

# Dilution effects on microzooplankton in dilution grazing experiments

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**ABSTRACT:** In dilution experiments, filtered seawater is used to create a gradient of grazing pressure on phytoplankton. Microzooplankton grazing is estimated by examining phytoplankton growth within the gradient. However, the dilution series also represents a resource gradient for microzooplankton. Here we report the effects of dilution on grazers. In 2 standard dilution experiments, using communities from the eutrophic (chlorophyll *a* = 12 to 15  $\mu\text{g l}^{-1}$ ) Rhode River Estuary, we examined the effects of dilution on different groups of microzooplankters: rotifers, tintinnid ciliates, oligotrich ciliates, predacious ciliates, and *Mesodinium rubrum*. Apparent growth rates of tintinnids and oligotrichs varied with prey concentration, decreasing with the dilution factor from about  $+0.5 \text{ d}^{-1}$  in undiluted whole water to about  $-1 \text{ d}^{-1}$  in the 5% whole water, closely resembling numerical response curves. Among tintinnids, there was an increase in the relative abundance of larger tintinnids in the time 24 h samples of dilute treatments compared to the less dilute treatments. No consistent dilution effect was shown by rotifers or predacious ciliates. The growth rates of the photosynthetic ciliate *M. rubrum* increased with dilution, resembling the typical pattern of chlorophyll *a* and autotrophic nanoplankton. Grazer growth in undiluted waters and grazer mortality in dilute water may be common and result in uncertainty in measured grazing rates. We urge that grazers be examined in grazing experiments not only to assess possible artifacts in grazing rate estimates, but also to provide information, beyond a simple grazing rate, on the grazer populations.

**KEY WORDS:** Ciliate · Oligotrich · Tintinnid · Phytoplankton · Nanoplankton · Rotifer · Flagellate

## INTRODUCTION

In 1982, Landry & Hassett introduced a dilution approach to the measurement of herbivory by microzooplankton in natural seawater communities (Landry & Hassett 1982). Dilution reduces encounter rates between phytoplankton and their microzooplankton grazers. The method consists of amending natural assemblages of phytoplankton and grazers with varying proportions of filtered seawater creating a dilution series, and then estimating the grazing rate as the increase in apparent phytoplankton growth rate with

the dilution factor. Specifically, the microzooplankton grazing rate is estimated as the slope of a regression of apparent phytoplankton growth in the various dilutions against dilution factor. The growth rate of the phytoplankton is estimated as the apparent growth rate extrapolated to 100% dilution (growth in the absence of grazers). The approach relies on relatively few assumptions: (1) phytoplankton growth rate is limited neither by nutrients nor by density dependence, (2) phytoplankton growth is exponential, and (3) consumption rates by microzooplankton are linear with respect to phytoplankton concentration.

The method requires little manipulation of natural communities other than dilution and the addition of nutrients when these are in short supply (to satisfy

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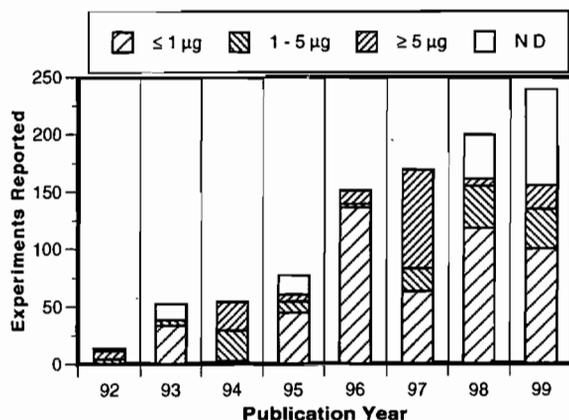


Fig. 1. Annual number of dilution experiments published from 1992 to 1999, reported in studies of marine and estuarine systems, classified by the initial chlorophyll concentration of the water used ( $\mu\text{g l}^{-1}$ ). Note the regular increases in the number of experiments reported and the large proportion conducted in waters with low chlorophyll concentrations ( $\leq 1 \mu\text{g chl l}^{-1}$ ). ND: no data or chlorophyll concentrations were not clearly relatable to individual experiments. Numbers of experiments include those with insignificant or negative grazing rates when reported. Data from 1992: Evans & Paranjape, McManus & Ederington-Cantrell, Verity & Vernet. Data from 1993: Burkill et al. b,c, Harrison et al., Landry et al., Verity et al., Weeks et al. Data from 1994: Kamiyama, Neuer & Cowles. Data from 1995: Burkill et al., Dagg, Fahnenstiel et al., Gifford et al., Landry et al. a,b, Sime-Ngando et al., Waterhouse & Welschmeyer. Data from 1996: Ayukai, Boyd et al., Chavez et al., Froneman & Perissinotto a,b, Froneman et al. a,b, Gallegos et al., Strom & Strom, Verity et al. Data from 1997: Froneman & McQuaid, Froneman et al., Gallegos & Jordan a, Latasa et al., Nejstgaard et al., Reckermann & Veldhuis, Scharek et al., Tamigneaux et al., Tsuda & Kawaguchi. Data from 1998: Ayukai & Miller, Cotano et al., Froneman & Balarin, James & Hall, Landry et al., Lessard & Murrell, Murrell & Hollibaugh, Ruiz et al., Schülter. Data from 1999: Brown et al., Caron & Dennett, Edwards et al., Gaul et al., Johnson et al., Kuipers & Witte, Lehrter et al., Rivkin et al.

Assumption 1). Given its simplicity, it is not surprising that the method has become increasingly popular in recent years, especially in open ocean waters of low chlorophyll content (Fig. 1). Dilution experiments are now a standard protocol for the estimation of microzooplankton herbivory (Burkill et al. 1993a, Landry 1993). Taxon- or pigment-specific mortality rates of phytoplankton have been estimated using the dilution method and HPLC measurements of pigments (e.g., McManus & Ederington-Cantrell 1992, Verity et al. 1993, Fahnenstiel et al. 1995, Waterhouse & Welschmeyer 1995, Verity et al. 1996, Latasa et al. 1997). The analysis of samples using flow cytometry has provided specific rates of mortality and growth for picoplankters such as *Synechococcus* and *Prochlorococcus* (e.g., Landry et al. 1995a, Reckerman & Veldhuis 1997, Johnson et al. 1999). The approach has also been

adapted for use in a wide variety of environments ranging from studies of bacterivory in the benthos (Kemp 1994) to use in anoxic waters (Detmer et al. 1993), sea-ice melt ponds (Wickham & Carstens 1998) and temperate lakes (Elser et al. 1995, Jones & Young 1998).

