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Quantitative and qualitative impacts of viral infection on a *Heterosigma akashiwo* (Raphidophyceae) bloom in Hiroshima Bay, Japan

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ABSTRACT: Several viruses specifically infecting the harmful bloom-forming raphidophyte *Heterosigma akashiwo* (Hada) Hada have been found recently. It has been reported that infection of a double-stranded DNA (dsDNA) virus (HaV) affects both the biomass and clonal composition of *H. akashiwo* blooms. To clarify the relationship between *H. akashiwo* and its viruses, both algal and viral dynamics were monitored in Hiroshima Bay, Japan from May through July 2000. To minimize any underestimation or overlooking of viruses lytic to *H. akashiwo*, 4 host clones with different virus sensitivity spectra were used for their enumeration and isolation. Because all 65 viral clones obtained were stainable with DAPI, the most dominant viruses lytic to *H. akashiwo* assessed during the survey were considered to be dsDNA viruses. The abundance of viruses lytic to *H. akashiwo* monitored by means of the most probable number (MPN) method using each host clone showed its own dynamics pattern, but the viruses shared similar trends with each other, exhibiting a marked increase accompanied by a sudden decrease in host abundance. Thus, different types of viruses lytic to *H. akashiwo* are considered to have coexisted and simultaneously affected the bloom to cause its decline. Based on the results of laboratory cross-reactivity tests between 90 *H. akashiwo* clones and 65 virus clones isolated from the bloom, they were divided into 6 and 3 groups, respectively, showing their high diversity with regard to their virus sensitivity and host specificity. Based on the viral dynamics and changes in host abundance and clonal composition from the peak over the end of the bloom, we concluded that the viral infection was one of the most important factors determining quantity (biomass) and quality (clonal composition) of the *H. akashiwo* population.

KEY WORDS: Viral infection · *Heterosigma akashiwo* · Harmful algal bloom · Algal virus · Host clonal composition

INTRODUCTION

Heterosigma akashiwo (Raphidophyceae) is a harmful bloom-forming phytoplankton in coastal waters of temperate and subarctic areas of the world, which often causes mortality of caged fish such as salmon and yellowtail (Honjo 1993, Smayda 1998). Recent research has focused on the contribution of viral infection to the disintegration of *H. akashiwo* blooms (Nagasaki et al. 1994a,b, Tarutani et al. 2000, Juneau et al. 2003). So far, at least 3 viruses infecting *H. akashiwo* have been reported: HaV (*H. akashiwo* virus) (Nagasaki & Yamaguchi 1997), HaNIV (*H. akashiwo* nuclear inclusion virus) (Lawrence et al. 2001), and HaRNAV (*H. akashiwo* RNA virus) (Tai et al. 2003). Among them, HaV is the largest virus and contains a dsDNA genome (Nagasaki & Yamaguchi 1997). HaV has a high interspecies specificity, and its intraspecies specificity is highly diverse among clones (Nagasaki & Yamaguchi 1998, Nagasaki et al. 1999a). Recently, through a field survey, Tarutani et al. (2000) found that the clonal composition as well as the biomass of a *H. akashiwo* bloom showed a drastic change as a result of HaV infection, and con-

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cluded that there were at least 2 types of HaV clones and 2 types of *H. akashiwo* clones in the bloom based on the results of a cross-assay between HaV clones and host clones isolated during the survey. However, because only 1 *H. akashiwo* strain (H93616) was used by Tarutani et al. (2000) to enumerate and isolate viruses, they presumably only enumerated and isolated viruses that were able to cause lysis of the given host strain (H93616), and viruses that did not lyse it, but lysed other *H. akashiwo* clone(s), were overlooked. Studies on cyanophages and *Micromonas pusilla* virus (MpV) have also shown that the viral titers estimated by means of the extinction dilution method using different host clones as hosts were variable (Waterbury & Valois 1993, Suttle & Chan 1994, Sahlsten 1998, Zingone et al. 1999). Therefore, to fully examine the dynamics of algal viruses in environmental waters, use of multiple algal host strains with different virus sensitivity spectra is considered a rational way of minimizing any underestimation or overlooking of viruses. In the present study, 4 *H. akashiwo* strains were used to enumerate and isolate viruses infecting *H. akashiwo*, which were expected to have different sensitivity spectra to viral infection based on a preliminary experiment and previous reports (Nagasaki et al. 1999a, Tarutani et al. 2000, unpubl.).

In fact, data showing the high diversity of algal hosts and their viruses have been gradually accumulated. MpV isolated from North Atlantic coastal water showed a diverse specificity to *Micromonas pusilla* strains isolated from different sites (Sahlsten 1998). *Heterosigma akashiwo* nucleic inclusion virus (HaNIV) isolated from British Columbia coastal water infected *H. akashiwo* strains isolated from the same area, but not those from other regions in the world (Lawrence et al. 2001). The infection specificity of both *Heterocapsa circularisquama* virus (HcV) and *H. circularisquama* RNA virus (HcRNAV) infecting *H. circularisquama* (Dinophyceae) was strain-specific, all of which were isolated from western Japan (Nagasaki et al. 2003, Tomaru et al. 2004, this issue). However, it has not been established to what extent and through what process the strain specificity of algal viruses works in maintaining intraspecies diversity of algal hosts. The present study aimed to answer the following questions through a field survey of a *H. akashiwo* bloom in Hiroshima Bay and a cross assay between host clones and virus clones isolated in the survey: (1) How do the distinct types of virus affect the dynamics of the *H. akashiwo* bloom? (2) How diverse are *H. akashiwo* clones from the viewpoint of virus sensitivity spectra? (3) How diverse are virus clones from the viewpoint of host specificity spectra? (4) How does the host clonal composition fluctuate during the bloom?

MATERIALS AND METHODS

Sampling. Water samples of the surface layer (0 m) and 0.2 m above the bottom (B–0.2 m) were collected 1 to 3 times weekly from 12 May through 28 July 2000 at a semi-enclosed basin (Itsukaichi Fishing Port; 34° 21.400′ N, 132° 21.864′ E) in northern Hiroshima Bay, Japan. In this area, *Heterosigma akashiwo* blooms occur around June to July (Imai & Itakura 1999). Sampling was conducted between 09:00 and 10:00 h because *H. akashiwo* migrates up to the surface in the early morning and down to the bottom layer in the afternoon (Nagasaki et al. 1996).

