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A modified dilution method reveals higher protozoan growth rates than the size fractionation method

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Abstract

Protozoan growth rates are typically estimated with the size fractionation method in which the organisms studied are separated from the larger grazers. This method was compared with a specially modified dilution method which removed not only the grazing pressure of larger grazers but also that of predators of the same size as the organisms studied. Five comparisons were performed under natural conditions with the following marine protozoa: *Balanion comatum* (3 sets of data), *Ebria tripartita*, and *Strombidium* sp. The dilution method revealed significantly higher growth rates (Wilcoxon's signed-rank test, $p = 0.04$), which underscores the importance of predation between organisms of the same size. It directly demonstrated that the size fractionation method may significantly underestimate protozoan growth rates.

Keywords: Marine protozoa; Growth rate; Size fractionation; Dilution method; Diel rhythm; Predation

Introduction

Ciliates and other large protozoa (heterotrophic protists) are top predators in microbial food webs in aquatic environments (Azam et al. 1983; Kirchman and Williams 2000). This means that the energy transmitted through the microbial food web emerges as the new biomass of these protozoa. Consequently, their production is a crucial parameter that describes energy flow through aquatic communities. In contrast to primary and bacterial production, which are easy to estimate based on the uptake of carbon dioxide or thymidine, there is no quick, reliable method for measuring protozoan production.

Direct estimates of protozoan growth are usually carried out through observations of the fraction of dividing cells or by using the size fractionation method. The first method (Coats and Heinbokel 1982; Reguera et al. 2003) requires large pools of cells for observation. Additionally, calculating growth rates requires detailed knowledge of the duration of cell division (i.e., precisely the phases which can be distinguished). The size fractionation method (e.g., Verity 1986) is performed by separating the organisms studied from their larger grazers. However, there is a substantial problem with this method: some protozoa can graze on other protozoa of the same size (Hansen 1991; Carrick et al. 1992; Leakey et al. 1994; Nielsen and Kiørboe 1994), and size fractionation does not eliminate this grazing.

Ciliate reproduction rates can also be estimated indirectly using laboratory-derived allometric formulae to calculate ciliate growth on the basis of cell volume and ambient temperature (e.g., Montagnes et al. 1988; Müller and Geller 1993). Growth rates estimated with these formulae are considered potential (maximal) values (Taylor and Johannsson 1991; Carrick et al. 1992; Leakey et al. 1994; Macek et al. 1996) since direct measurements with the size fractionation method usually yielded approximately 50% lower growth rates (Leakey et al. 1994; Macek et al. 1996). This

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discrepancy is most probably the result of predation among organisms of the same size. Carrias et al. (2001), who performed a careful study of whether or not separation of grazers during size fractionation was effective, observed that small pelagic ciliates (e.g., *Urotricha* spp., *Balanion planctonicum*) grew at rates comparable to those estimated with the theoretical models mentioned previously after grazing pressure had been successfully removed. It is also widely believed that protozoan growth rates under natural conditions are lower due to food limitation (e.g., Müller 1989).

The aim of the present study was to modify the dilution method (Landry and Hassett 1982) in order to estimate growth rates of protozoa after complete elimination of their grazing-induced mortality. Reproduction rates estimated with the modified dilution method were compared to those measured with the standard size fractionation method. I hypothesized that protozoan growth rates estimated with the dilution method would be higher. To the best of the author's knowledge, the dilution method has yet to be applied to measure protozoan growth rates. The dynamics of protozoan abundance during standard dilution experiments (i.e., those used for determining algal or bacterial grazing losses) have only been assessed to verify the experimental assumptions (Dolan et al. 2000; Agis et al. 2007).

