Dynamics of *Heterocapsa circularisquama* (Dinophyceae) and its viruses in Ago Bay, Japan

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ABSTRACT: To examine the relationship between the bloom-forming dinoflagellate *Heterocapsa circularisquama* and its infectious viruses, a field survey was conducted in Ago Bay, Japan, in 2001. A *H. circularisquama* bloom occurred in July. The bloom peaked in mid July and disintegrated within a few days at the end of July. The abundance of viruses infectious to *H. circularisquama* was high from the peak of the bloom and throughout the post-bloom period, but ceased by the end of August. At the peak of the bloom, 88% of the *H. circularisquama* cells in the population harbored small virus-like particles (VLPs). Based on transmission electron microscopic (TEM) observation, morphological resemblance between these VLPs and the single-stranded RNA (ssRNA) virus infecting *H. circularisquama* (HcRNAV: *H. circularisquama* RNA virus) isolated from the bloom was noticeable. The fluctuation patterns of the viruses indicated that at least 2 distinct types of virus with different host specificity spectra coexisted. A specific increase in viral abundance in the sediments was observed in the middle of the bloom, and these viruses were likely able to maintain their infectivity for at least 3 mo. The present study provides further evidence of the possible viral impacts on the biomass and clonal composition of algal populations in the natural environment, and offers support for the hypothesis that sediments are a reservoir of algal viruses.

KEY WORDS: Algal virus · ssRNA virus · *Heterocapsa circularisquama* · Population dynamics · Red tides · Harmful algal blooms

INTRODUCTION

Since the first reports on the high abundance of virus-like particles (VLPs) in natural seawater, several studies have highlighted their ecological significance (Bergh et al. 1989, Proctor & Fuhrman 1990, Thingstad et al. 1993, Suttle 2000, Wommack & Colwell 2000). Evidence indicating a possible relationship between viral infections and termination of some phytoplankton blooms has also accumulated. In particular, viral impacts on blooms of *Emiliania huxleyi* (Prymnesiophyceae) and *Heterosigma akashiwo* (Raphidophyceae) have been intensively studied. In both cases, bloom terminations were followed by specific in-

creases in viral abundance, suggesting that viral infection is a significant factor regulating bloom dynamics (Bratbak et al. 1993, Nagasaki et al. 1994a,b, Brussaard et al. 1996, Tarutani et al. 2000). The objective of the present study was to examine the relationship between a microalga, *Heterocapsa circularisquama* (Dinophyceae), and viruses which infect it in the natural environment.

Heterocapsa circularisquama is a harmful bloom-forming dinoflagellate that specifically kills bivalves (Horiguchi 1995), and is distributed on the west coast of Japan and Hong Kong (Tamai 1999, Iwataki et al. 2002). Since 1988, *H. circularisquama* blooms in Japan have frequently caused mass mortalities of bivalves

such as the pearl oyster *Pinctada fucata martensii*, short-necked clam *Ruditapes philippinarum*, mussel *Mytilus edulis*, and oyster *Crassostrea virginica* (Nagai et al. 1996, Matsuyama 1999). Until 2002, more than 40 cases of *H. circularisquama* red tide have been recorded, including 18 incidences leading to fisheries damages (Matsuyama 2003). Ago Bay, the field area for the present survey, is an inlet dotted with small islands, and it is famous for its pearl oyster production (~USD 64 million yr^{-1}). The first outbreak of a *H. circularisquama* bloom in this bay was in August 1992. The maximum cell abundance during the bloom was 87 420 cells ml^{-1}, and 30 to 90% of cultured pearl oysters were damaged. Since then, *H. circularisquama* blooms occurred in this bay almost every year. The maximum abundances of *H. circularisquama* were 980, 55 800, 5000, 20 000, 3580, 0.01, 3860 and 3056 cells ml^{-1} from 1993 to 2000, respectively, and serious damage to pearl oyster culture was also caused in 1994 and 1996 (Nakanishi et al. 1999, Tamai 1999, Matsuyama 2003). Indeed the abundance and the duration of *H. circularisquama* blooms in recent years in Ago Bay have been declining, but *H. circularisquama* is still regarded as one of the more noxious phytoplankton species which need to be vigilantly watched.

