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Research Paper

Dynamics of bivalve-killing dinoflagellate *Heterocapsa circularisquama* and its infectious virus-like agent in Ago Bay from 2005 to 2007

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ABSTRACT

We evaluated the dynamics of the bloom-forming dinoflagellate *Heterocapsa circularisquama* and its infectious virus-like agents (VLAs) in Ago Bay, Japan from 2005 to 2007. Cell density of *H. circularisquama* and various environmental factors were monitored one to four times per month; simultaneously, VLA titers in the water and sediment were also measured. The heavy bloom was observed in 2005 (> 10^3 cells mL^{-1}); the 2006 and 2007 blooms were lighter at < 10^2 cells mL^{-1}. In the present survey, each bloom was accompanied by specific increase in abundance of VLAs. This strongly suggests the intimate host-parasite relationship between *H. circularisquama* and the VLAs, as was observed in the case of previous surveys. Whereas, not as expected, abundance of VLAs in the sediment before the host bloom season did not show correlation with the *H. circularisquama* population size. For extracting laws concerning their relationship in natural water, more intensive and repetitive field survey will be required.

Key words: lytic virus, microalgal bloom, Mie prefecture, sediment

INTRODUCTION

Heterocapsa circularisquama is a bloom-forming small thecate dinoflagellate that kills bivalves (Horiguchi 1995). This species was first detected in 1988 in Uranouchi Inlet, Kochi Prefecture, Japan. Since then, its distribution has expanded rapidly throughout the central and western part of Japan. *H. circularisquama* often forms dense blooms that cause mass mortality in bivalves, such as oysters and short-necked clams (Matsuyama 2003, Kondo et al. 2012).

To date, two distinct viruses which infect *H. circularisquama* have been reported: a large icosahedral DNA virus (Heterocapsa circularisquama DNA virus [HcDNAV], 197 ± 8 nm in diameter [mean ± standard deviation]) and a small icosahedral RNA virus (Heterocapsa circularisquama RNA virus [HcRNAV], 34 nm in diameter) (Tarutani et al. 2001, Tomaru et al. 2004, Miller et al. 2011). HcDNAV harbors a double-stranded DNA (365 kbp) genome and is suggested to be the sole member of an unranked virus of genus Dinodnavirus (ICTV: https://talk.ictvonline.org/taxonomy/). HcRNAV has a single-stranded RNA (4.4 kb) genome encoding replicases and capsid proteins (Tomaru et al. 2004, Nagasaki et al. 2005). Recently, this virus has been classified into a new genus (Dinornavirus) and a new family (Alvernaviridae) in the order Sobelivirales based on a phylogenetic analysis of its RNA-dependent RNA polymerase domain (Wolf et al. 2018, Lang et al. 2021) (ICTV: https://talk.ictvonline.org/taxonomy/).

Considerable efforts have been paid to elucidate the ecology of *H. circularisquama* and its infectious viruses so far

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(Nagasaki et al. 2004, Tomaru & Nagasaki 2004, Tomaru et al. 2007, Fujimoto et al. 2013). Through the survey, viral infection was suggested to significantly regulate *H. circularisquama* bloom, especially in the termination phase. Another interesting hypothesis is that viruses in the sediment just before *H. circularisquama* bloom season might be a key factor regulating the host population size; i. e., large amount of viruses in sediments can reduce the abundance of *H. circularisquama*, and vice versa (Tomaru et al. 2007). However, this hypothesis is yet to be verified and validated in long-term field studies. The present study aimed to examine whether the above latter hypothesis is supported also based on the field data collected in Ago Bay in Japan, from 2005 to 2007.

