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A cautionary note: Examples of possible microbial community dynamics in dilution grazing experiments

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Abstract

Dilution experiments are used commonly to provide estimates of grazing pressure exerted on phytoplankton and bacterioplankton as well as estimate their growth rates. However, very little attention has been given to the dynamics of grazers, especially heterotrophic nanoflagellates (HNF), in such experiments. We found temporal changes in concentrations of ciliates and HNF in a dilution experiment using water from the oligotrophic N.W. Mediterranean Sea. Ciliates decreased markedly over 24 h when held in seawater diluted with particle-free water (60% and 20% final conc whole seawater) while HNF increased in concentration in the same treatments. Using a time-course approach in a second experiment, we monitored changes in HNF and bacterioplankton concentrations in 20% whole seawater (80% particle-free seawater). Both HNF and heterotrophic bacteria displayed stable concentrations for the first 12 h and then grew rapidly, especially HNF, from 12 to 24 h. Examination of bacterial community composition using denaturing gel gradient electrophoresis (DGGE) showed a change in community composition over the 24 h incubation period. Dilution can have differential effects on the distinct components of the marine microbial food web.
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1. Introduction

Dilution is a time-honored method in plankton ecology. Kirchman et al. (1982) proposed diluting natural planktonic communities with consumer or predator-free water to estimate growth in bacterioplankton. Landry and Hassett (1982) introduced the use of a dilution series to measure phytoplankton growth in situ and grazing losses. It is the most widely employed method to estimate grazing of microzooplankton and usually chlorophyll concentrations are employed as a metric of phytoplankton biomass (e.g., Bamstedt et al., 2000). In recent years, it has become very common to employ the dilution method of Landry and Hassett (1982) to estimate growth and grazing losses of picoplankton, especially heterotrophic bacteria but also that of phototrophic prokaryotes *Synechococcus* or *Prochlorococcus*. In 2005 reports alone, dilution grazing experiments were used to estimate grazing losses and growth rates in a large variety of marine and estuarine systems ranging from estuaries and coastal areas to open ocean waters (e.g., Bec et al., 2005; Berninger and Wickham, 2005; Collos et al., 2005; Fileman and Leakey, 2005; Garces et al., 2005; Jochem

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et al., 2005; Leising et al., 2005; Strzepek et al., 2005; Troussellier et al., 2005; Umani et al., 2005; Yokokawa and Nagata, 2005).

The dilution method, like any other method, can yield uninterpretable results. For dilution grazing experiments, this can take the form of insignificant correlation between prey growth rate estimates and dilution factor; variable proportions of uninterpretable results have been reported (Dolan et al., 2000; Worden and Binder, 2003). Attempts have been made to refine the dilution grazing method by selecting certain data, (either 3 or 2 dilution treatments) when the dilution series gives a non-linear result (e.g., Gallegos, 1989; Evans and Paranjape, 1992; Worden and Binder, 2003).

Non-linear relations between prey growth rates and dilution factor are typically ascribed to changes in the feeding behavior of grazers when prey availability is altered (e.g., Moigis, 2006). However, dilution may be associated with more than changes in feeding behaviour of grazers. For herbivorous ciliates, changes in the concentrations and composition of ciliate microzooplankton in typical dilution experiments have been documented in estuarine (Dolan et al., 2000) and marine communities (Dolan and McKeon, 2005). Dilution is known to affect heterotrophic bacteria. Changes occur in the community composition of bacterioplankton in seawater diluted with particle-free water over periods ranging from 24 to 48 h (e.g., Franklin et al., 2001; Beardsley et al., 2003; Fuchs et al., 2000). Surprisingly, there is to our knowledge no data on the responses of heterotrophic nanoflagellates to dilution. In fact, to our knowledge, the dynamics of all the three 'microbial loop' populations, ciliates, heterotrophic nanoflagellates and bacteria, have not been examined together in these experiments.