A large icosahedral double-stranded DNA (dsDNA) virus infecting *Heterocapsa circularisquama* (HcV) was the first dinoflagellate-infecting virus to be isolated and characterized (Tarutani et al. 2001, Nagasaki et al. 2003). Just recently, another small icosahedral ssRNA virus (HcRNAV) causing lysis of *H. circularisquama* has been isolated from the western part of Japan (Tomaru et al. 2004, this issue). As the algicidal activity of both viruses is highly specific to *H. circularisquama*, their possible control of its natural populations is suggested. However, no conclusive evidence of their impact has

been obtained, as the ecological link between *H. circularisquama* and viruses in the natural environment has scarcely been elucidated so far. In the present paper, we report the dynamics of a *H. circularisquama* bloom and co-occurring viruses in Ago Bay in 2001, and discuss the possible relationship between them.

MATERIALS AND METHODS

Field survey and sampling. Field sampling was carried out at the Tategami Station in Ago Bay, Japan (Fig. 1), once a week from April through November 2001. This station is located in a semi-enclosed area in a water depth of ca. 10 m. Pearl-oyster aquaculture is a major industry in the area. Water temperature, salinity, and dissolved oxygen concentration of the 0.5, 5 m and bottom minus 1 m (B-1m) layers in the water column were measured by use of a Chlorotec Model ACL1150-DK (Alec Electronics) and a dissolved oxygen meter, YSI Model 58, according to manufactures' recommendation, respectively. Simultaneously, seawater samples were collected from each layer using a Kitahara's water bottle (1 l), and sediment samples (0 to 1 cm depth) were also collected using an Ekman-Birge bottom sampler equipped with a 36 mm ∅ corer tube (Yokoyama & Ueda 1997). They were immediately sent to the laboratory without fixation. Phytoplankton abundance was immediately assessed by direct counting with optical microscopy, and the titration of viruses infecting *Heterocapsa circularisquama* was carried out within 24 h of collection (see below). The sediment samples were stored at 10°C in the dark until titration in December 2001, i.e. 1 to 7 mo after collection. To evaluate the effect of storage to viral titer, both titration just after collection and re-titration after 7 mo storage
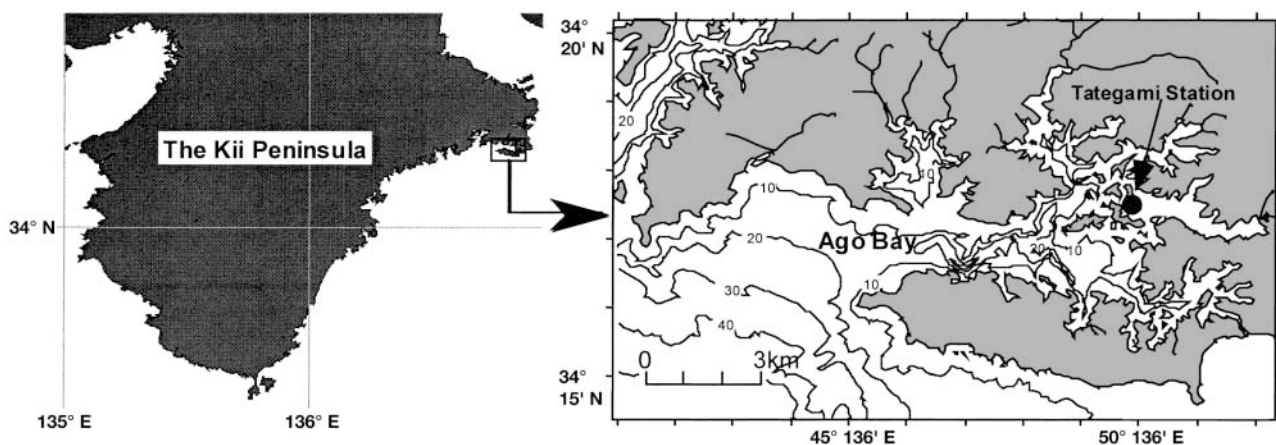

Fig. 1. Sampling station (Tategami Station; ●) in Ago Bay, Mie Prefecture, Japan. In the detailed map (right), the depth contour line (m) is also shown

were conducted on a sediment sample collected on 5 August 2002 in Ago Bay.