MATERIALS AND METHODS

Field survey and sampling

Field sampling was performed at Tategami Station (at approximately 10 m depth) in Ago Bay from January 2005 through December 2007 (Fig. 1). Sampling was done one to four times per month. The water temperature and salinity were measured at 0.5 m, 5 m, and 1 m above the bottom (B-1 m) layer of the water column using a Chlorotec Model ACL200-DK analyzer (Alec Electronics Co., Ltd., Kobe, Japan). Simultaneously, seawater samples were collected from each depth using a Kitahara water bottle (1 L). Sediment samples

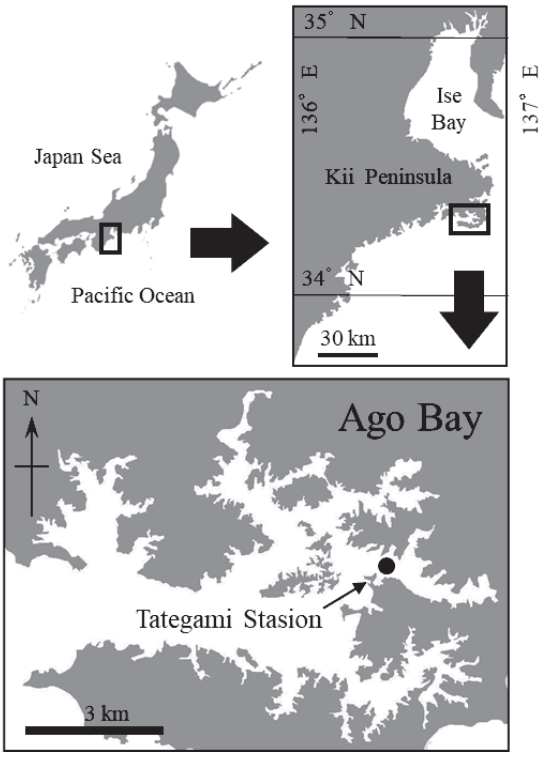

Fig. 1. Location of Tategami station in Ago Bay, western Japan.

(at 0-1 cm depth) were collected using an Ekman-Birge bottom sampler equipped with a 36 mm Ø-corer tube (Yokoyama and Ueda 1997). *H. circularisquama* abundance was directly measured immediately after sampling using optical microscopy. The lytic agent enumeration samples were sent to our laboratory at Hatsukaichi in Hiroshima, Japan, without fixation at 4 ˚C. The *H. circularisquama* lytic agent titers were determined within 24 h of collection. Furthermore, the water samples were filtered through a glass fiber filter (GF/C; Whatman, Little Chalfont, United Kingdom) to determine dissolved inorganic nutrients (ammonia, nitrate, nitrite [DIN], and phosphorus [PO_4-P]). The filtrates were stored at -30 ˚C until analysis, and their measurement was performed on an autoanalyzer (Bran-Luebbe, TRAACS 2000, Norderstedt, Germany).

Virus-like agent (VLA) titration

Before lytic agent titration, seawater samples were prefiltered through 0.8 µm pore-sized polycarbonate membrane filters (Nuclepore, Whatman) to remove zooplankton, phytoplankton, and most bacteria. Three grams of each sediment sample were shaken with 3 mL of modified SWM-3 media (Imai et al. 1996) enriched with 2 nM Na_2SeO_3 at 400 rpm for 30 min, and then centrifuged at 716 *g* for 10 min at 4 ˚C. Then, the supernatant was filtered through a 0.2 µm pore-sized Dismic-25cs filter (Advantec, Tokyo, Japan) to remove bacteria; these filtrates were used for further experiments.

Lytic agent titrations of the seawater (0.5 m, 5 m, and B-1 m) and sediment samples were conducted using the extinction dilution method (i.e., the most probable number [MPN] assay) proposed by Nagasaki et al. (2004). In this case, *H. circularisquama* HU9433-P and HA92-1 (type UA HcRNAV-sensitive strains), and HCLG-1 and HY9423 (type CY HcRNAV-sensitive strains) were used while titrating host strains for viral populations in the water samples (Tomaru et al. 2004); only HU9433-P and HCLG-1 were used as host strains for the sediment samples. Briefly, the filtrates were serially diluted with modified SWM-3 medium in 10-fold dilution steps. Aliquots of each dilution (100 µL) were added to 8 wells of a 96-well round-bottom cell culture plate (Falcon Brands, Irvine, CA, USA) and mixed with 150 µL of an exponentially growing *H. circularisquama* culture. The cell culture plates were incubated under a 12 h: 12 h light/dark cycle of 130 to 150 µmol photons m^{-2} s^{-1} under cool white fluorescent illumination (FL40S D EDL D65, Toshiba, Tokyo, Japan) at 20 ˚C. Algal lysis was monitored every alternate day for 14 days by optical microscopy. The MPN of the lytic agents for each host strain was calculated using a computer program developed by Nishihara et al. (1986). Thus, the abundances of the type UA and CY lytic agents were

