Nanoparticles and Ocean Optics

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LONG-TERM GOALS

Our long-term goal is to understand the role of the two most abundant nanoparticles in the sea, viruses and polymer aggregates and their impact on the inherent ocean optical properties of seawater.

OBJECTIVES

There are two objectives to this work that deal with the role of nanoparticles and ocean optics:

1. To determine the importance of viruses (and other biological processes) in the formation of chromophoric dissolved organic matter (CDOM) in the ocean through lysis of host cells.

2. To quantify the importance of aggregate formation to seawater optical properties.

APPROACH

The initial focus for the virology portion of this project (start date January '05) was the acquisition and characterization of virus/host assemblages for use in lab-based dilution experiments. We tested methods for the separation of naturally occurring virus and host from seawater samples in preparation for dilution experiments with natural assemblages.   Next, we examined the role of viruses in production of CDOM using a modified version of the Landry dilution method (Landry and Hassett 1982; Landry et al. 1995). Rather than using the dilution technique to focus on phytoplankton growth and mortality from grazing, we targeted CDOM production by all living cells, using UV absorption as the indicator of CDOM production.   We tested this dilution approach on heterotrophic bacterial hosts and their specific viruses, in simplified experimental

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systems, to see if we could see CDOM release induced by viral lysis and if we could control infection by the dilution process. Subsequent dilution experiments have included eukaryotic hosts (*Emiliania huxleyi*) and its specific viruses. We then did experiments with field samples from the Bigelow Laboratory dock as well as from shipboard experiments.

WORK COMPLETED

We transitioned these experiments to the field, where we applied the Landry dilution technique and diluted field samples containing a variety of hosts with virus-free seawater. We separated natural sea water assemblages off the Bigelow Lab dock into a concentrated suspension of free viruses (20-200nm), and a fraction with particles >200nm that contained mostly hosts (prokaryotes and eukaryotes). This was accomplished using tangential flow ultrafiltration, which enhanced the concentration of <u>only</u> particles <200nm (such as viruses) in one fraction and concentrated larger "host" cells (like phytoplankton, heterotrophic flagellates, heterotrophic dinoflagellates, ciliates, etc.) in another. For the actual dilution experiment, we diluted the "host" particles with virus-free seawater, hypothesizing that the some fraction of the hosts were already be infected with viruses, which means we have not only the hosts, but their specific viruses in our concentrated suspension. Dilution of these infected hosts should then regulate the subsequent infection cycles of the viruses, achieving our goal, which is to regulate natural virus infection via dilution and better understand its optical consequences. Instead of making diluent from 0.2μm-filtered seawater, we made it with 0.02μm-filtered seawater, to insure the diluent was virus-free. Ultra-filtration has been used before to sequester and concentrate free-floating viruses in natural assemblages so that one could examine associated effects on their hosts (Suttle et al. 1990; Proctor and Fuhrman 1992; Suttle 1992; Peduzzi and Weinbauer 1993; Weinbauer and Peduzzi 1995; Noble et al. 1999). The difference here, is that we applied Landry's dilution method using diluent prepared with ultrafilters that have 10X smaller porosity than used previously. Ultimately, this allowed derivation of rates of production of CDOM associated with particles in the virus size range (e.g. viruses). Time courses of absorption, scattering, fluorescence, particle size distribution and virus concentration were performed. The simplicity of this experimental design is that we were able to directly regulate the role of viral lysis in the production of CDOM.

This project also involved the examination of the optical properties of nanometer-sized aggregates in experiments in which we augmented 20 nm filtered seawater with natural, filter-sterilized CDOM. We then incubated this mixture in the presence or absenced of sodium azide (to inhibit biological reactions) or EDTA (to regulate chelation, which controls aggregate formation) (Chin et al. 1998) and parallel measurements of volume scattering and absorption were performed. Shipboard experiments were performed with this same basic experimental design, except no augmentation of DOC was made to the samples and the experiments were run with only the naturally-occuring DOC. Optical changes were followed using WETLabs ac-9s measuring spectral absorption and attenuation, as a function of time.