Despite wide-spread adoption, the method is not without problems. Publications commonly report instances of uninterpretable results, i.e. plotting apparent phytoplankton growth against dilution factor does not yield a significant regression (0.05 level). When this phenomenon is reported, frequencies range from 6 to 66% of the experiments run (Kamiyama 1994, Gifford et al. 1995, Landry et al. 1995a, Reckermann & Veldhuis 1997, Lessard & Murrell 1998, Murrell & Hollibaugh 1998, Caron & Dennett 1999, Gaul et al. 1999, Kuipers & Witte 1999). Some of these problematic results are likely due to the fact that slight slopes, or low grazing rates, are difficult to detect with regression analysis using the small 'n' values commonly employed (8 to 15). Other possibilities concern violation of the assumptions underpinning dilution assays. As nutrients are often added or measurable, nonlinear relationships between phytoplankton mortality rates and the dilution factor appear the most probable explanation.

Problems which have received particular attention with regard to the consumption rates of microzooplankton include differences in the per-capita feeding rate of grazers in the different dilution treatments (Gallegos 1989, Evans & Paranjape 1992) and the growth of grazers in the undiluted samples (Gallegos et al. 1996). Interestingly, the response of the grazer community to dilution in terms of apparent growth and mortality, although recognized as a possible problem (Landry et al. 1995b), has not been explored in any detail. To our knowledge, only a single study has provided data on apparent changes in microzooplankton concentrations; all non-tintinnid taxa were combined and no consistent patterns were noted (Gifford 1988).

In this study, we examined the microzooplankton community in 2 dilution experiments in all the treatments ranging from 100 to 5% whole water. We found that (1) oligotrich and tintinnid ciliates, which generally dominate microzooplankton communities, showed apparent growth rates which varied in proportion to available nanoplanktonic prey, (2) there were obvious differences in the species composition of the tintinnid community, (3) apparent growth of *Mesodinium rubrum* increased with dilution factor, and (4) predacious ciliates (*Didinium*, *Cyclotrichium*) and rotifers (*Synchaeta* spp.) showed no consistent changes in relation to dilution. The combined effects of grazer mortality in dilute treatments and growth in undiluted treatments confound estimates of grazing rate and the phenomenon may be common.

## METHODS

**Study site.** Dilution experiments were run in the Rhode River Estuary (38° 53' N, 76° 32' W), a eutrophic subestuary of the Chesapeake Bay. Detailed data on the compositions, concentrations, and seasonal cycles of nutrients, phytoplankton, and zooplankton have appeared previously (Dolan & Gallegos 1991, 1992, Gallegos et al. 1992, 1997, Gallegos & Jordan 1997a,b, Jordan et al. 1991a,b).

**Experimental protocol.** A standard dilution experiment protocol, used regularly for several years, was employed (see Gallegos & Jordan 1997a). Briefly, duplicate 2 l polycarbonate bottles were used to incubate whole estuarine water at 5 dilution levels: 100, 80, 40, 20, and 5 % whole water, diluted with GF/F-filtered water. All whole water aliquots were drawn from a single, well-mixed carboy, including samples for initial concentrations of nutrients, chlorophyll and microorganismal abundances. Nutrient salts (NaNO<sub>3</sub> and Na<sub>2</sub>HPO<sub>4</sub>) were added to the bottles to yield final concentrations of 60 µM NO<sub>3</sub> and 6 µM PO<sub>4</sub>. In Expt 1 additional bottles of whole water without nutrients added were incubated to check for nutrient effects on grazers. The bottles were incubated *in situ* at a depth of 1 m for 24 h, retrieved and returned to the laboratory for time 24 h ( $t_{24}$ ) sampling.

**Sample analysis.** Samples for chlorophyll analysis were filtered onto GF/F glass fiber filters and, following acetone extraction, the concentrations were determined using a Turner Designs 10-AU fluorometer. Nutrient analysis methods followed standard protocols outlined in Jordan et al. (1991a). Sample aliquots of 20 or 40 ml for enumerations of nanoplankton and bacteria were fixed with ice-cold glutaraldehyde (1 % final conc.). Bacteria, and autotrophic and heterotrophic nanoplankton were counted using an epifluorescence microscope and DAPI-stained preparations following standard protocols (e.g., Dolan & Gallegos 1991). For ciliate enumerations, sample aliquots of 50 ml (Initial,  $t_{24}$  100, and 80% whole water) or 500 ml ( $t_{24}$  samples for 40, 20, 5 % whole water) were fixed with acid Lugol's (2 % final conc). Prior to settling in inverted microscope sedimentation chambers, the 500 ml samples were pre-concentrated to 75 ml volumes by first settling in 500 ml graduated cylinders for 48 to 72 h, followed by slowly siphoning off the top 425 ml. We have used this pre-concentration method extensively and found no significant loss of ciliates (Dolan & Marsé 1995, Dolan et al. 1999). Single or multiple 10 ml subsamples, providing raw counts of at least 100 oligotrichs and 100 tintinnids, were examined using an inverted microscope. Parallel samples of whole water (25 ml) from initial water samples were fixed with glutaraldehyde and Lugol's and then examined with an

inverted microscope equipped with epifluorescence to permit the classification of oligotrich morphotypes, distinguishable in Lugol's-fixed material, as either mixotrophic or heterotrophic, based on chlorophyll-fluorescence visible in glutaraldehyde-fixed individuals. Ciliates were placed in the categories of: mixotrophic oligotrichs, heterotrophic oligotrichs, tintinnids, predacious ciliates (*Didinium* spp., *Cyclotrichium* spp.), *Mesodinium rubrum* and others (mostly *Balanion* spp., *Euplotes* spp.). For rotifers, samples ranging from 200 to 400 ml were concentrated to 20 ml through a 20 µm mesh nitex screen and 2 to 20 ml aliquots of this concentrate, providing raw counts of at least 100 rotifers, were settled and examined using an inverted microscope.