respectively estimated as MPN values of infectious units lytic to each agent-sensitive host strain.

Algal lysates in the most diluted wells were filtered through a 0.2 μm pore-size filter and inoculated into a fresh algal culture (1 % v/v) to verify their algicidal activity. After inoculation, the cultures were incubated under the conditions as mentioned above for 14 days. As the lytic agent likely has a diameter < 0.2 μm, the resultant MPN is considered to indicate the density of infectious viruses (i. e., VLA concentration). In the present study, no molecular biological method was used to verify if the lytic agents were viruses.

RESULTS

Dynamics of *H. circularisquama* and its VLA

During the field survey, relatively large-scale *H. circularisquama* blooms occurred from June 30 to August 8, 2005; the maximum abundance was 2.1 × 10^3 cells mL^{-1} at 5 m depth on July 20 (Fig. 2A). In 2006 and 2007, moderate *H. circularisquama* blooms occurred, and the maximum abundances were 20 cells mL^{-1} at B-1 m on August 24, and 9.7 cells mL^{-1} at B-1 m on July 27, respectively (Fig. 2A).

The abundance of VLAs causing lysis of *H. circularisquama* increased in the water column and sediment when *H. circularisquama* blooms occurred, then gradually decreased after the bloom (Fig. 2A-E). The water column had two distinct types of VLAs: 1) type UA-VLA, comprising VLAs from host strains HU9433-P or HA92-1; and 2) type CY-VLA, comprising VLAs from host strains HCLG-1 or HY9423 (Fig. 2B-D); these results were similar to that of a previous study in Ago Bay (Tomaru et al. 2007).

VLAs in water column and sediment

In 2005 summer, type UA- and CY-VLAs were detected in the water column on July 11 (Fig. 2B-D). The average abundance of type CY-VLA in the water column was > 10^3 infectious units mL^{-1}, higher than that of type UA. However, it decreased by two orders of magnitude in the following week

