, form the base of the ocean food web, and are drivers of major ocean biogeochemical cycles. Zooplankton grazing is thought to be one of the dominant drivers of plankton mortality (Sherr and Sherr, 2002; Calbet and Landry, 2004); and is a core process within the marine microbial loop (Azam et al., 1983; Turner, 2015). However, other processes such as nutrient limitation, sinking and viral lysis compete and interact with grazers as sources of plankton mortality (Barbeau et al., 1996; Kirchman, 2010; Weitz and Wilhelm, 2012; Brum et al., 2014). Understanding the dynamics of microbial food webs is therefore key to understanding their role in oceanic biogeochemical fluxes (Falkowski et al., 1998; Follows et al., 2007; Lima-Mendez et al., 2015).

Estimates of the relative importance of grazing versus other mortality drivers depends on the quality and robustness of experimental techniques. The dilution method (Landry and Hassett, 1982) is a long-established and popular technique used to measure the impact of micro- and nano-zooplankton on plankton communities. The method is outlined in Figure 1. A sample of seawater is taken and prefiltered so only the microbial fraction remains. Some of this whole seawater (WSW) sample is further filtered, to remove plankton and zooplankton, creating a diluent. The dilution method procedure creates a series of bottles each containing a different proportion of WSW mixed with diluent to create a dilution series.

By measuring the differences in plankton population sizes between two time points within each bottle, the corresponding approximate per capita growth rates can be calculated. The dilution curve represents a plot of the approximate growth rates within each bottle against the dilution level (the proportion of WSW within a bottle). The plankton growth rate and the grazing rate are reported as the intercept and slope, respectively, of the dilution curve. This is due to the underlying theoretical model of plankton dynamics presumed to operate in the bottle. This model introduced by Landry and Hasset is:

$$\frac{dP}{dt} = \overbrace{rP}^{growth} - \overbrace{aPZ}^{grazing} \tag{1}$$

in which $P$ is the density of plankton, which grow at rate $r$; and are grazed upon by zooplankton $Z$ at a rate of $a$. Consider that the whole seawater sample initially contains a plankton density of $P_0$ and a zooplankton density of $Z_0$. A particular bottle within the dilution series containing a proportion $F$ of whole seawater will initially contain plankton and zooplankton densities of $FP_0$ and $FZ_0$ respectively. Evaluating the apparent growth rate, calculated as the population change per capita, under these conditions shows that:

$$\frac{1}{P}\frac{dP}{dt}\bigg|_{FP_0,FZ_0} = r - (aZ_0)\,F. \tag{2}$$

Equation (2) implies that the relationship between dilution level and apparent growth rate will be linear with an intercept equal to the intrinsic per capita growth rate and a gradient equal to the initial per capita grazing mortality rate. However, the model given by equation (1) may be an insufficient description of bottle plankton dynamics for several reasons.

Zooplankton grazing may respond non-linearly with respect to plankton densities in the bottle for example due to feeding saturation. This could significantly alter the shape of the dilution curve and how grazing mortality estimates can be made (Evans and Paranjape, 1992; Landry et al., 1995; Chen et al., 2014). Additional sources of plankton mortality such as losses due to sinking or viral lysis (e.g. Evans et al. 2003; Kimmance and Brussaard 2010) are not accounted for in this model, yet incorporating these processes may be important for interpreting empirical data from dilution experiments. We also note that the model intends to describe bulk community dynamics. Diversity is not considered as plankton and zooplankton communities are each treated as a single population, neglecting potential important functional differences between species. Calbet and Saiz (2013) consider trophic chains; but overall limited attention has been given to diversity.

