Effect of viral infection on sinking rates of *Heterosigma akashiwo* and its implications for bloom termination

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ABSTRACT: Viruses play many important roles in the ecology of marine microbes, including that of mortality agents of phytoplankton. The widespread abundance of viruses in sediments, coupled with observations that some flagellated algae lose motility when infected, prompted this investigation into the fate of infected cells and the propagation of infection throughout a population *in situ*. We used 2 unrelated viruses that infect the bloom-forming alga *Heterosigma akashiwo* as model systems. Settling columns were used to determine the impact of viral infection on the vertical movement of *H. akashiwo*. Within 24 h following infection with HaRNAV 263 or *H. akashiwo* virus strain OIs1, 20.9 ± 5.8 and 12.2 ± 1.9 % of the populations were sinking at rates of 1.25 and 0.73 m d^{-1}, respectively. By 48 h post-infection approximately 50 % of cells infected with either virus had lysed. As sinking cells would encounter decreasing levels of light in the natural environment, we incubated infected cultures in complete darkness and compared the length of the lytic cycle to that of cultures incubated under a L:D cycle. Again, approximately 50 % of the population lysed by 48 h, regardless of the light conditions or type of virus, and lysis of the entire population was complete by 144 h. Based on these results, we propose that, depending on the characteristics of the water column, there are 3 potential fates for viruses from cells infected *in situ*. In water with a deep mixed layer (>8 m), lysis of infected cells would occur within the mixed layer and the infection would be propagated within the euphotic zone. If the water is relatively deep (100s of meters), and the mixed layer shallow (<8 m), cell lysis and viral release would occur below the mixed layer, where viral propagation would not occur. Finally, in shallow waters (10s of meters) lysis of infected cells would occur at the sediment surface, resulting in the accumulation of a high abundance of viruses.

KEY WORDS: Algal bloom termination · Algal virus · *Heterosigma akashiwo* · Sinking rates · Bloom dynamics

INTRODUCTION

Viruses play many important roles in marine microbial ecology. In addition to affecting community composition and diversity (Bratbak et al. 1996), nutrient cycling (Wilhelm & Suttle 1999), and potentially, genetic transfer (Suttle et al. 1990), viruses are important agents of cell death for phytoplankton and may contribute to algal bloom dynamics (Suttle et al. 1990, Bratbak et al. 1993, Nagasaki et al. 1994, Suttle 2000).

Lytic viruses infect many bloom-forming species, including *Aureococcus anophagefferens* (Sieburth et al. 1988, Gastrich et al. 1998) *Chrysochromulina* sp. (Suttle & Chan 1995), *Emiliania huxleyi* (Bratbak et al. 1993), *Phaeocystis pouchetii* (Jacobsen et al. 1996), *Heterocapsa circularisquama* (Taruntani et al. 2001) and *Heterosigma akashiwo* (Nagasaki et al. 1994, Lawrence et al. 2001, Tai et al. 2003). Although it is clear that viruses infect many bloom-forming species, little is known of the ecology of infected blooms. Most

research to date has involved establishing that viruses are mortality agents for phytoplankton and, more recently, attempting to determine the extent of their effect.

Heterosigma akashiwo is a raphidophyte that episodically undergoes population explosions to form economically and environmentally detrimental blooms. These blooms are seeded by the germination of benthic resting cysts into flagellated phytoplankton (Yamochi 1984). Establishment of bloom-forming populations is correlated with increases in river run-off and the formation of a pycnocline (Yamochi 1989). These processes are believed to increase the availability of macronutrients, trace metals such as chelated iron and manganese, and Vitamin B_{12} in bottom waters (Yamochi 1989, Honjo 1992). Diel vertical migrations of up to 10 m d^{-1} allow *H. akashiwo* to form dense blooms under these conditions by exploiting deep nutrient pools (Yamochi & Abe 1984). Thus, vertical migration is thought to be essential for bloom maintenance. The severity and duration of these blooms is unpredictable from year to year and the factors controlling them have remained elusive. However, the isolation of lytic viruses from areas where blooms are endemic indicates that viruses are involved in *H. akashiwo* mortality.