Environmental factors. Measurement of water temperature and salinity was conducted during the survey. For the determination of dissolved inorganic nutrients (ammonia, nitrate, nitrite [DIN] and phosphorus [PO_4-P]), water samples filtered through a glass fiber filter (Whatman GF/F) were frozen at –20°C until analysis using an autoanalyser (Bran-Luebbe, TRAACS 2000).

Abundance of phytoplankton and lytic viruses. Cell counts and taxonomic identification of *Heterosigma akashiwo* and other phytoplankton species were carried out with a Sedgewick-Rafter chamber under optical microscopy on the sampling days without fixation of the sample waters. The abundance of viruses lytic to *H. akashiwo* in seawater samples was estimated by means of the most probable number (MPN) technique (= extinction dilution method: Cottrell & Suttle 1995, Nagasaki & Yamaguchi 1997). Water samples were passed through a glass fiber filter (Whatman GF/F) and diluted with modified SWM3 medium (Chen et al. 1969, Itoh & Imai 1987) in a series of 10-fold dilution steps. Aliquots of each dilution (100 µl) were added to 8 wells in cell culture plates with 96 round bottom wells (Falcon) and mixed with 150 µl of exponentially growing culture of *H. akashiwo*. As hosts, clonal strains previously isolated from Itsukaichi Fishing Port *H. akashiwo* H93616, H94608, H98603-1 and H98603-4, each of which was expected to have different virus sensitivity spectra, were used. H93616 is sensitive to the 13 HaV clones used in the previous studies (Nagasaki & Yamaguchi 1998, Nagasaki et al. 1999a), while H94608 is resistant to all 13 tested HaV strains. H98603-1 and H98603-4 were isolated during the *H. akashiwo* bloom in early summer of 1998, and were found to be resistant to most HaV clones isolated using H93616 as a host in a previous study (Tarutani et al. 2000). The cell culture plates were incubated at 20°C under a 12:12 h light:dark cycle of 130 to 150 µmol photons m^{-2} s^{-1}, with cool white fluorescent illumination, and were monitored for the occurrence of culture lysis by optical microscopy for 14 d. The abundance of lytic viruses was calculated with a BASIC program

from the number of wells in which lysis occurred (Nishihara et al. 1986).

Isolation of *Heterosigma akashiwo* and virus clones. From each surface water sample, 10 *Heterosigma akashiwo* cells were randomly isolated on the day of sampling by the micropipetting method from 7 June through 28 July, and transferred to SWM3 medium. When growth of the isolated algal cell was observed, it was made free from bacterial contamination using the washing method given by Imai & Yamaguchi (1994). From the surface water samples, 76 host clones were obtained and maintained in culture. In addition, 48 cells of *H. akashiwo* were isolated from each bottom water sample from 16 June through 23 June and treated as described above. Then, 10, 9, 7, and 2 host clones from the bottom water samples on 16, 19, 21, and 23 June, respectively, were successfully made into culture, from which 3, 9, and 2 clones isolated on 16, 19, and 23 June, respectively, were selected. Consequently, 90 host clones (76 from the surface water samples and 14 from the bottom water samples) were examined in the present study.

Between 1 and 4 virus clones were isolated from the most diluted wells of each water sample when the abundance of viruses lytic to *Heterosigma akashiwo* was determined by the MPN method. The clonal isolation was carried out with 2 series of the extinction dilution procedure (Nagasaki & Yamaguchi 1997). Briefly, the algal lysate was diluted in modified SWM3 medium in a series of 10-fold dilution steps. Aliquots (100 µl) of each dilution step were added to 8 wells in cell culture plates with 96 round bottom wells containing 150 µl of an exponentially growing host culture. Then, the algal lysate in the most diluted well in the first assay was carried over to the second extinction dilution procedure. Finally, the resultant lysate in the final end-point dilution was used as a clonal lysate, in which the probability of 2 or more viruses occurring (i.e. failure in cloning) was estimated to be <0.0106. The viral cultures were filtered through 0.2 µm membrane filter (Dismic-25cs, Advantec) and made axenic. To check their genome type, clonal pathogen was observed using epifluorescence microscopy after staining with DAPI as described in Suttle (1993) and Weinbauer & Suttle (1997). Briefly, the clonal pathogen suspension was fixed with glutaraldehyde at a final concentration of 1%, and DAPI solution was added to each fixed sample at a final concentration of 1 µg ml^{-1}. The stained samples were filtered onto 0.02 µm pore size Anodisk filters (Whatman); then, the filters were mounted on a glass slide with a drop of low fluorescence immersion oil, and covered with another drop of immersion oil and a cover slip. The slides were viewed at a magnification of 1000× with an Olympus BX50 epifluorescence microscope, and compared to DAPI-stained HaV01 (Nagasaki & Yamaguchi 1997). Consequently, 65 axenic virus clones were isolated. They were cryopreserved according to Nagasaki & Yamaguchi (1999), and fresh viral suspensions were used in the host range tests.

Transmission electron microscopy (TEM). TEM preparation was conducted according to Tarutani et al. (2001). Briefly, *Heterosigma akashiwo* cells in 400 ml of water from the B–0.2 m layer on 19 June were harvested by centrifugation at 460 × *g* for 10 min at 4°C, and the resultant pellet was fixed with 1% glutaraldehyde in 0.1 µm-filtered seawater. The cell pellets were post-fixed for 3 h in 2% osmic acid in 0.1 M phosphate buffer (pH 7.2 to 7.4), dehydrated in a graded ethanol series, and embedded in Quetol 653 resin (Nisshin EM). Thin sections were stained with 4% uranyl acetate, 3% lead citrate, and observed at 80 kV using a JEOL JEM-1010 TEM.

Host range tests. To examine the intraspecies host specificity of the virus strains isolated in the present survey, supernatant of algal lysates centrifuged at 4500 × *g* for 5 min at 4°C was used. An aliquot of each lysate was inoculated (v/v = 10%) independently into exponentially growing cultures of the 94 *Heterosigma akashiwo* strains (4 strains used for virus isolation and 90 strains isolated during the survey). The culture plates were incubated under the same conditions of light and temperature as shown above, and the occurrence of algal lysis was monitored by optical microscopy. For comparison, growth of host cultures without pathogen inoculation was also monitored. Algal cultures in which the majority of cells lost mobility and degraded were scored as suitable hosts to the inoculum. The resultant data sets were converted to a Euclidean distance matrix and analyzed by means of unweighted pair-group method analysis (UPGMA) of clustering in PHYLIP (Phylogeny Inference Package, Version 3.5; Felsenstain 1993).