Here we present results from an examination of the dynamics of the three major components of the microbial loop: ciliates, heterotrophic nanoflagellates (HNF), generally assumed to be the major grazers of picoplankton, and heterotrophic bacteria (HB) in 2 dilution experiments. Our goal was examine the dynamics of microbial populations when diluted with particle-free seawater. In our experiments we found that nanoflagellate concentrations may, over a 24 h incubation time, be unrelated to dilution factor (but perhaps related to ciliate growth or mortality) and the community composition of the bacterioplankton can shift during a dilution experiment.

2. Materials and methods

The study was carried out in September–October 2004. Water for experiments was collected at 10 m depth from 'Point B', a standard oceanographic station at the mouth of the Bay of Villefranche (43°41′10′′N, 7°19′0′′E) using Niskin bottles. Filtered sea water was prepared using GF/F and 0.2 μm filters and a peristaltic pump and then mixed with appropriate volumes of whole seawater. The seawater used was not filtered to remove metazoan zooplankton for 2 reasons. Firstly, screening can damage ciliate microzooplankton and secondly, copepod abundances were very low, 0.2 individuals l^{-1}, based on plankton tows taken nearly simultaneously (Gasparini and Antajan, in press). For each 'dilution level' 10 l was prepared in a single carboy; the water was gently mixed and a set of 3 polycarbonate incubation bottles filled with 2.4 l of the solution.

2.1. Experiment 1

The first experiment, performed the 20–21st of September, consisted of 3 dilution levels: 20%, 60% and 100% whole seawater. From each dilution level carboy, a single sample was taken for the determination of initial chlorophyll concentration. Individual bottles were sampled immediately prior to (t_0) and at the end (t_{24}) of the incubation period, to determinate the abundance of ciliate microzooplankton, heterotrophic nanoflagellate (HNF) and heterotrophic bacteria (HB).

Incubations took place on the dock of the Station Zoologique. Bottles were placed in a flow-through seawater bath, covered with a neutral density filter yielding 50% incident illumination (measured inside filled incubation bottles using a LICOR instrument) similar to 10 m depth light conditions at the Pt B sample point (Dolan, unpublished observations). After 24 h, samples were taken from each bottle to determine t_{24} chlorophyll *a* concentration and abundance of micro-organisms.

Samples for fluorometric determination of chlorophyll *a* were filtered through GF/F filters, frozen and extracted in 10 ml acetone 90% (Lorenzen, 1967). Chlorophyll *a* concentration was determined by fluorimetry on a Turner Designs fluorometer. The apparent growth rate of phytoplankton (k) and grazing rate of microzooplankton (g) were calculated following Landry and Hassett (1982).

To determine concentrations of ciliate microzooplankton, 200 ml samples were taken from each dilution bottle, at t_0 and t_{24}, fixed in Lugol's solution (2% final conc) and refrigerated. Either aliquots (100 ml) or the entire sample was settled and material examined using inverted microscopy. To determine concentrations of HNF and heterotrophic bacteria, 200 ml samples were taken, fixed with EM-grade gluteraldehyde (1% final conc), and stored refrigerated. Aliquots (20–100 ml) of these samples were stained with diamidino-2-phenylindole (DAPI), filtered onto 0.2 μm black polycarbonate

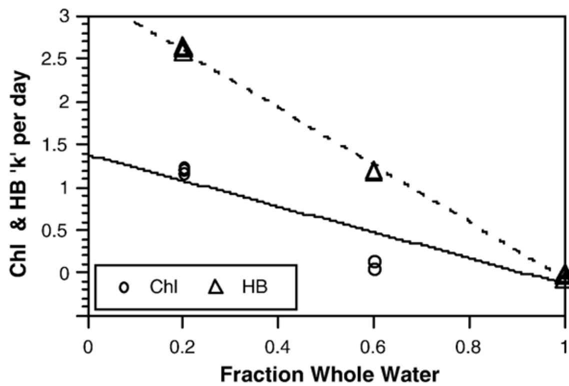


Fig. 1. Experiment 1 — Plot of apparent growth rate, per day, of phytoplankton (as chlorophyll) and heterotrophic bacteria (HB) based on changes in concentration over 24 h in the 3 dilution treatments as a function of the dilution factor employed. See Results for details.

filters mounted on slides. Slides were prepared and examined within 2 weeks of running the experiment. Counts were made at magnification of 1000× using a Zeiss Axiophot microscope. Heterotrophic bacteria (HB) abundance was determined from the same slides used for HNF estimates. Statistical analyses consisted simply of comparisons of the means of t_0 and t_{24} concentrations.