As with many species of bloom-forming algae, *Heterosigma akashiwo* is intermittently dormant between annual blooms and remains in the ecosystem by overwintering as resting cysts in sediments. Free virus particles are subject to various mechanisms of removal from the water column, such as adsorption to particulate matter, grazing by protists, UV decay, or decay by dissolved substances (Suttle & Chen 1992, Gonzalez & Suttle 1993, Noble & Fuhrman 1997). Therefore, the annual maintenance of viral populations may be dependent on environmental reservoirs that permit free viruses to persist until they contact suitable hosts. As high abundances of *H. akashiwo* viruses have been detected in sediments where blooms are endemic (Lawrence et al. 2002), and in bottom waters during the decline phase of *H. akashiwo* blooms (Taruntani et al. 2000), sediments may provide a reservoir for viruses when their hosts are dormant. The mechanisms that result in the accumulation of viruses which infect *H. akashiwo* are not known. Hypotheses include deposition inside infected cells, adsorption to sinking particulate matter, and sinking inside faecal pellets. Sinking of infected cells would propably result in the highest pelagic–benthic transfer, since an individual cells can contain hundreds of thousands of viral particles (Lawrence et al. 2001); however, this has not been examined. Also, it is not known if viral replication can occur in infected algae under the dark conditions they would encounter while sinking through the water column.

We examined the effect of infection on the vertical movement of *Heterosigma akashiwo* and the impact of darkness on the lytic cycle. The goal was to determine whether infected cells would sink in the dark and, if so, whether viral replication would occur. *H. akashiwo* can be infected by at least 3 unrelated viruses (Nagasaki et al. 1994, Lawrence et al. 2001, Tai et al. 2003), and we took the opportunity to determine if populations infected with different viruses behaved similarly. These data were used to develop a conceptual model that describes the fate of infected cells in nature.

MATERIALS AND METHODS

Cultures. *Heterosigma akashiwo* Strain 522 (Northeast Pacific Culture Collection), originally isolated from English Bay, Vancouver, Canada, was used in all experiments. Cultures of *H. akashiwo* were maintained in f/2-enriched seawater (Guillard 1975) (30‰) supplemented with 10 nM sodium selenite under continuous light or a 14:10 h light:dark (L:D) cycle (260 µmol m^{-2} s^{-1} photosynthetically active radiation), at 20°C. Growth of the cultures was estimated by measuring *in vivo* chlorophyll fluorescence (Turner Designs fluorometer) over time.

Virus HaRNAV 263 was isolated from the Strait of Georgia, British Columbia (Tai et al. 2003). It is a ssRNA virus that replicates in the cytoplasm of *Heterosigma akashiwo*. Virus OIs1 was isolated from sediment samples taken from the Okeeover Arm of Malaspina Inlet, British Columbia (Lawrence et al. 2002). It was cloned by diluting the virus to extinction twice (Cottrell & Suttle 1991). Virus stocks of each clone were produced by adding 0.1% (v/v) viral lysate to exponentially growing *H. akashiwo* cultures and monitoring for algal lysis by *in vivo* chlorophyll fluorescence. Lysed cultures were filtered through glass-fibre filters (Whatman GFF, nominal pore size = 1.2 mm) and polyvinylidine difluoride filters (Millipore, 0.45-µm pore size), and the filtrate was stored at 4°C until use (within 48 h).

Settling column experiments. Experiments were conducted using rapidly-growing cultures of *Heterosigma akashiwo* grown in 2.4 l low-form culture flasks under the conditions described above, except that illumination was provided from above on a 14:10 h L:D cycle. Viral lysate was added (1% v/v) at t = 0 to duplicate cultures. Duplicate uninfected cultures served as controls. Every 24 h, 6 h into the light period, each culture flask was gently mixed and 380 ml (V_{total}) was carefully poured into a 50 cm tall (L) Plexiglas® settling column (Bienfang 1981). At the same time, a 1 ml sub-sample for determining the initial abundance of *H. akashiwo* (A_{init}) was preserved with 10% Lugol's fixative, and a 50 ml sub-sample

fixed for transmission electron microscopy (TEM) with 1.5% glutaraldehyde in 0.2 M cacodylate buffer and 0.1 M sucrose (Lawrence et al. 2001). The settling columns were incubated with continuous overhead illumination under the temperature-controlled conditions described above. After 2 h (t), 50 ml (V_{bot}) was drained from the bottom of the columns, and a 1 ml aliquot was fixed with 10% Lugol's for determining the final abundance of sedimented cells (A_{bot} cells ml^{-1}).