Titration of viruses infecting *Heterocapsa circularisquama*. Before virus titration, seawater samples were prefiltered through 0.8 μm pore-size polycarbonate membrane filters (Nuclepore) to remove zooplankton, phytoplankton, and most bacteria. Three grams of each sediment sample were shaken with 3 ml of modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987) enriched with 2 nM Na_2SeO_3 at 400 rpm for 30 min, and centrifuged at $716 \times g$ for 10 min at 4°C. The supernatant was filtered through a 0.2 μm pore-sized Dismic-25cs filter (Advantec) to remove bacteria. The abundance of viruses infecting *H. circularisquama* in the seawater samples (5 m and B-1m) and the sediment samples was enumerated by means of the extinction-dilution method (Suttle 1993, Nagasaki & Yamaguchi 1997). Briefly, filtrates were diluted with modified SWM3 medium in a series of 10-fold dilution steps, and aliquots (100 μl) of each dilution step were added to 8 wells in cell culture plates with 96 round-bottom wells (Falcon) containing 150 μl of an exponentially growing host culture. As hosts, *H. circularisquama* strains HA92-1, HY9423, HU9433-P and HCLG-1 were used to enumerate viruses in the water samples, and HU9433-P and HCLG-1 were used to enumerate viruses in the sediment samples (Table 1). The cell culture plates were incubated under a 12:12 h light:dark cycle of 130 to 150 μmol photons $m^{-2} s^{-1}$ with cool white fluorescent illumination at 20°C. The occurrence of algal lysis was then monitored every other day for 10 to 14 d by optical microscopy, and the most probable number of lytic viruses was calculated using the computer program designed by Nishihara et al. (1986). In the procedure, 8 viral clones from the sediment samples were obtained by 2 cycles of the extinction-dilution procedure (Nagasaki & Yamaguchi 1997), using the same host strain of *H. circularisquama* that was initially used to obtain the pathogen. Their morphological features were examined by TEM as outlined below.

Transmission electron microscopy. *Heterocapsa circularisquama* cells from the seawater sample

obtained from the B-1m layer on 16 July 2001 were examined by TEM according to the method described by Tarutani et al. (2001). Thin sections were stained with uranyl acetate and lead citrate, and then observed under a JEOL JEM-1010 TEM. The intracellular structure of natural cells was compared to that of exponentially growing cells incubated under a 12:12 h light:dark cycle of 130 to 150 μmol photons $m^{-2} s^{-1}$, with cool white fluorescent illumination at 20°C. In the same way, thin sections of *H. circularisquama* cells at 2 d post-infection of the viruses from sediment samples and the negatively stained virus particles were also observed with TEM.

RESULTS

Hydrographic data

Hydrographic data collected at the Tategami Station are shown in Fig. 2. A *Heterocapsa circularisquama* bloom with a cell density of 10^{-1} to $\sim 10^3$ cells ml^{-1} occurred from 2 through 23 July (hereafter we define this as the bloom period), with bottom temperatures ranging from 20.5 to 22.8°C, salinity 34.3 to 34.4 psu, and dissolved oxygen 3.6 to 4.3 mg l^{-1} (Fig. 2).

Dynamics of *Heterocapsa circularisquama* and viruses in the water column

The peak of *Heterocapsa circularisquama* abundance was in the B-1m layer on 16 July (2450 cells ml^{-1}). Following the peak, *H. circularisquama* abundance rapidly decreased up to 30 July (Fig. 3A). During the survey, *H. circularisquama* cells were scarcely observed in the surface layer (0.5 m), and the cell abundance in the 5 m layer was 3 orders of magnitude lower than that of the B-1m layer at the peak of the bloom (16 July).

