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ECOLOGICAL ASPECTS OF VIRUSES IN MARINE SYSTEMS

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Janet Michelle Rowe
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DEDICATION

*To Moomah, Doodah, and my wonderful 'evil' twin,
a crazy and ever-loving dog,
and a librarian who works for tips.*

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ABSTRACT

The study of viral ecology in aquatic microbial communities is a relatively young field. The expanse of viral diversity has yet to be fully understood and there are still numerous questions concerning the roles that viruses play in aquatic communities and the constraints placed on these roles by co-occurring biotic and abiotic factors. Understanding how viruses interact with their hosts and how these interactions affect aquatic systems as a whole is vital to comprehending global-scale influences of viruses. In aquatic systems, and particularly in the world's oceans, microbes drive the cycling of important elements such as C, S, N and P; viruses, through a predatory, top-down effect, are major drivers of this process.

As a first step toward examining virus-host interactions, the isolation and identification of viral strains is vital as it provides a foundation for further studies. Through the adaptation of Koch's postulates and use of electron microscopy, an *Aureococcus anophagefferens*-infecting virus was isolated, clarifying confusion from earlier conflicting results. Subsequent analyses focused on the total viral population across large spatial scales, covering 3 major oceanic regimes. Total viral abundance and production rate were measured along with other biotic and abiotic factors such as size-fractionated chlorophyll *a*, total cell abundance, bacterial biomass production rate, salinity, temperature, etc. These variables were examined individually, pair-wise, and collectively to elucidate any significant trends and relationships among them. Results indicated three distinct trends. First, in the Sargasso Sea, viral parameters were fairly predictable. Second, in the North Atlantic, a threshold effect was reached. And third, in the Western Pacific a changing microbial community structure is likely a major influential factor. Lastly, a search for a new phylogenetic marker for viruses examined the major capsid protein (MCP) gene sequences of viruses from the North Atlantic as a proxy for the diversity of Phycodnaviridae (a major family of eukaryotic algae-infecting viruses). This analysis provides a foundation for future work aimed at teasing apart viral populations so that relative abundances and production rates can be determined and compared with total abundances and rates as well as with other parameters of the ecosystem in question.

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LIST OF ABBREVIATIONS

- CCN, Cloud Condensation Nuclei
- CTD, Conductivity, Temperature, Density (computer package)
- DAPI, 4',6-diamidino-2-phenylindole
- DGGE, Denaturing Gradient Gel Electrophoresis
- DIN, Dissolved Inorganic Nitrogen
- DIP, Dissolved Inorganic Phosphorus
- DMS, Dimethylsulphide
- DMSP, Dimethylsulphoniopropionate
- DOC, Dissolved Organic Carbon
- DOM, Dissolved Organic Matter
- EhV, *Emiliana huxleyi* Virus
- FSU, Fluorescence Spectral Units
- HNLC, High Nutrient, Low Chlorophyll
- MCP, Major Capsid Protein
- MOI, Multiplicity of Infection
- MPN, Most Probable Number
- NASB, North Atlantic Spring Bloom (NASB2005 = cruise name)
- PCR, Polymerase Chain Reaction
- % IC, Percentage of Infected Cells
- % MVL, Percentage of Mortality due to Viral Lysis
- PFGE, Pulsed Field Gel Electrophoresis
- POM, Particulate Organic Material
- qPCR, Quantitative Polymerase Chain Reaction
- RFLP, Restriction Fragment Length Polymorphism
- SEM, Scanning Electron Microscopy
- TCA, Trichloroacetic Acid
- TEM, Transmission Electron Microscopy
- VIC, Visibly Infected Cells
- VLP, Virus-like Particle
- WP2, Western Pacific Warm Pool (cruise name)

SECTION I

LITERATURE REVIEW

A tenant readily recognized in nature is that the small are often preyed upon by the large. In aquatic systems, this can be easily demonstrated by fish who prey on any other fish or creature, which can fit into their maw. Likewise, one can see this on the microbial scale as well, with microzooplankton devouring smaller particles such as bacteria and unicellular eukaryotes (Strom 2002). However, there is a limit as to how large a predator can grow; physics, energy considerations, and carrying capacity present limiting factors (Carbone et al. 2007). Some predators have taken advantage of an alternate strategy, being small enough to feed on prey from the inside. The studies of these predators, (e.g. viruses), while undoubtedly important in the medical field, are also invaluable in the environmental sciences. Just as large predators help to maintain population diversity and stability within their prey (as illustrated so well by Robert Paine's famous work with *Pisaster ochraceus*, 1966), smaller predators do the same in their microscopic communities (Azam et al. 1983; DeBruyn et al. 2004; Hutchinson 1961; Thingstad and Lignell 1997). Moreover, with a growth in our understanding of how microbial communities drive the biogeochemical and nutrient cycles that maintain the Earth's ecosystems in balance, the significance of these predators cannot be overstated.

The smallest predators, viruses, were first discovered in the late 19th century by Mayor, Ivanovsky, and Beijerinck (Bos 1995), while it was nearly two decades into the 20th century before bacteriophages were identified (Duckworth 1976). However, freshwater cyanophages (Safferman and Morris 1963) and later viruses infectious to freshwater and marine eukaryotic algae (Van Etten et al. 1991) were not reported until 1963 and the 1970's, respectively. Moreover, marine cyanophages (Proctor and Fuhrman 1990) and a wider range of marine eukaryote-infecting viruses (Suttle et al. 1990) were not reported until less than 2 decades ago. Due to the "newness" of these observations with respect to marine microbial phototrophs, it has been the focus of this dissertation to facilitate the development of proper isolation techniques for individual viruses, to query the dynamics of viral populations across open ocean systems, and to examine the diversity of viruses infectious to photosynthetic eukaryotic microbes.

Presently a number of marine microbe-infecting virus families have been described, though more families should be assigned and described in the future as investigations progress. Marine bacterio- and cyanophages fall within 3 morphologically distinct, main families with dsDNA genomes (Suttle 2005). Siphoviridae (λ -like) members have long, non-contractile tails, and so far researchers have observed moderately broad host ranges and that these phages can integrate into the host cell's genome (lysogeny) (Suttle 2005). Myoviridae (T4-like), have contractile tails and have been observed as having very broad host ranges, which is in contrast to podoviridae (T7-like), which have short non-contractile tails and narrow host ranges (Suttle 2005). However, both of these latter two families tend to be lytic (Suttle 2005).

Viruses that infect eukaryotic marine microbes have been categorized based on host species and their genetic material, though there are virus strains that remain unassigned. Reoviridae members generally infect multicellular hosts such as arthropods and vertebrates (ICTV 2006a), but recently it was proposed that a newly isolated double-stranded RNA (dsRNA) virus infectious to *Micromonas pusilla* be assigned to a new genus within Reoviridae, *Mimoreovirus*, based on morphological and molecular analyses (Attoui et al. 2006). Reoviridae members have dsRNA genomes composed of 10-12 segments, totaling ~18.2-30.5 kb (ICTV 2006a). Marnaviridae is a family of single-stranded RNA (ssRNA) viruses established after the discovery of a *Heterosigma akashiwo*-infecting virus. This group contains just this single member, a positive sense RNA virus with a genome ~8.6 kb (Lang et al. 2004). *Mimivirus*, a genus unassigned to any family, is also represented by one member (ICTV 2006d). Though this *Acanthamoeba polyphaga*-infecting, double-stranded DNA (dsDNA) virus was isolated from a cooling tower (La Scola et al. 2003), an oceanic survey suggests that mimiviruses could be abundant in marine waters (Monier et al. 2008). In the future, it is possible that the recently isolated, mamavirus could be considered a second mimivirus strain given the high degree of similarity between genomes of these two viruses (La Scola et al. 2008). Lastly, the virus family about which the most is known, is Phycodnaviridae. Presently, there are 6 genera, 5 of them for marine members, though there are still members that are unassigned (ICTV 2006b; Dunigan et al. 2006). Phycodnaviruses share in common

dsDNA genomes ranging from 160-560 kb, are non-enveloped, ~130-200 nm in diameter, and are icosahedral in shape (ICTV 2006b; Dunigan et al. 2006).

