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Infectious titers of *Emiliania huxleyi* virus 86 are reduced by exposure to millimolar dimethyl sulfide and acrylic acid

Abstract—We examined the ability of dimethylsulfoniopropionate (DMSP), its cleavage products dimethyl sulfide (DMS) and acrylic acid (AA), and the oxidized form of DMS dimethylsulfoxide (DMSO), to inhibit infection of *Emiliania huxleyi* virus 86 (EhV-86). Infectivity was assessed by plaque assay of viral stock that had been exposed to these compounds. The initial concentrations of the compounds tested were 250 mmol L⁻¹ for DMSP, DMS, and AA, and 14 mmol L⁻¹ for DMSO. These are the maximum concentrations thought to occur in *E. huxleyi* and therefore the highest EhV-86 might encounter. DMSP and DMSO had no effect on EhV-86; however, both DMS and AA diminished viral titers. Further experiments established that both DMS and AA significantly reduced titers from a concentration of 100 mmol L⁻¹ and that they had a greater antiviral effect when applied in combination. The DMSP system in algae could function as a chemical defense against viral infection that would benefit the surviving cells in the population by reducing infective titers of progeny viruses and therefore decreasing the probability of infection of further cells.

Viruses are abundant in the sea and are considered to influence ecological processes and the major biogeochemical cycles (Suttle 2005). Studies have shown that despite the decimation of algal populations by viruses, algal cells may remain after the population crash (Jacquet et al. 2002), and some species of phytoplankton are able to coexist with their pathogenic viruses (Thyrhaug et al. 2003). Despite indications that defense mechanisms against virus infection may exist in phytoplankton, no compounds have been identified that could fulfill this important role.

Emiliania huxleyi is a widely distributed, biogeochemically significant species of coccolithophore that forms large-scale bloom that may be decimated by viral infection (Wilson et al. 2002). A study of six *E. huxleyi* strains showed that the activity of their dimethylsulfoniopropionate (DMSP) lyase, the enzyme responsible for cleaving DMSP to dimethyl sulfide (DMS) and acrylic acid (AA), varied by more than 6,000-fold, and they were classified as either “low lyase” and “high lyase” strains (Steinke et al. 1998). Despite extensive screening, to date no viruses have been isolated that are capable of infecting the high DMSP lyase activity strains (Evans 2005). This has led to the suggestion that high DMSP lyase activity may be implicated in an antiviral defense mechanism (Schroeder et al. 2002). Previously the DMSP system has been proposed to serve a number of roles including compatible solute, antioxidant, overflow for excess reduced sulfur and energy,

and a chemical defense against grazing (Simo 2001). The grazing chemical defense hypothesis was first suggested after it was observed that microzooplankton were able to clear cultures of *E. huxleyi* with lower DMSP lyase activity much faster than those with high DMSP lyase activity (Wolfe and Steinke 1996). Further studies revealed that when offered a choice of prey, grazers preferentially selected those *E. huxleyi* strains with lower DMSP lyase activity (Wolfe et al. 1997). This led to the suggestion that the acrylate produced during grazing by mixing the DMSP lyase with its substrate was harmful to the protists (Wolfe et al. 1997), which is in line with the antimicrobial properties of this compound (Sieburth 1960). More recent studies suggest that prey selection may be directed by signaling molecules, possibly DMSP or related compounds, present on the cell surface or in the near-cell dissolved phase (Strom et al. 2003a, 2003b).

Studies of *Phaeocystis pouchetii* have revealed that DMS is produced and DMSP released during viral infection (Malin et al. 1998). Furthermore, our laboratory data have shown that during viral infection of *E. huxleyi*, AA and dimethylsulfoxide (DMSO), the oxidized form of DMS, are produced in addition to DMS (Evans 2005). This, in combination with the failure to isolate any viruses against high DMSP lyase activity strains, led us to consider whether the DMSP cleavage pathway could be linked to an antiviral defense mechanism within algal cells. Here we report on the effect of DMSP and the related compounds DMS, AA, and DMSO on the infectivity of *E. huxleyi* viruses.

Materials and methods—*E. huxleyi* CCMP 1516 (Provasoli-Guillard Centre for the Cultivation of Marine Phytoplankton), a low DMSP lyase activity strain (Steinke et al. 1998), was used in this study along with virus strain *E. huxleyi* virus 86 (EhV-86). All cultures were maintained in *f*/2 medium at 15°C under a light:dark cycle of 14:10 h and at an illumination of 250 μmol photons m⁻² s⁻¹. Prior to use in the experiments, approximately 50 ml of fresh virus stock was dialyzed twice against 1 liter *f*/2 medium at 4°C for 1 h to reduce the background concentration of DMSP and related compounds using a 10,000 nominal molecular weight cutoff and regenerated cellulose dialysis membrane prepared according to the protocol of Harris and Angal (1990).

