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Effect of heterotrophic nanoflagellates on the loss of virus-like particles in pond water

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A decrease in the abundance of virus-like particles (VLP) by heterotrophic nanoflagellates (HNF) was examined using size-fractionated water samples taken from a hypereutrophic pond in December 1999, and in March and July 2000. We recorded a considerable decrease in the abundance of VLP in the 5.0 μm filtrate relative to the 0.2–0.8 μm filtrates. Decrease rates of VLP were reduced in a parallel 5.0 μm filtrate treated with cycloheximide. The loss rates of VLP in 5.0 μm filtrate varied in each experiment, and a high rate of loss was found when the growth rate of HNF was high. These results suggested that HNF consumed the VLP and that HNF is an important factor for decreasing viral abundance in freshwater environments.

Key words: cycloheximide; heterotrophic nanoflagellates; hypereutrophic pond; ingestion; virus-like particles.

INTRODUCTION

Viruses are now recognized as the most abundant and biologically active components in aquatic ecosystems, which suggests that they probably influence various biochemical and ecological processes (Torrella & Monta 1979; Bergh *et al*. 1989; Borsheim *et al*. 1990; Proctor & Fuhrman 1990a; Suttle *et al*. 1990; Paul *et al*. 1991; Suttle & Chen 1992; Fuhrman 1999; Wilhelm & Suttle 1999; Tarutani *et al*. 2000; Wommack & Colwell 2000). Field studies of viruses have indicated that the majority were probably bacterial viruses (i.e. bacteriophages) (Cochlan *et al*. 1993; Waterbury & Valois 1993), and that a large proportion of the phytoplankton in surface waters contained virus-like particles, suggesting that infection by viruses is a significant cause of loss of bacteria and phytoplankton in marine (Proctor & Fuhrman 1990a; Heldal & Bratbak 1991; Waterbury & Valois 1993; Nagasaki *et al*. 1994) and freshwater (Safferman & Morris 1963, 1964; Daft *et al*. 1970; Fox *et al*. 1976; Leach *et al*. 1980) environments.

Heldal and Bratbak (1991) reported that the disappearance of natural communities of virus particles from seawater was extremely rapid, with measured decrease rates of up to 1.1 h^{-1} recorded. The decrease of viruses in aquatic environments is due to both physical and chemical factors, such as solar radiation (Noble & Fuhrman 1997; Wilhelm *et al*. 1998), temperature (Bratbak *et al*. 1990), heavy metals (Noble & Fuhrman 1997), organic chelators (Proctor & Fuhrman 1990b) and hydrolytic enzymes (Noble & Fuhrman 1997). Although some biological factors such as ingestion by protozoa (Suttle & Chen 1992; Gonzalez & Suttle 1993) have been examined, loss of viruses due to grazing by protozoa is still poorly understood and there is no information available for freshwater environments.

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Manage *et al*. (1999) and Manage *et al*. (2001) reported the sudden disappearance of infectious cyanophages of *Microcystis aeruginosa* after the abundance of cyanophages had peaked. This suggested that their disappearance was due to ingestion by protozoa. In the present study, we examined the effect of heterotrophic nanoflagellates (HNF) on the loss of virus-like particles (VLP) in the laboratory using fractionated pond water.

METHODS

Study area

Furuike Pond in Sancho, Matsuyama City, Ehime Prefecture, Japan is hypereutrophic because of anthropogenic loading from the watershed. The pond has a surface area of *ca* 7400 m^2 with an average depth of 0.97 ± 0.24 m during summer and 0.38 ± 0.13 m during winter (Nakano *et al*. 1998). The surface water temperature and pH ranged between 28.8°C and 4.0°C and 6.5 and 10.3, respectively, throughout the year. Chlorophyll *a* concentration varied between 1620 $\mu g\ l^{-1}$ and 47 $\mu g\ l^{-1}$ (Manage *et al*. 2001).

Sampling

Surface water samples were collected with acid-cleaned (1.2 NHCl) bottles on 20 December 1999, 20 March 2000 and 18 July 2000 from Furuike Pond. Water temperature was measured simultaneously using a thermistor (TOA Electronics Co., DKK, Tokyo, Japan).