**Data analysis.** The apparent growth rates in individual bottles were calculated as  $\ln(\text{conc}_{24}/\text{conc}_0)$ , where  $\text{conc}_{24}$  and  $\text{conc}_0$  are, respectively, the final measured concentration and the initial concentration, based on initial whole water concentration and dilution factor. Regression analysis of apparent growth rates against 'fraction unfiltered water' was run using Statview 4.5. Regression analysis was also used to relate apparent phytoplankton (as chlorophyll) growth rates to different measures of grazing pressure (as opposed to fraction unfiltered or dilution factor) in the individual containers. As measures of grazing pressure we considered: (1) ciliate concentration at  $t_{24}$  as %  $t_0$  whole water concentration, and (2) a measure of average ciliate concentration, following Gallegos et al. (1996), estimated as the geometric mean predator density (GMPD) calculated as  $[\text{conc}_0 \times \text{conc}_{24}]^{0.5}$ , converted to %  $t_0$  ciliate concentration. For these calculations, ciliate concentration was considered as total ciliates, with the exception of *Mesodinium rubrum*. We recognize that both apparent growth rates and ciliate concentrations, unlike 'fraction unfiltered', cannot be measured without error, and therefore correlation rather than regression should be used—we did so only to estimate potential error in ignoring actual predator concentrations.

We also estimated grazing rates by comparing the 2 extremes of the dilution series. The additional grazing estimates, which we term '100 versus 5%', were calculated using data only from the 100 and 5 % whole water treatments, by considering the containers, respectively, as representing vessels with and without grazers. A 'raw 100 versus 5%' grazing rate,  $g\ d^{-1}$ , was calculated as the difference between apparent chlorophyll growth rates in each container with and without grazers (average apparent growth rate in the 0.05 dilution factor containers). An 'adjusted 100 versus 5%' rate, corrected for grazer growth during the experiment was also calculated. The correction for grazer growth consisted of first calculating a per-capita graz-

ing rate by dividing the 'raw 100 versus 5%' grazing rate by the average number of grazers in the containers (GMPD), and then multiplying the resulting per-capita rate by the initial concentration of grazers (for our purposes only ciliates were considered) to obtain a grazing rate corrected for the growth of grazers in the containers. Finally, in the second experiment the plot of phytoplankton apparent growth rate against dilution factor indicated nonlinear feeding kinetics by the microzooplankton (Gallegos 1989). For that experiment we also estimated  $g$  using Eq. (11) in Gallegos (1989).

## RESULTS

Physical and chemical data for the dilution experiments are given in Table 1. End of experiment nutrient samples were not taken for Expt 1, but Expt 2 samples indicated that most of the added nutrients were utilized in all the dilutions (Table 1). Initial and final organismal concentrations for undiluted samples appear in Table 2. The comparison of ciliate concentrations in bottles with and without added nutrients (Expt 1) showed no differences (data not shown).

In both Expt 1 (Fig. 2) and Expt 2 (Fig. 3), apparent growth rates of phytoplankton (based on chlorophyll),

photosynthetic nanoplankton-ANAN, and the photosynthetic ciliate *Mesodinium rubrum* were highest in dilute waters and decreased with the proportion of whole water or 'fraction unfiltered'. For chlorophyll growth rates in Expt 2, this decrease appeared nonlinear with no further decrease above dilution factor = 40% (Fig. 3). In contrast, an opposite trend of growth rate (increasing with fraction unfiltered) was evident for tintinnids and heterotrophic oligotrichs. The apparent growth rates of mixotrophic oligotrichs (Expt 1 only), predacious ciliates, heterotrophic nanoplankton and rotifers varied irregularly. Regression statistics of the relationships with 'fraction unfiltered' given in Table 3 indicated the strongest relationships between 'fraction unfiltered', or dilution factor, and growth rates of oligotrichs and tintinnids. In both experiments, the overall range of net growth rates was wider for ciliates than for chlorophyll (Figs. 2 & 3; note change in scale for chlorophyll plots).

For the dominant taxa of oligotrichs and tintinnids in each experiment, apparent growth rates plotted against initial nanoplankton prey concentration (PNAN + ANAN) in the various treatments yielded numerical response curves with a typical hyperbolic shape (Fig. 4). Converting nanoplankton concentrations into carbon units by assigning the value of 11 pg C nanoplankton<sup>-1</sup> (average biovolume of 55  $\mu\text{m}^3 \times$

Table 1. Physical and chemical characteristics of waters employed in the dilution experiments. For Expt 2, final ( $t_{24}$ ) nutrient concentrations are given  $\pm$ SE of the 10 bottles. nd: no data

	T (°C)	Salinity (‰)	NH <sub>4</sub> ( $\mu\text{M}$ )	NO <sub>3</sub> ( $\mu\text{M}$ )	PO <sub>4</sub> ( $\mu\text{M}$ )
<b>Expt 1: October 10, 1999</b>					
Initial	18.6	13.2	9.6	0.5	0.2
$t_0$ after adding nutrients			9.6	60	6
$t_{24}$	17.9	nd	nd	nd	nd
<b>Expt 2: October 19, 1999</b>					
Initial	16.4	13.1	4.5	0.3	0.1
$t_0$ after adding nutrients			4.5	60	6
$t_{24}$	16.1	nd	4.8 $\pm$ 0.21	2.8 $\pm$ 1.75	0.6 $\pm$ 0.08

Table 2. Organismal concentrations in Expts 1 and 2, in undiluted waters at the beginning ( $t_0$ ) and end ( $t_{24}$ ) of the experiments of chlorophyll  $a$  in  $\mu\text{g l}^{-1}$  (Chl  $a$ ), phototrophic nanoplankton  $\times 10^{-3} \text{ ml}^{-1}$  (PNAN), heterotrophic nanoplankton  $\times 10^{-3} \text{ ml}^{-1}$  (HNAN), heterotrophic bacteria  $\times 10^{-6} \text{ ml}^{-1}$  (Bacteria), total ciliates excluding *Mesodinium rubrum*  $\text{ml}^{-1}$  (Ciliates), *M. rubrum*  $\text{ml}^{-1}$  (*M. rubrum*) and rotifers  $\text{ml}^{-1}$  (Rotifers). Concentrations at  $t_{24}$  are averages of the duplicate bottles

