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Control of nanoflagellate abundance by microzooplankton and viruses in a coastal ecosystem of the subtropical western Pacific

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ABSTRACT

Little is known about the factors controlling variations in nanoflagellate abundance in oceanic environments. In this study, combined size-fractionation with dilution experiments were performed to measure nanoflagellate growth rates, microzooplankton grazing, and virus-mediated mortality collected three times during the summer of 2013. Heterotrophic and pigmented nanoflagellate growth rates varied from 0.39 to 0.49 d^{-1} and 0.39 to 0.52 d^{-1}, respectively. Moreover, PNF and HNF loss rates varied from 0.19 to 0.23 d^{-1} and 0.17 to 0.25 d^{-1}, respectively. In these cases, a significant impact of microzooplankton grazing was detected, highlighting the important role of microzooplanktons in transferring carbon bound in nanoflagellates to higher trophic levels in this study area. Nanoflagellate net growth rate was not increased in the virus-diluted treatments, suggesting that there was no impact of viral lysis on the mortality of nanoflagellates during summer periods. Furthermore, these results showed a decrease of *Synechococcus* spp. and bacterial abundance with removal of viruses, and a subsequent decrease in nanoflagellate growth. This result implies that viral infection is an important mechanism in nutrient recycling under oligotrophic oceanic conditions in summer.

1. Introduction

Heterotrophic nanoflagellates (HNFs) are efficient bacterivores in most aquatic environments (Berninger et al., 1991; Hall et al., 1993; Almeida et al., 2001), but they are also important grazers on picophytoplankton and other protozoa (Sherr et al., 1991; Sherr and Sherr, 1994). Pigmented nanoflagellates (PNFs) can also contribute greatly to bacterivory at certain times or at certain depths within the water column (Bird and Kalff, 1986; Sanders et al., 1989; Hall et al., 1993; Hitchman and Jones, 2000; Tsai et al., 2011). These mixotrophic nanoflagellates are ecologically significant as both primary producers and consumers, contributing up to 50% of the total phototrophic nanoplankton biomass and up to 79% of the total bacterivory in marine and freshwater environments (Berninger et al., 1992; Sanders et al., 2000; Bell and Laybourn-Parry, 2003; Tsai et al., 2011).

The microzooplankton fraction constitutes an important group of heterotrophic and mixotrophic organisms in the size range of 20 to 200 μm, which includes many protists (ciliates and dinoflagellates) as well as small metazoans (copepod nauplii and some invertebrate larvae) (Calbet, 2008). Because they often play a dominant role within the microzooplankton community, ciliates and dinoflagellates have recently received much attention, mainly focusing on their roles as primary consumers of pico- and nano-sized primary producers (Vargas et al., 2007; Vargas and Martínez, 2009). In oligotrophic environments, 62%–97% of ciliates are reported to be <50 μm in equivalent spherical diameter (ESD), most often ranging between 20 and 40 μm (Ota and Taniguchi, 2003; Chen et al., 2012). Assuming the optimum predator: prey size ratio of about 8:1 established by Jonsson (1986), prey for these ciliates should range within 2.5–5 μm in size. Therefore, ciliates may be key grazers of nanoflagellates in oligotrophic regions (Kivi and Setälä, 1995; Suzuki et al., 1998). Although one previous study has reported ciliates to be the most important predators of nanoflagellates, consuming 32–80% of nanoflagellate production in a freshwater environment (Nakano et al., 2001), it is unclear how significant ciliate grazing pressure may be on the nanoflagellate communities of other ecosystems.