To determine whether DMSP, DMS, AA, or DMSO had any effect on EhV-86 infectivity, virus stock was incubated with either 250 mmol L⁻¹ DMSP, DMS, or AA, or

14 mmol L⁻¹ DMSO before being plaque assayed to assess virus titer. The concentrations selected are in the upper range that the viruses are likely to encounter within *E. huxleyi* cells (Steinke et al. 1998; Evans 2005). Immediately prior to use in the experiments, DMSP (CAS, Groningen, the Netherlands), DMS (Sigma-Aldrich, 98%), AA (Sigma Aldrich, high performance liquid chromatography grade), and DMSO (Sigma Aldrich, 99.5%) were made up in *f*/2 medium and the pH was adjusted to 7, since this is typical of the intracellular pH found within *E. huxleyi* cells (Nimer et al. 1994). The virus stock was then divided into 100 μ L aliquots and enclosed with the appropriate compound in 2-cm³ Alltech gas-tight vials equipped with Teflon lined caps. A minimum of six vials was set up for each treatment and incubated for 24 h at 15°C in the dark.

In a second plaque assay EhV-86 was combined with either AA or DMS over a range of concentrations from 10 to 250 mmol L⁻¹ to determine the lowest effective concentration that reduces EhV-86 infectivity. For each treatment three replicate vials were set up. Finally, to investigate whether DMS and AA act synergistically to reduce the infectivity of EhV-86, a plaque assay was done with viral stock that had been incubated with either DMS, AA, or both. The final concentration used for all compounds was 100 mmol L⁻¹, and a minimum of four vials was set up per treatment.

Results—Virus titers recorded were between 24 and 300 plaque forming units mL⁻¹. DMSP and DMSO had no effect on EhV-86 titers, but in contrast DMS and AA reduced titers of EhV-86 by an average of 87% and 70%, respectively, compared to the controls (Fig. 1). An independent sample *t*-test showed that these results were highly significant with *p* values of less than 0.001. Investigations to identify the lowest effective concentrations of DMS and AA that reduce viral infectivity revealed significant effects at concentrations of 100 mmol L⁻¹ with reductions of 64.6% and 32.3%, respectively, and this antiviral effect intensified with increasing concentrations (Fig. 2). Furthermore, DMS and AA caused a greater reduction in virus infectivity at 97% when applied together at 100 mmol L⁻¹ than when added individually where the reduction for DMS was 90.6% and for AA was 80.5% (Fig. 3). During this latter experiment it was observed that both DMS and AA yielded a greater reduction in viral titer at a concentration of 100 mmol L⁻¹ than was observed in the previous experiment. This effect was probably a result of the use of a different batch of lysate.

Discussion—Titers of EhV-86 were clearly reduced by exposure to DMS and AA. However, the levels required to cause a significant decrease were higher than the 1 to 3 nmol L⁻¹ bulk DMS concentrations typically measured in the oceans. Even during the decline of phytoplankton blooms, levels of DMS rarely exceed the low hundreds of nmol L⁻¹ (e.g., Malin et al. 1993). Comparatively little is known about the distribution and concentration of AA in the oceans, although studies in the Antarctic have reported maximum concentrations of 500 to 1,000 nmol L⁻¹ (e.g., Hefu et al. 1992). Thus, it is clear that reduction of

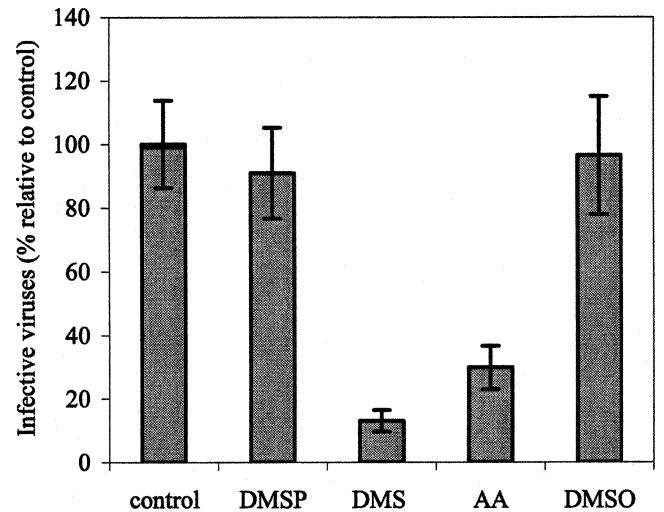


Fig. 1. Percentage of infective *Emilia huxleyi* virus 86 relative to a no-addition control after 24 h incubation with either 250 mmol L⁻¹ dimethylsulfoniopropionate (DMSP), dimethyl sulfide (DMS), and acrylic acid (AA) or 14 mmol L⁻¹ dimethylsulfoxide (DMSO). Error bars represent the standard deviation for six replicate samples.

infectivity by exposure to DMS and AA is only likely to be of significance for viruses while they are associated with a phytoplankton cell, i.e., during the infection process.