The abundance of *Heterosigma akashiwo* at $t = 0$ (A_{init} cells ml^{-1}) and in the sedimented fraction (A_{bot}) were determined by counting a 0.1 ml sample in a Palmer-Maloney chamber under 100× magnification. The percentage of cells sedimented in a culture was determined using the following formula adapted from Bienfang 1981):

$$\text{Percentage of sedimented cells (\%)} = \frac{(V_{bot} \times A_{bot}) - (V_{bot} \times A_{init})}{(V_{total} \times A_{init})}$$

The sinking rate was then determined as:

$$\text{Net sinking rate (m d}^{-1}\text{)} = \frac{[(V_{bot} \times A_{bot}) - (V_{bot} \times A_{init}) \times L]}{(V_{total} \times A_{init}) \times (t \div 24\text{h})}$$

Light:dark experiments. L:D experiments with *Heterosigma akashiwo* were conducted in 5 ml borosilicate culture tubes. Exponentially growing cultures were divided into the following 4 treatments: uninfected L:D, uninfected dark, infected L:D, and infected dark. At $t = 0$, cultures were inoculated with 1% v/v HaRNAV or OIs1 lysate and allowed to adsorb for 30 min under 260 µmol m^{-2} s^{-1} photosynthetically active radiation. Triplicate series of each treatment were then incubated under a 14:10 L:D cycle or constant darkness. Dark conditions were achieved by wrapping the culture tubes in 2 layers of aluminium foil and placing them in the L:D incubator. Growth and lysis was monitored by destructively sampling triplicate cultures from each treatment and measuring *in vivo* fluorescence. The relative biomass of infected cultures was determined by calculating the percentage of the fluorescence of infected cultures relative to the fluorescence of the control cultures (fluorescence $_{infected}$ ÷ fluorescence $_{control}$ × 100%).

Thin-sections of *Heterosigma akashiwo* prepared for TEM (Lawrence et al. 2001) were viewed with a Zeiss 10C-TEM at an accelerating voltage of 80 kV. At each time point a minimum of 100 cells were examined and scored as infected if viral particles were visible in the cytoplasm and/or if the cytoplasm was vacuolated. Care was taken to ensure that cells were only examined once. The percentage of visibly infected cells was then calculated by dividing the number of visibly infected cells by the total number of cells examined and multiplying by 100%.

RESULTS

Cell abundance in cultures of *Heterosigma akashiwo* infected with either HaRNAV or OIs1 increased during the first 24 h after inoculation with viral lysate and then began to decline (Figs. 1A & 2A). By 48 h, approximately 50% of the cells in each treatment had lysed, and complete lysis had occurred by 96 h. As there were no cells left to sample at 96 h, there are no data points at that time point. Cell abundance in control cultures grown under the same conditions increased until the experiment was terminated 96 h post-inoculation (Figs. 1A & 2A).

The net movement of uninfected *Heterosigma akashiwo* populations 6 h into the light period was towards the surface at approximately 0.7 m d^{-1}, and was relatively consistent throughout the experiments. Within the populations infected with HaRNAV, 24 h after infection, 20.9 ± 5.8% of the cells had sedimented
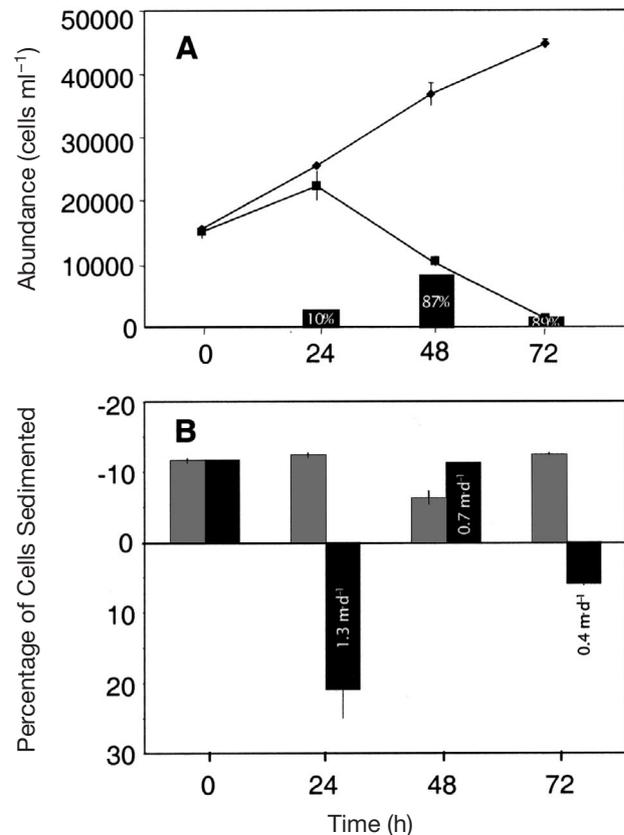