Material and Methods

The study was performed in the coastal zone of the southern Baltic Sea (54°38′N, 16°59′E). The study site was distant from freshwater input and rather stable with respect to environmental conditions. Annual salinity ranged from 6.6 to 7.7 PSU, which was slightly less than the offshore waters of the southern Baltic Sea at 7.5–8.0 PSU (Matthäus et al. 2008). The mean annual values of chlorophyll *a*, biochemical oxygen demand (BOD_5), and total suspended matter (TSM) were 2.38 mg m^{-3}, 1.45 mgO_2 l^{-1}, and 26.4 mg l^{-1}, respectively. This indicates that the environmental parameters resembled those observed in the open Baltic Sea (e.g., Wasmund et al. 2001).

Two times, in March and October 2009, the size fractionation and dilution methods were compared after short (3.5–4.1 h) and long (approx. 24 h) incubation periods. The short incubation periods were conducted around noon. The incubation periods were carried out under environmental conditions since the author's unpublished observations indicate that artificial mixing and illumination disrupts many non-acclimated protozoan cells (see also Stoecker et al. 1986). To obtain detectable and statistically significant growth only the most abundant organisms were counted. Prior to the experiments, all bottles, tubings etc. used in both the size fractionation and dilution experiments were soaked in a 10% HCl solution, and then washed with demineralized water as advised by Landry (1993).

The seawater used in the size fractionation experiments was sieved through 100-μm mesh gauze, then 50-μm mesh for larger organisms (SF50) or 25-μm mesh for smaller organisms (SF25). All mesh gauzes were made of Nylon (Hayward filter bags). Water (200 ml) was incubated in polyethylene bottles on a floating experimental set under environmental conditions. Before taking the initial and final samples, in which abundance of organisms were compared to calculate growth rates, the bottles were mixed by gently rotating them manually 50 times. Samples were fixed with acid Lugol's solution to a final concentration of 0.5% and analyzed under an inverted microscope with the Utermöhl (1958) method within a few weeks. Large Utermöhl chambers (50 ml or 100 ml) were applied to collect significant number of specimens. Net growth rates (k, h^{-1}) were calculated as follows:

$$k = \frac{\ln(A_1/A_0)}{t}$$

where A_0 and A_1 are abundance (cells ml^{-1}) before and after incubation; t is the time of incubation (h^{-1}).

The dilution method consists of creating a gradient of dilutions in which grazing pressure is gradually reduced (Landry and Hassett 1982; Landry 1993). The standard dilution method was modified to measure protozoan growth by replacing the particle-free filtrate (0.2 μm) with 10-μm filtrate. As a result, the growth of organisms with diameters larger than 10 μm that feed on particles smaller than 10 μm could be observed. This method relieves the grazing pressure of predators of the same size, because all protozoa larger than 10 μm are diluted, i.e. both the target organisms and their predators. Comparisons of nanoflagellates' (<10 μm) and bacteria abundances done for unfiltered water and 10-μm filtrate did not demonstrate changes in abundance of nanoflagellates and a noticeable decrease in bacterial abundance, which was most probably due to the removal of detritus particles to which some bacteria were attached. The filtrate used for dilutions was prepared by sequential sieving through 100-μm, 50-μm, and 10-μm mesh gauzes (made of Nylon, Hayward filter bags). Filtration was done gravitationally without a pump; this step is crucial to avoid breaking up of cells and releasing nutrients and substrates. Each time, unfiltered water and 10-μm filtrate were used to prepare gradient of dilutions containing 100% (duplicated), 80%, 60%, 40%, and 20% of unfiltered seawater. No supplements such as nutrients or organic matter were added. The water was incubated similarly to the procedure used for the size fractionation experiments. For each dilution the initial (before incubation) and final (after incubation) samples were collected, fixed, and analyzed as described above. Net growth rates (k) were calculated for every dilution (as above, Table 1), and all growth rates observed were plotted against the fraction of unfiltered seawater (Fig. 1) to estimate the intrinsic growth rate (r) for the theoretical situation of ideal dilution (0% of unfiltered seawater, equivalent to the Y-axis intercept of the linear regression). Since I did not observe any non-linear responses to dilution (see Gallegos 1989), a simple least-squares linear regression was used to analyze the