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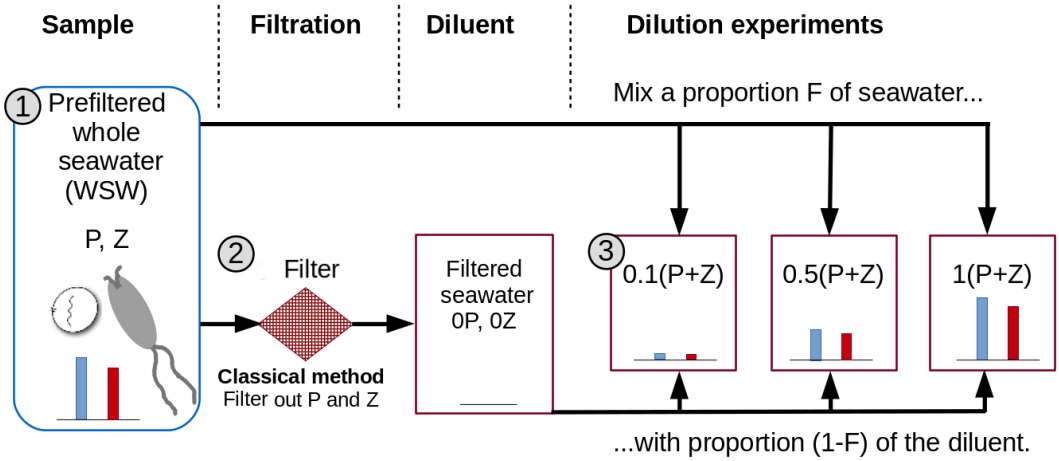


Figure 1: Schematic of the classical dilution method of Landry and Hassett. **1. Sample** Environmental samples are prefiltered to focus on microbial communities - this is termed whole seawater (WSW). Dilution method theory assumes WSW contains zooplankton and the plankton they graze upon. **2. Filtration** The classic dilution method filters some WSW to create a diluent containing no plankton or zooplankton. **3. Dilution series** A series of bottles are filled with a proportion $F$ of WSW and mixed with a proportion $(1-F)$ of the diluent creating a dilution series. The blue and red bars represent the relative abundance of plankton and zooplankton. Apparent growth rates are calculated by measuring the differences in plankton population sizes in each bottle across the dilution series at two time points (the beginning and end of an incubation period). The zooplankton grazing rate is estimated by finding the gradient of a linear regression model between the dilution level $F$ and the apparent growth rate.

Another critique of the method is that it does not take into account the resource requirements for plankton growth. In the absence of zooplankton ($Z_0 = 0$) this model predicts the plankton population will grow without bounds to infinity as niche competition between plankton is not considered. Subsequent modifications of the dilution method included a nutrient enrichment step so that plankton could grow near their idealized maximum rates (Landry et al., 1995). Even if nutrients are added to bottles to attempt to keep plankton growing in the exponential growth phase, this does not eliminate the potential of competition occurring between plankton. In this paper we focus on how the inclusion of niche competition may alter the interpretation of dilution experiment measurements.

A common way to represent niche competition between plankton is by using a logistic growth model (Verhulst, 1845). Logistic growth is a phenomenological model used to implicitly represent competition for resources, whilst not tracking those resources explicitly. It has long been used in mathematical ecology and has been used to describe plankton growth dynamics (e.g. Lewis et al., 2012). Using a logistic growth function has the effect of bounding plankton populations to a carrying capacity $K$. We note that the inclusion of logistic growth could be interpreted to mean that plankton mortality increases as $P$ approaches $K$ or that plankton growth decreases as $P$ approaches $K$, or a combination thereof. A candidate model considering the effect of niche competition is:

$$\frac{dP}{dt} = \overbrace{rP\left(1-\frac{P}{K}\right)}^{logistic\,growth} - \overbrace{aPZ}^{grazing}. \tag{3}$$

Using equation (3) as the description for plankton dynamics we find the apparent growth rate within a bottle in the dilution series is predicted as:

$$\frac{1}{P}\frac{dP}{dt}\bigg|_{FP_0,FZ_0} = r - \left(\frac{rP_0}{K} + aZ_0\right)F. \tag{4}$$

This model, including niche competition, also predicts a linear relationship between dilution level and apparent growth rates. Here, the intercept of the dilution curve represents the maximum growth rate, just as in the classical

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model. However, the slope is now interpreted as the combined effect of niche competition and grazing, both of which necessarily have positive signs. Therefore, dilution experiments may overestimate grazing rates should niche competition be important.

In this manuscript we demonstrate potential limits of the performance and robustness of the classical dilution method using *in silico* simulations of dilution experiments. We do so while varying the relative strength of zooplankton grazing to niche competition. Next, we propose a revision to the dilution method which, in theory, should provide robust estimates of zooplankton grazing. We discuss how the effects of niche competition and zooplankton grazing could be estimated by using the two approaches in tandem. We close by discussing how these results affect the interpretation and implementation of dilution method experiments.