2.2. Experiment 2

In the first experiment marked changes occurred in the concentrations of HNF and HB over the 24 h incubation period in the 20% whole water treatment. Thus a second experiment was run to determine time-course changes in a 20% whole water treatment. The experiment was run on the 7–8th of October. Bottles of 20% whole water from the same site were prepared and incubated as in the first experiment. Samples for estimates of HNF and HB abundance were taken every 6 h and processed as in experiment 1. Chlorophyll analysis was made at the beginning and at the end of the incubation period.

For the second experiment, bacterial community composition was examined using denaturing gradient gel electrophoresis (DGGE). A single sample was taken from the 10 l carboy of 20% whole water before the solution was distributed into the 3 incubation bottles. At the end of the experiment a sample was taken from each of the incubation bottles.

For these analyses, water samples were drawn down on filters and nucleic acids from filters were extracted as described elsewhere (Winter et al., 2001). Briefly, after four freeze thaw cycles in liquid nitrogen and in a water bath at 37 °C, an enzyme treatment with lysozyme (1.25 mg/ml final concentration; Fluka BioChemika #62970) at 37 °C for 30 min and Proteinase K (100 µg/ml final concentration; Fluka BioChemica #82456) for 2 h at 55 °C was performed. In contrast to the phenol–chloroform extraction step of the original protocol, nucleic acids were extracted with 4.5 M NaCl and chloroform, followed by isopropanol precipitation. This modified procedure avoids a toxic chemical and yields genetic fingerprints identical to those obtained by the original protocol (data not shown). The pellets were then re-suspended in 60 µl of water.

For PCR and DGGE, with 1 µl of template DNA a 566 bp product of the 16S rRNA gene was amplified in 50 µl reactions (1.5 mM $MgCl_2$, 0.25 µM of each primer and 2.5 U *Taq* polymerase; Sigma; #D 5930) together with a positive and a negative control using the primer pair 341F-GC/907R (Schäfer and Muyzer, 2001). Conditions of the touchdown PCR were set as described in Schäfer and Muyzer (2001). PCR products were then separated on a DGGE gel (Muyzer et al., 1993) made with a DCode Universal Mutation Detection System (Bio-Rad). The gel was imaged with the