Fig. 1. *Heterosigma akashiwo.* (A) Cell abundance of uninfected (♦) and HaRNAV-infected (■) cultures over time; percentages inside bars: percentage of infected population in which virus particles were visible by TEM. (B) Percentage of cells settled to the bottom in uninfected (grey) and HaRNAV-infected (black) cultures throughout lytic cycle; negative values indicate migration towards surface; positive values migration towards bottom. Values inside bars: sinking/swimming rates of infected cultures. Error bars are range, n = 2
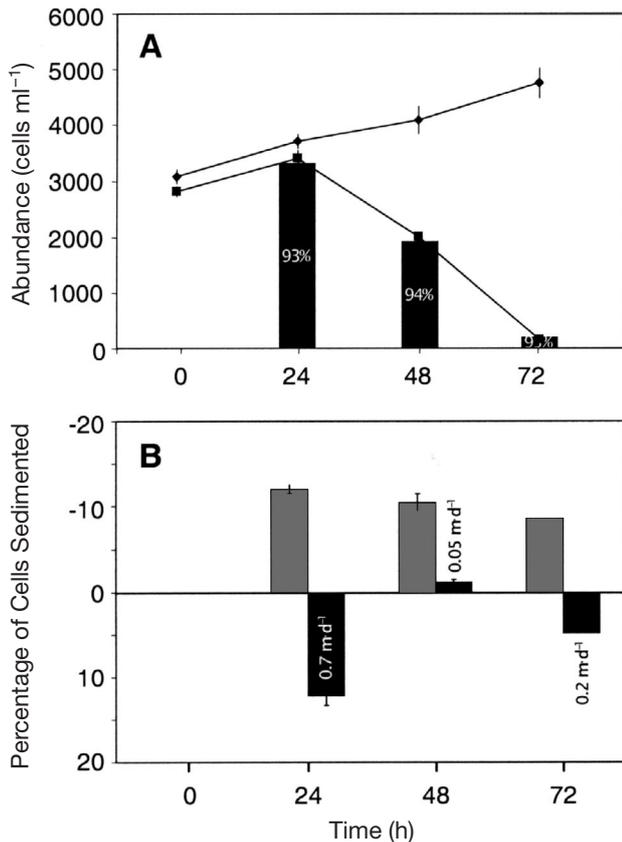
Fig. 2. *Heterosigma akashiwo.* (A) cell abundance of uninfected (♦) and OIs1-infected (■) over time; (B) percentage of cells settled to bottom in uninfected (grey) and OIs1-infected (black) cultures throughout lytic cycle. Further details as in Fig. 1
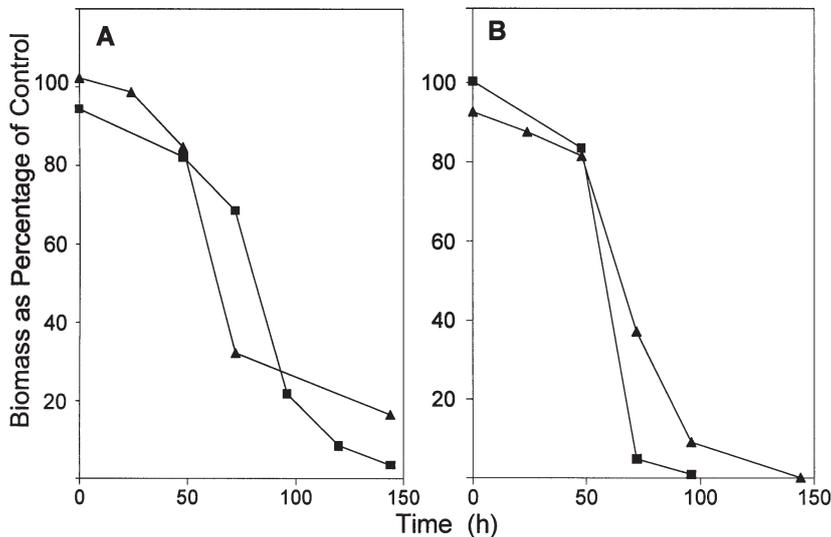
Table 1. *Heterosigma akashiwo.* Percentage of visibly infected cells throughout lytic cycle determined by TEM. A minimum of 100 cells was examined at each time point, and graded as either visibly infected or uninfected by either HaRNAV or OIs1. Percentage of infected cells was calculated by dividing number of visibly infected cells by total number examined and multiplying by 100%. p.i.: post-infection