During 2006-2007, we completed processing of our field data from the *R/V Cape Hatteras* cruise. Moreover, we began experiments on a new virus that we isolated from the Gulf of Maine. This virus (ø 43) infected the coccolithophore, *Emiliania huxleyi*, at a rapid rate that we have never observed in a eukaryotic virus. Optical experiments demonstrated major optical changes on the time scale of hours (described below) instead of days (which has been the norm in previous work). We also devoted time towards publishing the results of our work on the in-

fection of various strains of the prokaryote, *Synecchococcus* sp., and associated optical consequences. Following the completion of that work, we are preparing the next paper on the optical consequences of viral infection of a eukaryotic phytoplankton species, *E. huxleyi* which will include these new exciting results.

RESULTS

Significant results from virus characterization

Virus characterization is a critical part of this work since the kinetics of the infection are directly linked to subsequent optical changes. Infection of susceptible hosts has traditionally been associated with host destruction leading, in liquid cultures, to lysis of host cultures (and strong optical changes (Fig. 1)). The specific kinetics have varied with the virus-host systems involved, with viruses of heterotrophic marine bacteria possessing the highest rates of replication, followed by cyanophage and viruses of eukaryotic organisms. Our ONR-supported work with viruses of marine vibrios, *Pseudomonas* sp., *Synechococcus* sp., *Micromonas* sp. and the coccolithophorid, *E. huxleyi*, have generally supported this traditional view. Infection cycles for viruses of heterotrophic bacteria are in the range of 1 to 2 hr, while those for *Synechococcus* viruses ranged from 1 to 2 days. Typically, the pattern for most of our *E. huxleyi* virus isolates (eukaryotes) was somewhat more complicated. Following a three-hr latent period, an abrupt rise in virus numbers (~ 1000 X) was observed between 5 and 14 hr. Virus numbers then stabilized until 18 hr, when a second burst (10X increase) was observed through 22 hr when counts stabilized at ~ 10^9 ml^{-1} through the rest of the experiments. Host numbers did not change significantly during the initial burst and decreased only slightly during the second. The cultures did not completely clear until after 4-10d, considerably slower than for infected heterotrophic and autotrophic prokaryote hosts. The eventual lysis of the host was not accompanied by any significant increase in virus numbers, suggesting that most had been released earlier in the cycle.

A notable exception to this infection cycle was encountered with our recent isolate (ø 43), which caused complete lysis of the eukaryotic coccolithophore, *E. huxleyi*, in less than 24 hr. Growth studies revealed that virus progeny were released after ~1.5h post infection, and host lysis was evident at 10hr. By 18 to 20 hr, complete lysis of the host culture had occurred, making this the most rapid lytic cycle encountered for any *E. huxleyi* virus, or nearly any viruses infecting eukaryotic marine algae (Fig. 1A), an unprecedented observation.

Significant optical results

In laboratory experiments with the rapidly-infecting virus ø 43, inelastic scattering from chlorophyll *a* fluorescence was significantly affected after ~20h (Fig. 1B). Backscattering (b_b) of the host particles showed significant oscillating behavior 10h after infection, first exceeding, then decreasing below the b_b of the control. This probably was associated with the virus burst cycles. Light absorption at 254nm in the UV showed only subtle changes (with absorption of infected cells exceeding the control cells and dissolved absorption accounting for a third to half of the total absorption (Fig. 1D). At 350-680nm, the absorbance decreased below the control after ~10-12h, ultimately falling to values 5X less than the control. Dissolved absorbance was a lower fraction of the total absorbance at these wavelengths (Fig. 1D-G).