In addition to microplankton grazing, viruses can be an important causal factor of nanoflagellate mortality, producing changes in the dynamics and structure of protist communities (Brussaard et al., 2004). Viruses have been found to prevent or terminate blooms of important algal species (Suttle and Chan, 1995; Bratbak et al., 1996; Tarutani et al., 2000; Evans et al., 2003). Experimental studies with natural communities that examine the direct lysis of HNF by viruses, there are only four studies so far (Garza and Suttle, 1995; Massana et al., 2007; Saura et al., 2011; Weinbauer et al., 2015). A recent study suggested that giant viruses (head diameter of 405 ± 31 nm) could cause between 10 and 60% of the mortality of the total nanoflagellate community (Weinbauer et al., 2015). Although, there are some evidences of direct virus–HNF interactions (Garza and Suttle, 1995; Massana et al., 2007; Saura et al., 2011; Weinbauer et al., 2015), very few studies have investigated viral

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infection in marine HNF and PNF and its effects on their role within the microbial food web.

Knowledge of the relative contribution of these loss factors (grazing and viral lysis) to nanoflagellate mortality is critical for an optimal understanding of the flow of energy and nutrients in marine environments. However, these loss factors to nanoflagellate mortality are still poorly understood. In this study, combined size-fractionation with dilution experiments were performed to measure microzooplankton grazing, and virus-mediated mortality to compare their relative contributions of both to HNF and PNF mortality in a coastal ecosystem of the subtropical western Pacific.

2. Material and method

2.1. Sampling

Samples from surface waters were collected at an established coastal station (25° 09.4′ N, 121° 46.3′ E) along a rocky shore in northeastern Taiwan where seawater temperatures ranged from 28 to 30 °C over the sampling period (July to September 2013). The environment at this site has been previously described using data gathered from 1999 to 2001 (Tsai et al., 2005).

2.2. Nanoflagellate mortality and growth rates

We estimated microzooplankton grazing rates (m_g) and viral lysis (m_v) using the dilution and reoccurrence technique and the size-fractionation method (Shelford et al., 2012; Tsai et al., 2015) (Fig. 1) for three experiments during the summer. Prior to experiments, all filter holders and incubation bottles were acid-cleaned with 10% HCl and rigorously rinsed with Milli-Q water. To estimate the effect of microzooplankton grazing on nanoflagellates (Treatment 1), we set up size fractionation experiments. Firstly, we passed an 8 L sample of seawater through 100 μm mesh (to remove larger zooplankton), then we filtered subsamples through a 47 mm Nuclepore filter (type PC, pore size of 10 μm) (Treatment 2). The fractionation size used for the growth of nanoflagellates (10 μm filter) was demonstrated by a previous study at this site to remove microzooplankton but not nanoflagellates (Tsai et al., 2011) (Treatment 2). In the dilution and reoccurrence technique treatments, 500 mL of seawater was serially filtered through 10 μm and 0.2 μm, 47 mm diameter polycarbonate filters (AMD Manufacturing) operated at low pressure (<50 mm Hg), with the first filter removing

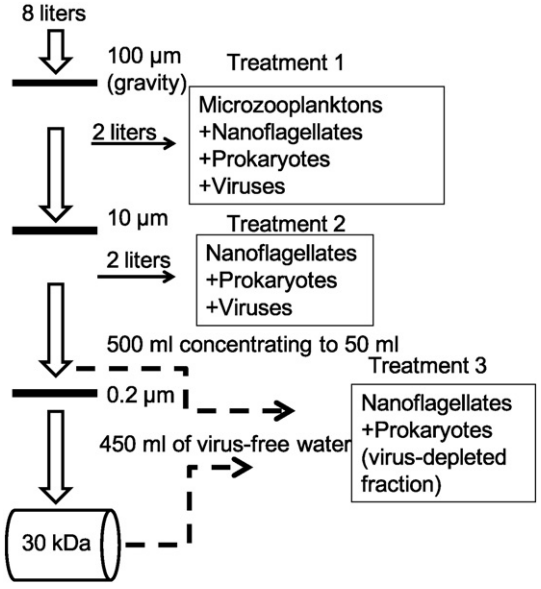

Fig. 1. Flow chart of the experimental design. For details, see the main text.