Research has demonstrated that viruses can play several roles in aquatic microbial ecosystems. Perhaps the most obvious role is as an agent of mortality for host populations (Weinbauer 2004; Wommack and Colwell 2000); through this predation viruses can control the diversity of the host population as described by the “kill the winner” hypothesis (Thingstad and Lignell 1997). This hypothesis suggests that community members that could out-compete others would be kept in check by viruses that infect them. As time progresses, the dominating community members change as the abundance of one falls allowing for another to rise that then falls when it succumbs to virus-induced mortality. However, the overall diversity remains the same as the cycling continues, providing a dynamic yet stable system (Thingstad and Lignell 1997).

Through this activity, which results in host cell lysis, nutrients are released to surviving members of the aquatic community in bioavailable forms (Wilhelm and Suttle 1999). Examinations of nutrients released from virally lysed cells of *Aureococcus anophagefferens*, revealed that several nutrients such as C, P, N, Fe, and even Se can be of benefit to the surviving community members (Gobler et al. 1997). It has also been found that virally released Fe was bioavailable to plankton native to a coastal, high nutrient low chlorophyll (HNLC) area, suggesting a means for local plankton to survive where Fe is limiting (Poorvin et al. 2004). Subsequently it was shown that this form of lysis-released Fe was more bioavailable to surviving cells than other forms of Fe (Mioni et al. 2005). More recently, it has been demonstrated that even free amino acids released upon viral lysis were bioavailable (Middelboe and Jorgensen 2006).

It is also possible that aquatic viruses may be agents of horizontal gene transfer. Work done with terrestrial and pathogen-infecting phages has provided substantial evidence for transduction (Canchaya et al. 2003), and recently this possibility for marine cyanophages that contain host photosynthetic genes was shown (Lindell et al. 2004).

In addition to the general roles that all marine microbe-infecting viruses can play, viruses that infect photosynthetic microbes play several roles specific to them. When host cells proliferate to an abundance noticeable on the macroscopic scale (better known

as an algal bloom), viruses have been found to influence the ontogeny of these blooms. Shortly before termination of a *Heterosigma akashiwo* bloom (red tide), cells under transmission electron microscopy were found to display a significant increase in infection from earlier time points, suggesting a link between viral activity and bloom termination (Nagasaki et al. 1994). Also, increased virus abundances toward the end of a bloom of *A. anophagefferens*, has suggested that viral activity may terminate these algal blooms (Gastrich et al. 2004). However, other work done with this same organism has suggested that perhaps viruses may also help perpetuate or even initiate bloom formation by removing competition and/or providing nutrients to the bloom-formers (Gobler et al. 2004; Gobler et al. 1997).

Another possible role of algal viruses involves the infection and lysis of hosts capable of producing dimethylsulphoniopropionate (DMSP). The role of this osmolytic compound is uncertain, though the most recent evidence suggests it, and its derivatives, may be important antioxidants (Sunda et al. 2002). DMSP is converted to dimethylsulphide (DMS) through DSMP-lyase activity either within the host cell or by other community members when released (Liss et al. 1997; Thornton 2002). DMS released to the atmosphere is hypothesized to become cloud condensation nuclei (CCN), which in turn stimulate cloud formation that can impact climate by increasing the Earth's albedo (*i.e.* reflecting sunlight) (Thornton 2002). Questions have been raised regarding how viral infection affects DMSP production and release. In one study, the flux in particulate and dissolved DMSP during infection of an axenic strain of *Micromonas pusilla* was examined and it was found that upon virus-mediated cell lysis, dissolved DMSP increased significantly; suggesting that viral activity may be important for releasing DMSP to surviving community members for conversion to DMS in nature (Hill et al. 1998). In another study, *Emiliania huxleyi* strains with high DMSP-lyase activity were found to be resistant to infection by 10 different virus isolates (Schroeder et al. 2002). While more work needs to be done to test the hypotheses that this resistance could be related to the high DMSP-lyase activity and that viruses can mediate a significant release of DMSP in nature, these findings do bring attention to the potential global-scale effects that viruses may have.

With such a variety of roles, marine viruses are clearly influential members of microbial communities. However, to begin to understand their potential global significance, one must assess the global influence of their coexisting microbial community members. The World's Ocean houses 1.3 Zg (1.3×10^{21} g) of S, of which, the predominant means of removal is through bacterial sulfate reduction with rates around 30×10^{13} g S yr⁻¹ (Bottrell and Newton 2006). Biologically driven P cycling in the surface waters of the ocean is $\sim 28 \times 10^{12}$ mol yr⁻¹ (~ 0.87 Pg yr⁻¹) (Troppe 1998). While estimates of Fe uptake/assimilation by phytoplankton in the open ocean range from 12 to 21.5×10^9 mol yr⁻¹ (0.67-1.2 Tg yr⁻¹) (Fung et al. 2000; Moore et al. 2002; Moore et al. 2004) and from 90 to 130 Tg N yr⁻¹ for N₂-fixers (Arrigo 2005). Additionally, the World's Ocean hosts $\sim 50\%$ of the global carbon fixation (104.9 Pg y⁻¹), resulting in nearly 48.5 Pg of carbon cycled on an annual basis (Field et al. 1998). The ocean is also a sink for $\sim 1/3$ of anthropogenic sources of CO₂ (Siegenthaler and Sarmiento 1993), which only serves to underscore the importance of these microbial recyclers. Unquestioningly, marine microbes have a strong global impact through their biogeochemical cycling, fixation of carbon, and subsequent production of oxygen. It therefore stands to reason that any organism(s) that can exert influence over these globally important microbes could also have globally reaching influences: a top-down effect (Wilhelm and Suttle 1999). However, before too many speculations are made, it is important to first establish estimates of the basic viral parameters in these systems and evaluate the relationships between them and with host or other oceanic parameters.

To this end, it is valuable to first remember the questions in viral ecology that have been posed, based on what this research has already revealed. Mann (2003) posed several questions concerning the persistence of cyanophages, how these phages act during infection inside the host, and whether or not host photosystems function during infection. Mann also asked at what point would the pressure of resistance selection become significant, which goes along with a question posed by Fuhrman and Schwalbach (2003) asking if when viruses deplete the abundance of a particular host strain, is that strain replaced by a resistant one or one that is very different from the originally dominant strain? Fuhrman and Schwalbach went on to ask, when increases in viral

abundance are observed, what organisms are being infected? Additionally, Hendrix (2003) presented a query about the differences between lytic and temperate phages and whether they had different accesses to evolutionary exchange, providing for different trajectories, evolutionarily speaking. Similarly, Weinbauer (2004) presented questions concerning the favorable environments and influences over lysogenic and lytic cycles, the deciding factors that push viruses toward or away from either lifestyle, and the relative abundances of each type. Weinbauer went on to raise a question about the extent to which viral lysis of host cells contributes to the composition of dissolved organic carbon and a question of whether the role(s) that viruses play in geochemical cycling is stabilizing or destabilizing for the ecosystem.

Suttle (in his section of Cypionka et al. 2005) and Brussaard et al. (2008b) have presented a summary of questions and current needs to those who do research on aquatic viruses. Suttle proposed inquiries on the significance of the abundance of aquatic viruses and of the wide-ranging diversity of their genetic information. The need for documenting the genetic richness of viruses in natural environments and to isolate and sequence more viruses were mentioned, as well as the need to supplement the lack of quantitative data on nutrient recycling mediated by viral lysis. This latter need ties into the greater vision of understanding and quantifying the impact of nutrient release on ecosystems. Additionally, Suttle pointed out that viruses in terrestrial systems and the processes that they mediate outside of disease roles should also be investigated. More recently, Brussaard et al. questioned how viruses may affect biological production, presenting three possibilities. Viruses may short-circuit the biological pump by returning elements, released through lysis, to the dissolved phase. Furthermore, accelerating the export from hosts may prime the biological pump. Finally, the release of “sticky” cellular components may drive the aggregation and subsequent transport of carbon to deeper waters, thereby hindering the biological pump. It was also pointed out that how the functions of physiological genes encoded in viral genomes can affect biogeochemical cycles is a question of current interest too.

