

## NOTES

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### Preferential grazing of *Oxyrrhis marina* on virus-infected *Emiliana huxleyi*

**Abstract**—We examined whether virus infection of *Emiliana huxleyi* with the lytic *E. huxleyi*-specific virus, EhV-86, influenced its palatability to the heterotrophic dinoflagellate *Oxyrrhis marina*. *E. huxleyi* mortality was assessed by comparing changes in the algae's concentration between four different incubations: (1) Grazer *O. marina* plus an equal concentration of infected and healthy *E. huxleyi* prey (grazed-infected); (2) the same equal concentration of healthy and infected *E. huxleyi* prey without grazer *O. marina* (infected); (3) healthy *E. huxleyi* prey (no infected cells) with grazer *O. marina* (grazed); and (4) healthy *E. huxleyi* prey alone (no grazer or infected cells; control). Grazing rates of *O. marina* increased in the presence of virus-infected *E. huxleyi* prey. An adapted plaque assay protocol revealed that the amount of infected cells in grazed-infected cultures was approximately one-quarter (0.28) of that observed in infected (minus grazer) controls. Given that these assays were normalized to cell number, they demonstrate that *O. marina* was preferentially feeding on virus-infected *E. huxleyi* over their healthy counterparts. The effects of marine viruses may have been misinterpreted since the likelihood of them being grazed during infection has not been previously considered. Preferential grazing of infected cells in the ocean would sequester more carbon in particulate form, making it available to higher trophic levels. Consequently, these results should be taken into consideration when modeling the ocean carbon budget.

The fate of carbon fixed by primary production shapes the biogeochemistry and ecology of the oceans. Organic matter and energy may be lost to the sea floor by sedimentation, passed to higher trophic levels by grazing or converted to the dissolved phase by lysis. Within the marine environment viruses are responsible for the infection and subsequent lysis of a substantial proportion of the unicellular eukaryotes (Brussaard 2004a). It has been estimated that as much as 25% of the photosynthetically fixed carbon may pass through the viral shunt daily (Wilhelm and Suttle 1999). Currently it is believed that viral lysis short-circuits the transfer of biomass to higher trophic levels of the food web by the conversion of particulate to dissolved organic matter, not readily available for grazing (Fuhrman 1999). A net effect of this process is to sequester matter and energy in the euphotic zone and increase carbon respiration in the surface waters (Suttle 2005). Since viruses are host-specific they may shape the food web both directly, via the lysis of certain species, and indirectly by the reduction of biomass available to be grazed and subsequently passed to higher trophic levels. This has biogeochemical consequences as viral lysis

prevents carbon and other elements being passed through the biological pump which is where elements are lost to the seabed when larger organisms die and sink (Suttle 2005). The sequestration of dissolved organic matter in the euphotic zone increases the amount of carbon, nutrients, and other important elements, such as sulphur, within the surface ocean and subsequently influences their ocean-atmosphere balance.

Microzooplankton grazing on marine algal cells may be selective, whereby there is an imbalance between the proportion of prey types in a predator's diet and the proportion of the same prey type in the environment (Chesson 1983). Studies have shown that predators can distinguish between prey types independent of total prey density or feeding history and that selective grazing is constant (Hamels et al. 2004). Factors such as prey size and motility, which influence a cell's susceptibility to predation, may passively govern selective grazing. However, studies have demonstrated that selective grazing may also be an active process based on nutritional value and palatability of the prey and is driven by chemical cues and the production of deterrent compounds (Verity 1988; Müller and Schlegel 1999; Hamels et al. 2004).

During viral infection, phytoplankton undergo physiological and biochemical changes (Brussaard et al. 2001; Evans et al. 2006, 2007) which could influence their susceptibility to predation, and studies have speculated on the occurrence of selective grazing on virally infected algal cells (Evans 2005). Despite its potential biogeochemical significance, to our knowledge the occurrence of positive or negative selection of virally infected algae by grazers has not been previously demonstrated. *Emiliana huxleyi* is a widely distributed, biogeochemically significant species of coccolithophore, which forms large-scale blooms that may be decimated by viral infection (Wilson et al. 2002) or microzooplankton grazing (Nejstgaard et al. 1997). Here we report on the influence on grazing rate and preference of *Oxyrrhis marina* when fed on a mixture of virally infected and healthy *E. huxleyi*.