Size fractionation

Six 500 ml subsamples of the pond water were fractionated, two through a plankton net of mesh size 5.0 μm and two each through sterilized Nuclepore filters of 0.8 μm and 0.2 μm. We assumed that the filtrates of the 0.2 μm filters contained VLP, those of the 0.8 μm filters contained VLP and bacteria, and those of the 5.0 μm mesh plankton net contained VLP, bacteria, small phytoplankton cells and flagellates. Each 500 ml of fractionated pond water was placed in a 1 litre Erlenmeyer flask, incubated in the dark and adjusted to the natural pond water temperatures on the days that the samples were taken. On 20 March and 18 July 2000, in a parallel experiment, cycloheximide (200 $mg\ l^{-1}$, Suttle & Chen 1992), a specific inhibitor of protein synthesis in eukaryotes, was added to additional duplicated samples of 5.0 µm filtered pond water. To enumerate VLP, bacteria and HNF, subsamples were collected from each fraction. In the December and March experiments, sampling was carried out at 48 h intervals after the set-up of the experiment and in the July experiment, samples were collected at 24 h consecutively for a period of 2 weeks.

Abundance of organisms

For the enumeration of VLP, 2 ml of the water sample from each fraction was filtered through a 0.2 µm Nuclepore filter and immediately fixed with 0.02 µm filtered formalin at a final concentration of 2%. The total number of VLP were enumerated using epifluorescence microscopy under blue excitation, using the fluorescent dye SYBR Green 1 (Noble & Fuhrman 1998). To enumerate total bacteria and HNF, 4 ml each of the 0.8 µm and 5.0 µm filtrates were fixed with glutaraldehyde at a final concentration of 1% and were stored at 4.0°C and enumeration was carried out within 24 h. To enumerate total bacteria and HNF, the samples were filtered through 0.2 µm and 0.8 µm Nuclepore filters (black) and counted using an epifluorescence microscope under ultraviolet excitation after staining with the fluorescent dye 4′,6-diamidino-2-phenylindole (DAPI) (Porter & Feig 1980) and the primuline method (Caron 1983), respectively. We counted nanoflagellates as HNF if they showed no obvious red chlorophyll *a* fluorescence under the green excitation.

Determination of growth and loss rates of the organisms

Loss rates *(h)* of VLP and bacteria, and the growth rate of HNF in each fraction, were calculated by $h = \{ln\,(C/Co\}/t$, where Co and C are the abundance of VLP, bacteria or HNF at the beginning and at the end of the time interval t, respectively. We calculated the mean values of loss and the growth rates of the organisms in each fraction.

RESULTS

The abundance of VLP in the 0.2 μm and 0.8 μm filtrates ranged between 10^8 and 10^9 particles ml^{-1} throughout the incubation periods on all three sampling dates (Fig. 1). In the 5.0 μm filtrates the abundance of VLP declined sharply in the first 2 days and then continued to decline throughout the incubation, reaching 3.1×10^6 particles ml^{-1}, 1.1×10^6 particles ml^{-1} and 4.8×10^5 particles ml^{-1} in December, March and July, respectively. The abundance of VLP in the 5.0 μm filtrate with cycloheximide added did not decrease significantly, but fluctuated around the same abundance as in the 0.2 μm and 0.8 μm filtrates of both March and July (Fig. 1).

Bacterial abundance was very similar in both the 0.8 μm and 5.0 μm fractions for the first 10 days of the incubation in December, but after 10 days the abundance in the 5.0 μm fraction started to decline (Fig. 2). In March, a similar pattern was observed in the 0.8 μm fraction to that in December,