Intracellular DMSP, the precursor of DMS and AA, is present in many phytoplankton species (Keller et al. 1989), including *E. huxleyi* (Steinke et al. 1998), at mmol L⁻¹ concentrations. Therefore, it is possible that DMS and AA could also occur at these concentrations if the intracellular DMSP is cleaved by DMSP lyase. This is less likely to happen during active growth, since DMSP and DMSP lyase are thought to be segregated within the cell (Wolfe and Steinke 1996) and DMS exudation has been shown to constitute only a small proportion of the total DMSP (e.g.,

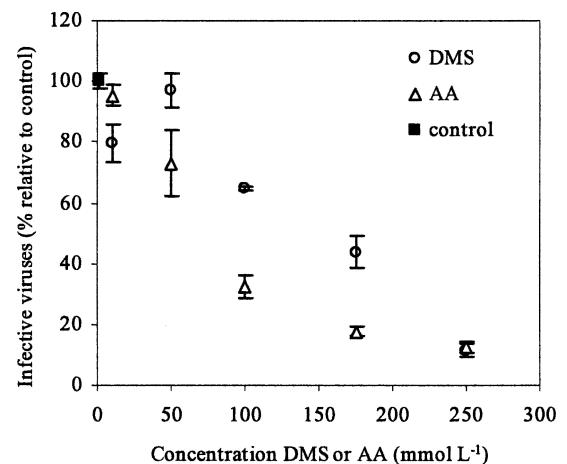


Fig. 2. Percentage of infective *Emilia huxleyi* virus 86 relative to a no-addition control after 24 h incubation with either dimethyl sulfide (DMS) or acrylic acid (AA) at a range of concentrations from 10 mmol L⁻¹ to 250 mmol L⁻¹. Error bars represent the standard deviation for three replicate samples, but for 100 mmol L⁻¹ DMS it is too small to be visualized.

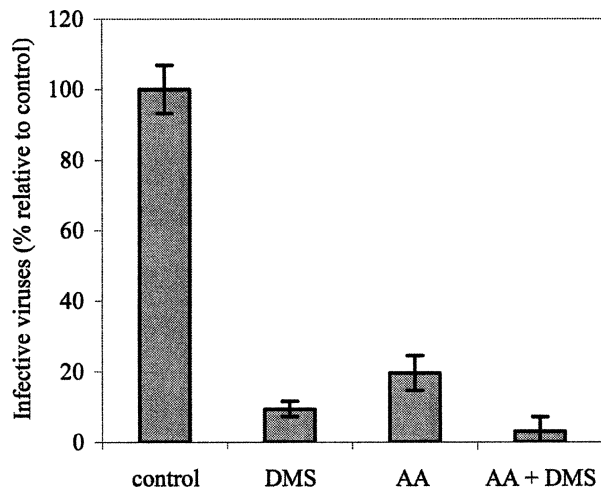


Fig. 3. Percentage of infective *Emiliana huxleyi* virus 86 relative to a no-addition control after 24 h incubation with either dimethyl sulfide (DMS), acrylic acid (AA), or both DMS and AA. All compounds used were at a final concentration of 100 mmol L⁻¹. Error bars represent the standard deviation for four replicate samples.

Stefels and Van Boekel 1993). Viral lysis results in increased DMS production from algal cells (Malin et al. 1998), and we have observed production of AA during viral infection of *E. huxleyi* (Evans 2005), so these compounds might increase to levels within algal cells that are sufficient to reduce viral titers during viral infection. As with grazing (Wolfe and Steinke 1996), high concentrations are more likely during the final stages of lytic infection when cellular integrity is lost and DMSP is mixed with DMSP lyase. This would suggest that if DMSP cleavage is an antiviral strategy it functions by decreasing the number of infectious viral progeny produced during lytic viral infection. Clearly the host cell would not benefit, but the production of noninfective viruses will reduce the chance of other potential hosts becoming infected. Viruses contact their hosts by passive diffusion, and viral infection is dependent on the collision of an infective virus with a susceptible host. Reducing the ratio of infective to uninfected viruses increases the amount of collisions required to result in successful infection, thereby reducing the probability of infection. In addition, the longer they are outside the cell the more likely the viruses will succumb to decay processes. Furthermore, if exposure to DMS and/or AA does not inhibit the ability of EhV to bind with receptors on the host cell but blocks virus-cell adsorption, as has been shown with influenza virus after treatment with sulfated polysaccharides and polymers (Hosoya et al. 1991), then uninfected viruses could further decrease the probability of infection. This is because uninfected viruses would block receptor sites on the host cell, rendering fewer sites available to their infective counterparts. Under these circumstances production of uninfected viruses would act as a negative feedback mechanism on the rate of viral infection. A similar mechanism was suggested by Thyrhaug et al. (2003) whereby viral inhibitors are produced alongside viruses during infection of cells that serve to