#### Methods

**Culturing**—Phytoplankton cultures of *Emiliana huxleyi* CCMP 1516 and *Dunaliella minuta* PCC 430 were obtained from Brian Palenik (Scripps Institution of Oceanography) and the Plymouth Culture Collection (Marine Biological Association), respectively. The heterotrophic dinoflagellate *Oxyrrhis marina* CCAP 1133/3 was obtained from the Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory). Stock cultures were maintained in *f/2*

medium at 15°C under a light:dark cycle of 16:8 h at 80  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ . *O. marina* cultures were fed with *D. minuta*, and several days prior to the experiment they were transferred to the dark to allow the grazers to remove the prey and induce a state of starvation. This was confirmed by examining the *O. marina* under blue-light excitation to check their food vacuoles were predominately clear of prey organisms and observing the grazer when combined with healthy *E. huxleyi* to ensure they were feeding. *E. huxleyi* virus 86 (EhV-86), the lytic viral pathogen used, was isolated during an *E. huxleyi* bloom in the Western English Channel during July 1999 (Wilson et al. 2002). Lysates were generated by the addition of one milliliter to a 1-liter culture of *E. huxleyi*. After lysis, the culture lysate was filtered (0.22  $\mu\text{m}$ ) to remove cellular debris and stored in the dark at 4°C until required.

**Incubation experiments**—*E. huxleyi* mortality was assessed by comparing changes in the algae's concentration between four different incubations: (1) grazer *O. marina* plus an equal concentration of infected and healthy *E. huxleyi* prey (grazed-infected); (2) the same equal concentration of healthy and infected *E. huxleyi* prey without grazer *O. marina* (infected); (3) healthy *E. huxleyi* prey (no infected cells) with grazer *O. marina* (grazed); and (4) healthy *E. huxleyi* prey alone (no grazer or infected cells; control). The four incubations were set up in triplicate in 500-milliliter polycarbonate bottles. Virus-infected *E. huxleyi* prey were sourced from a pre-infected culture which was set up 4 d in advance by adding 2.5 milliliters of viral stock to 4 liters of *E. huxleyi* culture with a density of  $1.5 \times 10^5 \text{ cells mL}^{-1}$  (which equated to an approximate ratio of 0.4 viruses per host) and in the early exponential stage of growth. From previous characterization of the growth curve of cultures set up in an identical manner, we could predict the proportion of infected cells present at any particular time, and this was confirmed retrospectively by monitoring cell mortality in the culture (Fig. 1). The incubation experiment was set up when the culture contained an estimated 70% infected cells and appropriate volumes of the infected culture were combined with healthy *E. huxleyi* culture in mid-exponential growth to create a 1:1 ratio of infected to healthy cells.

Grazer *O. marina* was added to the required treatments at a ratio of one healthy grazer (assessed by motility as observed by microscopy) to 50 prey. Prior to combination, all the stock cultures used were gently but thoroughly mixed to ensure homogeneity. Samples for *E. huxleyi* enumeration were taken ~0.5 h, then 14 h, after set-up from each incubation. Also at 15 h, a modified plaque assay was conducted from the treatments containing virus-infected *E. huxleyi* to investigate the ratio of infected to uninfected cells. The experiment was conducted within the time required for one growth cycle of *E. huxleyi* as the reproduction of the healthy cells would have changed the ratio of healthy to infected cells and made it complex to determine whether the *O. marina* were grazing selectively when offered the choice of infected or healthy *E. huxleyi*.