Virus	Visibly infected cells (%)		
	24 h p.i.	48 h p.i.	72 h p.i.
HaRNAV	10	87	89
OIs1	93	94	95

(Fig. 1B) at a rate of approximately 1.3 m d^{-1} while 12.2 ± 1.9% of cells infected with OIs1 were sinking (Fig. 2B) at a rate of ca. 0.7 m d^{-1}. By 48 h the net movement of infected populations was towards the surface, at a rate of ca. 0.7 m d^{-1} for HaRNAV-infected cells, and 0.05 m d^{-1} for OIs1-infected cells. Net movement switched again to downwards 72 h post-inoculation, and 6 ± 0.3% of populations infected with HaRNAV were sinking (Fig. 1B) at ca. 0.4 m d^{-1}, while 4.8 ± 0.4% of populations infected with OIs1 were sinking (Fig. 2B) at ca. 0.2 m d^{-1}.

When incubated under L:D conditions, the relative fluorescence of HaRNAV- or OIs1-infected cultures began to decrease relative to that of control cultures 48 h after inoculation with viral lysate (Fig. 3A). Lysis of the entire population was complete 144 h after inoculation. The biomass of infected cultures incubated under continuous dark conditions also began to decrease relative to control cultures at 48 h (Fig. 3B), and lysis was complete by 144 h.

Examination with TEM revealed that only 10% of cells infected with HaRNAV showed visible signs of infection 24 h post-infection (Table 1). At this time, 93% of cells infected with OIs1 were visibly infected. By 48 h, infection could be detected visually in 87% of the HaRNAV- and 94% of the OIs1-infected cells that were examined; however, it is important to note that 50% of the populations infected with either virus had lysed by this point (Figs. 1A & 2A). Infection was visible in ca. 90% of the cells examined for either system by 72 h post-infection.

Fig. 3. *Heterosigma akashiwo.* (A) Biomass inferred by relative fluorescence) of cultures infected with (■) HaRNAV and (▲) OIs1 viruses and incubated under 14:10 h L:D conditions; (B) relative fluorescence of cultures infected with (■) HaRNAV and (▲) OIs1 viruses incubated the dark. Biomass expressed as a percentage relative to uninfected cultures; n = 3

DISCUSSION

Vertical migration allows flagellated phytoplankton to acquire subsurface nutrients that are otherwise inaccessible to their non-migrating counterparts. Therefore, in

stratified water swimming is believed to be of competitive advantage to populations of flagellated over non-flagellated algae (Smayda 1998). Blooms of *Heterosigma akashiwo* occur under stratified conditions, and cells undergo daily migrations at 1 to 1.3 m h^{-1} (Yamochi & Abe 1984) to reach nutrient-rich water up to 10 m deep (Smayda 1998). The results of this study indicate that once infected, *H. akashiwo* cells sink at rates of up to 1.3 m d^{-1}. There are no other reports in the literature for sinking rates of virally infected phytoplankton. However, using Stoke's law we can calculate the theoretical sinking rate of an *H. akashiwo* cell:

$$\omega_s = \frac{(\rho_s - \rho)gd^2}{18\mu}$$

where ω_s is the sinking rate of a sphere (cell), ρ_s is the density of the sphere (assumed to be approximately the density of marine phytoplankton cytoplasm, 1065 kg m^{-3} (Smayda 1970), ρ is the density of the fluid through which the sphere is falling (1027 kg m^{-3}), g is the force of gravity (9.8 m s^{-1}), d is the diameter of the sphere (10^{-5} m), and μ is the molecular viscosity of seawater (1.15 × 10^{-3} kg m^{-1} s^{-1}; at 20°C, 29 psu). This calculation provides an estimate that non-motile *H. akashiwo* cells will only sink ~0.2 m d^{-1} at 20°C, 29 psu. The estimated density for a *H. akashiwo* cell does not account for physiological and biochemical changes that may accompany infection. Also, the calculation does not account for other factors such as aggregation, which would result in more rapid sinking rates than those achieved by individual cells. These factors could result in a sinking rate more rapid than that calculated by Stoke's law, and may account for the difference between the observed sinking rate and the calculated sinking rate. Alternatively, factors such as altered behaviour (ie. downwards swimming) could also be responsible for the sinking rates observed in this study.