However, perhaps of most relevance to the work discussed in subsequent sections, here are some of the questions and needs put forth earlier by Brussaard (2004b). Key

questions regarding the qualitative effect that viruses can have on phytoplankton highlighted the need to know what factors influence host specificity and how multiple algal viruses can coexist on one host population. Understanding the factors that regulate genotypic richness, infectivity and resistance of hosts, abundance, and fate of viruses over spatial and temporal scales was distinguished as being necessary for future research. Additionally, because phytoplankton naturally experience nutrient limitation on some scale, and because this has been linked to bloom development, an understanding of the interactions between virus and host under such conditions is also important. Model systems would provide the needed format to investigate this and other factors regulating viral control on host populations, a vital component of aquatic virus research. Moving more towards *in situ* studies, Brussaard points out the ecological interest in understanding the influences over viral infection in natural conditions, which could answer questions such as how and when are viruses able to infect their hosts. Lastly, Brussaard placed a great emphasis on using current and identical methods to compare and contrast the viral and virus-host activities across differing aquatic environments, such as eutrophic coastal waters and oligotrophic open ocean waters. Searching for answers to these questions, and more, has undoubtedly proven to be a formidable task for researchers, requiring multiple avenues of research: small- to large-scale work, working with model systems and natural communities, as well as molecular-scale approaches designed to tease apart natural populations.

Efforts in global- or even simply in large-scale work could not be made without the foundation that small-scale work, focusing on individual virus-host systems has provided. Numerous marine virus-host systems have been isolated and characterized since this area of research began. Because of the significant global impact that marine algal viruses may have due to their hosts' activities, these viruses are of particular interest and those with eukaryotic hosts (namely Phycodnaviridae) are a focus of the research described herein. To compare these algal viruses, along with other marine microbial eukaryote-infecting viruses, Table 1.1 lists characterized isolates, their hosts, sampling origin, current taxonomic placement, and genetic material. These virus isolates consist of single- and double-stranded RNA and DNA genome-types with wide host ranges. It is

Table 1.1. Lists characterized viruses of marine eukaryotic microbes, their host, location of isolation, present day taxonomic classification according to the International Committee on Taxonomy of Viruses (2006b; 2006c; 2006d) and the proposal by Lang, et al. (2004), and genetic material.

Reference(s)	Virus	Host	Place of Isolation	Family and Genus	Genetic Material
(Attoui et al. 2006; Brussaard et al. 2004a)	MpRNAV-01B (aka MpRNAV and MpRV)	<i>Micromonas pusilla</i> LAC38	Norwegian Coast	<i>Mimoreovirus</i> within Reoviridae proposed	dsRNA
(Lang et al. 2004; Tai et al. 2003)	HaRNAV-SOG-263 (aka HaRNAV strain 263)	<i>Heterosigma akashiwo</i>	Fraser River Plume, Strait of Georgia, British Columbia	Single Member of Marnaviridae	+ sense ssRNA
(Nagasaki et al. 2004)	RsRNAV	<i>Rhizosolenia setigera</i>	Ariake Sea, Japan	Not Assigned	+ sense ssRNA
(Takao et al. 2006; Takao et al. 2005)	SssRNA	<i>Schizochytrium</i> sp. strain NIBH N1-27	Kobe Harbor, Japan	Not Assigned	+ sense ssRNA
(Mizumoto et al. 2007; Tomaru et al. 2004)	HcRNAV (107 isolates with 2 host-specific ecotypes, UA and CY)	<i>Heterocapsa circularisquama</i>	Coast of Japan	Not Assigned	+ sense ssRNA
(Shirai et al. 2008)	CtenRNAV01	<i>Chaetoceros tenuissimus</i> Meunier	Shiotsuka River (Ariake Sound), Japan	Not Assigned	+ sense ssRNA
(Nagasaki et al. 2005)	CsNIV	<i>Chaetoceros salsugineum</i>	Mouth of Shiotsuka River (Ariake Sea), Japan	Not Assigned	ssDNA
(Bettarel et al. 2005)	CspNIV	<i>Chaetoceros</i> cf. <i>gracilis</i>	Chesapeake Bay, U.S.A.	Not Assigned	Not Determined

Table 1.1. Continued.

Reference(s)	Virus	Host	Place of Isolation	Family and Genus	Genetic Material
(Lawrence et al. 2001)	HaNIV	<i>Heterosigma akashiwo</i>	Discovery Passage, British Columbia	Not Assigned	dsDNA
(Takao et al. 2007)	SmDNAV	<i>Sicyodochytrium minutum</i>	Skukugawa River (Hyogo Prefecture), Japan	Not Assigned	dsDNA
(La Scola et al. 2003)	Mimivirus (aka APMV)	<i>Acanthamoeba polyphaga</i>	Cooling Tower, Bradford, England	<i>Mimivirus</i> within proposed Mimiviridae	dsDNA
(Wilson et al. 2002)	EhV86 and EhV84	<i>Emiliana huxleyi</i> CCMP 1516	Western English Channel, UK	<i>Coccolithovirus</i> (type species and tentative species, respectively) within Phycodnaviridae	dsDNA
(Castberg et al. 2002)	EhV-99B1, EhV-2KB1, and EhV-2KB2	<i>Emiliana huxleyi</i>	Marine Biological Field Station in Espeland, Norway	Tentative species of <i>Coccolithovirus</i> within Phycodnaviridae	dsDNA
(Schroeder et al. 2002)	EhV88, EhV163, EhV201, EhV202, EhV203, EhV205, and EhV207	<i>Emiliana huxleyi</i> (differing strains)	Western English Channel off coast of Plymouth, UK or Raunefjorden, Western Norway	Tentative species of <i>Coccolithovirus</i> within Phycodnaviridae	dsDNA
(Cottrell and Suttle 1991)	SP1	<i>Micromonas pusilla</i>	Pacific Ocean, Scripps Pier, La Jolla, California	Type species of <i>Prasinovirus</i> within Phycodnaviridae	dsDNA

Table 1.1. Continued.

Reference(s)	Virus	Host	Place of Isolation	Family and Genus	Genetic Material
(Cottrell and Suttle 1991)	PB6, PB7, PB8, SP2, GM1, PL1, and SG1	<i>Micromonas pusilla</i>	Peconic Bay, New Suffolk, New York, Pacific Ocean, Scripps Pier, La Jolla, California, Gulf of Mexico, Marine Science Pier, Port Aransas, Texas, and Strait of Georgia, British Columbia	Tentative species of <i>Prasinovirus</i> within Phycodnaviridae	dsDNA
(Suttle and Chan 1995)	CbV-PW1	<i>Chrysochromulina</i> spp.	Gulf of Mexico (Laguna Madre, Aransas Pass)	Type species of <i>Prymnesiovirus</i> within Phycodnaviridae	dsDNA
(Baudoux and Brussaard 2005; Brussaard et al. 2004b)	PgV-01T, PgV-03T, PgV-07T, PgV-09T, PgV-14T	<i>Phaeocystis globosa</i>	Southern North Sea	Tentative species of <i>Prymnesiovirus</i> within Phycodnaviridae	dsDNA
(Wilson et al. 2006)	PgV-102P	<i>Phaeocystis globosa</i>	Western English Channel off coast of Plymouth, UK	Tentative species of <i>Prymnesiovirus</i> within Phycodnaviridae	dsDNA
(Muller et al. 1996)	EsV and EfasV	<i>Ectocarpus siliculosus</i> and <i>E. fasciculatus</i> (respectively)		<i>Phaeovirus</i> within Phycodnaviridae	dsDNA
(Maier et al. 1998)	PlitV-1	<i>Pilayella littoralis</i>	Alaska	<i>Phaeovirus</i> within Phycodnaviridae	dsDNA
(Nagasaki and Yamaguchi 1997)	HaV GSNOU-30	<i>Heterosigma akashiwo</i>	Nomi Bay, Japan	<i>Raphidovirus</i> within Phycodnaviridae	dsDNA

Table 1.1. Continued.