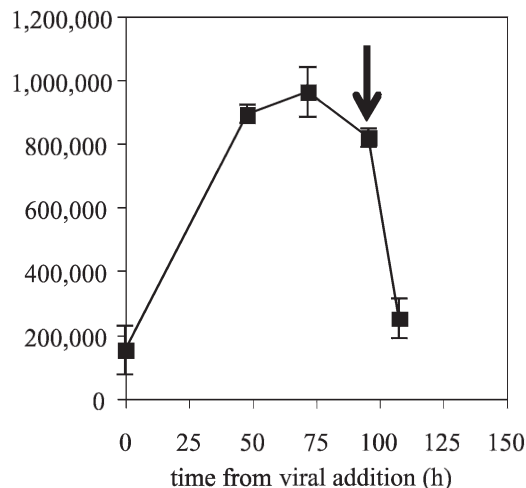


Fig. 1. Dynamics of the *E. huxleyi* cells in a virally infected culture used to source pre-infected *E. huxleyi* prey for the experiment. The arrow indicates the point at which *E. huxleyi* were taken from the experiment, ~4 d after the initial infection, when the culture had a density of  $8.16 \times 10^5 \text{ cells mL}^{-1}$  where it was estimated that 70% were infected.

An incubation period of ~15 h was used; this allowed the maximum period for the *O. marina* to graze whilst still allowing time for the set up of plaque assays and completion of the other measurements before the *E. huxleyi* began to divide.

**Enumeration**—*E. huxleyi* counts were done using a Beckman Coulter Multisizer™ 3 with a 100- $\mu\text{m}$  orifice. Autoclaved, 0.2- $\mu\text{m}$  filtered seawater was used as the electrolyte. Samples were analyzed in triplicate and data was collected and interpreted using Coulter Multisizer3 Version 3.01a software. For virus enumeration 1-milliliter samples were fixed in a final concentration of 0.5% glutaraldehyde for 30 min at 7°C. Samples were then snap-frozen in liquid nitrogen and stored at -80°C prior to analysis using SYBR Green I nucleic acid gel stain (Molecular Probes) according to method of Brussaard (2004b). *O. marina* samples preserved with Lugol's iodine were counted in triplicate using a Sedgwick-Rafter counting chamber. One milliliter of the preserved sample was transferred to the counting chamber and left for 10 min to allow the protists to settle. Cell number estimates were calculated on the basis of 30 randomly selected fields of view per chamber.

**Plaque assay**—The number of virally infected cells was assayed by a modification of the plaque assay technique of Schroeder et al. (2002). In a traditional plaque assay, the number of infectious units in a sample is determined by incubation with a susceptible host present in excess and then registering subsequent infections. The technique was modified to determine the level of infection already occurring in situ in the experimental treatments. For each of the two treatments containing infected *E. huxleyi*, with and without grazer *O. marina*, six assays were conducted

with two aliquots removed from each replicate. The volumes removed were calculated to contain the same total amount of cells based on cell counts performed immediately prior to set up of the plaque assay. Free viruses were removed from the samples by washing via centrifugation at 4000 revolutions per min at 4°C for 5 min and resuspension in 1 milliliter of *fl2* media which was repeated twice. A final centrifugation step was then conducted and the sample pellets were resuspended in 2 milliliters of concentrated healthy *E. huxleyi* cells. The concentrated cells were prepared immediately prior to the plaque assay set up by centrifugation of 50-milliliter aliquots of a healthy *E. huxleyi* culture in the mid-exponential stage of growth and resuspension of the resulting pellets in 1 milliliter of *fl2* media. For each sample, 1-milliliter dilutions of 1, 0.5,  $10^{-1}$ ,  $10^{-2}$ , and  $10^{-3}$  were produced by combining with the appropriate volume of concentrated cells. A plaque assay was conducted for each dilution.

## Results

The cell counts determined after 0.5 h were  $3.4 \times 10^5 \text{ mL}^{-1}$ ,  $2.4 \times 10^5 \text{ mL}^{-1}$ ,  $3.0 \times 10^5 \text{ mL}^{-1}$ , and  $2.1 \times 10^5 \text{ mL}^{-1}$  for the treatments control, infected, grazed, and grazed-infected, respectively. This indicates that during the period between counting the cells in the stock cultures and setting up the treatments and completing the initial cell counts from the experimental incubations, mortality of a proportion of the *E. huxleyi* occurred due to grazing and viral lysis. Subsequently the concentrations at the 0.5-h time point were not even over the four treatments, indicating that the ratio of infected to uninfected was <50%. However, this would not have affected the conclusion that infected *E. huxleyi* were preferentially grazed. Lower numbers of infected *E. huxleyi* would have reduced the likelihood of them being grazed since their abundance would have been less than the uninfected algae. Consequently, the chance encounter rate of the grazers with infected cells would have been lower than with uninfected cells. Under these conditions, a positive result would have increased significance.