Lysis in both virus systems occurred from 48 to 96 h. The infected populations could be divided into 2 subpopulations: one that started to sink at 24 h, and one that started to sink at 72 h (Figs. 1 & 2). While sample limitations did not allow us to verify that the sinking cells were actually infected, it is probable that all cells were infected since the number of viruses added to the cultures should have resulted in synchronous infection (authors' unpubl. data). This probably indicates that the progression of the lytic cycle is dependent on the host's cell cycle, as is the case for many virus–eukaryotic cell systems (Knipe 1996). In a synchronous culture growing at a rate of 1 d^{-1}, as in this study, only a portion of the entire population would undergo mitosis each day, even though the cells that do undergo mitosis would do so at the same time. Therefore, the progression of the infection cycle, and therefore the onset of sinking, would be tied to the

circadian cell cycle of the cultured population. Thus, the onset of sinking and cell lysis is likely to depend on host-cell physiological status, as well as the timing of viral infection.

Packaged OIs1 virus particles within infected *Heterosigma akashiwo* cells were visible with TEM 24 h post-infection, while viruses were not visible in cells infected with HaRNAV until 48 h post-infection. This was probably due to differences in the replication and viral packaging of these viruses. Therefore, examinations of visibly infected cells is not a reliable method for estimating the proportion of infected cells and virus-mediated mortality in natural populations of *H. akashiwo*. Moreover, infected cells will sink and therefore not be detected in surface populations. These results indicate that estimates of virus-mediated mortality of *H. akashiwo* in nature are not yet possible with current techniques.

Dark post-infection incubation did not inhibit lysis of *Heterosigma akashiwo*, nor did it alter the length of the lytic cycle for either virus system examined. As nucleic acid and protein synthesis are energy-consuming processes, *H. akashiwo* cells must either have sufficient energy stores to allow the lytic cycle to continue, or they must produce enough energy in the dark to drive these processes. Some cyanophage–host systems produce viruses in the dark, using energy from cyclic photophosphorylation and/or oxidative phosphorylation for viral synthesis (Padan et al. 1970, Sherman & Haselkorn 1971, Adolph & Haselkorn 1972, Allen & Hutchison 1976, Bisen et al. 1988). The net result is that sinking infected cells will still undergo lysis in the dark and release progeny viruses into the environment.

Total population lysis in the L:D experiments did not occur until 144 h post-infection, while complete lysis was reached within 96 h during the sinking experiments. This difference was probably due to differing culture conditions, such as culture vessel and direction of illumination. This variability in the length of the lytic cycle suggests that environmental conditions and host physiology will affect the length of the lytic cycle in nature, and therefore also affect the depth of water an infected, sinking cell will reach prior to lysis. Variability in the length of the lytic cycle is therefore intrinsically linked to the fate of viruses in the ocean. Other factors that affect the sinking rate of infected cells will also impact the fate of viruses and their ability to propagate infection within a bloom. For example, aggregation has not been examined in infected *Heterosigma akashiwo* populations; however, it is well known to result in increased sinking rates of phytoplankton (Smayda 1970).

The detection of lytic viruses that infect *Heterosigma akashiwo* in the natural environment (Chan et al. 1998,

Taruntani et al. 2000, Lawrence et al. 2001) indicates that viruses are mortality agents. However, the extent of virus-mediated mortality is not easy to determine. The results of this study indicate that viral control of blooming populations is complex, as it is dependent on a number of factors. The propagation of infection through a bloom depends on the fate of the virus and infected cells and the strain-specificity of the virus. It is also very dependent on the physical oceanographic conditions. We can hypothesise at least 3 potential outcomes for infection of *H. akashiwo* (Fig. 4):