Reference(s)	Virus	Host	Place of Isolation	Family and Genus	Genetic Material
(Nagasaki and Yamaguchi 1998)	HaV01-HaV14	<i>Heterosigma akashiwo</i>	Unoshima Port (Fukuoka Prefecture), Nomi Bay, or Hiroshima Bay, Japan	<i>Raphidovirus</i> within Phycodnaviridae	dsDNA
(Jacobsen et al. 1996)	PpV01	<i>Phaeocystis pouchetii</i>	Raunefjorden, Norway	Unassigned within Phycodnaviridae	dsDNA
(Gastrich et al. 1998)	BtV	<i>Aureococcus anophagefferens</i>	East Coast of U.S.A.	Unassigned within Phycodnaviridae	dsDNA
(Tarutani et al. 2001)	HcV	<i>Heterocapsa circularisquama</i>	Wakinoura Fishing Port (Fukuoka Prefecture), Japan	Unassigned within Phycodnaviridae	dsDNA
(Sandaa et al. 2001)	CeV-01B	<i>Chrysochromulina ericina</i>	Norwegian Coastal Waters	Unassigned within Phycodnaviridae	dsDNA
(Sandaa et al. 2001)	PoV-01B	<i>Pyramimonas orientalis</i>	Norwegian Coastal Waters	Unassigned within Phycodnaviridae	dsDNA
(Derelle et al. 2008)	OtV5	<i>Ostreococcus tauri</i>	Bages Lagoon, France	Putative Member of Phycodnaviridae	dsDNA

worth noting that several are unassigned to any particular family, emphasizing that there is still much more to be learned about marine viruses.

These characterized virus-host systems, and likely many others to come will continue to expand our understanding of virus-host dynamics by yielding the means to investigate infection parameters as well as to develop methods aimed at discerning and teasing apart viral metrics in natural systems. Traditionally, measurements of viral parameters have centered on the following determinations (discussed in more detail later): total viral and host abundances using either epifluorescence microscopy or flow cytometry and chlorophyll *a* concentrations for photosynthetic hosts, virus production rates using one of three basic methods discussed below, bacterial biomass production using radioactive tracers, burst size and percentage of visibly infected cells using transmission electron microscopy, and percentage of mortality due to viral lysis, contact rate between virus and host cell, percentage of contact success, etc. based on calculations using the data collected from the aforementioned procedures. Lab-scale methods have been expanded for use *in situ*, allowing researchers the means to elucidate the influences of viruses in natural microbial communities. Presently, due to the time and expense involved in large-scale open ocean work, much of our understanding of viruses in marine systems is restricted to coastal systems and relatively short open ocean transects (Brussaard et al. 2008a; Hewson and Fuhrman 2007; Middelboe et al. 2006; Weinbauer et al. 1993; Wilhelm et al. 2003; Winter et al. 2008; Wommack et al. 1992).

When investigating the environmental parameters/variables that may be associated with viral activity in marine systems, abundance and production rate are two main factors examined; providing an estimate of the viral load and activity, respectively. However, burst size and the percentage of visibly infected cells are of strong importance too as they tie viral parameters to host parameters. Because of their size and inability to be cultured outside of a host, initial enumerations of viruses were based on transmission electron microscopy (TEM) or most probable number (MPN)-based assays, although given the difficulty in culturing hosts and the uncertainty for total counts, the former has been more practical for environmental samples (Weinbauer 2004; Wommack and Colwell 2000). Subsequently, epifluorescence microscopy, and more recently, flow

cytometry, have provided faster, more practical means of enumerating virus-like particles (Wommack and Colwell 2000). Currently, epifluorescence microscopy (Wen et al. 2004; Wommack and Colwell 2000), and flow cytometry (Brussaard 2004a; Chen et al. 2001) remain the predominant methods of enumerating viruses from aquatic samples, though TEM and MPN-based assays are still used when appropriate. TEM can reveal morphological information and MPN can reveal abundances of active, specific host-infecting viruses. For example, a study looking at phages titres of specific host strain-infecting cyanophages in the Sargasso Sea, determined abundances of this subset of the total viral population present to be $\sim 10^3$ phages mL^{-1} (Sullivan et al. 2003). Studies across a wide variety of aquatic habitats (see Table 1.2 for viral abundances from a subset of these habitats) have provided total viral abundances mL^{-1} ranging from $\sim 10^4$ to $\sim 10^8$ (Weinbauer 2004), though typically those between 10^5 - 10^7 have been observed.

As the fate of an individual virus particle is uncertain, it is important for the viral community to have production rates that can overcome the difficulties associated with coming into contact with the correct host. These factors include: damage from UV-light and other forms of decay, sinking of infected cells prior to lysis, and even grazing (Brussaard 2004b). Monitoring the production of viruses *in situ* has been challenging to researchers, and the use of differing methods has made comparisons between studies difficult. However, steps have been taken to evaluate and compare the top methods of determining virus production rates and further refinement is underway. Recently, the implementation and results of the fluorescently-labeled virus (Noble and Fuhrman 2000), dilution (Wilhelm et al. 2002), and thymidine incorporation (Steward et al. 1992a; Steward et al. 1992b) methods of determining virus production rate were compared (Helton et al. 2005). The results of this comparison recommend the dilution approach, now called the “virus reduction and reappearance assay” (Weinbauer, Rowe, and Wilhelm, In Press), which determines production rates from the reappearance of free virus particles after the lysis of cells already infected at the time when free virus particles were removed by means of selective filtration (Wilhelm et al. 2002). Additionally, one proposed improvement to this method has been the replacement of dead-end filtration with tangential flow filtration to reduce the host cells that are lost during the initial

Table 1.2. Abundances of viruses observed from a subset of the various aquatic locations sampled.

Reference	Location	Abundance (mL ⁻¹)
(Baudoux et al. 2007)	North Atlantic Ocean, Near Azores	1.6-2.5*10 ⁷
(Parada et al. 2007)	North Atlantic Ocean, Subtropical	1.4*10 ⁶ (at 100 m)
(Wilhelm et al. 2003)	Southeastern Pacific Ocean	~2-16*10 ⁶
(Clasen et al. 2008)	Coastal Pacific Ocean, Near Canada	0.4-39*10 ⁷
(Clasen et al. 2008)	Coastal Arctic Ocean (Southwestern Beaufort Sea)	0.2-10.9*10 ⁶
(Ortmann and Suttle 2005)	Hydrothermal Vents (several)	9.12-61.89*10 ⁶
(Ortmann and Suttle 2005)	Hydrothermal Vents, Plumes (several)	2.03-6.18*10 ⁶
(Wells and Deming 2006)	Arctic Sea-Ice Brines	1.6-82*10 ⁶
(Alonso et al. 2001)	Alboran Sea	< 10 ³ – 1.8*10 ⁶
(Bettarel et al. 2002)	Mediterranean Sea	~6-11*10 ⁶
(Weinbauer et al. 2002)	Baltic Sea	23.4-32.9*10 ⁶
(Winter et al. 2005)	North Sea	2.1-184*10 ⁶
(Hwang and Cho 2003)	East Sea	~1-10*10 ⁶
(Bratbak et al. 1996)	Raunefjorden (Norway)	~2-5*10 ⁷
(Bratbak et al. 1996)	Osterfjorden (Norway)	~2-8*10 ⁷
(Wommack et al. 1992)	Chesapeake Bay (U.S.A.)	2.6*10 ⁶ -1.4*10 ⁸
(Noble and Fuhrman 1997)	Santa Monica Bay (U.S.A.)	~1-5*10 ⁷
(Wilhelm et al. 1998)	Gulf of Mexico (U.S.A.)	3.0-65*10 ⁸
(Lymer et al. 2008)	Lake Erken (Sweden)	~12-37*10 ⁷
(Hennes and Simon 1995)	Lake Constance (Germany)	1-4*10 ⁷
(Vanucci et al. 2005)	Lake Ganzirri (Italy)	5.26*10 ⁴ -7.54*10 ⁸
(Dorigo et al. 2004)	Lake Bourget (France)	5.8-26*10 ⁷
(DeBruyn et al. 2004)	Lake Erie (North America)	3.0-40.7*10 ⁷
(Klut and Stockner 1990)	Sproat Lake (Canada)	~10 ⁷
(Brum et al. 2005)	Mono Lake (U.S.A.)	1.4-19*10 ⁸
(Schoenfeld et al. 2008)	Yellowstone Hot Springs (U.S.A.)	3.07-14.4*10 ⁵
(Kepner Jr. et al. 1998)	Antarctic Lakes (several)	4.2-33.5*10 ⁶

filtration step designed to remove the free virus particles (Winget et al. 2005). Though this is a worthwhile consideration, other comparisons suggest that there is no difference between these two filtration methods (Weinbauer, Rowe, and Wilhelm, In Press). It is interesting to note that while viral abundances are generally higher in more productive waters (e. g. coastal regions), virus production rates do not always follow that same trend, and have been found to vary between $\sim 10^2$ to $\sim 10^6$ particles mL^{-1} (Weinbauer 2004).