In the B-1m layer, viruses infecting *Heterocapsa circularisquama* strains HU9433-P and HA92-1

Table 1. *Heterocapsa circularisquama.* Strains used in the present experiments to enumerate the virus titer by means of the extinction-dilution method. All of them are axenic and clonal

| Strain	Origin	
	Location	Isolation date
H. circularisquama HU9433-P[a]	Uranouchi Bay (Kochi Prefecture)	Dec 1994
H. circularisquama HA92-1	Ago Bay (Mie Prefecture)	Jun 1992
H. circularisquama HCLG-1[a]	Gokasho Bay (Mie Prefecture)	Aug 1999
H. circularisquama HY9423	Yatsushiro Kai (Kumamoto Prefecture)	Sep 1994
[a]Strain used to enumerate the lytic viruses in the sediment samples		
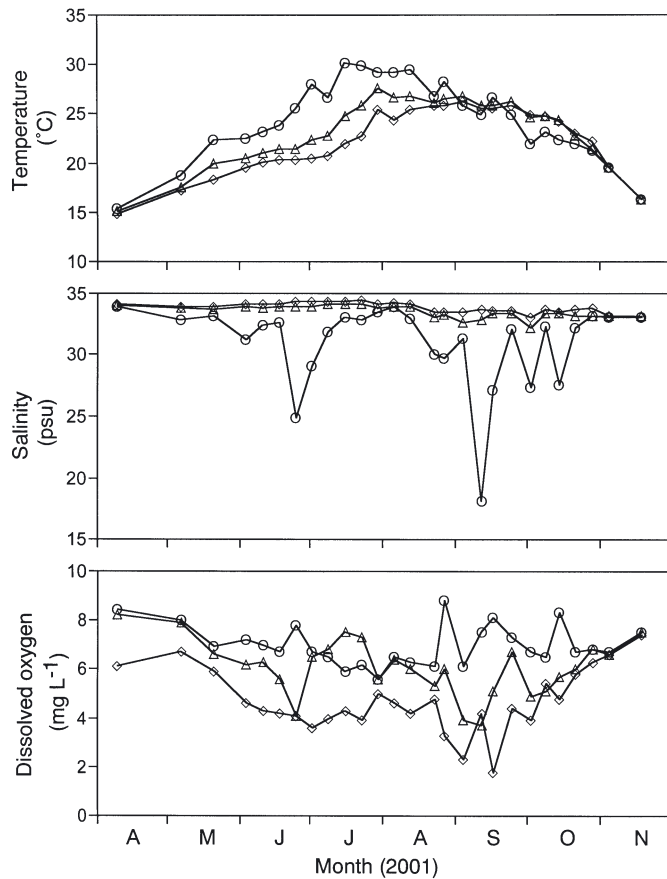
Fig. 2. Temporal changes in the hydrographic conditions in 0.5 m (O), 5 m (△), and bottom minus 1 m (B-1m; ◇) layers at the Tategami Station during the field survey

showed similar trends in terms of dynamics, i.e. following a rapid increase from 16 through 23 July, they gradually decreased from 23 July to the end of August (Fig. 3C). Also in the 5 m layer, similar trends of dynamics were noticeable between them (Fig. 3B). Two different trends of fluctuation in virus abundance were observed through the survey using 4 *H. circularisquama* strains as hosts. At B-1m, the abundance of viruses infecting Strains HU9433-P and HA92-1 increased from 16 through 23 July and then gradually decreased to the end of August (Fig. 3C). The same decline was observed at 5 m (Fig 3B). Viruses infecting Strains HCLG-1 and HA92-1 also showed the same development, both at B-1m and 5 m, but the decrease in abundance to <0.1 infectious unit ml^{-1} occurred 2 to 3 wk earlier than for the viruses infectious to HU9433-P and HA92-1 (Fig. 3B,C).

In conjunction with the present study, 52 virus clones were isolated from the same water samples (Tomaru et al. 2004). However, no large dsDNA virus, such as HcV (Tarutani et al. 2001), was found among them.