compete with healthy viruses for receptor sites on the host thereby reducing the chance of infection. However, it must be noted that nothing is currently known about EhV-86 receptor sites and further investigation is required to determine whether viruses rendered uninfected by DMS and/or AA are still able to bind to receptors sites. If one or both of these effects occur then DMSP cleavage by DMSP lyase during viral disruption could serve as an altruistic defense against viral infection.

Virus-induced collapse of *E. huxleyi* blooms has been well documented in the marine environment (Jacquet et al. 2002; Wilson et al. 2002). This raises the question: if *E. huxleyi* is in possession of an antiviral defense mechanism, how does it influence the ecology of virus-host interactions? Of course if this mechanism was completely effective, i.e., reduced infective viral titers to zero, then viruses of *E. huxleyi* would become extinct. Likewise if *E. huxleyi* specific viruses were too effective as pathogens, then *E. huxleyi* populations would not be able to reach the high concentrations observed during blooms that favor virus proliferation. It has been suggested that successful parasites are able to coexist with their hosts without doing them excess harm, and it may be that the DMSP antiviral defense mechanism promotes coexistence of *E. huxleyi* and its viruses. *E. huxleyi* has been shown to coexist with its pathogenic virus, and this has been attributed to phenotypic plasticity and diversity of both host and pathogen in terms of susceptibility to viral infection, in addition to the possibility of negative feedback mechanisms involving inhibitors of viral infection (Thyrhaug et al. 2003). The DMSP antiviral defense mechanism would complement these hypotheses, since although this mechanism would promote the development of blooms, increased host density would result in increased collision frequency and therefore infection, and so it would delay rather than prevent virus-induced bloom collapse. By delaying the time required for viruses to overwhelm a population there is an increase in the probability of phenotypic change within that population that may render the cells resistant to infection by the viruses present.

Since reduction of viral titers by the cleavage products of DMSP is proportional to their concentration, it would be beneficial to the organisms to express high DMSP lyase activity. This could explain why to date no viruses have been isolated against the high DMSP lyase activity *E. huxleyi* hosts CCMP 373 and 379, despite numerous attempts in our laboratory (Schroeder et al. 2002; Evans 2005; Martinez-Martinez pers. comm.), since viruses of high DMSP lyase activity hosts would be less successful and therefore, less common in the oceans.

Both cleavage products of DMSP were shown to have potent antiviral effects, which strongly suggests that the DMSP system could serve as an algal chemical defense mechanism against viruses. However, it should be noted that the current study is preliminary and additional work will be required to confirm this hypothesis and determine the precise modus operandi. It will be necessary to establish whether DMS and/or AA reach the required concentrations within virally infected algal cells and the length of exposure required. Currently, technical limitations prevent

the acquisition of such data: (1) during a culture crash both infected and uninfected cells are present and currently there are no conclusive methods to distinguish between them, making an accurate estimate of compound per infected cell difficult to achieve. (2) A reliable method for the analysis of DMS in the particulate phase is lacking. Despite this, our data show for the first time strong evidence for a chemical defense against viruses in phytoplankton, which has clear implications for the ecology of virus–host interactions.

Claire Evans¹ and Gillian Malin

Laboratory for Global Marine and Atmospheric Chemistry
School of Environmental Sciences
University of East Anglia
Norwich NR4 7TJ, United Kingdom

William H. Wilson¹

The Marine Biological Association of the United Kingdom
The Laboratory
Citadel Hill
Plymouth PL1 2PB, United Kingdom

Peter S. Liss

Laboratory for Global Marine and Atmospheric Chemistry
School of Environmental Sciences
University of East Anglia
Norwich NR4 7TJ, United Kingdom

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¹Present address: Plymouth Marine Laboratory, Prospect Place, the Hoe, Plymouth, PL1 3DH, United Kingdom.

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