	Chl $a$	PNAN	HNAN	Bacteria	Ciliates	<i>M. rubrum</i>	Rotifers
<b>Expt 1: October 10, 1999</b>							
Whole water $t_0$	14.8	17.9	6.9	6.1	31.5	53.9	2.0
Whole water $t_{24}$	22.0	35.9	6.2	8.2	43.6	17.9	2.9
<b>Expt 2: October 11, 1999</b>							
Whole water $t_0$	12.7	53.9	12.5	6.3	30.4	38.8	2.4
Whole water $t_{24}$	19.4	90.3	4.7	4.8	33.3	20.4	2.1

## EXPERIMENT 1

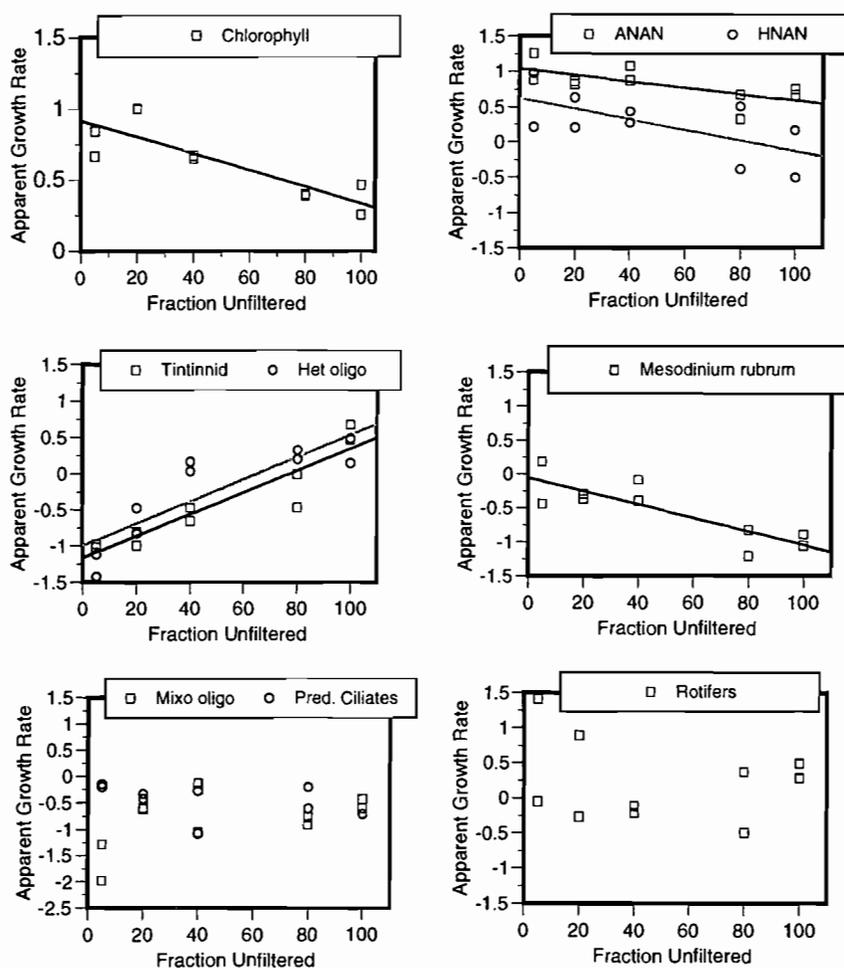


Fig. 2. Dilution plots from Expt 1. Apparent growth rates increased with dilution factor for chlorophyll, autotrophic nanoplankton (ANAN), heterotrophic nanoplankton (HNAN), and *Mesodinium rubrum*. In contrast, the growth rates of tintinnids and heterotrophic oligotrichs (Het oligo) declined and no clear effect of dilution was shown by rotifers, mixotrophic oligotrich (Mixo oligo) or predacious ciliates (Pred. Ciliates). Regression parameters are given in Table 3

$0.2 \text{ pg C } \mu\text{m}^{-3}$ ) indicates threshold prey concentrations (below which growth = 0) ranging from 20 to  $100 \text{ ng C ml}^{-1}$  and saturating prey concentrations (above which growth rate does not increase) of 100 to  $300 \text{ ng C ml}^{-1}$ .

The examination of  $t_{24}$  samples showed distinct differences between dilution treatments in the composition of tintinnid ciliate communities. There appeared to be a shift within the tintinnid community in both experiments, towards an increase in the dominance of species with larger loricas in the more dilute treatments, reflected in increases in average lorica length of the tintinnid community (Figs. 5 & 6). In the most dilute treatments, none of the tintinnid species showed positive apparent growth, therefore the changes in tintinnid community composition reflect differential survival of different species rather than differential growth.

As a metric of grazing pressure, using the regression of dilution factor against apparent chlorophyll growth rates gave higher grazing rate estimates than regressions against ciliate concentrations, either time-averaged or at the end of incubation (Table 4). Specifically, grazing rate estimates made using time-averaged (GMPD) ciliate concentrations, appropriate when the prey concentration is in the linear response range of the microzooplankton, were 93% of the rates estimated using the dilution factor for Expt 1. Regressions of end-point ciliate concentrations against apparent growth rates yielded grazing estimates equal to 81 and 63% of the dilution factor estimates for Expts 1 and 2, respectively. Grazing rate estimates based on the difference between phytoplankton growth in whole water and 5% whole water, i.e. 'raw 100 versus 5%', gave rates equivalent to 67 and 103% of the dilution