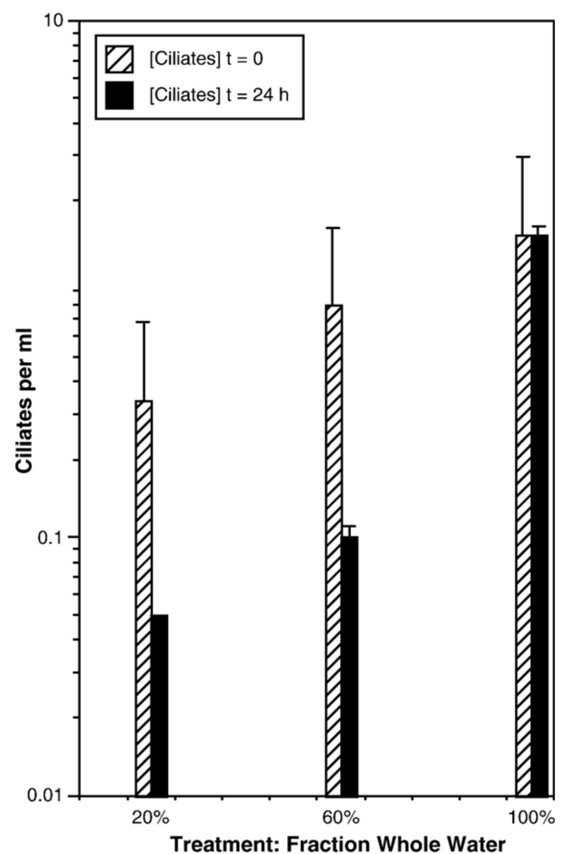


Fig. 2. Experiment 1 — Changes in the concentrations of ciliates over 24 h in each of the treatments within the dilution series. Error bars represent SD. See Results for details.

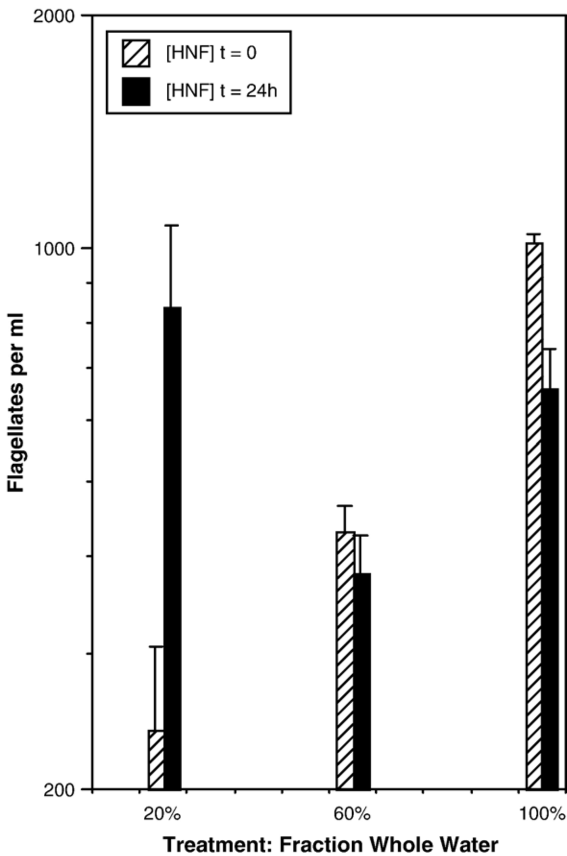


Fig. 3. Experiment 1 — Changes in the concentrations of heterotrophic nanoflagellates (HNF) over 24 h in each of the treatments within the dilution series. Error bars represent SD. See Results for details.

gel documentation system GelDoc EQ (Bio-Rad) after 15 min SYBR Gold staining (Molecular Probes; #S11494) by distributing a 10× SYBR Gold solution on the gel. DGGE banding pattern was analyzed with the Quantity One Software (Bio-Rad). DGGE analysis consisted of comparing bacterial community fingerprints of *in situ*, T_0 and the endpoints of the three incubations.

3. Results

3.1. Experiment 1

Apparent chlorophyll growth rate in the individual bottles (based on changes in concentration from t_0 to t_{24}) was significantly related ($r^2=0.847$) to dilution factor (Fig. 1). Following the method of Landry and Hassett (1982) the regression line provided an estimate of chlorophyll growth rate of 1.38 d^{-1} (the *y*-axis intercept) and a grazing rate estimate of 1.5 d^{-1} (the slope of the regression line). For bacteria, HB, the regression relationship was stronger ($r^2=0.997$) and provided higher estimates of growth and grazing mortality, both of about 3.3 d^{-1}.

Dilution had different effects on the two groups of potential grazers, ciliates and HNF. Ciliate concentrations did not change significantly over 24 h in the bottles of 100% whole water but declined dramatically in bottles of both 60% and 20% whole water (Fig. 2). Counts of ciliate cells in t_{24} samples were quite low but agreed remarkably well. Ciliates in the 20% whole water treatment at t_{24} were near the limit of detection in 200 ml volumes examined but precisely the same, 10 cells, in the samples for the 3 bottles. There was a similar agreement between samples at t_{24} for 60% whole water treatment bottles with raw counts of 22, 20, and 15 cells.