During the incubation period, *E. huxleyi* cell numbers changed negligibly in the healthy control. Grazing (grazed), and viral lysis (infected) alone caused the loss of 7000 and 19,000 *E. huxleyi*  $\text{mL}^{-1}$  respectively, although results of a Bonferroni correction indicated these reductions were not significant. Whereas, in the treatment containing both infected cells and grazers (grazed-infected), algal numbers decreased by 42,000  $\text{mL}^{-1}$  (Fig. 2). This result was highly significant when compared with all the other treatments with a *p*-value of <0.001. The latter result indicates that grazing and/or viral lysis proceeded at a faster rate when these two mortality processes occurred in parallel.

Free EhV-86 particles were present at concentrations within error of each other for the infected and the grazed-infected treatment at the start of the experiment with average  $\pm$  standard deviations of  $1.6 \times 10^8 \pm 2 \times 10^7 \text{ mL}^{-1}$  and  $1.5 \times 10^8 \pm 1.4 \times 10^7 \text{ mL}^{-1}$ , respectively. After the incubation period, EhV-86 were still present at equal amounts in the infected, and the grazed-infected treatments, although their concentrations had declined

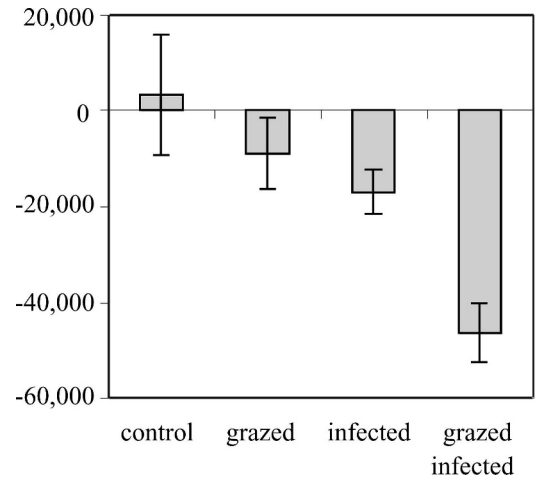


Fig. 2. Change in *Emiliana huxleyi* cell density over a 15-h incubation period in treatments containing: healthy *E. huxleyi* (control), a mixture of 50% virally infected and 50% healthy *E. huxleyi* (infected), healthy *E. huxleyi* and the microzooplankton grazer *Oxrrhysis marina* (grazed), and 50% virally infected and 50% healthy *E. huxleyi* and *O. marina* (grazed-infected). Changes in *E. huxleyi* density are the average and standard deviation of triplicate cultures set up for each treatment.

slightly at  $1.3 \times 10^8 \pm 0.8 \times 10^7 \text{ mL}^{-1}$  and  $1.3 \times 10^8 \pm 2 \times 10^7 \text{ mL}^{-1}$ , respectively. This decrease in EhV-86 over the incubation period indicates that rates of their loss, probably due to re-adsorption to uninfected cells, were greater than production. The identical levels of viruses recorded indicates that levels of new virus infections occurring over the course of the experiment would have been equal in the two treatments and, therefore, would not have interfered with the conclusions made.

Plaque assays conducted at the end of the 15-h incubation period revealed that the proportion of infected cells in the grazed-infected cultures treatment was approximately one-quarter (0.28) of that observed in the infected (minus grazer) control (Fig. 3), indicating that infected cells were preferentially grazed. An independent sample *t*-test showed that these results were highly significant with a *p*-value of <0.001. This was determined from the amount of plaque-forming units detected at  $10^{-3}$  level of dilution, and the number of plaques observed plate<sup>-1</sup> ranged from 26 to 201. Plates from the lower levels of dilution had too many plaques to count.