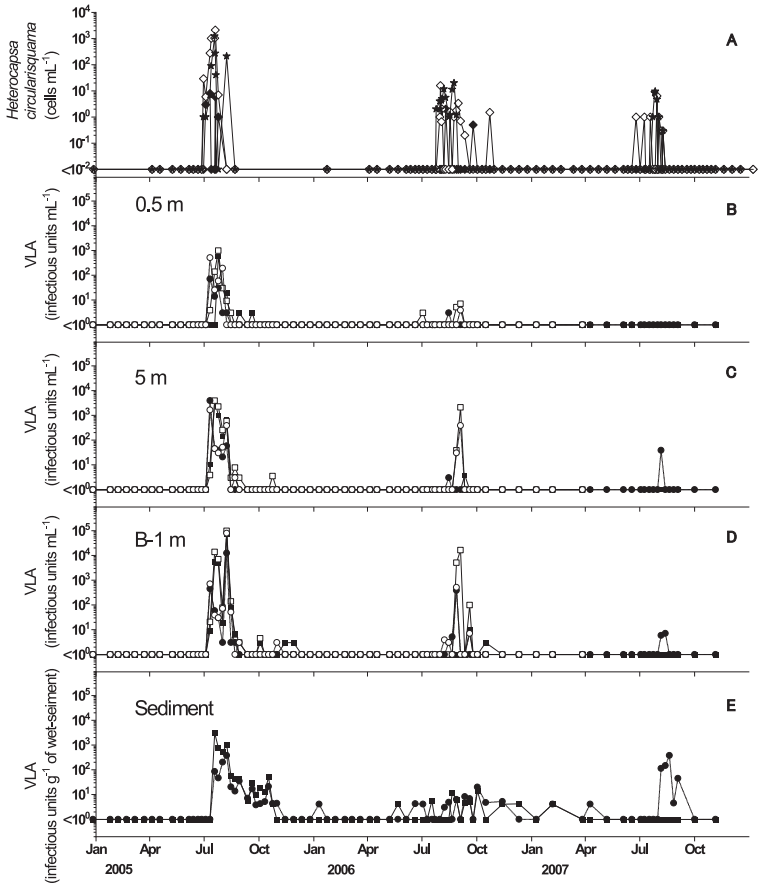

Fig. 2. Temporal changes in the abundance of *Heterocapsa circularisquama* at 0.5 m (◆), 5 m (◇), and 1 m above the bottom (B-1; ★) (A) and its virus-like agents (VLAs) at 0.5 m (B), 5 m (C), B-1 m (D), and in the sediment (E) in Ago Bay, Japan, from 2005 through 2007. The abundance of VLA was estimated by the extinction dilution method using four host strains: HU9433-P (■), HA92-1 (□), HCLG-1 (● with dotted line), and HY9423 (○ with dotted line). We treated HU9433-P and HA92-1 as host strains sensitive to type UA-VLAs and HCLG-1 and HY9423 as those sensitive to type CY-VLAs.

and maintained at the lower level until August 1 (Fig. 2B-D). The abundance of type UA-VLAs rapidly increased by three orders of magnitude from July 11 to July 18 and then gradually decreased, ranging from 1.9×10^1 to 2.6×10^2 infectious units mL^{-1} on August 1 (Fig. 2B-D). Both VLA types increased again to August 8, 2005, when a second *H. circularisquama* bloom peak occurred; the maximum abundances of UA- and CY-VLAs were 9.8×10^4 and 7.7×10^4 infectious units mL^{-1}, respectively, occurring at B-1 m (Fig. 2D).

VLAs were first detected in the sediment on July 19, 2005, one week after VLAs in the water column began to increase (Fig. 2E). The maximum abundances of type UA- and CY-VLAs were 3.0×10^3 infectious units g^{-1} of wet sediment on July 19, 2005, and 2.0×10^2 infectious units g^{-1} of wet sediment on August 1, 2005, respectively. After this date, the values gradually decreased (Fig. 2E).

Although only small-scale *H. circularisquama* blooms occurred in 2006 and 2007, higher abundance of VLAs were detected in both years. In the water column, type UA-VLAs peaked at 1.6×10^4 infectious units mL^{-1} on September 4, 2006, while type CY-VLAs peaked at 5.1×10^2 infectious units mL^{-1} on August 28, 2006 (Fig. 2D). Furthermore, in 2007, type CY-VLA peaked in the water column at 3.9×10^1 infectious units mL^{-1} on August 6, but type UA-VLA did not show increase in abundance (Fig. 2C).

VLA concentrations in sediments were low in 2006; type CY-VLA peaked at 2.0×10^1 infectious units g^{-1} of wet sediment on October 2, 2006. After that, both VLA types fluctuated but maintained lower concentrations until April 2007 (Fig. 2E). In 2007 summer, increase in sediment VLAs began on August 6. The maximum concentration of type CY-VLA was 3.8×10^2 infectious units g^{-1} of wet sediment, on August 20, 2007 (Fig. 2E); type UA-VLAs went undetected in the sediment in 2007.