For HNF, marked declines occurred in the 100% whole water; no significant change occurred in the 60% whole water bottles, and remarkable increases occurred in incubations of 20% whole water (Fig. 3). The distinct trends of ciliates and HNF within the dilution series led to opposite relationships of apparent growth rates of these grazers with the growth rates of chlorophyll. Interestingly, ciliate growth appeared to be inversely related to chlorophyll growth while HNF

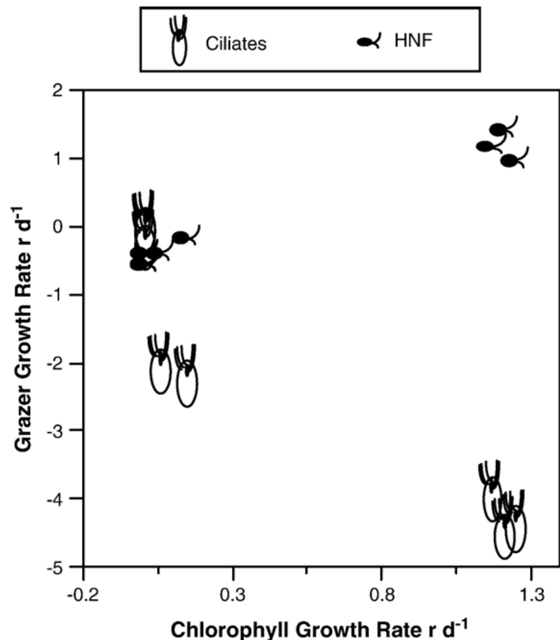


Fig. 4. Experiment 1 — Scatter plot of apparent net rates of exponential change among populations of ciliates and heterotrophic nanoflagellates (HNF) in the experimental bottles plotted as a function of the corresponding rate of change in chlorophyll concentration. Note that ciliate populations declined as both chlorophyll and HNF increased. Exponential rates of change were assumed.

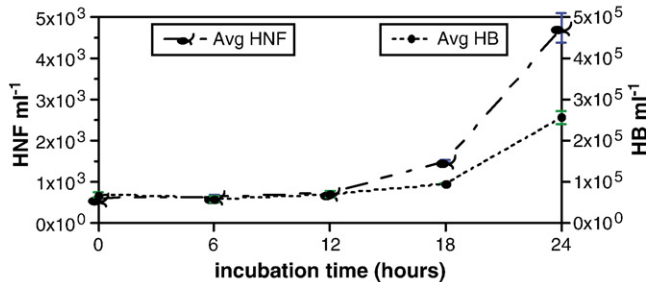
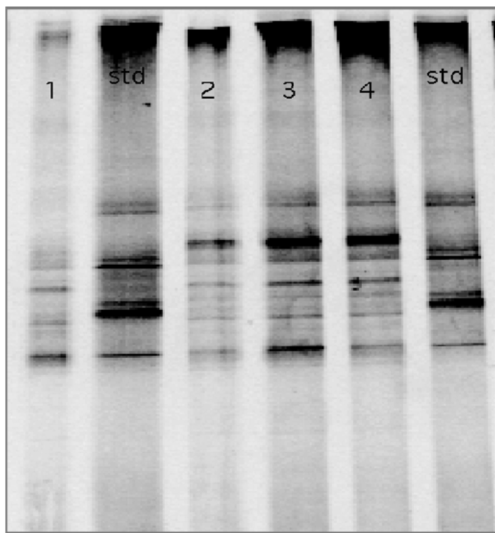


Fig. 5. Experiment 2 — Temporal changes in the concentrations of heterotrophic bacteria (HB) and heterotrophic nanoflagelldates (HNF) over 24 h among populations diluted 4 to 1 with particle-free seawater. Symbols represent average values±SD from the 3 bottles. See Results for details.

growth appeared positively related to the apparent growth of chlorophyll (Fig. 4). Given these apparently divergent trends, we then further investigated the high growth rates of HNF and their prey, HB, in 20% whole water incubations in the second ‘‘time-course’’ experiment.