Viral dynamics in the sediments

Fluctuation in viral titer of the sediment samples is shown in Fig. 3D. As a preliminary experiment revealed that storage at 10°C in the dark considerably affected the viral titer of sediment samples (93 to 98% decrease within 7 mo), this graph should be regarded as data reflecting an approximate trend in viral dynamics of the sediments, but not reflecting the absolute titers. The abundance of lytic viruses in the sediments showed a rapid increase during the *Heterocapsa circularisquama* bloom, from 16 through 23 July, and then gradually decreased from August through November (Fig. 3D).
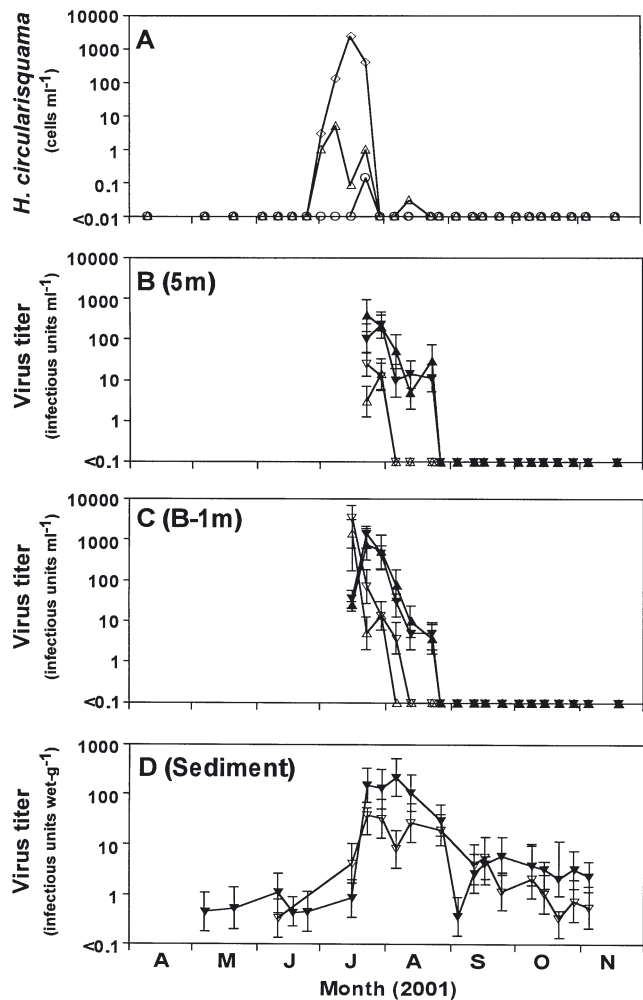

Fig. 3. *Heterocapsa circularisquama.* Temporal changes in the abundance in 0.5 m (O), 5 m (△), and bottom minus 1 m (B-1m; ◇) layers at the Tategami Station (A), and the lytic viruses in 5 m layer (B), B-1m layer (C), and in the sediments (D). Abundance of viruses lytic to *H. circularisquama* was enumerated by the extinction-dilution method using the 4 typical host strains shown in Table 1: HU9433-P (▼), HA92-1 (▲), HCLG-1 (▽), and HY9423 (△). Error bars are 95% CIs