RESULTS AND DISCUSSION

Dynamics of *Heterosigma akashiwo* and its viruses

In the surface layer, *Heterosigma akashiwo* abundance gradually increased from 19 May, exceeded 10^4 ml^{-1} on 7 June, and sustained a high abundance ranging from 1.9×10^4 to 2.2×10^5 cells ml^{-1} until 19 June (Fig. 1A). During the dense bloom period, both in the 0 m and B–0.2 m layers, *H. akashiwo* accounted for >90% of the phytoplankton community in terms of cell abundance, while diatoms accounted for <1% (data not shown). Then, in the surface layer, *H. akashiwo* showed a sudden decrease by 4 orders of magnitude between 19 to 23 June and a gradual increase in surface water from 30 June until the end of

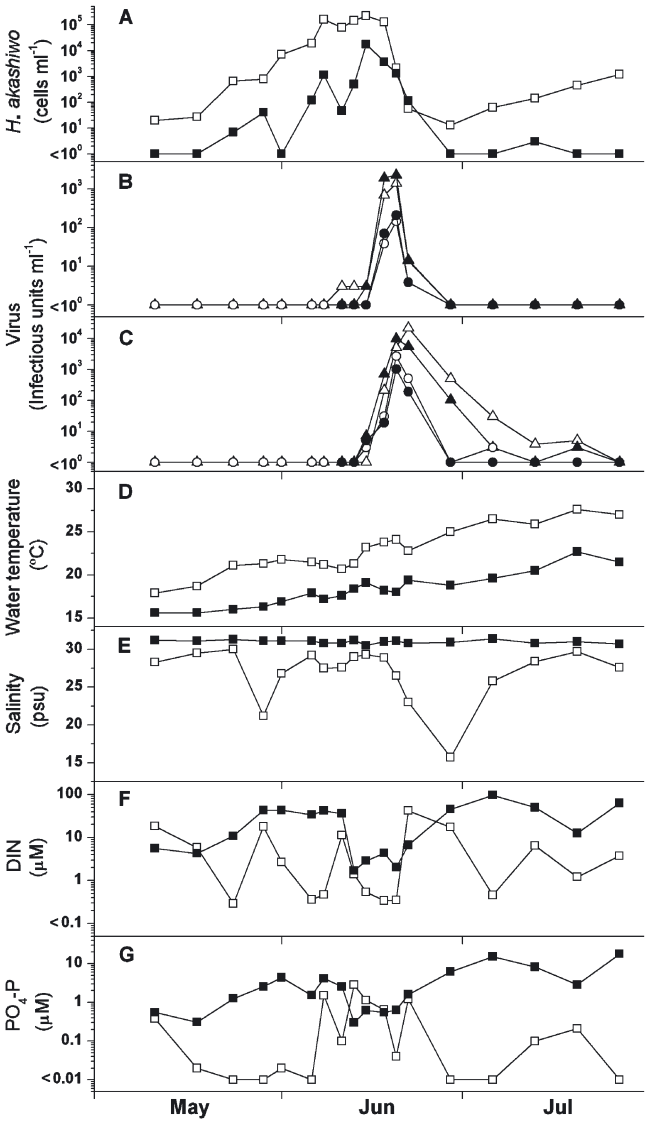

Fig. 1. (A) Changes in abundances of *Heterosigma akashiwo*, (B) virus abundances determined by the MPN method using 4 distinct host strains at the surface and (C) 0.2 m above the bottom (B–0.2 m), (D) water temperature, (E) salinity, (F) dissolved inorganic nitrogen (DIN), and (G) phosphorus (PO_4-P) in northern Hiroshima Bay, during the period mid-May to July 2000. (□) and (■) indicate data at the surface and the B–0.2 m layer, respectively (A,D–G). (△), (▲), (○), and (●) indicate the virus abundances measured by use of *H. akashiwo* strain H93616, H94608, H98603-1 and H98603-4, respectively (B,C)

July. In the bottom layer, *H. akashiwo* increased from 25 May through 16 June accompanied by intermittent reductions in abundance, and then rapidly decreased by 4 orders of magnitude over the end of June.

In the previous studies, viral infection was shown to be one of the main factors causing the termination of *Heterosigma akashiwo* blooms based on the observations of the specific increase of *H. akashiwo* cells harboring virus particles (Nagasaki et al. 1994b) and viral titer in the water column in the final stage of blooms (Tarutani et al. 2000). Also, the abundance of viruses lytic to *H. akashiwo* showed a marked increase accompanied with a sudden decrease in host abundance (Fig. 1A–C). Furthermore, by means of TEM, virus particles similar to HaV in shape and size were observed in the cytoplasm of *H. akashiwo* cells collected from the B–0.2 m layer on 19 June (Fig. 2). In addition, all of the virus clones isolated in the present survey were stainable with DAPI and looked similar to DAPI-stained HaV01 particles in appearance under an epifluorescence microscope (Nagasaki & Yamaguchi 1997). Based on these data, it is suggested that the most dominant viruses infecting *H. akashiwo* assessed during the survey were dsDNA viruses, and that HaV was at least one of the main constituents of the virus population leading to the decline of the *H. akashiwo* bloom in Hiroshima Bay in the early summer of 2000. Recent studies, however, reported the existence of non-HaV *H. akashiwo* infectious viruses, HaNIV (Lawrence et al. 2001) and HaRNAV (Tai et al. 2003). Therefore, it is also possible that DAPI positive *H. akashiwo* infectious viruses other than HaV were included in the viruses isolated here.

In the present survey, abundances of viruses causing lysis of the 4 different *Heterosigma akashiwo* clones were measured independently. It was noticeable that the virus abundance in the water column estimated by using each host clone showed its own dynamics pattern (Fig. 1B,C). It was not until the peak of the bloom that viruses infecting H93616 and H94608 in the surface layer were detected. They reached 10^3 infectious units ml^{-1} from 19 through 21 June, and then rapidly decreased (Fig. 1B). In contrast, those infecting H98603-1 and H98603-4 in the surface layer were first detected just prior to the sudden decrease of the host cell density, and reached 10^2 infectious units ml^{-1} from 19 through 21 June followed by a rapid decrease (Fig. 1B). In the B–0.2 m layer, the abundance of viruses infecting H93616 and H94608 was also higher than those of H98603-1 and H98603-4, but the decrease in viral abundance was more gradual than in the surface layer. Through the gradual decrease in the B–0.2 m layer, the abundance of viruses infectious to H93616 was 1.7- to 10 times higher than that infectious to H94608 (Fig. 1C). The trends of virus dynamics were thus divided into 3 groups, viruses lytic to (a) H93616, (b) H94608 and (c) H98603-1 and H98603-4, suggesting that at least 3 groups of viruses lytic to *H. akashiwo* bearing different host infectivity coexisted in the same water during the bloom period.