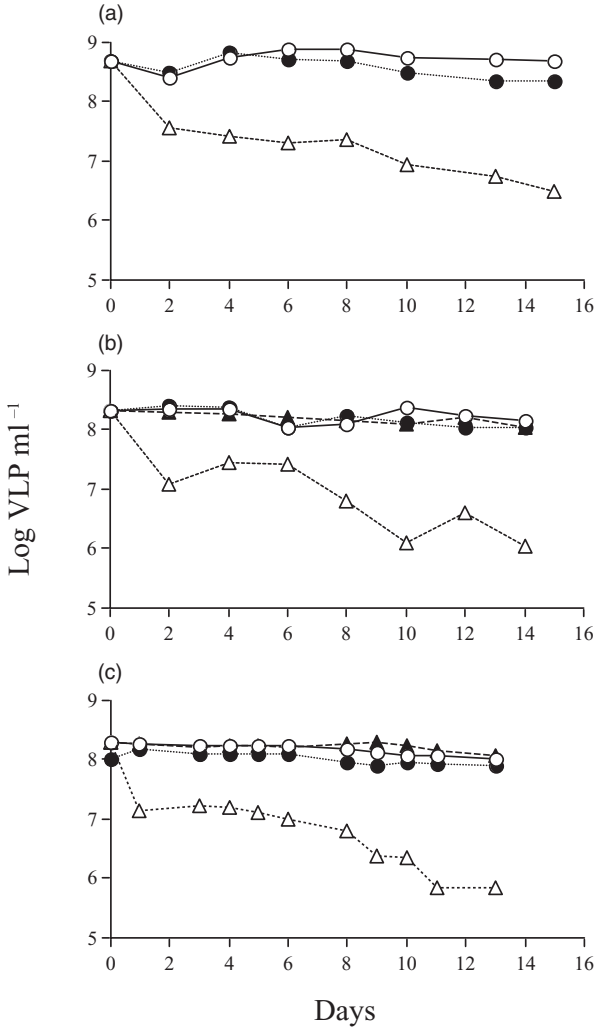

Fig. 1. Changes in abundance of virus-like particles (VLP) in natural freshwater samples filtered through 0.2 μm (○), 0.8 μm (●) and 5.0 μm (△) pore filters and cycloheximide treated 5.0 μm (▲) pore filters. The differences between replicates were within the size of the symbols for all data plots. (a) December 1999; (b) March 2000; (c) July 2000.

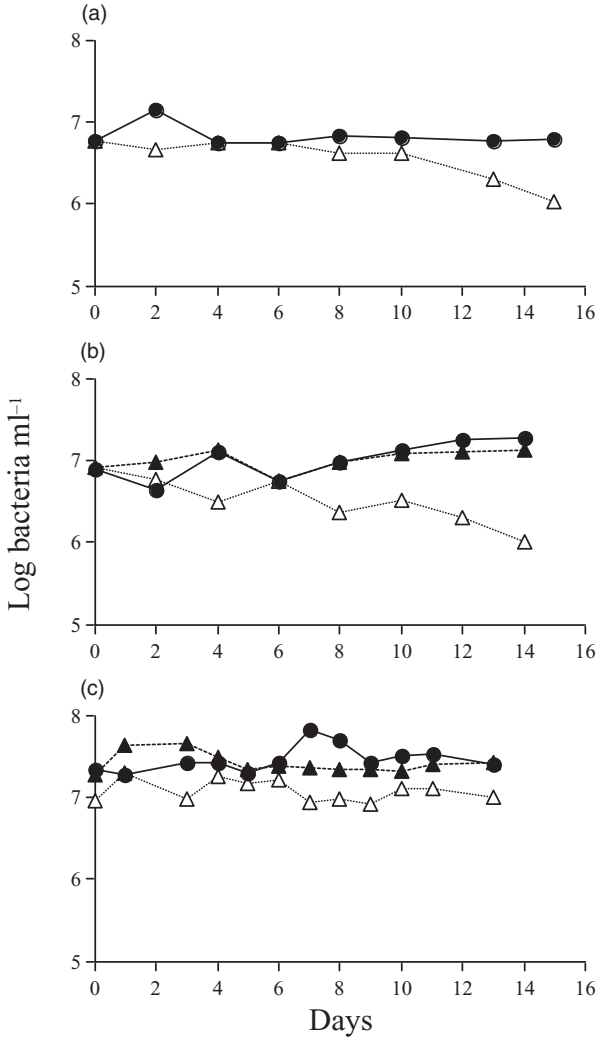

Fig. 2. Changes in abundance of bacteria in natural freshwater samples filtered through 0.8 μm (●) and 5.0 μm (△) pore filters and cycloheximide treated 5.0 μm (▲) pore filters. The differences between replicates were within the size of the symbols for all data plots. (a) December 1999; (b) March 2000; (c) July 2000.

although abundance in the 5.0 μm fraction started to decline earlier. The 5.0 μm sample treated with cycloheximide showed bacterial abundance very similar to that of the 0.8 μm fraction. In July, all three fractions (0.8 μm, 5.0 μm and 5.0 μm treated with cycloheximide) maintained very similar abundance of bacteria throughout the incubation with rather lower abundance in the 5.0 μm fraction throughout (Fig. 2).