We have also made optical observations associated with viral infection in dilution experiments. We performed virus characterization and infection experiments with cultures and field microbial populations in which we used tangential flow cartridges to concentrate particles > 0.2um (representing "host" particles) as well as particles between 50 Kdaltons and 0.2um (representing viruses and colloids). We used the dilution technique (Landry et al. 1995) to manipulate the subsequent contact rate between the infected host particles and their progeny viruses (in these cases, the diluent was virus-free, having passed a 50Kdalton filter. Note, however, we did not re-add the concentrated free viruses back to infect the host assemblage. Instead, we assumed that some fraction of the hosts were already infected with specific viruses, such that dilution would slow subsequent infection cycles by reducing the contact rate. This indeed was the case, since after diluting purified host cells in virus-free seawater, new virus production was documented within 3h of the host isolation. We performed experiments in three different ocean environments of the NW Atlantic (oligotrophic, mesotrophic and eutrophic).

Our results demonstrated moderate effects of dilution on infection and subsequent variability of absorption and scattering, particularly in the UV portion of the spectrum, the spectral region most affected by CDOM. Absorption and scattering changes occurred at rates characteristic of microbial growth in the sea (up to $2d^{-1}$). Microscopic counts with SYBR green dye demonstrated significant enhancement of virus concentrations as a function of dilution, concurrent with the optical changes. For all experiments, we observed changes in backscattering and absorption (particularly in the UV, between 250-285nm), indicative of CDOM release. One of the most striking results was that there was a general "homogenizing of the UV absorption spectra with time in the various infection experiments, such that at time zero, there was more variability in the UV spectrum, but after one day, most of the spectra converged to a single spectral shape. We believe that these results are reflecting the microbial recycling of CDOM (uptake of CDOM by bacteria, subsequent release following host lysis by viruses, and subsequent re-uptake of CDOM by other uninfected bacteria). This optical effect has never been demonstrated before. Moreover, our results highlight the fact that these were not simple host-virus infection experiments such as in the laboratory; the field experiments measured the optical consequences of infection within the entire microbial community of field populations.

Significant results of aggregation experiments

The size distribution of particles in the uninfected controls, and in the ø43 viral infected cultures of *E. huxleyi* was measured using a Flow Field Flow Fractionator. These measurements allowed us to follow changes in the formation of sub-micron particles and their behavior at different times during the experiments (Fig. 2). The earliest signs of infection of *E. huxleyi* by ø43, were observed 8 hours post infection, which is the fastest we have observed thus far of all the viral phages we have examined. This period was marked by a large decrease in the amount of >1μm particles. However the largest deviation in the size distribution of particles between the control and the infected cultures was observed at the end of the 9th hour of the experiment. This period was marked by a dramatic increase in the amount of particles ranging in size from 100 to 200nm in the infected cultures and a decline in particles > 1μm in size, clearly a sign of cell lysis and release of viral phage into the medium. By the end of 19.5h, we began to observe signs of destruction of cellular pigments and ghosting of the infected cells as evident from absorption spectra of the > 1μm particles (Fig. 2H). This period was marked by a rapid aggregation of the smaller particles into larger > 1μm aggregates whose absorption spectra resembled that of