T_0 "1"	bottle 1 "2"	bottle 2 "3"	bottle 3 "4"
+	-	-	-
+	+	+	+
-	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	+	+	+
+	-	-	-
-	+	+	+
+	+	+	+
+	+	+	+
+	-	-	-
+	+	+	+
+	+	+	+

Fig. 6. Experiment 2 — Results of DGGE analysis of bacterial community composition at the beginning and end of Experiment 2 in which a natural community was diluted 4 to 1 with particle-free seawater. Note that the banding patterns of three bottles at the end of the experiment correspond with one another closely and differ from the time zero sample. See Results for details.

3.2. Experiment 2

Time-course changes in the concentrations of HNF and HB in 20% whole water (80% filtered seawater) were very similar in the 3 bottles. For the first 12 h, bacterial and HNF concentrations were stable and then between 12 and 24 h of incubation both populations grew at high rates (Fig. 5). After a 12 h lag period, HNF populations grew at a rate equivalent to a daily rate of $3.8 \pm 0.29\ d^{-1}$ and bacteria at an equivalent daily rate of $2.6 \pm 0.12\ d^{-1}$.

Analysis of heterotrophic bacterial community composition using DGGE showed shifts in the composition of the community over the 24 h period (Fig. 6). Richness measured as the number of 16S rRNA gene fragments on a DGGE gel was 19 at T_0 and 18 at the end of the incubation period (24 h). Despite the rather similar richness between the beginning and the end of the experiment the microbial community changed with respect to presence/absence of bands. At the end of the 24 h incubation we observed two additional bands compared to T_0 and three bands could no longer be detected. A similarity analysis showed 76% similarity between the fingerprints at T_0 and T_{final}. The replicates themselves were identical. Comparing the relative intensity of bands we detected differences in some of the bands with respect to T_0. For one band for example, intensity increased from 12.5% to 30.3%±2.7 in the three incubations during the 24 h.

4. Discussion

In the first experiment, we ran an ordinary dilution grazing experiment. We were not attempting to obtain an estimate of microzooplankton grazing nor phytoplankton growth. Hence, we will discuss neither the adequacy of using chlorophyll as a biomass indicator of phytoplankton nor linear vs. curvi-linear relationships between apparent growth rates and dilution factor. Our purpose was to examine the dynamics of ciliates, HNF and HB in dilution experiments.

We found that the consumers of phytoplankton and bacteria, ciliates and HNF, can vary in concentration with time within a dilution series. HNF appeared to increase as ciliates decreased, consistent with a predator–prey relationship between the two. The time-course monitoring in Experiment 2 showed that both HB and HNF can grow in diluted (80% filtered seawater) but at different rates and that there was a time-lag in both HB and HNF growth. This time lag likely varies with temperature and the trophic state of the system. Thus, the general effects on estimates of growth and grazing losses of phytoplankton and bacterioplankton, as an example of picoplankton, are difficult to predict.

Admittedly, our data is derived from 2 small experiments with small sample sizes. Hence, it would be hazardous to use our data for quantitative rate estimations. In particular, our raw counts of ciliates in the dilute treatment carry a large counting error. The rates of decline of ciliates were assumed to be exponential (Fig. 4) but we lack the time-course data needed to support the assumption. However, to our knowledge the data presented are unique, the agreement between replicates was good, and the trends among the distinct microbial groups, given commonly accepted trophic relationships were coherent.

It is generally accepted that there is a predator–prey relationship between ciliate microzooplankton and HNF as well as between HNF and HB or other picoplankton. Therefore, one may expect that mortality of ciliates in dilute treatments will be accompanied by increases in HNF concentrations. Conversely, growth of ciliates in undiluted waters can yield lower than expected HNF concentrations in the whole water bottles. Shifts in HNF concentrations may be reflected in shifts in the grazing mortality of picoplankton both heterotrophic and autotrophic. Lower than expected HNF abundance may allow greater than expected growth of picoplankton in the bottles. Higher HNF concentrations than expected in a given treatment, as result of mortality among ciliate grazers, would yield artificially low rates of picoplankton growth.