# 2 Methods

For the *in silico* dilution experiments, whole seawater is mixed with the diluent at 10 dilution levels (with proportions $F$ = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1 of WSW) to create a dilution series with 10 bottles. The apparent growth rate, $\widetilde{r}$ , within each bottle is calculated from recordings of the simulated population dynamics as:

$$\widetilde{r} = \frac{1}{T} \log \left( \frac{P_0}{P_T} \right) \tag{5}$$

where $P_0$ is the plankton density at the beginning of the simulation and $P_T$ is the plankton density after time $T$ (Landry and Hassett, 1982). Here the apparent growth rate is calculated using an incubation time of 24h in length. Dilution method estimates of grazing mortality are then calculated by finding the slope of the linear regression fit of apparent growth rates within each simulated bottle in the dilution series (response) against the proportion, $F$, of WSW it contains (predictor). The intercept of this regression is understood as representing plankton growth rate, whilst the slope is interpreted as the grazing mortality rate. Model parameters for *in silico* dilution experiments are shown in Table S1.

# 3 Results

## 3.1 Top-down pressure as indicator of control of plankton by grazers

In the absence of zooplankton the steady state solution for plankton population density within the bottle is $P_A^* = K$. Similarly, the steady state in the presence of zooplankton is $P_Z^* = K\left(1 - \frac{aZ_0}{r}\right)$ . Neither of these densities are necessarily reached during the dilution experiment. Nonetheless, these densities provide a means to quantify the relative importance of grazing. To see why, note that increasing zooplankton pressure will lead to a reduction in $P_Z^*$ , away from $P_A^*$ . Zooplankton pressure is maximised when the per capita mortality rate due to grazing, $aZ_0$ , is equal to the per capita intrinsic plankton growth rate, $r$ . We can quantify top-down control of plankton by the zooplankton by using $\delta_Z$ as a measure of **zooplankton pressure**:

$$\delta_Z = 1 - \frac{P_Z^*}{P_A^*} = \frac{aZ_0}{r}. \tag{6}$$

In the absence of zooplankton, all plankton mortality is driven by competition between plankton and $\delta_Z = 0$ . If top-down zooplankton grazing were to drive steady state plankton density to half of the resource limited density then $\delta_Z = 0.5$ . When zooplankton grazing results in the plankton population being drawn to extinction ( $P_Z^* = 0$ ), then top-down control by zooplankton is maximised and $\delta_Z = 1$ . We use three specific levels of zooplankton pressure (**low:** $\delta_Z = 0.048$ , $aZ_0 = 0.002$ , **intermediate:** $\delta_Z = 0.48$ , $aZ_0 = 0.02$ , **high:** $\delta_Z = 0.96$ , $aZ_0 = 0.04$ ) to highlight the performance and sensitivity of the dilution method to the dominance of zooplankton grazing as a driver of plankton mortality.

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### 3.2 Mortality rates inferred from classical dilution method experiments are sensitive to the relative importance of bottom-up and top-down pressure

The ability of the dilution method to estimate zooplankton associated mortality rates is potentially affected by the relative level of bottom-up (i.e., niche competition) and top-down (i.e., zooplankton) pressure. This is highlighted in Figure 2a using simulations with the three chosen levels of zooplankton pressure. Each level of zooplankton pressure corresponds to a steady state zooplankton density (see Table S1) from which a steady state plankton density, $P^*$, is calculated while keeping the growth rate, $r$, fixed at 1 per day. The grazing mortality rate was estimated using the dilution experiment, in order to compare to the baseline mortality value $aZ_0$. Estimates made using the classical dilution method were closest to the baseline rates when zooplankton pressure was high, but substantially higher than the expected baseline rates when zooplankton pressure was low. Mortality rate bias is calculated as the estimated grazing mortality rate (by the dilution method) divided by the baseline grazing mortality rate. Looking across the perceived bias in mortality rates (Figure 2b) we find that baseline mortality is overestimated by a factor of $\approx 20$ at the highlighted low zooplankton pressure, a factor of $\approx 2$ at the highlighted intermediate zooplankton pressure and by less than 5% at the highlighted high zooplankton pressure. Thus mortality rate bias is lowest when zooplankton pressure is highest, corresponding to situations where plankton densities approach 0. This suggests that the classical dilution method provides more accurate estimates of zooplankton associated mortality rates when zooplankton pressure is high *i.e., when grazing is significantly more important than niche competition*.