## Discussion

The sum of *E. huxleyi* cell losses caused by grazing and viral lysis in isolation was significantly lower than those losses observed when the mortality mechanisms took place in parallel (i.e., grazed-infected), suggesting that the rate of grazing (and arguably viral lysis) is increased in grazed-infected cultures (Fig. 2). Furthermore, infected cells were preferentially grazed over their noninfected counterparts (Fig. 3). The speed of lytic viral infection is dictated by the physiological condition of the host cell which, for phytoplankton, is influenced by factors such as light and

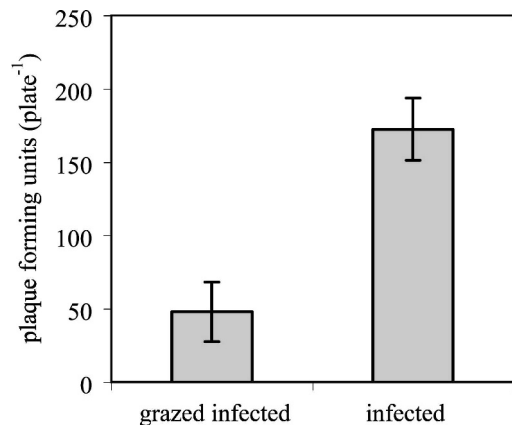


Fig. 3. The relative proportion of infected cells and at the end of the 15-h incubation period for *Emiliania huxleyi* cells from the treatments containing a mixture of 50% virally infected and 50% healthy *E. huxleyi* cells (infected), and 50% virally infected and 50% healthy *E. huxleyi* and *Oxyrrhis marina* (grazed-infected) as assessed by modified plaque assay. Identical amounts of cells were assayed from all the treatments examined. These results represent the average and standard deviation of plaque-forming units observed on six assays, two from each replicate, from the  $10^{-3}$  level of dilution. The lower levels of dilution exhibited too many plaques to enumerate.

nutritional status (Waters and Chan 1982; Bratbak et al. 1998). Therefore, it is unlikely that the duration of infection was shortened merely by presence of grazers. Instead, it is more likely that the rate of grazing increased in the presence of infected *E. huxleyi*. One hypothesis to explain this result is that physiological changes within infected cells increase the rate of feeding on the virally infected *E. huxleyi* and facilitate preferential selection by grazer *O. marina*. A second hypothesis is simply a change in size of the infected cells (Frost 1972).

During viral infection, phytoplankton cells undergo various biochemical and physiological changes (Brussaard et al. 2001; Evans et al. 2006, 2007), which could account for increased grazing rates and preferential grazing over healthy cells. We have observed that virally infected *E. huxleyi* exhibit increased cell size (data not shown) as determined by Coulter Counter analysis. It is known that phagotrophic protists generally preferentially graze on larger prey cells (Gonzalez et al. 1990). Studies have also shown that calcifying *E. huxleyi* are preferentially grazed over their naked counterparts, which was suggested to result from the larger size of calcified cells (Hansen et al. 1996). Larger cells would be easier to locate and also potentially confer a greater nutritional reward when compared with grazing on smaller cells, a process that would probably require the same amount of energy. Thus the effects observed could have been due to selection of the infected *E. huxleyi* as a consequence of their increased size.

Preferential consumption of the infected *E. huxleyi* cells could also have been as a result of an active mechanism whereby cells are selected due to their palatability. Infected cells will differ in their composition from noninfected cells by factors such as their nucleic acid content, and these differences could improve their nutritional value to the grazers. Active

grazing has also been linked to the production of toxic or inhibitory compounds (Verity 1988; Müller and Schlegel 1999; Hamels et al. 2004). It has been established that *O. marina* selects for *E. huxleyi* cells with lower dimethylsulfoxoniopropionate lyase (DMSP lyase; the enzyme responsible for cleaving DMSP to dimethyl sulphide and acrylate) activity; it was suggested that these cells would produce less acrylate, a potentially toxic compound (Wolfe et al. 1997). Previously, we have shown that virally infected *E. huxleyi* exhibit lower levels of DMSP lyase activity (Evans et al. 2007) which could make them more attractive to grazers by reducing their exposure to the harmful acrylate.