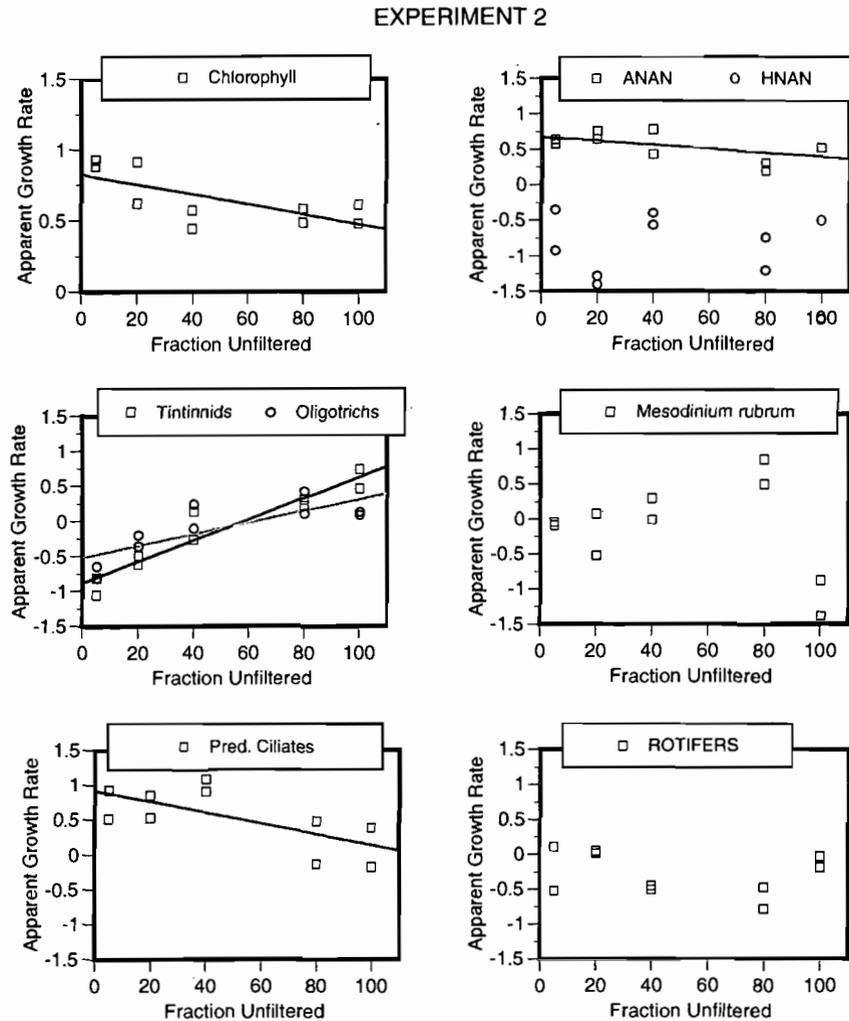


Fig. 3. Dilution plots from Expt 2. Apparent growth rates increased with dilution factor for chlorophyll, autotrophic nanoplankton (ANAN), and predacious ciliates (Pred. Ciliates). As in Expt 1, the growth rates of tintinnids and heterotrophic oligotrichs declined and no clear effect of dilution was shown by heterotrophic nanoplankton (HNAN), or *Mesodinium rubrum*. All oligotrichs were grouped in the experiment as mixotrophic forms were not easily distinguished from heterotrophic forms. Regression parameters are given in Table 3

Table 3. Parameters of regression relationships between dilution (fraction unfiltered) and apparent growth rates of phytoplankton (Chl a), phototrophic nanoplankton (PNAN), heterotrophic nanoplankton (HNAN), all ciliates excluding *Mesodinium rubrum* (Total ciliates), all oligotrichs (Oligotrichs), all tintinnids (Tintinnids), *Mesodinium rubrum* (*M. rubrum*), predacious ciliates (Pred. cils) and rotifers (Rotifers). Significant relationships are shown in **bold**; n = 10 for all analyses

	Chl a	PNAN	HNAN	Total ciliates	Oligotrichs	Tintinnids	<i>M. rubrum</i>	Pred. cils	Rotifers
<b>Expt 1: October 10, 1999</b>									
Slope	<b>-0.6</b>	<b>-0.4</b>	<b>-0.7</b>	<b>1.3</b>	<b>1.5</b>	<b>1.5</b>	<b>-1.0</b>	-0.4	-0.3
r <sup>2</sup>	0.73	0.45	0.41	0.83	0.63	0.87	0.72	0.23	0.20
Probability	0.002	0.033	0.045	0.001	0.007	0.001	0.002	0.166	0.582
<b>Expt 2: October 11, 1999</b>									
Slope	<b>-0.3</b>	-0.3	-0.3	<b>1.1</b>	<b>0.9</b>	<b>1.4</b>	-0.5	<b>-0.8</b>	-0.2
r <sup>2</sup>	0.50	0.33	0.25	0.85	0.65	0.89	0.05	0.47	0.05
Probability	0.023	0.083	0.546	0.001	0.005	0.001	0.145	0.028	0.519

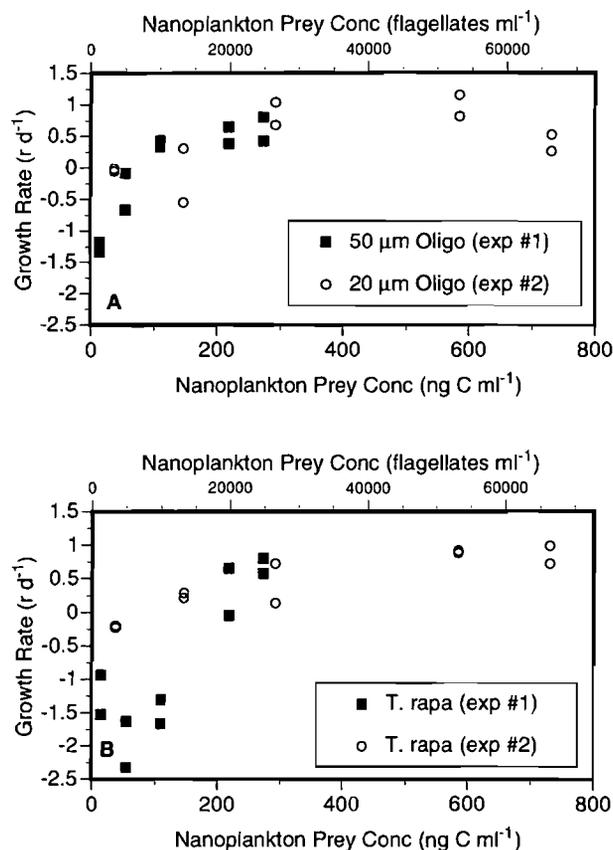


Fig. 4. Apparent growth rate of numerically dominant oligotrichs and tintinnids in the dilution experiments as a function of initially available nanoplankton prey. (A) Oligotrich growth rates: in Expt 1 for a 50 µm long conical *Strombidium* sp., in Expt 2 for a spherical 20 µm diameter *Strombidium* sp. (B) Growth rates of the tintinnid *Tintinnopsis rapa* in Expts 1 and 2

factor rates, and the adjusted 100 versus 5% rates were 67 and 86% of the dilution factor rates. In contrast, both the phytoplankton growth rate and microzooplankton grazing rate in Expt 2 were higher when calculated according to nonlinear feeding kinetics (Table 4). This appears to result from the formulation of Eq. (11) of Gallegos (1989), which calculates  $g$  for initial conditions of the experiment, while accounting for the fact that per-capita grazing declines during the course of the experiment when feeding is nonlinear and phytoplankton prey density is increasing (i.e. there was a positive net growth rate of phytoplankton in Expt 2, Fig. 3).