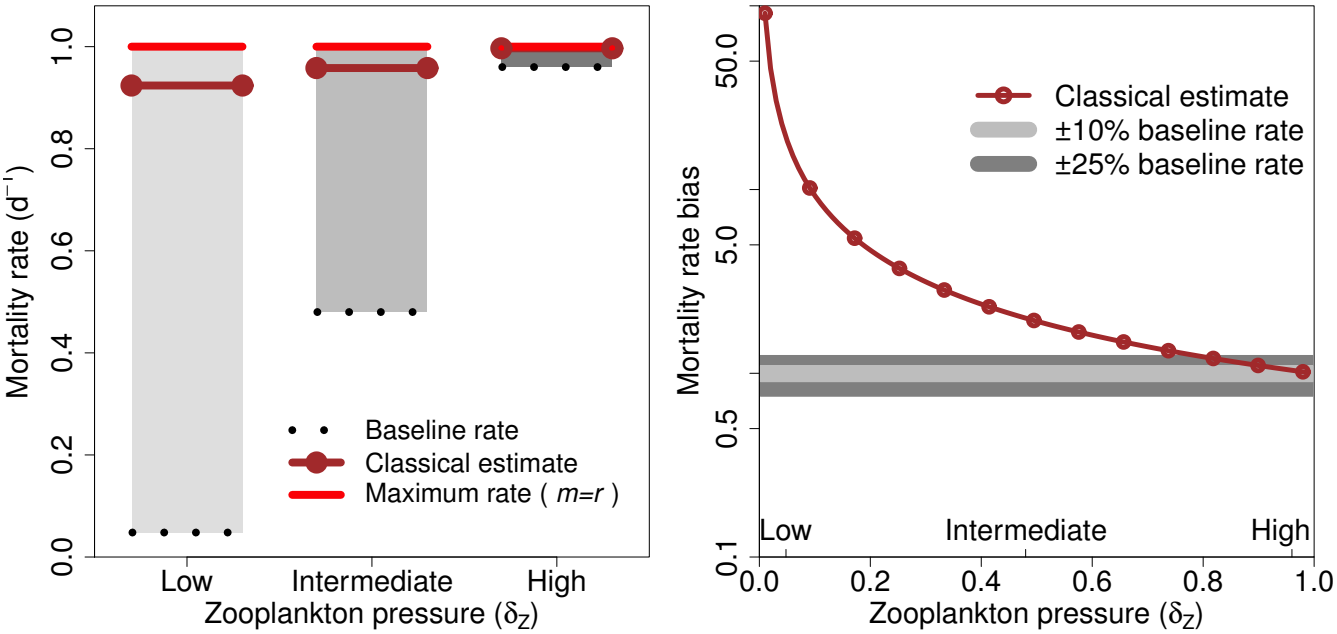


Figure 2: The classical dilution method may overestimate rates of mortality via grazing. (a) Expected baseline zooplankton associated mortality rates and rates estimated using the classical dilution method for three levels of zooplankton pressure; low zooplankton pressure ($1000$ zooplankton ml$^{-1}$), intermediate zooplankton pressure ($10000$ zooplankton ml$^{-1}$) and high zooplankton pressure ($20000$ zooplankton ml$^{-1}$). The maximum mortality rate is calculated for the condition when total mortality, $m$, is equal to the plankton growth rate $r$. (b) Mortality rate bias across the full gradient of zooplankton pressure. The zooplankton pressure associated with each of the examples given in (a) are shown on the x-axis.

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### 3.3 The classical dilution method is an estimator of both grazing and population growth inhibition

Grazing mortality rates are overestimated by the classical dilution method when zooplankton pressure is low. Indeed, the estimated grazing mortality rates appear closer to the maximum plankton growth rate (see Figure 2a). This feature of the classical dilution method arises due to the inability of the method to disentangle grazing and niche competition as shown in equation (4). As a simple modification, we assume that the WSW sample is dynamically at steady state; $P^*$ and $Z^*$ denoting the steady state densities in the environment. Therefore, the initial conditions for our *in silico* experiments for a bottle with dilution factor $F$ are $P = FP^*$ and $Z = FZ^*$ , where $Z^* = Z_0$ and $P^* = K\left(1 - \frac{aZ_0}{r}\right)$. Substituting these quantities into equation (4) we find:

$$\frac{1}{P}\frac{dP}{dt}\bigg|_{FP^*,FZ^*} = r\left(1 - F\right). \tag{7}$$

In the limit when $F = 0$ , we find that the per capita rate of change is $r$ ; whilst at $F = 1$ , the per capita rate of change is $0$. At steady state plankton growth is balanced by plankton mortality. When zooplankton pressure is high, mortality due to grazing is much greater than mortality due to niche competition and so $r \approx aZ_0$ . However, as zooplankton pressure decreases the relative importance of mortality due to competition between plankton increases and $r$ is no longer a good estimate of grazing mortality. Thus, an alternative interpretation for the slope calculated by the classical dilution method is that it represents **the rate of plankton mortality by both zooplankton grazing and by niche competition.**

### 3.4 Diluting zooplankton alone can yield accurate grazing mortality estimates

In the classical dilution method the incubated samples contain diluted levels of both plankton and zooplankton (Landry and Hassett, 1982). Rather than enriching the medium (see Landry et al. 1995) we propose a new approach: altering the filter to exclude zooplankton but not plankton cells. Thus, altering the proportion of WSW used within each treatment will only change the initial zooplankton density – representing a linear gradient between no zooplankton and ambient levels of zooplankton; whilst maintaining ambient levels of plankton. The proposed "Z-dilution" method, is depicted in Figure 3.

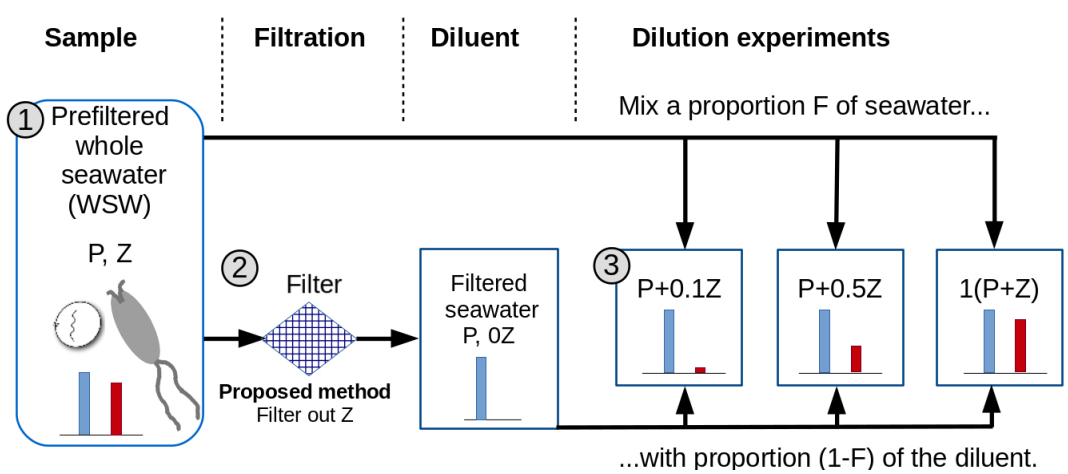


Figure 3: Proposed revision to the classical dilution method. Whilst the classical dilution method (see Figure 1) uses a filter excluding plankton and zooplankton, the proposed method instead uses an alternative filter, able to exclude zooplankton, but through which plankton can pass. Thus constituent levels of zooplankton and plankton within each bottle, shown by red and blue bars respectively, differ to those in the classical dilution experiment.

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In this case, we expect the per capita bottle population dynamics for a dilution level $F$ to be:

$$\frac{1}{P}\frac{dP}{dt}\bigg|_{P_0,FZ_0} = r\left(1-\frac{P_0}{K}\right) - (aZ_0)\,F \tag{8}$$

where the slope of the dilution curve is here found as the grazing rate. This slope is precisely the baseline grazing