TEM observations

TEM observations revealed that the existence of small VLPs was restricted to the cytoplasm of the *Heterocapsa circularisquama* cells in the B-1m layer on 16 July (Fig. 4C,D), but no large HcV-like particles were detected. Small VLPs were detected in 88% of the thin sections of *H. circularisquama* (n = 202), and their distribution was restricted to within the cytoplasm. Although no crystalline array was observed, the VLPs were considered to be similar in appearance to the ssRNA virus HcRNAV simultaneously isolated from the same seawater sample (Tomaru et al. 2004). In addition, the intracellular structure of natural cells was remarkably different from that of vigorously growing cells incubated under suitable conditions, i.e. largely
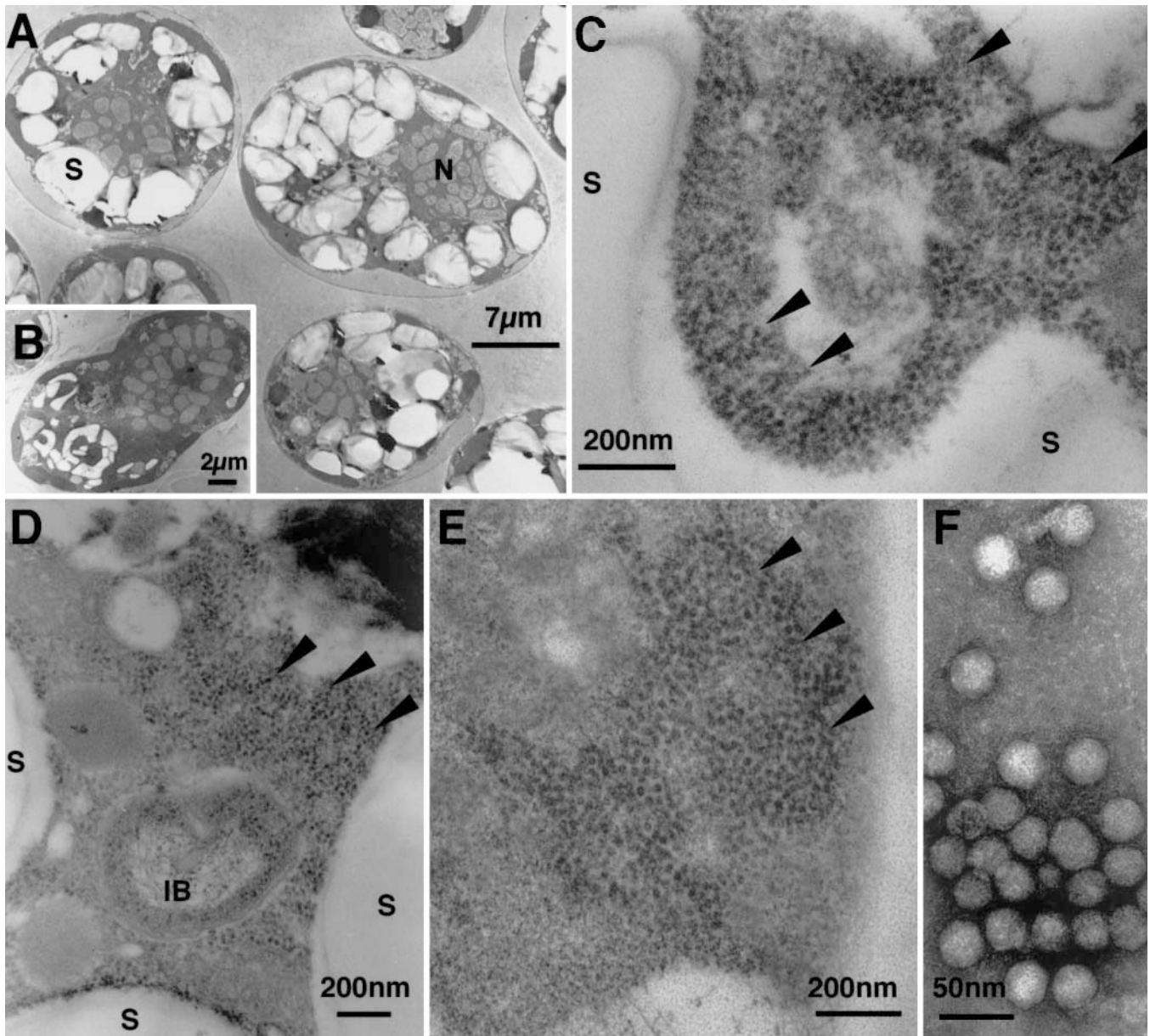

Fig. 4. *Heterocapsa circularisquama*. Transmission electron micrographs. (A) Cells from the natural population sampled from the bottom minus 1 m (B-1m) layer at the Tategami Station on 16 July 2001. For comparison with (A), a thin section of a vigorously growing cell incubated under suitable conditions (see 'Materials and methods') is shown (B). (C,D) Higher magnifications of virus-like particles randomly dispersed within the cytoplasm of *H. circularisquama* cells in (A) (arrowheads). (E) Aggregation of viruses within the cytoplasm of a *H. circularisquama* cell infected with a HcRNAV-like virus isolated from the sediment sample, and (F) negatively stained virus-like particles (VLPs) in the suspension of (E). S = starch grain, N = nucleus, IB = intracellular bacterium

occupied with vacuoles filled with an electron-lucent material (Fig. 4A), which are likely starch grains (Horiguchi 1995). In contrast, vigorously growing cells contained a smaller number of vacuoles (Fig. 4B).