## DISCUSSION

Our experimental results show that within a dilution series considerable apparent growth and mortality can

occur among ciliates, and different functional groups of ciliates display distinct patterns of growth and mortality. Typical grazers of nanoplankton, oligotrichs and tintinnids, showed rates of growth and mortality which co-varied with the availability of nanoplankton prey (e.g., Fig. 4). Clear differences between dilution treatments were evident in the composition of the tintinnid community at the end of the experiments (Figs. 5 & 6), likely due to lower mortality rates of larger tintinnids compared to small species in the dilute treatments. Predacious ciliates, raptorial feeders on dinoflagellates and small ciliates (Dolan 1991), exhibited a different pattern, showing either no clear trend (Fig. 2) or increases in apparent growth rates with dilution (Fig. 3). As we did not sample all the dilution treatments at  $t_0$  we can only describe growth rates as apparent growth rates. However, at the end of the experiments, the ciliate community composition certainly differed between the different dilution treatments, and to the extent that different ciliate taxa ingest different prey items, grazing patterns would vary between dilution treatments.

Attempts to extrapolate experimental findings of patterns of selective grazing to *in situ* populations would be complicated considerably by qualitative differences in grazer communities between different dilution treatments, and between the  $t_0$  and  $t_{24}$  communities. Dilution experiments have often been employed to examine not only rates, but also specific patterns of microzooplankton grazing. Dilution experiments have been used to provide estimates of mortality rates with regard to different size-fractions of phytoplankton (Kamiyama 1994, Gifford et al. 1995, Froneman & Perissinotto 1996a,b, Froneman et al. 1996a,b, Gallegos et al. 1996, Strom & Strom 1996, Froneman & McQuaid 1997, James & Hall 1998, Kuipers & Witte 1999). For individual phytoplankton taxa, many mortality rate estimates have been from dilution experiments with microscopically identified taxa (e.g., Landry et al. 1993, Fahrenstiel et al. 1995, Murrell & Hollibaugh 1998, Rivkin et al. 1999) using pigment data (e.g., McManus & Ederington-Cantrell 1992, Verity et al. 1993, 1996, Waterhouse & Welschmeyer 1995, Latasa et al. 1997) or by flow cytometry (Landry et al. 1995a,b, 1998, Brown et al. 1999). Our results show distinct differences between grazer communities at the beginning and end of dilution experiments. One might expect the relative importance of grazing by 'starvation-resistant forms' (e.g., small metazoans, large tintinnids, predacious ciliates) to be exaggerated compared to the natural grazer communities in dilution experiments. Therefore, evidence of selective grazing by microzooplankton, based on differences in mortality rates of certain size-fractions of chlorophyll or individual taxa, in dilution experiments

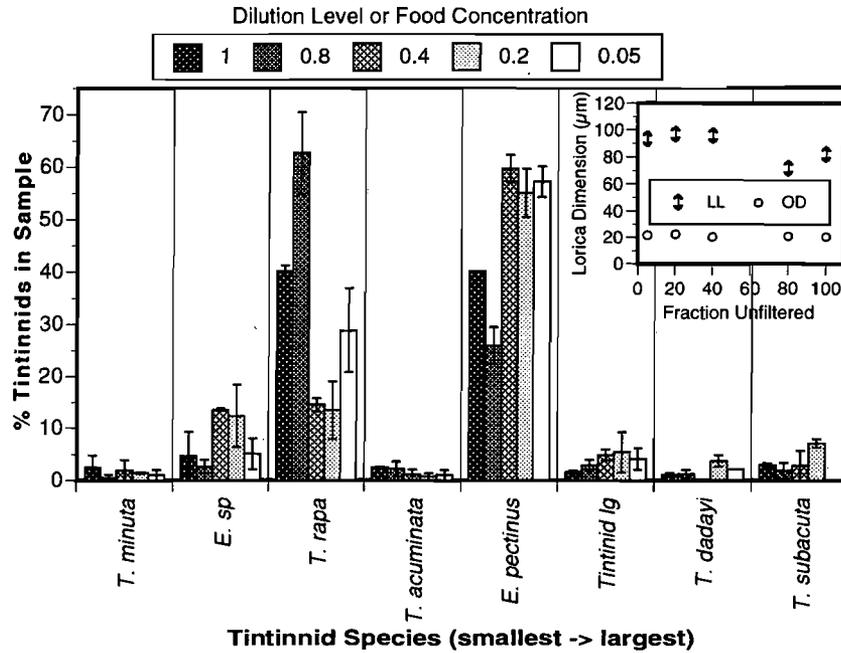


Fig. 5. Expt 1. Composition of the tintinnid ciliate community in the different dilution treatments (1 to 0.05) in the  $t_{24}$  samples. Error bars show the range of the % values in the duplicate samples. *Tintinnopsis* species appear as 'T.', *Eutintinnus* species as 'E.', *E. sp* is about 50  $\mu\text{m}$  in length. *Tintinnidium lg* is a large *Tintinnidium* species, possibly *T. mucicola*. Note the declines in the relative importance of most of the smaller species with dilution factor. Inset graph shows overall averages of tintinnid lorica dimensions in the dilution treatments, oral diameter (OD) and lorica length (LL). Average LL was greatest in highly diluted waters with low food concentrations