Environmental factors

The temporal changes in water temperature, salinity, and water density in Ago Bay, from 2005 to 2007, are shown in Figure 3. In each year, the water temperatures during the bloom season ranged from 21 to 30 ℃ (Fig. 3A). During the

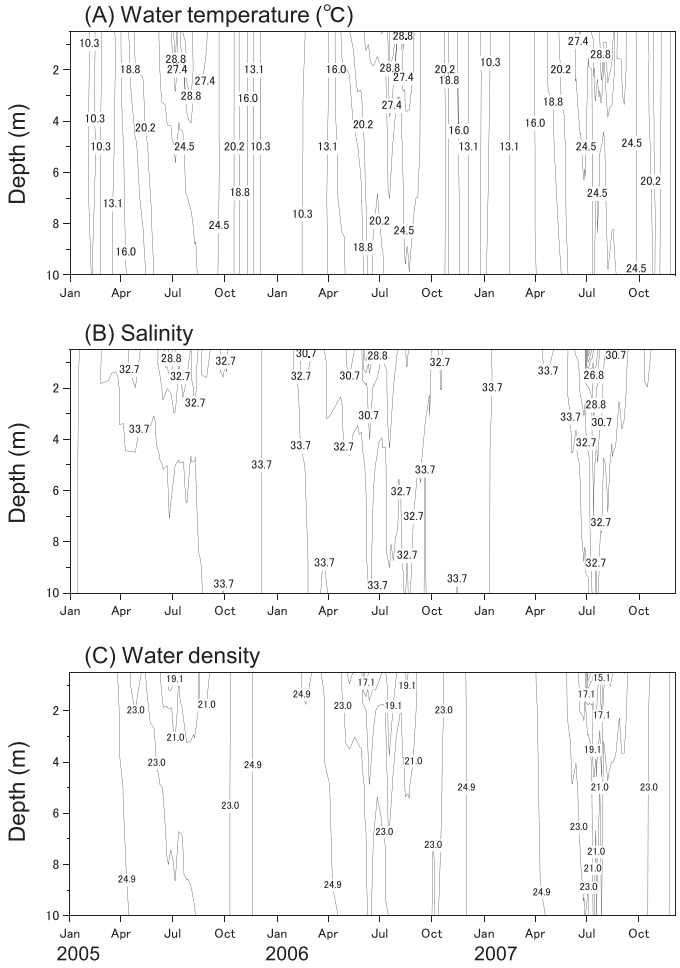

Fig. 3. Temporal changes in water temperature (A), salinity (B), and water density (C) in Ago Bay, Japan, from 2005 to 2007.

2005 and 2006 bloom periods, the salinities were > 29, whereas those in 2007 were relatively lower (Fig. 3B). On July 17, 2007, the surface layer salinity was 19, after which it gradually increased but remained below 30 during the bloom period (Fig. 3B). Furthermore, the water density was lower in 2007, reflecting lower salinity (Fig. 3C). Finally, the DIN and PO_4-P concentrations ranged between 0.39-7.36 μM (N) and < 0.01-0.72 μM (P) in 2005, 0.87-15.54 μM (N) and < 0.001-1.92 μM (P) in 2006, and 0.33-14.94 μM (N) and < 0.001-1.31 μM (P) in 2007, respectively (data not shown).

DISCUSSION

Effects of hydrographic factors on *H. circularisquama* blooms

Throughout the observation period, *H. circularisquama* blooms occurred every summer from July till September. In this study, the hydrographic parameters during the bloom period were within their reasonable ranges for *H. circularisquama* growth (i. e., 20-30 °C, salinity ≥ 15) (Yamaguchi et al. 1997). The point of note was that the nutrient concentrations were the lowest in 2005, when *H. circularisquama* density was the highest. Although, dynamics of phytoplankton (ex. bloom size) is not easily explainable only based on nutrient conditions, accumulation of field data analysis is essential to find a law between bloom dynamics and its ambient hydrographic conditions.

Viral dynamics

In 2005 and 2007, the VLA abundance increased both in the water column and sediment after initiation of *H. circularisquama* blooms (Fig. 2). However, in 2006, low abundance of VLAs was continuously detected in the water column and sediment from May through July, before the *H. circularisquama* bloom (Fig. 2B, E). Furthermore, sediment VLAs accumulated in 2005 summer, gradually decreased in late October, and then were sporadically detected on January 10, 2006 (Fig. 2E). However, no sediment VLAs were detected from late January through early May of 2006 (Fig. 2E).