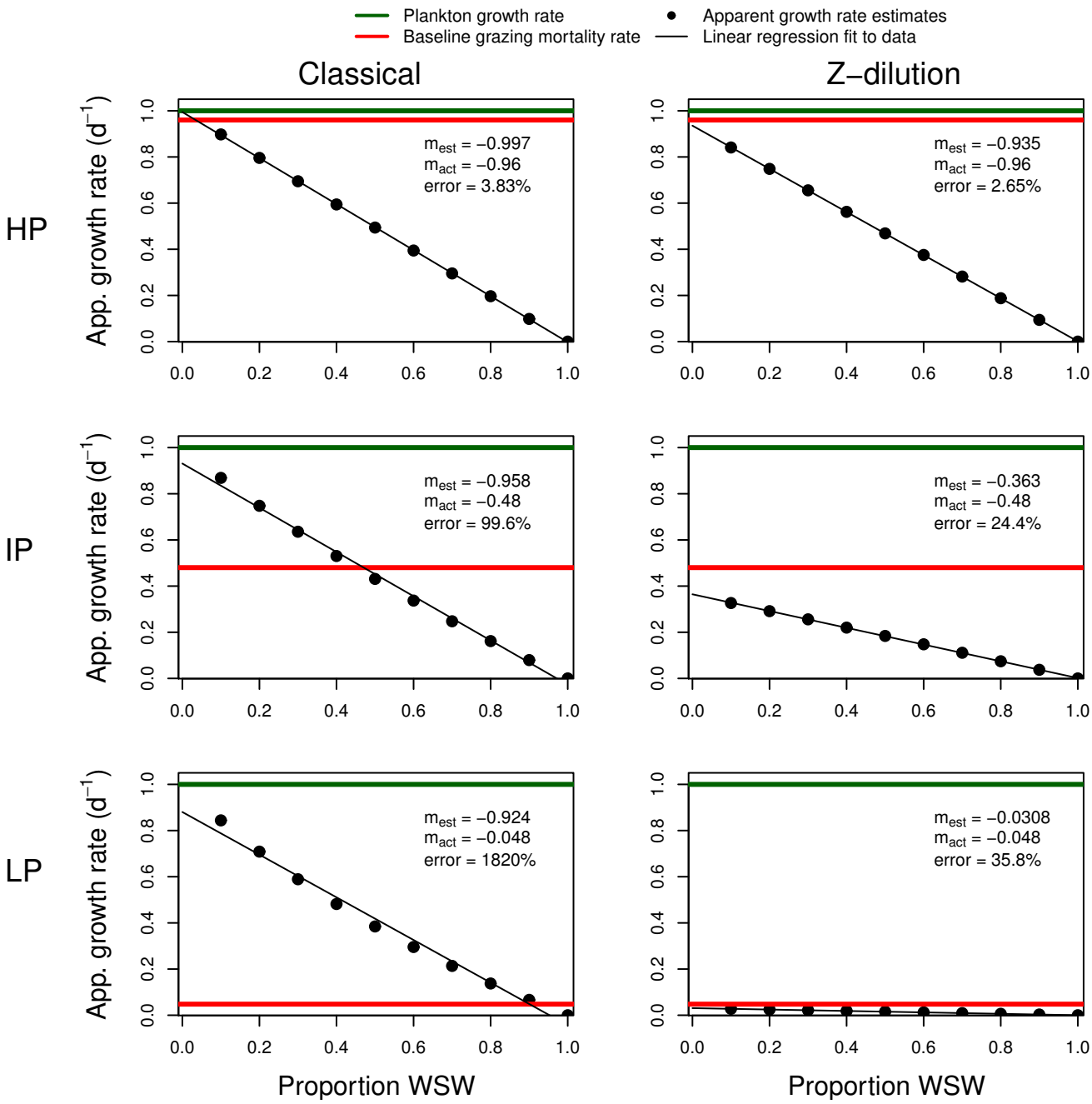


Figure 4: Apparent growth rates for each experimental bottle after a 24h incubation period when using the classical dilution method and the revised dilution method for three environments with different zooplankton pressure conditions (LP: Low pressure, IP: Intermediate pressure and HP: high pressure, as defined in Figure 2). The estimated mortality rate ($m_{est}$) found as the linear regression slope, the baseline mortality rate ($m_{act}$) and the percentage error in estimation are shown for each subplot (all rounded to 3 s.f.).

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mortality rate we hope to estimate using the dilution method. This analytical result suggests this revised method should accurately estimate grazing mortality rates, regardless of the level of zooplankton pressure.

As before, we can investigate the expected per capita growth rate *in silico* using the Z-dilution method under steady state dynamics. By substituting the initial conditions for this revised dilution method, $P = P^*$ and $Z = FZ^*$ , into equation (4) we obtain the per capita plankton rate of change as:

$$\frac{1}{P}\frac{dP}{dt}\bigg|_{P^*,FZ^*} = aZ_0(1-F). \tag{9}$$

Under steady state conditions we expect the dilution curve to have a slope of $aZ_0$ and an intercept of $aZ_0$.