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CDOM. Another increase in the amount 150- 200nm particles was observed 24h into the experiment suggesting a cyclical behavior in the lysis and release of viral phages in the infected cultures. This period was marked by a large change in the optical properties of the $> 1\mu$m aggregates, which displayed a large absorption peak between 240 and 290nm. At the end of 31h, the FFFF fractograms within the control and infected cultures converged to reveal similar size distribution of particles. Particles between 100 and 200nm were conspicuously absent but there appeared to be an accumulation of larger ($>1\mu$m) particle aggregates and particles of between 20 to 50 nm. What was also conspicuous was the difference in the absorption spectra of the $> 1\mu$m aggregates between the infected cultures and the controls. The spectra of particles in the control sample, for instance, resembled typical phytoplankton absorption spectra whereas particles within infected cultures were clearly devoid of pigments and resembled typical CDOM spectra.
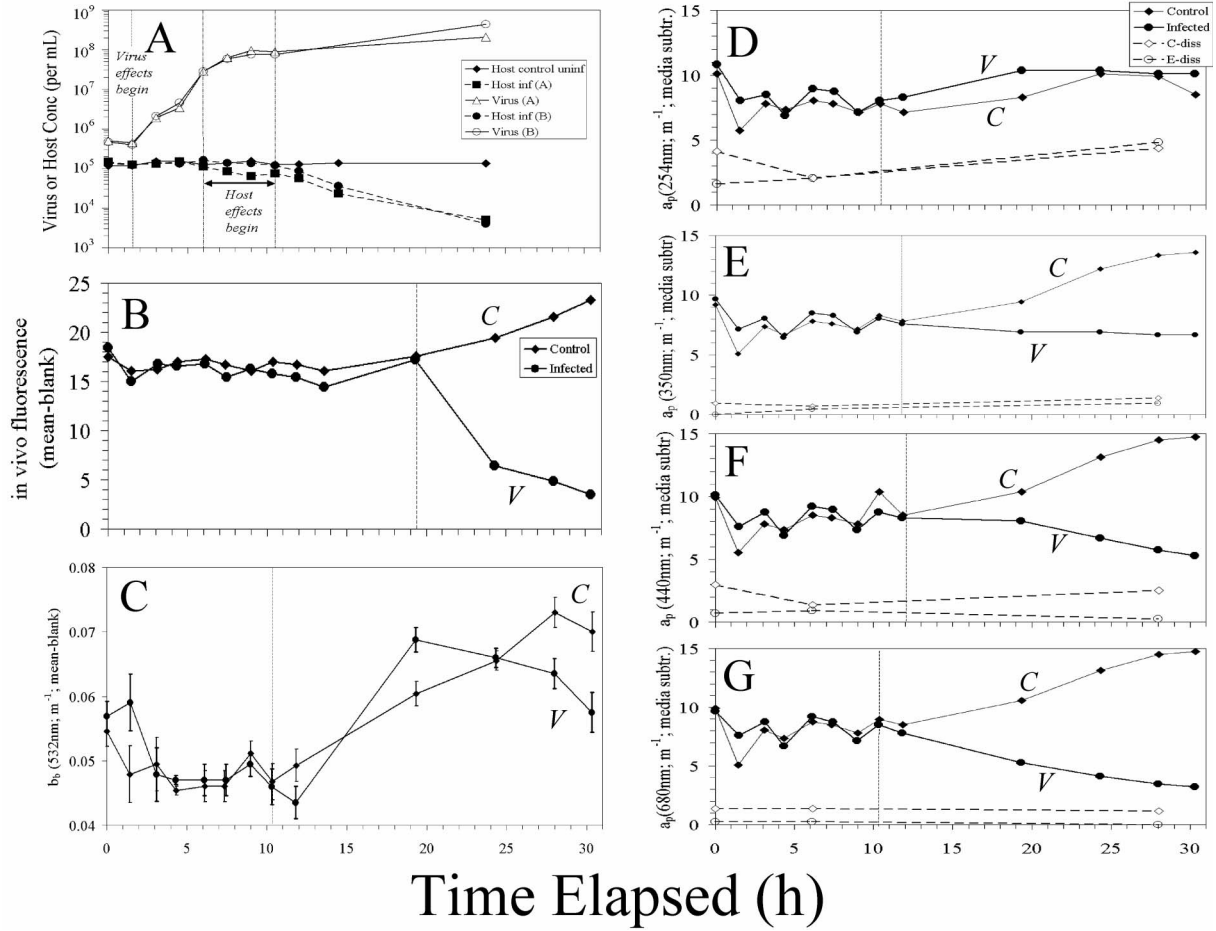
Fig. 1*-Results of infection of eukaryotic coccolithophorid, *Emiliania huxleyi*, with new virus isolate ø 43, which demonstrates rapid optical changes, unprecedented for eukaryotic phytoplankton infection. A) Change in particle concentration following infection in replicate flasks. Solid diamonds show the lack of change in a control, uninfected culture, open triangles and open circles show the log-linear increase in viruses after 1.5h infection, leveling after about 8h, solid squares and solid circles show log-linear decrease in host concentration after 8-11h. B) Chlorophyll a fluorescence (inelastic scattering) in control (uninfected; diamonds) and infected (circles) aliquots of E. huxleyi, following subtraction of blank. Results show strong drop in fluorescence after about 20h. C) Particle backscattering at 532nm for control (diamonds) and infected cells (circles). Error bars represent one standard deviation. Results demonstrate significant divergence after 10h, but with oscillating behavior, in which b_b of infected cells first exceeded, then ultimately decreased below control b_b levels after 1d. D-G) Total absorption in control (solid diamonds) and infected (solid circles) flasks at 254nm, 350nm, 440nm and 680nm respectively (in units of m^{-1}). Media blanks have been subtracted. Open symbols with dashed lines show the absorption following filtration of samples through 0.2μm filters (open diamond = control; open circle=infected). Symbol