Tarutani et al. (2000) reported that HaV abundance in the bottom layer was higher than that in the surface

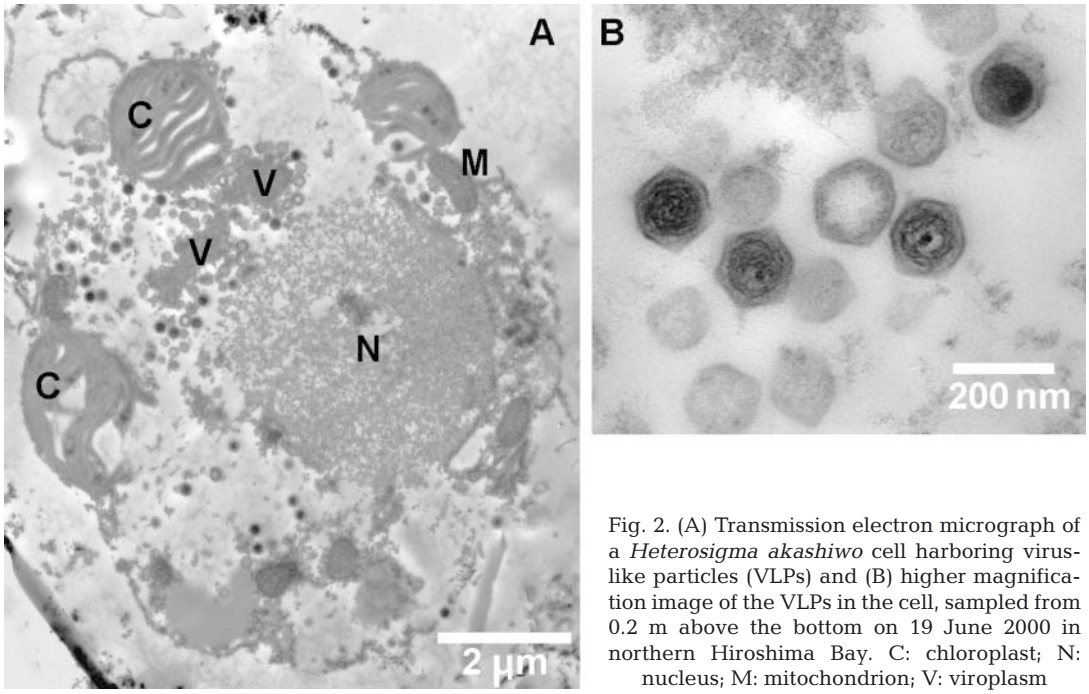

Fig. 2. (A) Transmission electron micrograph of a *Heterosigma akashiwo* cell harboring virus-like particles (VLPs) and (B) higher magnification image of the VLPs in the cell, sampled from 0.2 m above the bottom on 19 June 2000 in northern Hiroshima Bay. C: chloroplast; N: nucleus; M: mitochondrion; V: viroplasm

layer at the termination stage of the *Heterosigma akashiwo* bloom in Hiroshima Bay, 1998. They suggested that infected cells had sunk to the bottom, and that HaV was supplied to the bottom layer by their lysis (Tarutani et al. 2000). Higher viral titer at the bottom layer detected in the present study was also likely due to a similar mechanism.

Environmental factors

Hydrographic data collected in the present survey are also shown in Fig. 1. During the peak of the *Heterosigma akashiwo* bloom, from 9 through 19 June, water temperature and salinity ranged from 20.7 to 23.8°C and 27.5 to 29.3 psu in the surface layer, and from 17.2 to 19.1°C and 30.5 to 31.2 psu in the B–0.2 m layer, respectively (Fig. 1D,E). Considering that optimum temperature and salinity for the growth of *H. akashiwo* is 15 to 25°C and 10 to 40 psu (Tomas 1978, Honjo 1993), respectively, the environmental factors were suitable for its growth during the peak and decline (Fig. 1D,E).

DIN concentration and PO_4-P concentration ranged from 0.34 to 11.28 µM and 0.1 to 2.83 µM in the surface layer, and 1.7 to 42.9 µM and 0.3 to 4.13 µM in the B–0.2 m layer, respectively (Fig. 1F,G). *Heterosigma akashiwo* has relatively higher nutrient requirements: K_S values for NO_3, NH_4 and PO_4-P uptake of *H. akashiwo* are 2.0 to 2.5, 2.0 to 2.3 and 1.0 to 2.0 µM, respectively, and growth limitation occurs at <0.5 µM PO_4-P (Tomas 1979, Smayda 1998). Thus, it is noticeable that the nutrient levels just prior to the bloom termination were relatively low for the growth of *H. akashiwo* (Fig. 1F,G). However, even when an inflow of freshwater from land adjacent to the bloom supplied nutrients from 21 through 23 June, the *H. akashiwo* population kept decreasing in abundance. Considering the increase in viral abundance at the termination stage of the bloom, it is concluded that the *H. akashiwo* population was suppressed by viral infection and its replication was not significantly activated, even when sufficient nutrients were added. The decrease of N and P from 23 through 30 June in the surface layer (Fig. 1F,G) was presumably caused by the growth of *Prorocentrum* spp. (data not shown).