The abundance of HNF in the 5.0 μm filtrate was initially very low in December, rather higher in March and much higher in July. Abundance then increased to maximum, before falling to a lower level (Fig. 3). Towards the end of the incubation their abundance began to increase on all three dates, earliest in July and less steeply in December (Fig. 3).

Table 1 lists the loss rates of VLP and bacteria in those samples where significant increases in the abundance of HNF were detected. In general, VLP abundance did not decrease, or decreased only very slowly, in the 0.2 μm filtrate (Fig. 1 and Table 1). When particles >0.8 μm in size were removed, the loss rates of VLP were substantially reduced (Table 1), but bacterial abundance remained high (Fig. 2). The range of measured loss rates of VLP in the 5.0 µm fraction from 0 days to 2 days and from 10 days to 15 days of incubation were between 0.053 h^{-1} and 0.054 h^{-1} (mean 0.054 h^{-1}) and between 0.006 h^{-1} and 0.012 h^{-1} (mean 0.009 h^{-1}) in December. In March, the equivalent mean figures were 0.039 h^{-1} and 0.027 h^{-1}, and in July they were 0.034 h^{-1} and 0.013 h^{-1}. Virus-like particle abundance throughout the incubation decreased with correlation coefficients generally greater than 0.90 (except March) ($r=0.91$ in December, $r=0.89$ in March and $r=0.98$ in July) when they were plotted against time on a semi-logarithmic axis (data not shown). Thus, a significant loss of VLP was attributable to eukaryote organisms in the 5.0 µm size fraction because loss rates were reduced by the addition of cycloheximide (Fig. 1 and Table 1). Bacterial abundance decreased very slowly in both the 0.8 µm filtrate and the 5.0 µm filtrate with cycloheximide. The abundance of bacteria decreased gradually in the 5.0 µm filtrate in December (0.011 h^{-1}) and March (0.014 h^{-1}) during the late growth phase of HNF (Fig. 2 and Table 1).

Two periods of growth were detected in each of the HNF incubations in December, March and July (Fig. 3), which may have occurred because of a succession of dominant flagellate species that coincided with decreases in VLP (Figs 1,3). The loss rate of VLP by HNF was high during the first growth period, whereas it was lower during the second growth period of HNF (Table 1).