Virus production rates depend upon many other factors that are valuable to examine as well, namely burst size, the percentage of visibly infected cells (both generally gathered by means of TEM (Weinbauer and Suttle 1996), and calculations such as host mortality due to viral lysis, percentage of infected cells, and contact rate. Burst size informs the researcher of how many particles are produced before cell lysis and this in turn depends upon the host cell and its capabilities during infection. For example, it has been observed that burst sizes of cells cultured in the laboratory are higher than those *in situ*, which can range from as low as 6 to high as 500 (Wommack and Colwell 2000). Also, when examining cells for virus-like particles, researchers can note the percentage of cells that are visibly infected. This in turn can be adjusted to include the cells that are infected, but not yet at a visibly infected stage so that the viral burden on the microbial community can be more fully understood (Proctor and Fuhrman 1990). Additionally, factors such as host mortality due to viral lysis (Proctor and Fuhrman 1990) and contact rate (Murray and Jackson 1992) can be determined. While these calculations can provide valuable information, for *in situ* determinations, their conclusions should be regarded as rough estimates to the assumptions that must first be made. An initial study reported that cells are only visibly infected during the last $\sim 10\%$ of infection (Valentine and Chapman 1966), however as discussed more recently (Mann 2003), other studies have found different results (e.g. that cells were visibly infected for the last $\sim 60\%$ of infection (Waterbury and Valois 1993)) and these differences in laboratory-based experiments raise questions over the proper assumptions to be made when calculating for mixed communities in natural samples. Along those same lines, questions have been raised (Binder 1999) over the assumption that absolute mortality is equal to twice the amount of infected cells when making calculations of host mortality due to viral lysis (Proctor and

Fuhrman 1990). Certainly as more is learned and extrapolated from laboratory-based experiments, these calculations will become more refined.

Though there may be challenges in making calculations between viral and host parameters, direct measurements can be made of other host parameters, such as abundance and biomass production rates. These in turn can provide the researcher with an understanding of the available host population and its activities, which can directly influence the viral population. Chlorophyll *a* concentrations, particularly when size-fractionated (Welschmeyer 1994), provide an indication of the phytoplankton biomass, while TEM, epifluorescence microscopy, and flow cytometry can provide direct counts of total cells (Heldal et al. 1994). Fluorescent *in situ* Hybridization (FISH) can be used to identify cells based on genetic markers (Amann et al. 2001; Congestri 2008), while flow cytometry has the added benefit of being able to distinguish cells based on size and fluorescence (e.g. Olson et al. 1985), which allows one to distinguish specific populations. This information is particularly beneficial when considering that the host-specificity of viruses can vary from being highly specific to crossing genera (Sullivan et al. 2003; Suttle and Chan 1993).

Additionally, to understand the activity of the host populations, heterotrophic biomass production rates can be obtained using either or both of the H³-leucine and H³-thymidine uptake assays (Fuhrman and Azam 1982; Kirchman 2001). Primary production rate measurements (Steigenberger et al. 2004; Wilhelm et al. 2004) can elucidate the activity of photosynthetic hosts. From previous studies examining these parameters in marine environments, researchers have observed that chlorophyll concentrations can range from ~0.2mg m⁻³ in oligotrophic ocean gyres to > 5.0 mg m⁻³ in areas with higher nutrient levels (Falkowski et al. 1998). Bacterial or total cell abundances are generally ~1-2 orders of magnitude less than viruses in the same area and range from ~10⁴-10⁷ cells mL⁻¹, with 10⁶ cells mL⁻¹ generally found in coastal waters (Wommack and Colwell 2000). Primary production rates range from < 100 mg C m⁻² d⁻¹ in open ocean gyres to as high as ~1,000 mg C m⁻² d⁻¹ in upwelling coastal areas (Behrenfeld et al. 2006), while bacterial biomass production rates can reach rates > 250 mg C m⁻² d⁻¹ in the euphotic zone of the World's Ocean (Ducklow 1999). Comparing

these results with measurements of viral parameters in these same areas provides for an important step towards understanding how viral and host populations interact with one another.

Though clearly much has already been learned about viruses in marine systems, more information is still needed to view the full picture and assess their global impact. Perhaps the next step in examining virus-host dynamics could be considered a marriage of small-scale laboratory work and large-scale natural community work; molecular tools developed from model systems in the lab can be used to tease apart the total virus population in natural communities. Progress in this area is just beginning and therefore little is known about the development of methods.

Currently, work has progressed in the use of Pulsed Field Gel Electrophoresis (PFGE) (Steward 2001) and Denaturing Gradient Gel Electrophoresis (DGGE) (Short and Suttle 1999) to examine diversity within the sampled viral population. Viral (measured with PFGE) and bacterial (measured with DGGE) community compositions have been demonstrated to change together over days and months in an investigation that covered 4 different marine environments (Steward et al. 2000). However, similar methods used in a limnic system revealed that changes in host and viral community composition did not always correlate with each other (Tijdens et al. 2008). While PFGE is a great tool for assessing diversity and monitoring changes in diversity, it should be kept in mind that it cannot perfectly resolve a viral community's composition. Comparisons with Restriction Fragment Length Polymorphism (RFLP) analysis (Jiang et al. 2003) and TEM (Santos et al. 2007) have shown that the full diversity is not represented with PFGE alone. For a more targeted approach, DGGE was used to examine viral (qualified by the DNA polymerase) and eukaryotic (qualified by 18S rDNA) communities, and it was found that not always did changes in either community coincide with each other or with measured physical parameters, suggesting a greater complexity to virus-host interactions (Short and Suttle 2003).

Additionally, quantitative PCR (qPCR) is being explored as a means to quantify specific members of the total viral population, providing a measure of species evenness to compliment the richness determined by the aforementioned techniques. With qPCR,

marine cyanophage abundance has been assessed using the conserved g20 (capsid assembly protein) gene as a biomarker (Sandaa and Larsen 2006). Additionally, this approach has been combined with PFGE and DGGE to provide a more complete look at true cyanophage diversity (Sandaa and Larsen 2006). qPCR for viruses in aquatic systems has been a focus more so for those viruses that infect humans and other macroeukaryotes (e.g. Fong and Lipp 2005; Haramoto et al. 2007; Hirayama et al. 2005; Pepin et al. 2008), but it is reasonable to assume that more studies like that of Sandaa and Larsen (2006) will soon be underway. Progress for qPCR, and for standard PCR approaches, has been aided by genomic sequencing of aquatic phages and viruses. At present, there are > 30 marine viral genomes sequenced and publicly available for the design of molecular probes. Of those, 4 are characterized as Phycodnaviridae: EsV-1 (Delaroque et al. 2001), EhV-86 (Wilson et al. 2005), OsV5 (published as OtV5 in Derelle et al. 2008), and *Feldmannia* sp. Virus (FsV) (GenBank: EU916176). For freshwater Phycodnaviridae (*Chlorella* viruses) there are 5 genomes currently available: PBCV-1 (Van Etten 2003), ATCV-1 (Fitzgerald et al. 2007c), FR483 (Fitzgerald et al. 2007a), NY-2A (Fitzgerald et al. 2007b), and AR158 (Fitzgerald et al. 2007b). Though MT325 has also been sequenced (Fitzgerald et al. 2007a), it is not publicly available at present.