In addition, selective grazing is thought to be mediated by chemical cues, and we have shown that viral infection of *E. huxleyi* results in the production of low molecular weight and rapidly diffusive compounds such as dimethyl sulphide (Evans et al. 2007) and hydrogen peroxide (Evans et al. 2006). It is highly likely that these or other organic compounds released during infection could generate a gradient to their source of origin, the infected cell. This would make infected *E. huxleyi* easier for *O. marina* to locate over their healthy counterparts, which produce much lower concentrations of these compounds. This theory is supported by the fact that concentrations of EhV-86 were identical in the grazed-infected and infected treatments at the final time point, despite a reduction in the amount of infected cells present in the grazed-infected treatment. This suggests that grazing of the infected cells occurred after or during the release of viral progeny. The accumulation of free viral progeny has been shown to occur in parallel with a reduction in membrane integrity and the accumulation of organic compounds in the dissolved phase of infected *E. huxleyi* cultures (Evans et al. 2007). In addition to preferential selection, chemical cues could also account for the increased rate of grazing observed in the cultures containing virally infected cells. This could result from increased prey-location speed facilitating the location and, therefore, consumption of more cells by the grazer in a given period. Alternatively, chemical cues may simply stimulate *O. marina* to graze at an increased rate. However, it should also be noted that chemical cues may be implicated in the inhibition of grazing. For example DMSP, a compound produced during viral infection of *E. huxleyi* (Evans et al. 2007), has been shown to inhibit grazing on *E. huxleyi* by a number of microzooplankton species (Strom et al. 2003).

Further investigations are required to determine the precise mechanism by which infected *E. huxleyi* are preferentially selected over uninfected *E. huxleyi* for consumption by *O. marina*. Because chemical cues are likely to be key factors in preferential selection, studies to determine the identities of these compounds and their effect on grazer behavior are warranted. In particular, it would be pertinent to examine whether grazing rates are altered by the presence of lysis products and, more specifically, whether grazers are influenced, and therefore potentially guided, by gradients in the concentration of these compounds.

Preferential grazing of infected cells will have implications for the ecology and biogeochemistry of the oceans primarily mediated by its influence on the flow of matter and energy through the microbial loop and the marine food

web. If infected cells are grazed, they will not complete the lytic cycle, and the percentage of carbon passing through the viral shunt will be reduced. Thus, current flux calculations indicating that one-quarter of the photosynthetically fixed carbon flows through the viral shunt per day (Wilhelm and Suttle 1999) may be overestimated. Furthermore, the efficiency of the biological pump would increase as more matter and energy passes to higher trophic levels via grazers rather than being transferred to dissolved organic matter, not readily available for grazing.

To our knowledge this study is the first to demonstrate increased rates of grazing in the presence of virally infected algae and the preferential selection of infected cells by grazers over their uninfected counterparts. Further studies will be required to determine whether selective grazing occurs in other virus–host systems. Crucially, methods need to be developed to look at this phenomenon in natural systems. However, if the factors proposed here such as production of signaling compounds or increased cell size during infection are responsible for preferential grazing, then it is likely to be widespread in aquatic environments. Particularly since studies have demonstrated changes in physiology during virus infection in other phytoplankton (Malin et al. 1996; Brussaard et al. 2001) and it is known that many micro- and macrozooplankton species are able to exercise selective preferences during grazing (Hamels et al. 2004 and references therein). Furthermore, grazing and viral lysis have been shown to occur simultaneously during the decline of phytoplankton blooms (Evans et al. 2003; Baudoux et al. 2006), indicating it is likely this process could occur, certainly in the marine environment. Clearly, the interaction between grazers and viruses is more complicated than the simplistic view that cells are either grazed or lysed, and the implications for the marine food web are far-reaching. This process could present yet another pathway that must be carefully considered when modeling the flow of carbon in the ocean.

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