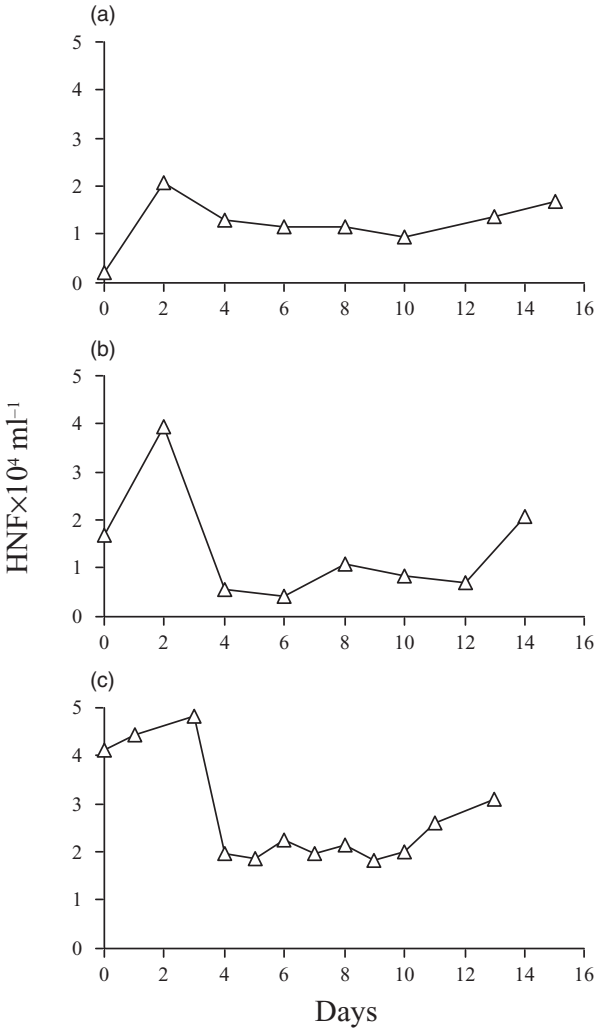

Fig. 3. Changes in heterotrophic nanoflagellate (HNF) abundance in natural freshwater samples filtered through 5.0 μm pore filters. The differences between replicates were within the size of the symbols for all data plots. (a) December 1999; (b) March 2000; (c) July 2000.

DISCUSSION

The present study showed the effect of HNF on the loss rates of VLP. This study provided the first indirect indication of the grazing effect of HNF

Table 1 Effect of cycloheximide and size fraction on loss rates of VLP and bacteria

Experiment date	Growth phase of HNF (days)	Growth rate of HNF (d^{-1})	Loss rate (h^{-1}) 0.2 μm	0.8 μm	5.0 μm	5.0 μm (Cycloheximide)
VLP						
20 December 1999	0–2	1.17	0.013	0.009	0.054	ND
	10–15	0.11	0.001	0.003	0.009	ND
20 March 2000	0–2	0.42	−0.002	−0.005	0.039	0.000
	12–14	0.55	0.003	0.000	0.027	0.008
18 July 2000	0–3	0.05	0.002	−0.003	0.034	0.003
	9–13	0.13	0.003	0.000	0.013	0.005
Bacteria						
20 December 1999	0–2			−0.018	0.005	ND
	10–15			0.001	0.011	ND
20 March 2000	0–2			0.012	0.008	−0.002
	12–14			−0.001	0.014	−0.001
18 July 2000	0–3			−0.003	0.000	−0.012
	9–13			0.000	−0.002	−0.002

Mean values of the duplicates are shown.
HNF, heterotrophic nanoflagellate; ND not determined; VLP, virus-like particles.

on VLP abundance in freshwater, although we did not show direct evidence of ingestion of VLP by HNF.

We detected that the loss rates of VLP was reduced after the removal of bacteria in the 0.2 μm filtrate (Fig. 1, Table 1) and were higher in the 0.8 μm filtrate compared to those in the 0.2 μm filtrate, except on 20 March, after 12–14 days of HNF growth. Thus, digestion by bacterial enzymes could be one reason for the loss of VLP (Suttle & Chen 1992).

Suttle and Chen (1992) reported that the titers of the phage PWH3a-Pl on PWH3a bacteria was significantly reduced after the addition into their culture of marine phagotrophic flagellates. In the present study, loss rates of VLP in the 5.0 μm filtrate were considerably greater than those in the 0.2 μm and 0.8 μm filtrates (Fig. 1 and Table 1), but were much reduced by the addition of cycloheximide (Table 1), and there was a tendency for the loss rates of VLP to be higher when HNF growth rate was high (Fig. 3 and Table 1). Thus, we suggest that the observed loss of VLP may result from grazing by HNF.

Bitton and Mitchell (1974), Suttle and Chen (1992), and Noble and Fuhrman (1997) reported that viruses irreversibly bind to heat-labile, loosely associated aggregates, and that the aggregates are responsible for the loss of the viruses. It was found that loss rates of VLP were high on December 8–10 and on July 6–8 (Fig. 1) despite the low growth of HNF (Fig. 3). This suggested that other factors contributed to the decrease in viral abundance. In the present study, the 5.0 μm filtrate may contain not only HNF but may also contain other flagellates, bacteria, small phytoplankton cells, small ciliates and other non-living particles. Thus, the loss of VLP in the present study may not only be due to grazing by HNF. However, the noted decreases in VLP abundance were observed when HNF increased from 0 days to 2 days (Figs 1,3) and the loss rates of VLP were reduced in the 5.0 μm filtrate with cycloheximide added. This suggested that HNF was an important biological agent in the loss of viruses in this freshwater environment. Further ecological studies are needed to ascertain the direct impact of HNF on the loss of VLP *in situ.*

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