Viruses isolated from the sediment samples were icosahedral and 31 ± 2 nm (mean ± SD, n = 30) in diameter, and formed random aggregations in the cytoplasm of *Heterocapsa circularisquama*; thus, they are considered to be morphologically similar to HcRNAV (Fig. 4E,F).

DISCUSSION

In the present survey, as virus titration of the water samples was not started until the peak of the bloom (Fig. 3B,C), viral dynamics before this time are unknown. However, considering that viral abundance in the water column was less than the detectable limit after September when host cells were not observed in the water column, it is presumable that the viral titer in the water column was low before the bloom and increased during the bloom period in July (Fig. 3). This conjecture was also supported by the results obtained through the field survey in 2002 at the same sampling station, in which viruses infecting *Heterocapsa circularisquama* were scarcely detected before the bloom, but showed a specific increase in its early stage (Tomaru et al. unpubl. data). Thus, a possible relationship between *H. circularisquama* and viruses was suggested: emergence of viruses in the bloom period, both in the water column and the sediments, most probably reflected the results of viral lysis of *H. circularisquama* cells (Fig. 3). The observation that a large proportion (88%) of *H. circularisquama* cells harbored VLPs (i.e. most likely infected by HcRNAV-like viruses) also strengthens the above speculation. In laboratory experiments, *H. circularisquama* cells infected by HcRNAV markedly lost their motility (Tomaru et al. 2004). Thus, it is likely that virus-infected *H. circularisquama* cells had sunk down to the bottom layer and sediments during the bloom, and then burst to supply newly produced viruses to the environment.

In conjunction with the present survey, only small types of virus were isolated by means of the extinction-dilution method from natural waters and sediments (Tomaru et al. 2004). By this method, the most abundant types of virus lytic to a *Heterocapsa circularisquama* strain (used as a host) tend to be selected and isolated more frequently, because viruses were picked up from the most diluted wells in the isolation procedure. The reason why no large viruses such as HcV were isolated might be due to the dominance of the HcRNAV-like virus. Another point to consider is that large virus particles were not detected in thin sections

of *H. circularisquama* at the peak of the bloom either. As for the viruses isolated from the sediments, they were also morphologically similar to those isolated from the water samples (Fig. 4E,F). Therefore, we consider that the small viruses were more abundant than the large ones in the present bloom.

Because high abundance of lytic viruses was detected in the sediments even after the viruses in the water column disappeared in August, they may be longer lived than in the water column once they are buried in the sediment. Based on the results of titration (Fig. 3D), it is probable that viruses remained infective in the sediments for more than 3 mo. These results are comparable to those of Lawrence et al. (2002), who detected viral agents lysing *Heterosigma akashiwo* as deep as 40 cm below the sediment-water interface. Considering that 93 to 98% of the viral titer was lost within 7 mo of storage at 10°C in the dark, the gradual decrease in virus titer in the sediments from August through November (Fig. 3D) might have been caused by the inactivation of viruses, i.e. loss of infectivity. Resuspension is also a possible explanation for the decrease in virus abundance in the sediments, but the low values found in the water above the sediment (B-1m) do not support this hypothesis.