Diversity of host clones and virus clones

Raw data of the cross reactivity test between host clones and virus clones isolated in the present survey are shown in Fig. 3. Virus sensitivity patterns of the host clones were diverse even among those isolated from a single seawater sample. Because of the complexity of the data, the susceptibility pattern of *Heterosigma akashiwo* clones to the virus clones and the lytic activity of virus clones to the host clones were analyzed by means of an UPGMA (Figs. 4 & 5). Host clones and virus clones were sorted based on the

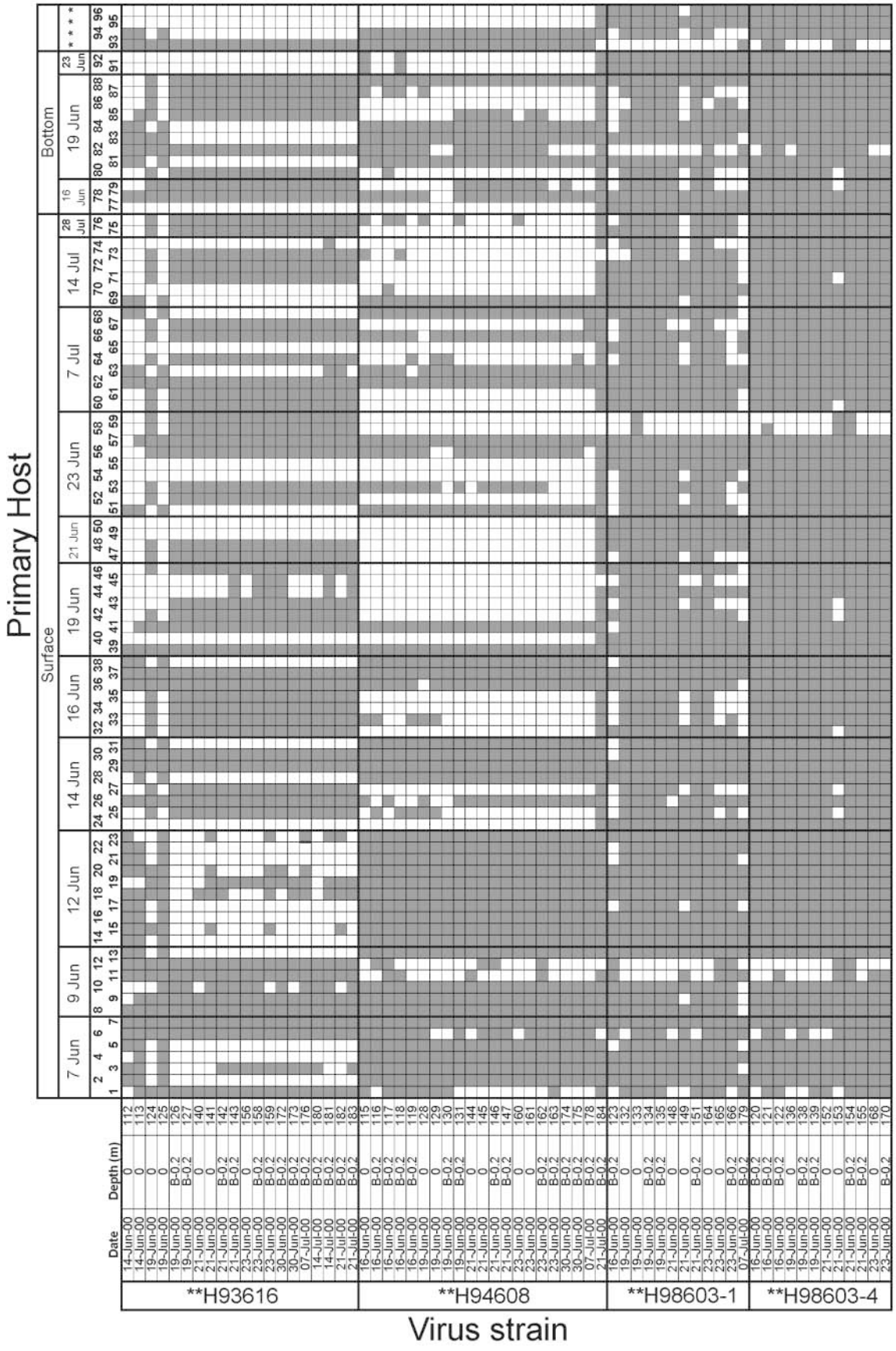

Fig. 3. Viral susceptibility of *Heterosigma akashiwo* clones to co-occurring virus clones from northern Hiroshima Bay during the period 7 June to 28 July, 2000, and that of 4 host strains used for virus isolation. Shaded and open columns indicate susceptibility (with cells lysed) and resistance (with cell growth almost equal to that of the controls) to each virus clone, respectively. *Host strain numbers 93, 94, 95, and 96 represent the *Heterosigma akashiwo* strains H93616, H94608, H98603-1, and H98603-4, respectively, used for virus isolation; **host strains used for virus isolation

results of UPGMA, when clones being <0.20 distant in terms of Euclidean distance were regarded as belonging to the same groups. Consequently, *H. akashiwo* clones and virus clones tested in the present experiment were divided into 6 (the Host Group A, B, C, D, E and F; Fig. 4) and 3 groups (the Virus Group I, II and III; Fig. 5), respectively. These data indicate that *H. akashiwo* clones and virus clones lytic to *H. akashiwo* that were highly diverse in terms of virus sensitivity and host specificity, respectively, coexisted in natural waters of Hiroshima Bay.

The raw data in Fig. 3 was also sorted in the same way as shown in Fig. 6. Although $2^3 = 8$ patterns of sensitivity were expected with regard to the host diversity assuming that there are 3 virus groups in the natural waters, only 6 were detected in the present survey (Fig. 6). As expected, virus clones categorized in each group were mostly composed of those isolated using the same host clone(s): the Virus Group I was composed of 24 clones, in which 3, 20 and 1 clones were isolated using H93616, H94608, and H98603-1 as hosts, respectively; as for the Virus Group II, 24 clones in which 1, 11, and 12 clones were isolated using H94608, H98603-1, and H98603-4, respectively; and all 17 clones in the Virus Group III were isolated by using H93616.

Based on the sensitivity patterns of the host clones used in the present survey, H93616, H94608, H98603-1, and H98603-4 were categorized in the Host Group D, E, B, and B, respectively. Although we first intended to prepare host clones for enumeration and isolation of viruses that were different in sensitivity spectra, H98603-1 and H98603-4 were categorized in the same host group (Fig. 6).