We also found a change in the community composition of the bacterioplankton over the 24 h incubation period. The changes we documented with DGGE agree with previous reports of shifts in bacterial community composition in incubations with predator-free water in both freshwater communities (e.g., Simek et al., 2001), or marine bacterioplankton (Fuchs et al., 2000). Changes in the community composition of bacterioplankton can occur rapidly when the either grazing mortality or growth rates are altered (Simek et al., 2002, 2003).

It is likely that the changes in bacterial community composition we detected resulted not from a decline in grazing losses in the bacterial community, but rather a containment effect, for example, provoking the growth of surface-associated forms. This conclusion is based on the growth rates estimated for the bacterial community compared to our rough estimates of HNF grazing rates (Fig. 7) based on a per cell HNF clearance rate of 12 nl $cell^{-1}$ h^{-1} (Dolan and Simek, 1999). The grazing pressure exerted by the *in-situ* concentration of HNF appears inadequate, by a factor of about 3, to control the growth of HB growing at the peak rates estimated from the ‘dilute’ bottles. Hence, a containment or bottle effect appears to be the most likely

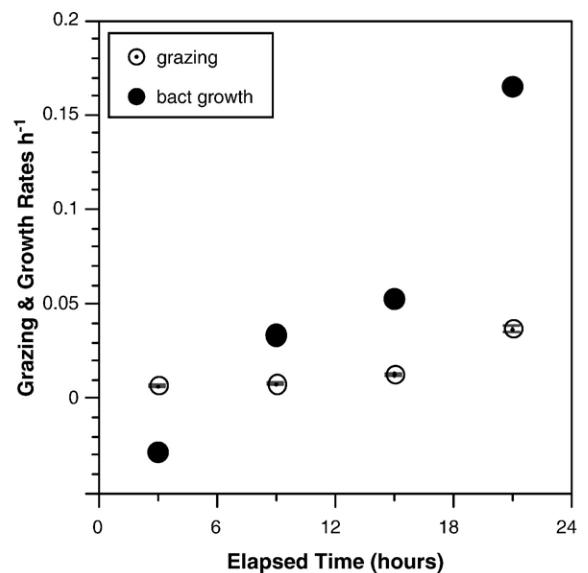


Fig. 7. Comparing estimates of grazing losses and growth rates of bacteria in diluted seawater in Experiment 2. HNF grazing rates were estimated for each bottle and each time-interval as average concentration of HNF for the interval, following Frost (1972), and a clearance rate of 12 nl HNF^{-1} h^{-1}, estimated previously for HNF from the Rade de Villefranche (Dolan and Simek, 1999). HB growth rates were estimated as exponential rates of change for each time interval and each bottle. Symbols show average values ± SD, error bars if not visible were smaller than the symbol.

explanation. Marked shifts in community composition of bacterioplankton are known to occur as a 'bottle effect' (e.g., Gattuso et al., 2002). Dilution experiments, as they inevitably involve relatively long incubations in containers, may give misleading results with regard to the factors regulating community composition of bacterioplankton communities.

Our data suggest that in dilution experiments distinct components of microbial community can display distinct changes. Ciliate microzooplankton may decline markedly in dilute treatments. HNF populations as well as bacterioplankton may increase and do so at different rates in the dilute treatments. Furthermore, the community composition of the bacterioplankton can shift during a 24 h incubation. While the phenomena we document may or may not occur in all dilution experiments, it is important to note that one or more could. To minimize shifts in grazer concentrations, deviations from dilution factors, we suggest that incubation times be minimized. If long incubations times (≥ 24 h) must be employed, time-course sampling to check for changes in grazer concentration would be prudent.

Acknowledgements

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