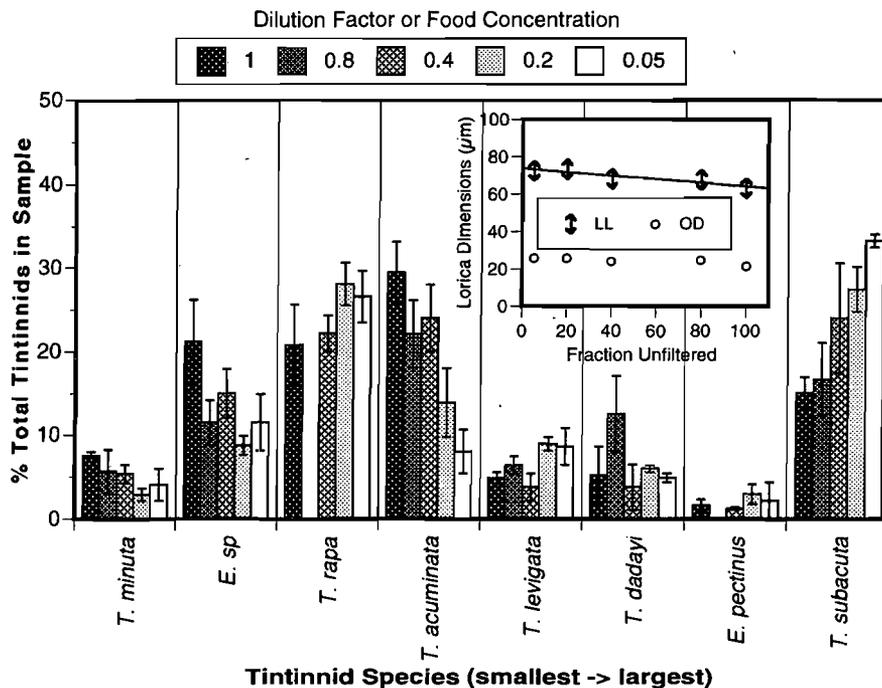


Fig. 6. Expt 2. Composition of the tintinnid ciliate community in the different dilution treatments (1 to 0.05) in the  $t_{24}$  samples. Error bars show the range of the % values in the duplicate samples. Species as in Fig. 5. Similar to data from Expt 1 (Fig. 5), smaller species formed a smaller portion of the tintinnid community in samples from the more dilute treatments. Inset graph shows overall community averages of tintinnid lorica dimensions in the dilution treatments, oral diameter (OD) and lorica length (LL). Similar to the trend found in Expt 1, average LL increased as food levels decreased, and in this case average LL was significantly related to the fraction unfiltered ( $p < 0.05$ )

Table 4. Comparison of estimates of grazing rates ( $g$ ), phytoplankton growth ( $k$ ) and the ratio of grazing to growth ( $g/k$ ) calculated by regression of apparent chl  $a$  growth against (1) dilution factor, (2) average ciliate concentrations (geometric mean predator density [GMPD]), (3) ciliate concentrations at the end of the experiment ( $t_{24}$  [ciliates]). Additional rate estimates were calculated comparing apparent chlorophyll growth in bottles of undiluted water versus 5% whole water, either uncorrected for ciliate growth (Raw 100 vs 5%) or corrected (Adj 100 vs 5%). For Expt 2, grazing was also calculated using the Gallegos (1989) equation which corrects for nonlinear feeding kinetics (NLFK). Rates given  $\pm$  SE

	Dilution factor	GMPD	$t_{24}$ [ciliates]	Raw 100 vs 5%	Adj 100 vs 5%	NLFK
Expt 1, $g$	$0.58 \pm 0.13$	$0.54 \pm 0.13$	$0.47 \pm 0.14$	$0.39 \pm 0.10$	$0.39 \pm 0.13$	
Expt 2, $g$	$0.35 \pm 0.12$	$0.27 \pm 0.10$	$0.22 \pm 0.09$	$0.36 \pm 0.07$	$0.30 \pm 0.05$	0.42
Expt 1, $k$	$0.92 \pm 0.08$	$0.87 \pm 0.08$	$0.82 \pm 0.08$	$0.75 \pm 0.09$	$0.75 \pm 0.09$	
Expt 2, $k$	$0.82 \pm 0.08$	$0.80 \pm 0.07$	$0.79 \pm 0.07$	$0.91 \pm 0.02$	$0.91 \pm 0.02$	0.91
Expt 1, $g/k$	0.63	0.62	0.57	0.52	0.52	
Expt 2, $g/k$	0.43	0.34	0.28	0.40	0.33	0.46

should be viewed with caution as the experiments may be biased in favor of starvation-resistant grazers.

In our experiments, not only did ciliate community composition differ among treatments, but the final ciliate abundance deviated considerably from dilution factor. A large part of the deviation of ciliate abundance from dilution factor was due to apparent growth and mortality of oligotrichs and tintinnids. In both experiments, the numerically dominant oligotrich and tintinnid showed differences in apparent growth rates among treatments, which closely resembled classic numerical response curves when growth rates were plotted as a function of prey concentration (Fig. 4). Despite slightly lower initial chlorophyll concentration (Table 2), the nanoplankton prey concentrations in Expt 2 were substantially higher than those in Expt 1 (Fig. 4), coinciding with the observation of nonlinear feeding kinetics (Fig. 3). Ciliate growth rates in Expt 2 appeared saturated at the 3 highest dilution factors (Fig. 4), which was the same range of dilution factors in which chlorophyll growth rates no longer declined. To our knowledge, this is the first demonstration of congruence between nonlinear feeding responses as inferred from apparent growth rates of phytoplankton with numerical response curves of ciliate grazers.

For the 2 numerically dominant oligotrich species, estimating threshold and saturating concentrations of prey gave values falling well within the range of reported values (Fig. 7). While it should be noted that published data may be biased towards 'easily cultured' oligotrichs, the growth responses of the oligotrichs in our experiments did not appear unusual.

Likewise, maximum mortality rates estimated as apparent growth rates of  $-1.0$  to  $-1.5$   $d^{-1}$  are not unusual, although little comparative data exist (see Montagnes 1996, Jeong et al. 1999, Montagnes & Lessard 1999). These high mortality rates are likely due to high weight-specific metabolic rates of oligotrichs. For example, the heterotrophic oligotrich *Strobilidium spiralis* respire 2.8% of cell carbon  $h^{-1}$  at 20°C (Stoecker & Michaels 1991), and in mixotrophic oligotrichs respiration rates range from 1 to 5% cell carbon  $h^{-1}$  (Crawford & Stoecker 1996). If ciliates are unable to replenish cell carbon due to insufficient prey and are respiring 3% of cell carbon  $h^{-1}$ , they would likely suffer high mortality rates within 24 h.