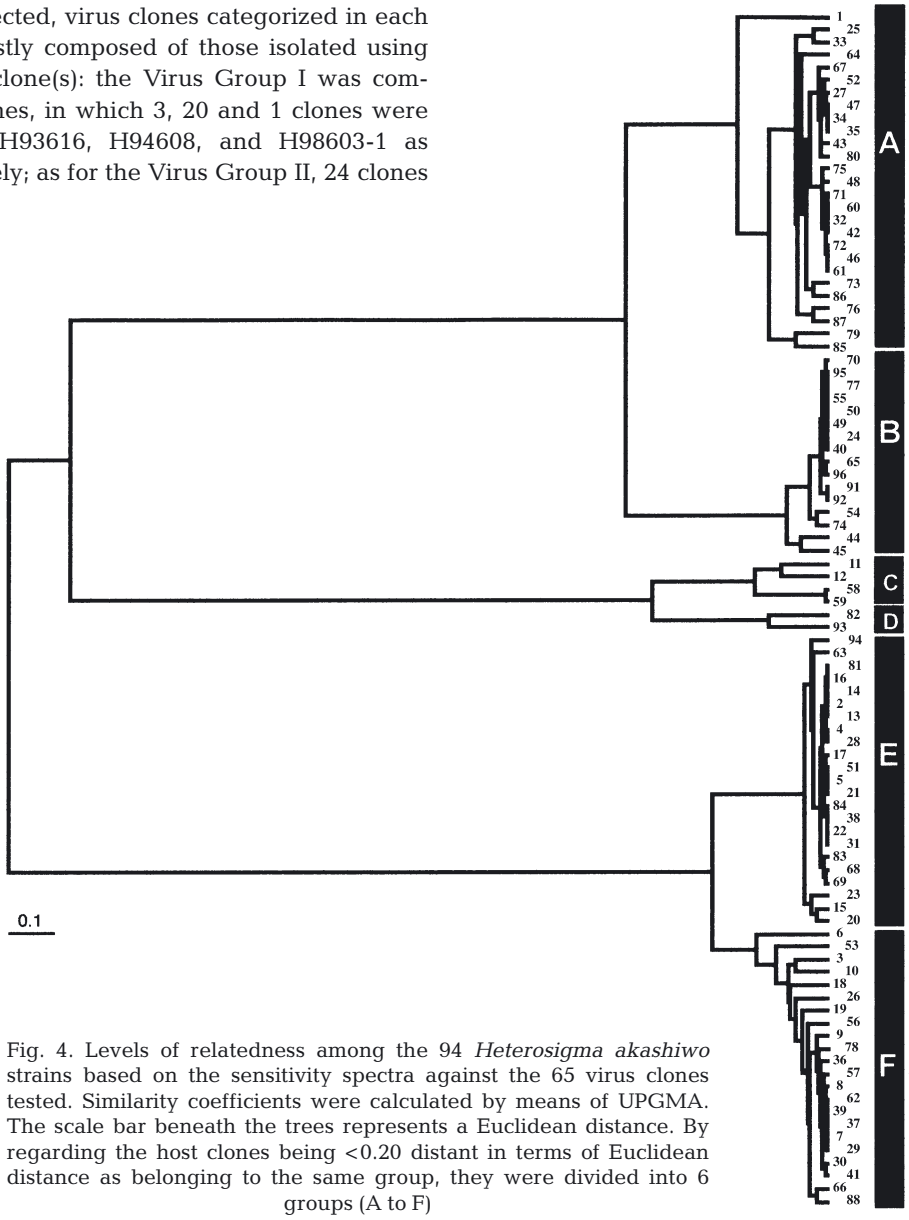

Fig. 4. Levels of relatedness among the 94 *Heterosigma akashiwo* strains based on the sensitivity spectra against the 65 virus clones tested. Similarity coefficients were calculated by means of UPGMA. The scale bar beneath the trees represents a Euclidean distance. By regarding the host clones being <0.20 distant in terms of Euclidean distance as belonging to the same group, they were divided into 6 groups (A to F)