Case I: In areas where the mixed layer is relatively deep, sinking cells may not reach the pycnocline prior to lysis. Under these conditions, progeny viruses would be released above the pycnocline where they could infect new cells and thereby propagate infection within the bloom. If these conditions persisted and/or the number of viruses available for infection was high enough, the bloom would terminate due to viral infection. This process may explain the detection of free virus particles in surface-water samples from the Strait
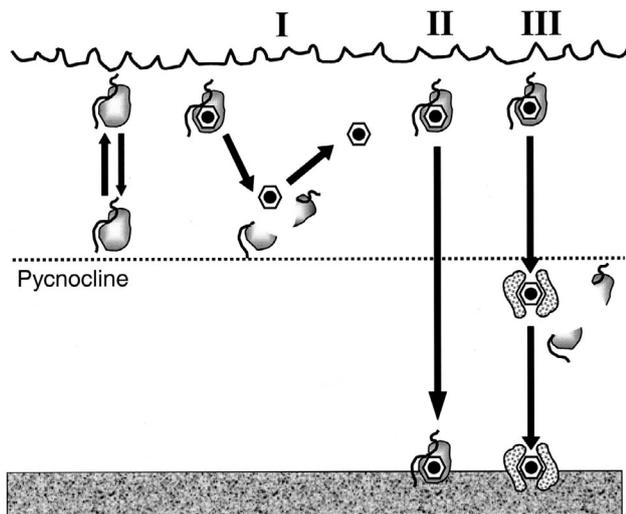
of Georgia (Chan et al. 1998) and the association of visibly infected cells with bloom termination in Japanese waters (Nagasaki et al. 1994).

Case II: In very shallow environments, infected cells may sink to the bottom prior to lysis. Under these conditions, viruses would accumulate at the sediment surface and could be resuspended to infect future populations of algae. At the sinking rates and conditions of this study, infected cells could reach the benthos prior to lysis (within 96 to 144 h) in a water column approximately 4 to 8 m deep. Under conditions that promote aggregation, sinking rates could be much higher, resulting in the transport of infected cells to much deeper sediments. This represents a rapid transport mechanism that could explain the abundance of infectious viruses (3210 agents cm^{-3} sediment) detected in shallow sediments, such as Malaspina Inlet (Lawrence et al. 2002), and the observed high concentration of infectious viruses near the sediment-water interface (Taruntani et al. 2000).

Case III: In deeper environments with a relatively shallow mixed layer, cells may sink below the pycnocline but not reach the sediments prior to lysis. This study has shown that, in darkness, lysis will continue in infected cells and produce infectious progeny viruses. This is an explains an increase in viral particles at the surface infecting *Heterosigma akashiwo* following deep tidal mixing (A. Chan & C. Suttle unpubl data). Virus particles released below the pycnocline are still subject to losses such as adsorption to particles (Suttle & Chen 1992, Noble & Fuhrman 1997), grazing by protists (Suttle & Chen 1992, Gonzalez & Suttle 1993), or decay by dissolved substances (Noble & Fuhrman 1997). Nonetheless, infectious virus particles are found in sediments collected at the deepest depths sampled (285 m) in the Strait of Georgia (Lawrence et al. 2002).

Phytoplankton mortality can be caused by numerous factors, including sedimentation, grazing (e.g. Walsh 1983), physiological stress due to nutrient or light limitation (Berges & Falkowski 1998) and viral lysis (Suttle et al. 1990, Bratbak et al. 1993, Suttle 2000). Cellular mortality resulting in the decline of an entire population, or the termination of a bloom, however, is a very different and ultimately a more complex process than the mortality of individual cells. The models of the fate of infected cells and release of viral particles presented here emphasise that the interactions between virus and phytoplankton blooms are also very dependent on the physical environment.

Fig. 4. *Heterosigma akashiwo.* Conceptual model depicting fate of virally infected cells and viruses that infect them. On the left, uninfected cells vertically migrate between surface and pycnocline. Depending on physical oceanographic conditions, infected cells and the viruses produced during infection succumb to 3 potential outcomes: In Case I, under highly stratified conditions or in areas where pycnocline is relatively deep, infected cells lyse above pycnocline and release progeny viruses where these may propagate infection within the bloom. In Case II, when infected cells are not retained within pycnocline and water column is relatively shallow, infected cells will reach the benthos prior to lysis, resulting in high abundances of viruses at the sediment–water interface. In Case III, infected cells are not retained within mixed layer but sink below the pycnocline where cell lysis occurs and the viruses are released, the released viruses are subject to various loss terms and may be adsorbed to particles and be transported to bottom

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