## 3.5 Comparison of classical and revised dilution methods

We now compare the performance and robustness of the classical and Z-dilution methods. We do so by varying the strength of zooplankton pressure. Figure 4 shows the dilution curves measured for both methods after 24h incubation for the three highlighted conditions of zooplankton pressure. The linear regression fits for the classical dilution method have a slope (and intercept) close to the intrinsic growth rate under all three conditions. In contrast, the slope (and intercept) found by the Z-dilution method is closer to the identified baseline rates of zooplankton associated mortality rate. This provides confirmatory evidence to support the analytical results found in equation (7) and equation (9).

As a consequence, the classical and Z-dilution methods give substantially different estimates of grazing mortality rates (Figure 5). When zooplankton pressure is high estimates made by both methods are within 10% of the true

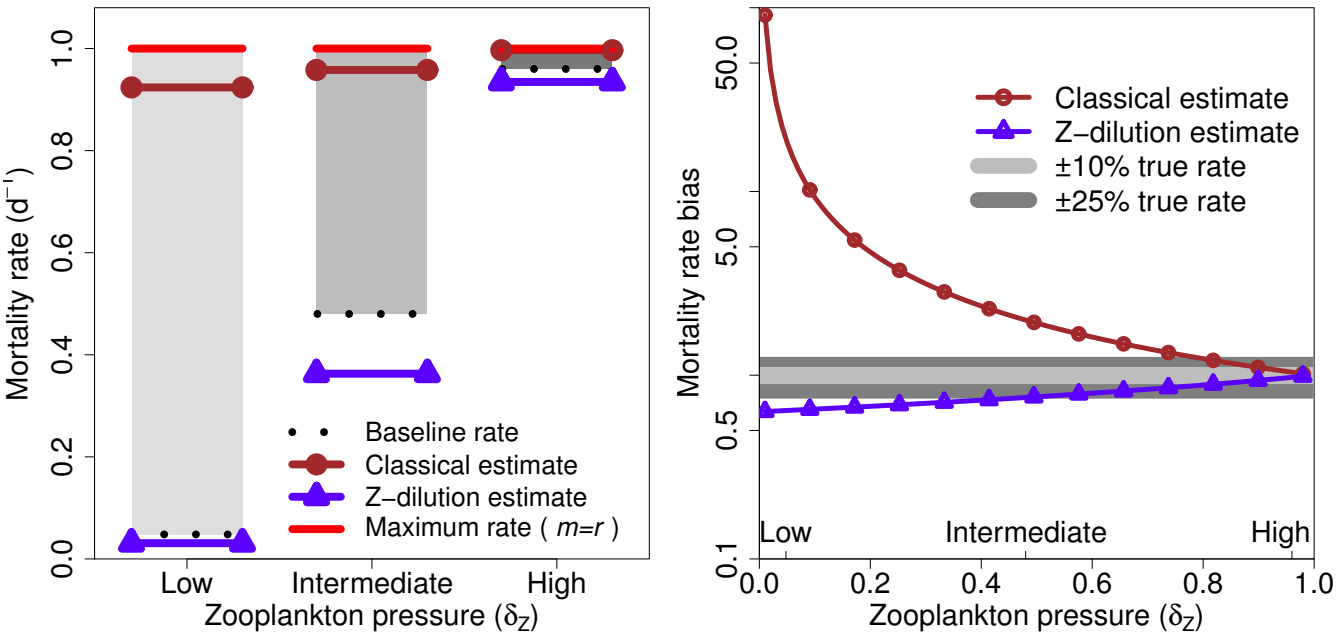


Figure 5: Mortality rate estimates for zooplankton associated plankton mortality made by the classical (Landry and Hassett) and revised dilution methods after a 24h incubation period. (a) Mortality rates and their estimates at three levels of zooplankton pressure. The maximum mortality rate is calculated for the condition when the mortality, $m$, is equal to the plankton growth rate $r$. Baseline mortality rates are shown for each condition. (b) Mortality rate bias is plotted against the level of zooplankton pressure ($\delta_Z$) - the three conditions shown in (a) are marked on the x-axis. Bands indicating $\pm$10% and $\pm$25% differences from the true mortality rate in the sample are shown.