From such molecular studies researchers can finally gain a look inside natural assemblages of viruses to learn about diversity and member-specific parameters and roles. Additionally, speculations can soon be tested properly with this new virus-specific community information. For example, researchers have assumed that heterotroph-infecting bacteriophages are more prevalent than algal viruses (a term inclusive of cyanophages) because of close correlations between bacterial biomass production rates and viral abundance (Weinbauer 2004). However, it is also likely that a) there are more phototroph-infecting viruses yet to be found, b) a relationship between bacterial and viral parameters could be a correlation resulting from the relationship between primary producer and viral parameters (Maranger and Bird 1995), and more importantly c) that the composition of the viral population is influenced by the environmental regime (Angly et al. 2006), which would provide for certain populations that are dominated by

heterotrophic bacteriophages with others dominated or co-dominated by algal viruses, depending on the environment. Also, due to an assumption that algal viruses cannot propagate within a non-photosynthesizing host, virus production assays using the dilution method are typically carried out in the dark, believing the algal virus population effectively removed. This again has not been sufficiently tested and evidence of certain algal viruses containing photosynthetic genes (Lindell et al. 2005; Mann et al. 2003; Sullivan et al. 2005) and of viral infection-induced photosynthesis impairment (Evans et al. 2006; Juneau et al. 2003; Seaton et al. 1995) might imply otherwise: that at least some algal viruses are forced to propagate without benefit of photosynthesis due to host system shutdown. It has also been shown in several studies that while light can affect activity for some viruses, for others light limitation had either no effect at all or only appeared to alter the burst size (Brussaard 2004b). Moreover in examining total virus production rates in the Western Pacific, no significant differences in rates were observed when comparing *in situ* light and dark bottle incubations that were otherwise identical (Weinbauer, Rowe, and Wilhelm, In Press). Improved molecular techniques could allow researchers to determine which are the more prevalent virus members in specific communities instead of relying on inferences based on correlations. Virus production methods could be more accurately compared to one another to assess specific virus production rates as well as what effects, if any, procedures such as incubating bottles in the dark may have on these specific production rates.

Moreover, another issue to be addressed here is the proper collection of new viruses to use in the laboratory setting for small-scale culture work as well as for the basis of large-scale and molecular work. Mimiviruses, the largest viruses known (Claverie et al. 2006), provide an excellent caution about virus isolation methods. The traditional 0.22 μm cutoff for isolating viruses is too small to allow a 400 to ~750 nm mimivirus (La Scola et al. 2003; Xiao et al. 2005) to pass through. Improved molecular techniques could highlight populations of viruses where researchers are missing information on key virus members and alert them to alter isolation techniques. Conversely, improved molecular techniques could allow researchers to determine that a significant portion of

the viral community in question is represented by isolated members in collection and by molecular analyses.

Unfortunately, virus-specific molecular work has its own unique problems. Virus gene and genome sequencing efforts have been disrupted in the past due to insufficient DNA concentrations, resistance to restriction enzyme digestion, and inability of certain genetic segments to be cloned due to their deleterious effects on the cell hosting the cloning vector (Paul et al. 2002). Virus populations have been observed to change rapidly, further complicating their study (Bratbak et al. 1990; Bratbak et al. 1996; Marston and Sallee 2003; Steward et al. 2000). Also, phylogenetic studies have had difficulties due to the lack of any universal viral genes equivalent to the 16S rRNA or 18S rRNA genes of bacteria and eukaryotes, respectively. However, despite these hurdles, molecular work has progressed to provide researchers with genetic and genomic data on viruses. The issue faced by this new direction of molecular studies is in choosing a biomarker with which to tease apart natural virus communities.

Presently, progress has been made in identifying suitable biomarkers from conserved structural and functional proteins, though only specific groups or families of viruses can be targeted in this way. For cyanomyoviruses, g20, the gene for the viral capsid assembly protein, has become a standard (Fuller et al. 1998; Sullivan et al. 2008; Zhong et al. 2002). From work with the DNA polymerase gene (DNA *pol*) for podoviruses, it was suggested that it could be a valuable marker (Chen and Lu 2002) and recently, DNA *pol* and g20 were both used to examine *Synechococcus*-infecting phage isolated from estuarine environments (Wang and Chen 2008). For freshwater cyanomyoviruses infecting *Anabaena* and *Nostoc*, the major capsid protein (MCP) gene was targeted to examine diversity (Baker et al. 2006). Additionally, for picorna-like viruses in marine samples, it was found that the conserved RNA-dependent RNA polymerase (RdRp) gene could be used for family assignment (Culley et al. 2003).

Studies on Phycodnaviridae have traditionally used the DNA *pol* gene to identify members and assess diversity (Chen and Suttle 1995; Chen et al. 1996; Van Etten 2006), though recently, the MCP gene has been examined as a potential additional biomarker using isolates and coastal water samples (Larsen et al. 2008; Schroeder et al. 2002). It is

important to note for clarity's sake, that DNA *pol* and MCP conservation here is not the same as discussed above for cyanophages. It was found that for *Emiliana huxleyi* viruses, MCP gene sequences were better able to distinguish isolates than DNA *pol*, suggesting that MCP could be a more appropriate biomarker for this subset of viruses (Schroeder et al. 2002). Looking at non-*Emiliana huxleyi*-infecting Phycodnaviridae, it was demonstrated that the MCP could still be a valuable biomarker, however it was noted that phylogenetic analyses performed with MCP and with DNA *pol*, despite similarities in the grouping of viruses, showed different ancestries (Larsen et al. 2008). This not only raises questions about the history of Phycodnaviridae, but about the accurate assessment of viral phylogeny in general. One possible solution that has been proposed, is to compare entire genomes instead of selected genes when examining phage phylogeny (Rohwer and Edwards 2002). After comparing 105 phage genomes, it was suggested that this genomic approach to viral phylogenetics may prove to be key for understanding ancestral relationships, after the refinement of algorithms and the sequencing of more genomes (Rohwer and Edwards 2002). Though there are still questions with this approach and with the molecular analyses of viruses in general, it is important to remember that these questions along with others will lead to a better overall understanding of virus-host interactions: their specific influences up to their global-scale effects.

HYPOTHESES

To qualify the objectives discussed below, the following specific, null hypotheses were established for testing in the research described in later sections. Null hypotheses were established so as to be rejectable, testable statements that provide for the necessary disproof for accurate scientific investigation (Popper 1959).

SECTION 1

i) The infectious agent pathogenic to *Aureococcus anophagefferens* is not a virus and does not fulfill Koch's postulates.

ii) Phage-like particles present in cultures of *A. anophagefferens* do not cause cell death and do not fulfill Koch's postulates.

SECTION 2

iii) Viral parameters are not correlated with host or oceanic variables in the Sargasso Sea.

iv) Viral parameters are not correlated with host or oceanic variables in the North Atlantic.

v) The Sargasso Sea and North Atlantic are not statistically distinct regimes.

SECTION 3

vi) Viral parameters are not correlated with host or oceanic variables in the Western Pacific.

vii) The Western Pacific is not statistically distinct from the Sargasso Sea or North Atlantic.

SECTION 4

viii) There is no difference in diversity of *Emiliania huxleyi*-infecting Phycodnaviridae among North Atlantic samples, lysates produced by infecting host cultures with the same North Atlantic samples, and coastal isolates in culture.

ix) The Major Capsid Protein gene cannot be used to assess *Emiliania huxleyi*-infecting Phycodnavirus diversity.

OBJECTIVES

The research described herein was designed to deliver information to each of the levels of research focusing on the role(s) and influences of marine viruses on the global ecosystem: small-scale laboratory work with model systems, large-scale work with *in situ* parameters of natural communities, and intra-community work using molecular tools designed from laboratory studies for use in field studies. Though the use of model virus-host systems is somewhat routine nowadays, section 2 presents a cautionary story of using proper isolation techniques. Koch's postulates (1884), despite being developed for bacterial pathogens, can be used *in sensu* to determine the pathogenic culprit of interest. Conceivably, a mistaken identification can have numerous negative effects down the line of research, hindering overall results. Isolations and characterizations of viruses allow not only for more model systems of study, but for an increase in the information available for genetic, genomic, and phylogenetic analyses.