larger nanoflagellate predators and the second concentrating bacteria, *Synechococcus* spp. and nanoflagellates (Wilhelm et al., 2002). A transfer pipette was used to keep the bacteria, *Synechococcus* spp. and nanoflagellates in suspension above the 0.2 μm filter (Shelford et al., 2012) approximately 50 mL. One subsample of the 0.2 μm filter was then 30 kDa-filtered to remove viruses and create virus-free water. The dilution and reoccurrence treatment was prepared by adding 50 mL of pico- and nanoflagellate concentrate to 450 mL of virus-free water (Treatment 3) (Fig. 1). This approach resulted in bacteria, *Synechococcus* spp. and nanoflagellate abundances similar to in situ abundances (Tsai et al., 2015). The seawater samples were incubated for 48 h in triplicate in 500-mL polycarbonate bottles under natural light in a water bath to mach in situ temperatures at the time of sampling. Samples for estimate of viral, picoplankton (bacteria and *Synechococcus* spp.), nanoflagellate and microzooplankton abundance were taken every 24 h. Furthermore, the net growth rate of nanoflagellates (k, h^{-1}) was calculated for each sample based on microscopic cell counts at the start and the end of the experiment (N_{t0} and N_t), assuming exponential growth:

$$k = \ln(N_t/N_{t0})/(t-t_0)$$

where t_0 and t are the start and end of the experiment.

2.3. Viral, bacterial, nanoflagellate and microzooplankton abundance counts

Viruses, bacteria, *Synechococcus* spp. and nanoflagellates were counted using an epifluorescence microscope (Nikon Optiphot-2) (1000×). Viruses were processed following a slight modification of a procedure described by Nobel and Fuhrman (1998). Briefly, samples from 0.5 to 1 mL were filtered through an Anodisc filter (0.02 μm pore size, Whatman) backed by a 0.45 μm pore size Millipore filter. The samples were then placed on drops of SYBR Green I (Molecular Probes) solution diluted at 1:400 in TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) and stained for 15 min in the dark. The filters were placed on glass slides and added with 25 μL of 50% glycerol 50% PBS buffer (0.85% NaCl, 0.05 M NaH_2PO_4, pH 7.5) containing 0.1% *p*-phenylenediamine as antifade and mounting agents. Subsamples of 1–2 mL or 20 mL were filtered onto 0.2 μm or 0.8 μm black Nuclepore filters for bacteria and nanoflagellates, respectively. Samples were stained with DAPI at a final concentration of 1 μg mL^{-1} (Porter and Feig, 1980) to count bacteria and heterotrophic nanoflagellates (HNFs). Pigmented nanoflagellates (PNF) and HNF were counted based on the absence or presence of chlorophyll autofluorescence using a separate filter set optimized for chlorophyll or DAPI under a 1000× epifluorescence microscope (Nikon-Optiphot-2). Bacteria and HNF were identified by their blue fluorescence under UV illumination. *Synechococcus* spp. and PNF were identified by their orange and red autofluorescence under blue excitation light. To obtain reliable estimates of abundance, at least 100 nanoflagellates, 400 *Synechococcus* spp., 800 bacteria and 1000 viruses were counted per sample. These estimates never amounted to more than 15% of coefficient of variation for nanoflagellates, and 5% for picoplankton and virus.

To determine abundance of ciliates and dinoflagellates at the start of the experiment in treatment 1, 100 mL water samples were fixed with neutralized formaldehyde (2% final concentration) (Stoecker et al., 1989) and preserved at 4 °C until analysis. The subsamples (100 mL) were then settled in an Utermöhl chamber (Utermöhl, 1958). The entire area of the Utermöhl chamber was examined at 200× or 400× using an inverted microscope (Nikon-TMD 300).

2.4. Statistical analysis

Data analyses compared the net growth rates of nanoflagellates in all treatments using the non-parametric Mann–Whitney test and Kruskal–Wallis ANOVA. All statistical operations were performed