The apparent result of ciliate growth in the undiluted or slightly diluted vessels, and mortality in the high dilution treatments was, that dilution factor was a poor

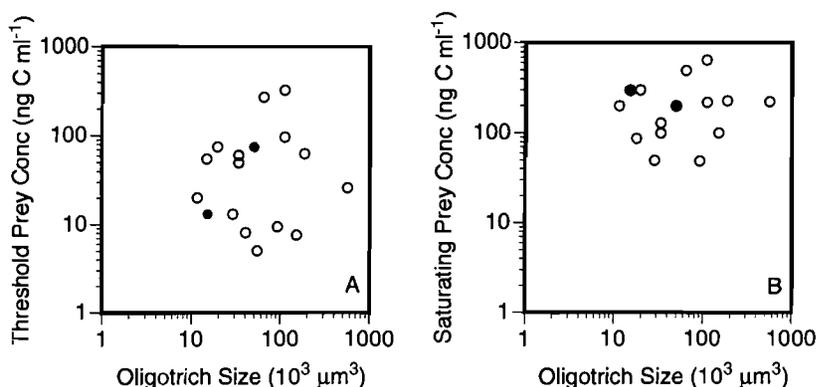


Fig. 7. Threshold and saturating prey concentrations of oligotrichs: comparison of the dominant oligotrichs (see Fig. 4) in the dilution experiments (●) with threshold and saturating prey concentrations (○) reported for a variety of oligotrichs (data from Montagnes 1996, Jeong et al. 1999, Montagnes & Lessard 1999). Note that growth response parameters of the oligotrichs from the dilution experiments do not appear unusual. Interestingly, for oligotrichs, neither minimum nor saturating prey concentrations appear to be related to oligotrich size; overall averages are 75  $ng\ C\ ml^{-1}$  for threshold prey concentration and 222  $ng\ C\ ml^{-1}$  for saturating prey concentration

predictor of ciliate concentration or ciliate grazing pressure. Comparison of grazing rate estimates made using dilution factor or estimates of ciliate concentration suggested that ignoring changes in ciliate concentrations overestimates grazing rates (Table 4). Rates based on simple differences between phytoplankton growth in whole water and growth in highly diluted water (100 vs 5%) were also lower than grazing rates based on dilution factor, with the exception of the 'raw 100 versus 5%' rate for Expt 2 (Table 4). In contrast, the grazing rate calculated on the basis of nonlinear kinetics in Expt 2 was the highest of all. Thus, while dilution is an elegant and relatively non-intrusive manipulation of the plankton community, the impacts of dilution on community structure are evidently more far ranging than commonly recognized. The effects of these changes on grazing rate estimates need further elucidation with modeling studies of more complex food webs.

For the estimates based on differences in 100 versus 5% whole water, the use of 5% whole water to measure phytoplankton growth in the absence of grazers might appear as perhaps providing underestimates. However, Gallegos & Jordan (1997a) demonstrated that apparent growth rate of phytoplankton in highly diluted incubations (fraction unfiltered = 0.05) was a close approximation to the intrinsic growth rate determined by solution of the growth equations, whether linear or nonlinear (Gallegos 1989). Our results showing grazer mortality at high dilution indicate that the approximation succeeds because 0.05 is close to 0 functionally as well as mathematically. That is, grazing pressure in a 95% diluted incubation is even closer to 0 than would be predicted for a dilution factor of 0.05. This result should be robust in waters of all trophic states. It might be difficult to apply growth measured by changes in pigment concentrations in waters of very low chlorophyll concentrations, but filtration or sample volumes are easily adjusted to increase detection limits when examining autofluorescent picoplankton by microscopy or flow cytometry.

The magnitudes of the differences found in grazing rate estimates, rates 63 to 93% of dilution factor rates (without the exception noted above), may appear modest but differences in growth and mortality we found in different dilution treatments, which occurred beginning with chlorophyll-rich waters, could be expected to be larger when working in waters with low chlorophyll concentrations. For example, consider an average threshold prey concentration, below which mortality occurs, of about 75 ng carbon ml<sup>-1</sup> for known oligotrichs (Fig. 7). If chlorophyll-containing prey represent but half the ciliate's ration and the carbon to chlorophyll ratio is 50, mortality will occur at chlorophyll concentrations below 0.75 µg chlorophyll l<sup>-1</sup>. It

should be admitted that such calculations involve a considerable amount of uncertainty and our knowledge of numerical response parameters is perhaps not based on forms characteristic of all wild populations. However, these rough calculations support the view that gradients of ciliate growth and mortality, which lead to an overestimation of grazing rates, possibly occur when dilution series bracket chlorophyll concentrations of 1 µg chlorophyll l<sup>-1</sup> or less.

We have argued that dilution experiments can yield grazing patterns different from natural communities due to changes in the grazer community during the experiment. This possibility has long been recognized (Landry 1993). We have also argued that dilution experiments probably overestimate ciliate grazing rates, especially in low chlorophyll waters, using our experimental data and consideration of known threshold prey concentrations of oligotrichs. Previously, potential problems identified with the dilution approach were the artifacts resulting from changes in grazer abundance in undiluted water (e.g., Gallegos et al. 1996) and changes in individual grazer activities (Gallegos 1989, Evans & Paranjape 1992). Thus, grazer growth in undiluted water is occasionally verified (e.g., Landry et al. 1993, Verity et al. 1993), and nonlinear feeding responses are now commonly considered. Here we have focused on the phenomenon of different changes in grazer abundances in the different dilution treatments.

Aggregate grazer activity, in the form of disappearance rates of fluorescently labelled bacteria (FLB), has been examined in different dilution treatments (Landry et al. 1995b). In experiments using waters of the central equatorial Pacific Ocean, in which *Prochlorococcus* and *Synechococcus* dominate the phytoplankton, average grazing rates from regressions of apparent phytoplankton growth against 'relative grazing' from FLB disappearance, while not significantly different, were lower than rates based on 'dilution factor' (Landry et al. 1995b, Table 3). Interestingly, such results suggest that 'dilution effects' may be less severe among grazers of picoplankton, presumed by most authors to be heterotrophic nanoflagellates (Dolan & Šimek 1999).

Beyond detection of possible artifacts in grazing experiments, the examination of the grazer community can provide a wealth of information. Temporal changes in grazer biomass can be used to infer the efficiencies of transfer between phytoplankton and grazers (e.g., Verity et al. 1993). Knowledge of the size-structure of the microzooplankton community can also be valuable as it likely influences exploitation of the microzooplankton by high trophic levels. For example, the grazing rates for copepods feeding on tintinnid ciliates or non-loricate ciliates apparently vary greatly

with ciliate size (e.g., Gifford & Dagg 1988, Cariou et al. 1999). We urge then that experiments designed to estimate microzooplankton grazing focus on the predators as well as the prey.

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