In sections 3 and 4, large-scale research is visited with the aim of supplementing what is known about viral parameters in open ocean systems. Three open ocean regimes were studied across two very distinct gradients. A research cruise crossing a productivity gradient took place in the Atlantic Ocean in the summer of 2005, allowing researchers to examine the Sargasso Sea and North Atlantic during an annual spring bloom (section 3): two regimes of contrasting trophic status. In addition, a separate research cruise crossing a temperature gradient took place in the Western Pacific Ocean during the winter of 2007 (section 4); providing a model for the effects of increased temperature on marine microbes. These two cruises, covering > 10,000 km (see Figure 1.1), provide a first-time look at several key pieces of information: the basic virus-host parameters (discussed previously) across large expanses of open ocean, the comparisons of these basic virus-host parameters across three distinct transects, and the comparisons of these basic virus-host parameters with other measured oceanic parameters. Important trends, such as regime-dependency, have been discovered from these data, and it has been shown that each transect is statistically distinct from the others.

Lastly, section 5 covers the use of molecular tools to tease apart natural virus communities, using Phycodnaviridae as a representative population. Due to the need to

better understand algal viruses and because for those with eukaryotic hosts, the most is known about Phycodnaviruses, Phycodnaviridae was the family of viruses on which this research focused. It should be noted that there is a sister project to this work with a focus on cyanophages. The examination of multiple eukaryotic algal viruses from the previously examined natural communities of the North Atlantic discussed in section 3, have provided a means to further test the use of the conserved major capsid protein gene as a biomarker to assess Phycodnaviridae diversity of *Emiliana huxleyi*-infecting viruses. Comparisons of nucleotide sequences have revealed new richness, distinct from what has been previously seen from coastal water isolates. While no overt spatial variations in diversity were evident, there were differences observed between sequences amplified from virus concentrates and those amplified from lysates generated by inoculating host cultures with the same virus concentrates. Such analyses have provided information on the use of the major capsid protein gene as a genetic biomarker, and have indicated that the true species richness of *Emiliana huxleyi* viruses is likely much higher than previously known. Future work using this biomarker in conjunction with DNA *pol* sequences to assess phylogeny and in solo to facilitate qPCR targeting species evenness is very promising at this point. Collectively, these objectives address valuable aspects of aquatic virus research and serve as a solid foundation for future work.

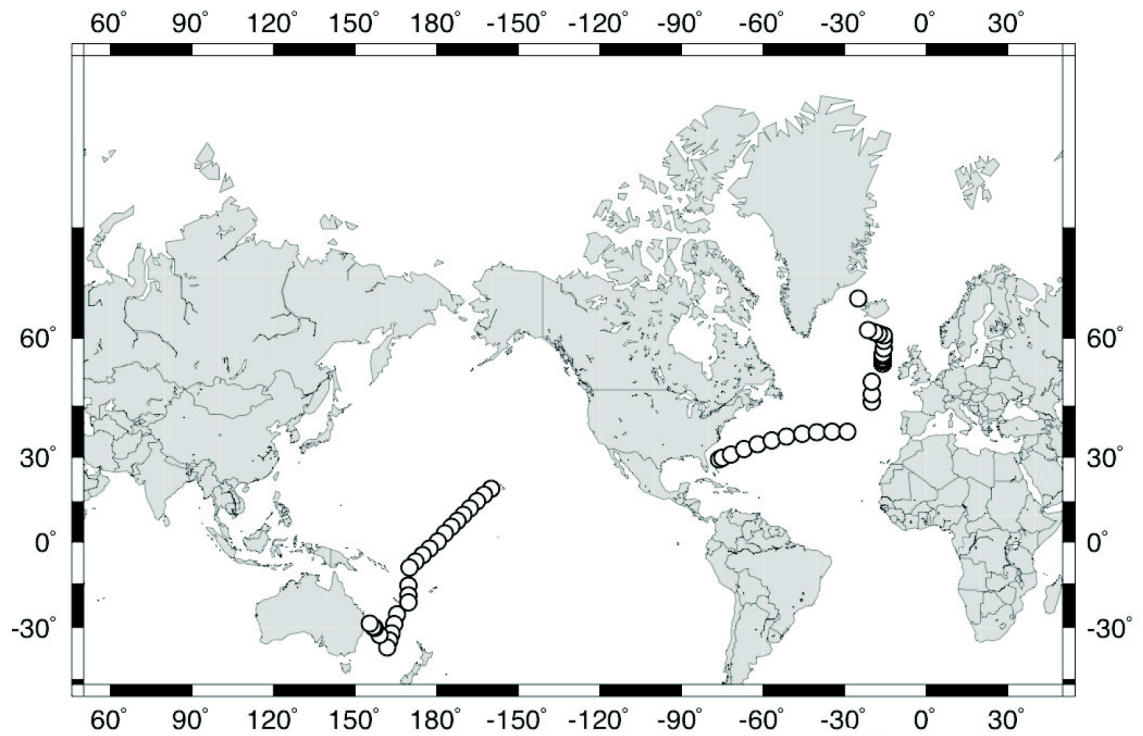


Figure 1.1. Locations of sampling stations across the Sargasso Sea, North Atlantic, and Western Pacific from two different research cruises.

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SECTION II

ISOLATION OF A NON-PHAGE-LIKE LYTIC VIRUS INFECTING
AUREOCOCCUS ANOPHAGEFFERENS

This section is a version of an article that first appeared in Journal of Phycology under the same title by Janet M. Rowe, John R. Dunlap, Christopher J. Gobler, O. Roger Anderson, Mary D. Gastrich, and Steven W. Wilhelm.

Rowe, J. M., J. R. Dunlap, C. J. Gobler, O. R. Anderson, M. D. Gastrich, and S. W. Wilhelm. 2008. Isolation of a Non-Phage-Like Lytic Virus Infecting *Aureococcus anophagefferens*. Journal of Phycology 44: 71-76.

My contribution to this paper was the discovery of agents pathogenic to *Aureococcus*, isolation of the *Aureococcus*-infecting virus, performing the controlled growth and lysis experiments, performing the transmission electron microscopy negative stain and scanning electron microscopy analyses under the guidance of J. R. Dunlap, and much of the background information gathering and writing.

ABSTRACT

We have been working to characterize viruses that infect the HAB-forming pelagophyte *Aureococcus anophagefferens* Hargraves et Sieburth. Field samples were collected during brown-tide events in 2002 and tested for the presence of lytic agents. Here, we describe a recently isolated, lytic virus-like particle (VLP) that is morphologically similar to particles observed in thin sections of infected *A. anophagefferens* cells from natural samples. TEM and SEM have revealed VLPs consistent with the morphological characteristics of previously described Phycodnaviridae. Large icosahedral particles (~140 nm) of similar shape and morphology dominate cell lysates and are accompanied by smaller phage-like particles and heterotrophic prokaryotes that appear to be incurable from our cultures. To determine which of these particles interacts with the *Aureococcus* cells, we preserved cultures during the early stage of infection so that SEM could be used to visualize those particles that attach to the surface of naïve cultures. SEM revealed that 63% of the large icosahedral-shaped particles attached to *A. anophagefferens* cells after only 30 min of exposure, while no significant frequency of attachment to the alga was observed for the phage-like particles. The results of these observations are in contrast to previous studies, where phage-like particles were reported to infect cells. When considered in conjunction with field observations, the results suggest that this newly isolated virus represents the dominant virus-morphotype associated with bloom collapse and termination.