key in panel D applies to panels D-G. Results show absorption decreases in infected cells after 10-12h for 350nm to 680nm wavelengths. Results from filtered samples show that most of absorption was associated with host particles, not the dissolved fraction. Significant dissolved absorption was seen in the UV (254nm), possibly from enhanced virus DNA absorption. In panels B-G, C denotes control, uninfected cells while V denotes cells infected with virus ø 43.
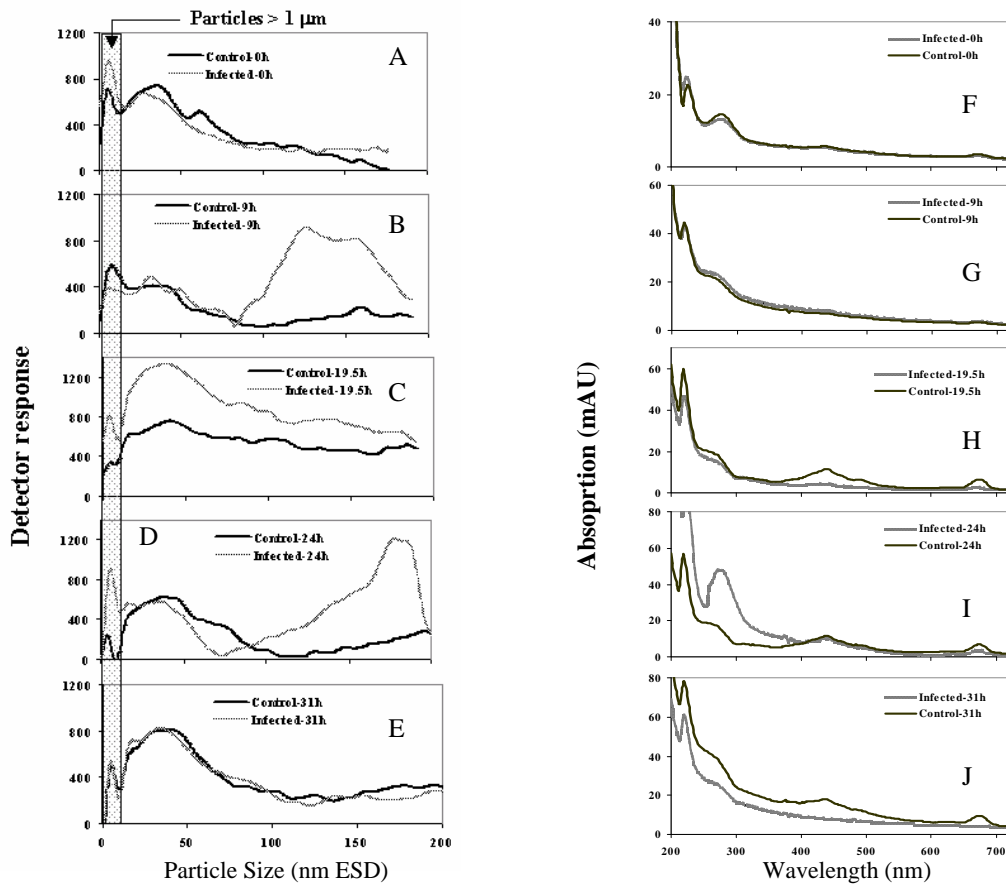
Fig. 2- Panels A-E) Flow Field Flow Fractograms (FFFF) showing changes in the size distribution of particles in the control and infected cultures of E. huxleyi at various time points during the experiment shown in Fig. 1. The hatched area to the left of each panel indicates particles with greater than 1 μm in diameter. The mechanism of particle migration and separation of large particles in the channel of the FFFF is different from that of normal FFFF. On account of their negligible diffusion, hydrodynamic forces tend to lift larger particles towards the centre of the channel. As a result these particles migrate out faster out of the channel. Results show that the time-zero fractograms (A) are virtually identical, but by 9h (B), particles between 100-200nm formed in the infected culture. By 19.5h (C), smaller 40nm particles dominated the infected flask. At 24h (D), 150-200nm particles again dominated the infected flask. By 31h (E), both size distributions were identical. Note the overall decrease in >1μm particles in the infected cultures over the experiment (data under hatched region of panels A-E. We postulate that the sub-micron particles were influenced by multiple burst cycles of the viruses and that differences between time points were due to the smallest particles rapidly aggregating to larger particles over time scales of hours, and eventually lost to the >1um size fraction (not visible on these plots). Panels F-J) The absorption spectra of the particles eluting out of the FFFF revealed initially identical absorption spectra (F), then a systematic loss of pigments and ghosting of the infected cells by 19 hrs into the experiment. Note that infected cells showed increased UV absorption at 24h (G). A similar trend was observed in bulk spectroscopy measurements (Fig. 1D) at 254nm from 12-22h. By the end of the experiment, absorbance of infected cells was lower than control cells over all UV and visible wavelengths (J). Black lines represent absorption spectra of control, uninfected cells and gray lines represent spectra of infected cells.