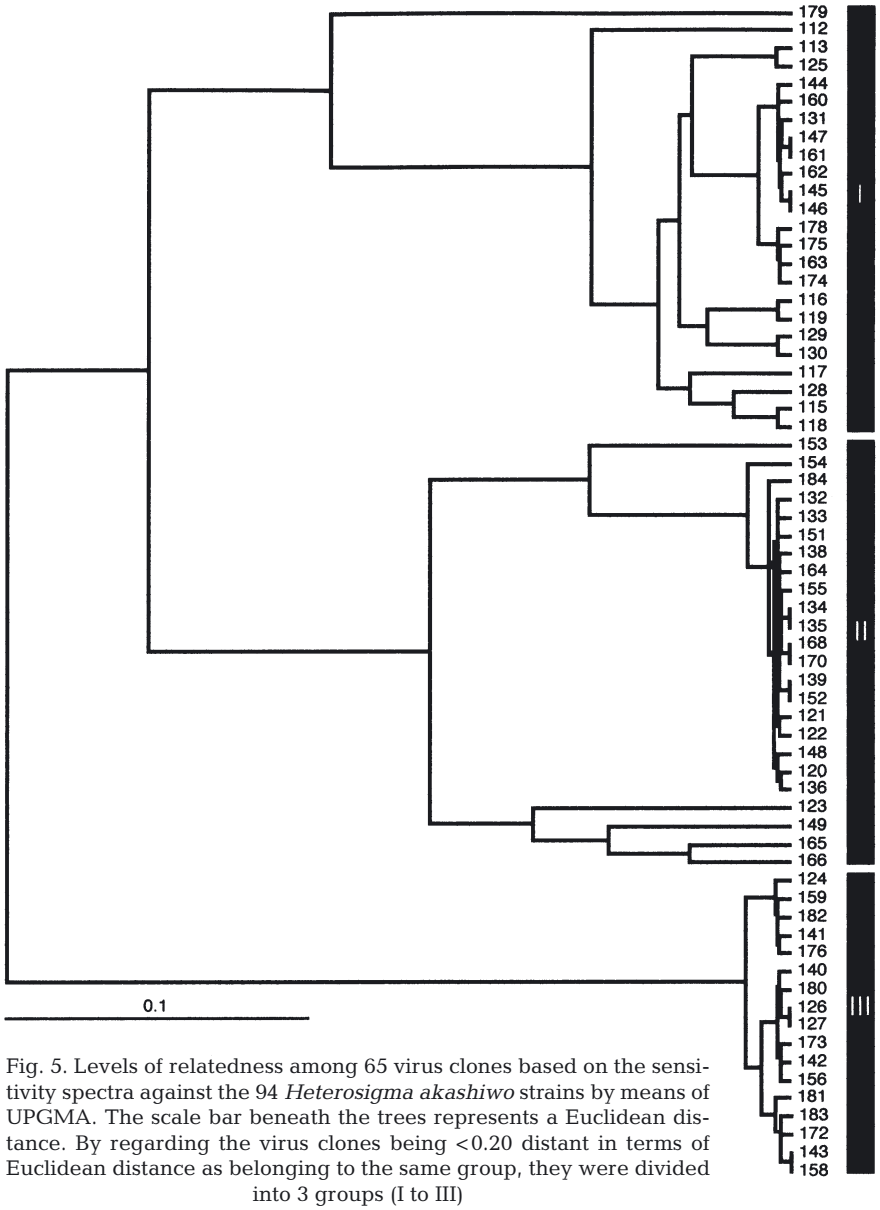

Fig. 5. Levels of relatedness among 65 virus clones based on the sensitivity spectra against the 94 *Heterosigma akashiwo* strains by means of UPGMA. The scale bar beneath the trees represents a Euclidean distance. By regarding the virus clones being <0.20 distant in terms of Euclidean distance as belonging to the same group, they were divided into 3 groups (I to III)

Dynamics of host clonal composition

The viral dynamics in the present experiment (Fig. 1B,C) indicates that viruses detectable by use of H93616 and H94608, but not by H98603-1 or H98603-4, were dominant throughout the bloom period. Therefore Virus Group I, detectable by H93616 and H94608 (Fig. 6), is considered to have been the most dominant in the bloom, and Virus Groups II and III were presumably more minor constituents. In which case, the dominant host groups should have been Host Groups D, E, and/or F, which could produce viruses belonging to Virus Group I. Because only 1 host clone isolated in this study clustered into Host Group D (Host Strain 82; Fig. 6), it is most probable that Host Groups E and F dominated in the bloom. In Fig. 7, the time course of changes in the host clonal composition is shown. These data clearly indicate that Host Groups E and F accounted for a large part of the population especially before the bloom termination, supporting the above speculation.

Here, a significant problem arises: even though Host Groups E and F could produce both Virus Groups I and II, what made the abundance of Virus Group II fluctuate at a lower level than that of Virus Group I in the bloom? One possible explanation is that the burst size of Virus Group II was smaller than that of Virus Group I, and then the former group could not dominate.

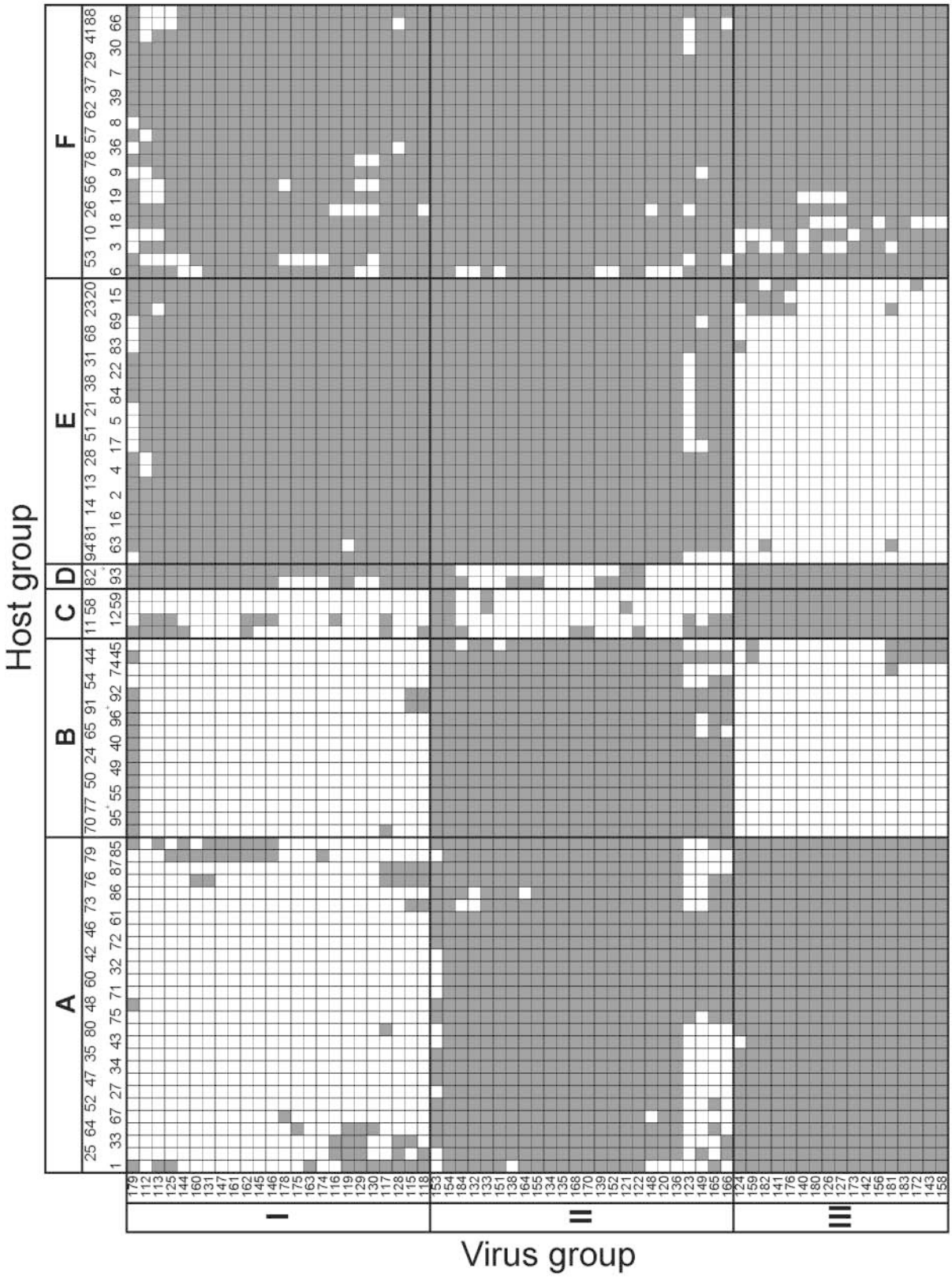

Fig. 6. Sorted data of the viral susceptibility (Fig. 3) based on the cluster analysis by means of UPGMAs (see Figs. 4 & 5). *Host strain numbers 93, 94, 95, and 96 represent the *Heterosigma akashiwo* strains, H93616, H94608, H98603-1, and H98603-4, respectively, used for virus isolation