Another noticeable point is that 2 apparently different trends in fluctuation of viral abundance were observed in the water column by using 4 *Heterocapsa circularisquama* strains as hosts in the survey (Fig. 3B,C). This suggests that there were 2 distinct types of viruses having different host specificity spectra; one is infectious to HU9433-P and HA92-1 (UA-type), and the other to HCLG-1 and HY9423 (CY-type). They were also detected in the sediments (Fig. 3D). These results agree well with the observation that HcRNAV strains isolated from western Japan were divided into 2 types (UA and CY types) based on their infection spectra (Tomaru et al. 2004). The infection specificity of each type of HcRNAV was strain-specific, and complementarily different to one other, i.e. UA-type viruses were lytic to some of the *H. circularisquama* strains, but not to those that were sensitive to CY-type viruses, and vice versa. Among 4 *H. circularisquama* strains isolated from the bloom (HCAG-2 to 5), 3 (HCAG-2, -3 and -4) were specifically sensitive to the CY-type virus, and 1 (HCAG-5) was specifically sensitive to the UA-type virus (Tomaru et al. 2004). Thus, because the distinct types of virus and host coexisted in the environments, it is probable that the dynamics of each type of virus reflected the dynamics of its respective host, and the 2 types of virus play a significant role in regulating the intra-species diversity as well as the biomass of *H. circularisquama* populations in the natural environment. Indeed, these observations do not completely rule out the possibility of simultaneous infection by the

2 types of virus, but *H. circularisquama* clones having apparent sensitivity to both types of virus were not isolated (n = 56). Hence, even though *H. circularisquama* clones sensitive to both types of virus had coexisted, it is unlikely that they were dominant in the population. Of course, the sensitivity spectrum of each host clone should be translated with care, as the effects of growth and physiological condition of hosts on their susceptibility to viruses was barely examined.

Physical conditions, nutrient conditions, and impacts by grazers are also considered to be important factors regulating algal blooms, but these factors were not examined sufficiently in the present survey. Physical parameters (temperature, salinity, and dissolved oxygen concentration) did not show any significant changes in the duration of the *Heterocapsa circularisquama* bloom (Fig. 2), which were in a suitable range for the growth of *H. circularisquama* (Yamaguchi et al. 1997, Nakanishi et al. 1999). With regard to nutrients in the bottom layer, concentrations of both dissolved inorganic nitrogen (N) and phosphate (P) ranged at a relatively low level: 0.16 to 0.52 and 0.03 to 0.13 μM, respectively (K. Nakanishi et al. unpubl. data). Thus, low nutrient conditions are considered to be another significant factor affecting the dynamics of the bloom. Although it is unknown what caused the apparent difference in intracellular structures between the natural cells observed at the peak of the bloom and vigorously growing cells in culture (Fig. 3A,B), one possible explanation is the physiological effects of the low nutrient condition, as HcRNAV-infected cells did not show the similar intracellular feature (i.e. maximizing of vacuoles) in laboratory experiments (Tomaru et al. 2004). Because the abundance of zooplankton was not monitored in the present survey, the grazing impact on the *H. circularisquama* population is unknown. Thus, only based on the present results, it is difficult to assess the extent of contribution of each factor in regulating the bloom dynamics and causing the bloom termination.

Nevertheless, the present study provides further evidence for the possible viral impact on the biomass and clonal composition of algal populations in the natural environment, and supports the hypothesis given by Tarutani et al. (2000): viruses are an important component in quantitatively and qualitatively controlling phytoplankton populations. Because up to 88% of the *Heterocapsa circularisquama* cells in the population contained VLPs in their cytoplasm, the impact of viral infection was considered exceedingly high compared to the cases of other algal viruses reported so far (Bratbak et al. 1993, Nagasaki et al. 1994a,b, Brussaard et al. 1996, Gastrich et al. 2002). The results of our field survey and the intra-species host range assay given by Tomaru et al. (2004) suggest that 2 possibly indepen-

dent host-virus systems were involved in the *H. circularisquama* bloom. To provide direct evidence of the viral impacts on host clonal composition, however, molecular probing techniques would be required to identify different types of host and to distinguish infection by different types of virus. The present study also offers support for the hypothesis that sediments are a reservoir of viruses (Lawrence et al. 2002). The presence of HcRNAV-like viruses in sediments provides indirect evidence that viral infection of *H. circularisquama* occurs in overlying waters. However, processes of viral recruitment into sediments, and viral release (resuspension) from sediments, have not been elucidated yet. Our present results will invite further investigation into this area in the future.

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