Two possible explanations are proposed for this phenomenon. First, *H. circularisquama* population had started propagation in May 2006, but remained under the detection limit (< 0.01 cells mL^{-1}), until initiation of the bloom. Under these conditions, VLAs may have occasionally infected host cells, supplying progenies to the water column and sediment. However, when the environmental conditions became suitable for growth and the host population increased, the level of VLAs also increased.

The second possibility is that VLAs originated from natural hosts other than *H. circularisquama* affected its dynamics. In most cases, algal viruses are species- and strain-specific (Tomaru et al. 2015). In the case of diatom-infecting viruses, their infection has been shown to be highly species-specific or inter-specific (Kimura and Tomaru 2015). Although infection of HcDNAV and HcRNAV in organisms other than *H. circularisquama* have not yet been found, this is to be noted as one of the possible schemes. In actual, recent bioinformatic studies suggested the existence of HcRNAV- or HcRNAV-like viruses in diverse environmental and organismal samples (Wolf et al. 2018, Karki et al. 2021, Takahashi et al. 2021).

In 2006 and 2007, the abundance of *H. circularisquama* in the blooms was < 10^2 cells mL^{-1} (Fig. 2A), and the maximum abundance of VLAs in the water column was two orders of magnitude higher in 2006 than in 2007 (Fig. 2C, D). Conversely, the maximum abundance of VLAs in the sediment was one order of magnitude lower in 2006 than that in 2007 (Fig. 2E). Thus, the sedimentation of infected *H. circularisquama* cells before cell lysis is considered to be the main cause of increase in abundance of VLAs in the sediment. In actual, virus-infected *H. circularisquama* cells are immotile (Tomaru et al. 2004), settling at the sea bottom (Nagasaki et al. 2004). Moreover, various factors may determine whether *H. circularisquama* cells lyse in the water column or sink to the sea bottom, including the depth at which the cells are infected, the latent period, and the sinking rate of the infected cells. Water density might also affect the sedimentation of infected cells. Generally, based on Stokes' law, the sedimentation velocity of plankton is higher in lower-density waters (Tanimoto and Hoshika 1994). In this study, the abundance VLAs increased in the water column in 2006 (August 21 till September 11) and 2007 (July 31 till August 20) (Fig. 2B-D). Notably, the average water density was lower in 2007 (19.3) than in 2006 (20.8) (Fig. 3C).

Moreover, the VLA burst sizes are important for understanding VLA abundances in the environments. Environmental factors, water temperature, light intensity, salinity, and nutrients affect the latent period and burst size, controlling physiological conditions (Mojica and Brussaard 2014). Therefore, adequately addressing viral accumulation patterns in water columns and sediments will require a detailed analysis of infected cell sedimentation and viral replication under various environmental gradients.

Sediment VLA before *H. circularisquama* blooms

Sediment VLAs were not detected before *H. circularisquama* blooms in 2005 and 2007, which were relatively heavy and light, respectively. In 2006, the *H. circularisquama* bloom was

relatively light, but the sediment VLAs gradually increased for almost two months before the bloom. The viruses lyse *H. circularisquama* cells even at low concentration level, although the annual bloom size was not controlled by the viral infection alone. In 2004, the sediment and the water above it in Ago Bay exhibited increased viral abundance just before the bloom. However, *H. circularisquama* caused a high-concentration bloom (> 10^3 cells mL^{-1}) during this season (Tomaru et al. 2007).

Our field survey in Ago Bay from 2005 to 2007 tested the hypothesis that the sediment virus abundance just before the host bloom regulates *H. circularisquama* population size in that season. Notably, however, our data did not support this hypothesis. In general, physical and chemical factors principally affect phytoplankton growth, such as water temperature, light intensity, salinity, and nutrients. Therefore, to better understand *H. circularisquama* and its relationship with its infectious virus in nature, we should continuously collate detailed field data from diverse environmental conditions for future analyses.

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