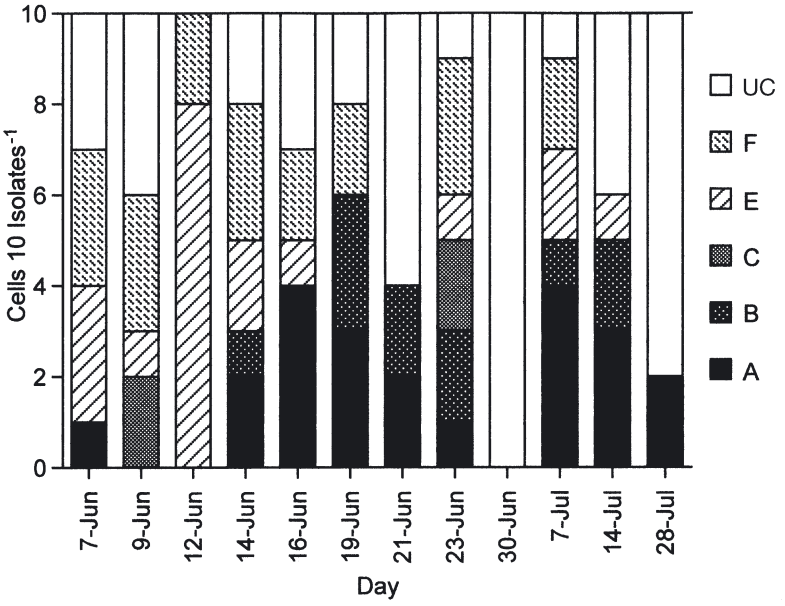

Fig. 7. Change of the host clonal composition in 10 host clones isolated from the surface waters in northern Hiroshima Bay from 7 June through 28 July. A, B, C, E, F, and UC indicate the Host Groups A, B, C, E, F, and host strains isolated but unculturable, respectively

Another possibility is that the adsorption efficiency of Virus Group II to Host Groups E and F were lower than that of Virus Group I, and the viral proliferation rate of the latter virus group was higher, making Virus Group I dominant. However, both these scenarios remain just as speculation and require further investigation.

The change of host clonal composition (Figs. 3 & 7) was not so drastic as that observed in the survey by Tarutani et al. (2000), when a change of virus sensitivities of dominant cells within the *Heterosigma akashiwo* population at the termination stage of the bloom was apparent. In their survey, only H93616 (categorized in the Host Group D here) was used as the host strain for enumeration and isolation of viruses. Considering that the high viral titer measured by using H93616 in their survey was as high as ~10^4 and ~10^6 ml^{-1} at the surface and bottom layer, respectively, and that 16 of the 17 virus clones isolated by use of H93616 in their survey were not infectious to H94608 in Host Group E (K. Tarutani unpubl.), it is probable that the dominant viruses in the 1998 bloom (Tarutani et al. 2000) were the Virus Group III and the dominant hosts were the Host Groups A, C, D and/or F, which could produce Virus Group III (Fig. 6).

Thus, comparing the possible composition of the *Heterosigma akashiwo* blooms in 1998 and 2000, it is probable that the combination of the host clones and the virus clones characterizing a series of *H. akashiwo* blooms is changeable year by year, even in an enclosed basin such as Itsukaichi Fishing Port.

Unculturable host clones

As shown in Fig. 7, it should be noted that the host clones that could not be cultured in the isolation procedure were ignored in the present experiments. Especially after the bloom termination (21 June), the ratio of culturable clones decreased as was previously observed by Nagasaki et al. (1996). The ratios of culturable clones isolated from the bottom water samples were also as low as 4.2 to 20.8% (see 'Materials and methods'). There are several possible causes for their unculturability: (1) they might have already been infected by viruses; (2) the prepared condition was not suitable for their growth; (3) the algicidal effect of contaminating bacteria; and/or (4) they had already been in the encystment stage, but this awaits elucidation in future studies. However, comparison of the clonal compositions between at the surface and at the bottom (Table 1) gives an idea of a possible explanation to this problem. On 19 June, smaller-sized rounded cells of *Heterosigma akashiwo* dominated within the host population in the bottom water, and most of the isolates were not culturable. Among the culturable clones, Host Group B accounted for 30% at the surface, but was not detected from the bottom water. Besides, Host Groups D and E found in the bottom population were absent in the surface layer. Nagasaki et al. (1996) also found the dominance of smaller-sized cells in a *H. akashiwo* population that had ceased upward migration at the final stage of the bloom, and also verified that most of them were not culturable. Considering the similarity between the smaller-sized cells found in the present field survey and the pre-encystment cells reported by Itakura et al. (1996), initiation of encystment might be one of the causes for the difference of the culturability and the host clonal composition between the surface and the bottom waters (Table 1). On the other hand, HaV-infected cells changed their form to roundish, lost their mobility, and consequently sank to the bottom of culture

Table 1. *Heterosigma akashiwo.* Clonal composition in the isolates at the surface and the bottom water on 19 June 2000 in Itsukaichi Fishing Port, northern Hiroshima Bay. Each character, A to F, indicates the host group shown in Fig. 4. *n = the total isolates number

(%)	A	B	C	D	E	F	Not grown	*n
Surface	30	30	0	0	0	20	20	10
Bottom	8.3	0	0	2.1	6.3	2.1	81.3	48

vessels (Nagasaki et al. 1999b), and were hardly distinguishable from the smaller-sized cells. Thus, the non-culturable clones observed in the present study may have included both viral infected cells and pre-encystment stage cells. The difference of host clonal composition among the culturable cells between the surface and the bottom waters might have reflected the impact by viral infection.

Conclusions

Based on the observations given above, we conclude that the viral infection affected the dynamics of the *Heterosigma akashiwo* bloom and that the diversities of both *H. akashiwo* and its viruses were high, which allowed the dynamic change of host clonal composition during the bloom. Therefore, viral infection is considered to be one of the most important factors determining quantity and quality of the *H. akashiwo* population. Based on the cross assay, *H. akashiwo* clones were divided into 6 groups. By selecting typical clones from each group, more intimate investigation on viral dynamics can be designed. Moreover, future studies will also focus on what determines the dominant host groups and virus groups in a bloom. Further analysis on viral infection and growth for each combination between host clones and virus clones would be required to answer this question.

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