IMPACT/APPLICATIONS

This work is important to our understanding of the impact of viral infection on the optical properties of seawater and its suspended microbial communities. In particular, it is critical to know the magnitude of changes in inherent optical properties, the rates of change and host specificity. Our field and laboratory dilution experiments have provided this. Along with changing the size spectrum of the particulate matter, viruses essentially convert Case I waters (where the optical properties can be easily indexed to chlorophyll *a*) to Case II-like waters (where the optics are dominated by dissolved organic matter, not chlorophyll *a*). Results of this work have provided important understanding of the time scales of optical changes for the numerically-abundant nano- and picoplankton following infection. The discovery of the virus ø 43 demonstrates that viral infection rates of eukaryotic hosts are even faster than we originally postulated. Our long-term goals are to better understand time-space optical variability associated with viruses, which ultimately provides improved observational capabilities, ocean color algorithms, and predictive-multidisciplinary models. This work also is important for understanding the optical properties associated with polymer-gel formation. Polymer gels begin as nanometer-sized colloids of dissolved organic matter (DOM) that aggregate, producing micron-sized colloids. Polymer gels and viruses represent two of the most abundant particles in the sea; thus, our results have far-reaching implications for ocean optical properties, for both molecular and particle scattering.

TRANSITIONS

Our dilution experiments have shown the power of the technique for understanding the role of virus infection in ocean optics. However, complex microbial communities, when diluted, are still complex microbial communities. In future experiments we plan to use flow cytometry sorts to simplify viral-host interactions in natural populations and better elucidate their impact on ocean optical properties.

RELATED PROJECTS

This ONR grant supplied funds for a high sensitivity digital microscope camera/stage manipulator, which has been used for high-resolution virus viewing with SYBR-stained viruses (a fluorescent DNA stain). We have developed software for a) automated focusing for microscopic samples and image acquisition of phytoplankton samples and b) image analysis algorithms (in conjunction with the Univ. Massachusetts Computer Sciences Department through a separate NSF grant) for automated analysis of particles based on their fluorescence or birefringence properties. The automated phytoplankton enumeration software is now running 24h per day, as we are processing thousands of samples from our field work and experiments. A complete description of this system will be published in the peer-reviewed literature.

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