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mortality rate after a 24h incubation period. However, the ability to estimate mortality rates declines with reduced zooplankton pressure. The classical dilution method overestimates zooplankton associated mortality. The revised method, in which only zooplankton are subject to dilution, tends to underestimate the true rate. The degree to which the revised method underestimates the expected rates is substantially less than that by which the classical dilution method overestimates the expected rates. This is particularly evident when zooplankton pressure is low.

# 4 Discussion

Estimated zooplankton grazing rates are central to efforts to understand the relative importance of top-down vs. bottom-up forces in the global oceans. We find that the performance of mortality rate estimates is dependent on the filtering apparatus used by the dilution method and species life-history traits. In this paper we reviewed the robustness of the classical dilution method and a proposed alternative, the "Z-dilution" method. Classical dilution theory works well when niche competition is low relative to zooplankton grazing. But, in circumstances when niche competition is an important control of plankton populations, we predict that the classical method will lead to an overestimation of grazing mortality. Instead, we find that diluting zooplankton, but not plankton, represents a feasible approach to isolate the effects of grazing from niche competition. In doing so, the method goes beyond efforts to estimate grazing exclusively - whether in the classical dilution method or in extensions that include enrichment (Landry and Hassett, 1982; Landry et al., 1995).

We found that the slope of the dilution curve in the classical dilution method is the combined effect of niche competition and zooplankton grazing (equation (4)). Our work suggests that if equation (3) is a reasonable description of the population dynamics within each dilution bottle, then finding the differences between the slopes (or equivalently the intercepts) of the classical and the Z-dilution methods would provide a way to quantify the effects of niche competition. We note that an alternative and complementary approach for assessing the relative impact of niche competition could be found by measuring the differences between the slopes of the classical (Landry and Hassett, 1982) and nutrient-enriched (Landry et al., 1995) dilution experiments. If niche competition is unimportant, then one should expect to recover the same dilution curve for the classical, the nutrient-enriched, and the Z-dilution methods. We also note that in the Z-dilution method we assume the use of a filter that can exclude zooplankton, but allow all plankton to pass through. In practice, it will be important to evaluate the extent to which size- or feature-specific filtering can be achieved.

This is not the first time that the use of the dilution method has been critically assessed (e.g. Evans and Paranjape 1992; Landry et al. 1995; Agis et al. 2007; Calbet and Saiz 2013; Chen et al. 2014) or suggested to overestimate the impact of grazing. Indeed, a recent meta-analysis found that many studies erroneously applied tests that assessed the significance of mortality rates based on whether they were greater than or less than zero, rather than strictly greater than zero (Latasa, 2014; Landry, 2014). Other studies have also suggested that grazing rates may be overestimated due to changes within the grazer communities (Dolan and McKeon, 2005). Assuming grazer communities are constant on the timescale of dilution experiments we found that zooplankton associated mortality rates, found via the classic dilution method, may be overestimated in environments when zooplankton pressure is low. However, we suggest that previous rate estimates made by the classical dilution method could potentially be used as an upper bound for the true grazing mortality rate. Similarly, estimates made via nutrient-enhanced approaches could be compared to those here as a means to gauge the *relative* importance of grazing with respect to niche competition. We encourage those who use the dilution method to investigate the Z-dilution method as a way to gain quantitative understanding of the importance of niche competition. The present analysis further supports the need to combine theory and experiments together to improve understanding of marine microbial ecology.

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# Supporting Information

| Symbol | Description | Value | Units |
|---|---|---|---|
| $r$ | Intrinsic per capita growth rate of plankton | $1$ | day$^{-1}$ |
| $K$ | Plankton carrying capacity | $2.2 \times 10^7$ | plankton ml$^{-1}$ |
| $a$ | Zooplankton attack rate | $4.8 \times 10^{-5}$ | ml/(zooplankton·day) |
| $Z_{0L}$ | Initial zooplankton density at low pressure | $1000$ | zooplankton ml$^{-1}$ |
| $Z_{0I}$ | Initial zooplankton density at intermediate pressure | $10000$ | zooplankton ml$^{-1}$ |
| $Z_{0H}$ | Initial zooplankton density at high pressure | $20000$ | zooplankton ml$^{-1}$ |
| $P_0$ | Initial plankton density | $K\left(1 - \frac{aZ_0}{r}\right)$ | plankton ml$^{-1}$ |

Table S1: Ecological parameters used in this study.