INTRODUCTION

Brown-tide events caused by *A. anophagefferens* have been observed in mid-Atlantic estuaries since 1985 and can be devastating to the surrounding environment because they attenuate sunlight that reaches submerged photosynthetic organisms (Bricelj and Lonsdale 1997; Gobler et al. 2005). One common photosynthetic organism affected is eelgrass, an important food source and habitat for members of higher trophic levels (Bricelj and Lonsdale 1997). Previous studies have investigated the factors that potentially influence *A. anophagefferens* bloom dynamics and have suggested that bioavailable dissolved organic matter (DOM) promotes blooms. As such, increased virus

densities during a bloom event may promote bloom growth by providing a source of DOM through cell lysis (Gobler et al. 2004). Companion studies have also examined factors that may limit bloom proliferation. In these studies, observations of thin-sectioned natural samples of *A. anophagefferens* taken during bloom events have revealed visibly infected cells, with a frequency of nearly 40% toward the end of the bloom event (Gobler et al. 2004). These findings have led researchers to believe that viruses play an integral role in bloom dynamics, regulation, and potentially termination. Previous studies (Garry et al. 1998; Milligan and Cosper 1994) have reported smaller, bacteriophage-like tailed particles in association with lysis of cultures of *A. anophagefferens*. These findings were not consistent, however, with subsequent published TEM data, which showed that natural samples of *A. anophagefferens* contain particles that are ~140 nm in diameter and icosahedrally shaped (Gastrich et al. 2002; Gastrich et al. 2004). These observations as well as the recent isolation of bacteria that also lyse *A. anophagefferens* (Frazier et al. 2007) have made the reexamination of this host-virus system a priority. To this end, we set out to identify particles consistent with those observed in nature that may be consistently associated with the lysis of *A. anophagefferens* cells.

MATERIALS AND METHODS

Sample collection and culturing

Water samples from a variety of locations have been collected as part of an ongoing study of the *A. anophagefferens* virus-host dynamics (see Gastrich et al. 2004). For the current study, samples collected at Quantuck Bay (40°49' N, 72°37' W) and Great South Bay (40°25' N, 73°08' W), New York, USA, were used to screen for naturally occurring viruses that can infect *A. anophagefferens*. Water samples were collected as previously described (Gobler et al. 2004), and the virus-size class (30 kDa, 0.20 µm) concentrated using an Amicon M12 ultrafiltration system (Wilhelm and Poorvin 2001). Virus concentrates were then returned to the lab and screened for lytic activity against cultures of *A. anophagefferens* CCMP 1784. *A. anophagefferens* cells were maintained in an alteration of a previously described brown-tide medium (Cosper et al. 1993) made with water from the Sargasso Sea (reduced to 28 psu with Milli-Q purified water, Academic

Gradient A10 Biocel Synthesis; Millipore, Sundbyberg, Sweden) and with a final selenium concentration of 10 μM . Cultures were maintained at 22°C and under a 12:12 light:dark (L:D) cycle ($\sim 50 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) in 50 mL glass culture tubes. Samples were screened daily for plankton growth (via chl production) using a Turner Designs TD-700 fluorometer (Sunnyvale, CA, USA) equipped with an in vivo chl *a* filter set (excitation $\lambda = 340\text{--}500 \text{ nm}$; emission $\lambda = >665 \text{ nm}$).

Virus infection and lytic cycle length

During screening, a sample from Quantuck Bay was demonstrated to contain a filterable lytic agent infectious to *A. anophagefferens*, and three samples from Great South Bay contained nonfilterable lytic agents (later identified as bacteria) also infectious to *A. anophagefferens* cells. The infectious agent isolated from the Quantuck Bay sample could pass through a low-protein binding, 0.2 μm nominal-pore-size polycarbonate filter (GE Osmonics Inc., Labstore, Minnetonka, MN, USA), while those from the Great South Bay samples could not (see Frazier et al. 2007). To determine an approximate length of the lytic cycle, a simple one-step growth curve was performed. Cells were infected with filtered virus at a minimum multiplicity of infection (MOI) of 1 (empirically determined, data not shown), and cell growth (or death), as compared to controls, was monitored over time as fluorescence spectral units (FSUs). To ensure that fluorescence was a valid measure of cell growth (not complicated by the process of infection and cell lysis), an additional assay was conducted at the same time. Twenty-one cultures of *A. anophagefferens* were initiated at the same time and allowed to recover from the stress of transfer. Control cultures received either no lysate ($n = 3$) or killed lysate (autoclaved to kill any remaining biotic factors, $n = 3$). Prior experiments indicated no difference between these two types of controls (data not shown). The remaining 18 cultures were inoculated at the same time from the same stock of lytic agent with 100 μL of 0.22 μm filtered lysate (again, minimum MOI of 1). After taking an FSU reading of all the cultures at specific time points (starting with inoculation time), three random treatment cultures were sacrificed, and cell abundance was determined by manual counting via epifluorescence microscopy. These cultures were fixed with glutaraldehyde (2% v/v final), and 2 mL of sample was collected onto 25 mm diameter, 0.22 μm nominal-pore-

size black polycarbonate filters (Osmonics Inc.). Autofluorescing cells were enumerated via epifluorescence microscopy using a Leica DMRXA microscope (Leica Microsystems, Wetzlar, Germany). Three control cultures and three random treatment cultures were allowed to run until the end of the experiment.

Sample preparation and TEM analysis of free VLPs (negative stain)

Virus-mediated cell lysates, generated from unhindered infections, were spotted onto carbon-coated collodion (2%; Electron Microscopy Sciences, Hatfield, PA, USA) films atop 400-mesh electron microscope copper grids and allowed to sit for 5 min (providing for attachment via Brownian motion). Grids were then stained with 0.75% uranyl acetate for 1 min and rinsed three times with glass distilled water. Samples were viewed with a Hitachi H-800 TEM (Hitachi High Technologies America Inc., Schaumburg, IL, USA) with an accelerating voltage of 100 KeV. This methodology provided a means to determine the presence and examine the basic morphology of the suspected infectious agent.

TEM—sample preparation and analysis of infected cells (thin section)

To confirm the morphology of VLPs within infected cells, separate cultures infected within the lab were also examined. Samples were prepared for TEM as previously published (Gastrich et al. 2002). Briefly, infected cells were prefixed in a 2% research grade buffered (0.2 M cacodylate, pH = 7.8) glutaraldehyde solution (Thermo Fisher Scientific, Waltham, MA, USA). Then specimens were postfixated in 2% osmium tetroxide (0.2 M cacodylate, pH = 7.8) (Electron Microscopy Sciences), dehydrated, and embedded in EPONTM resin (Hexion Specialty Chemicals, Columbus, OH, USA). Ultrathin sections were collected on copper grids (Electron Microscopy Sciences). *A. anophagefferens* cells were examined for the presence of VLPs using methods previously described (Gastrich et al. 2002).

Characterization of virus attachment to A. anophagefferens cultures

To determine which particles (if any) associated with *A. anophagefferens* cells during the infection process, we exposed naïve cultures (never having been exposed to a virus) of

this pelagophyte to 0.2 μm filtered lysates that had been generated by exposure to the lytic agent. Briefly, 1 mL of lysate (treatment) or 1 mL of killed lysate (control) was added to 25 mL of actively growing culture, and the mixture incubated under standard growth conditions for 30 min to allow for initial attachment. To prevent further infection and in an attempt to immobilize attached particles on the surface of host cells, cultures were fixed with glutaraldehyde (2% v / v final; TEM grade 70% glutaraldehyde from Ladd Research Industries, Williston, VT, USA) buffered in 0.05 M cacodylate 28 psu seawater (cacodylic acid sodium salt from Ladd Research Industries). Preserved samples were immediately placed on ice and allowed to settle overnight at 4 °C (Nudelman et al. 2006). Subsequently, these samples were prepared for SEM. Excess fixative was gently vacuumed off, and samples were washed in water and transferred onto freshly glow discharged and poly-l-lysine coated glass coverslips. Samples, attached to coverslips, were dehydrated in a graded acetone series (25%, 50%, 75%, 90%, 100%, 20 min each step) and critical-point-dried in liquid CO₂ with a Ladd Research Industries critical point dryer. Prior to viewing, the coverslips were attached to aluminum specimen stubs with double-sided copper tape and coated with a thin layer of gold in a Structure Probes Inc. (SPI) sputter coater (West Chester, PA, USA). Samples were viewed using a LEO 1525 field emission scanning electron microscope (now under Carl Zeiss MicroImaging Inc., Thornwood, NY, USA) using 3–5 kV accelerating voltage. All images were recorded digitally.

RESULTS

Growth curves

To confirm the use of FSUs as an accurate means to assess *A. anophagefferens* cell abundance and to establish a means to estimate cell number based on FSU readings, culture samples at different times in their growth were enumerated just after fluorescence readings were taken. Plots of cells per mL versus FSUs clustered around a straight line

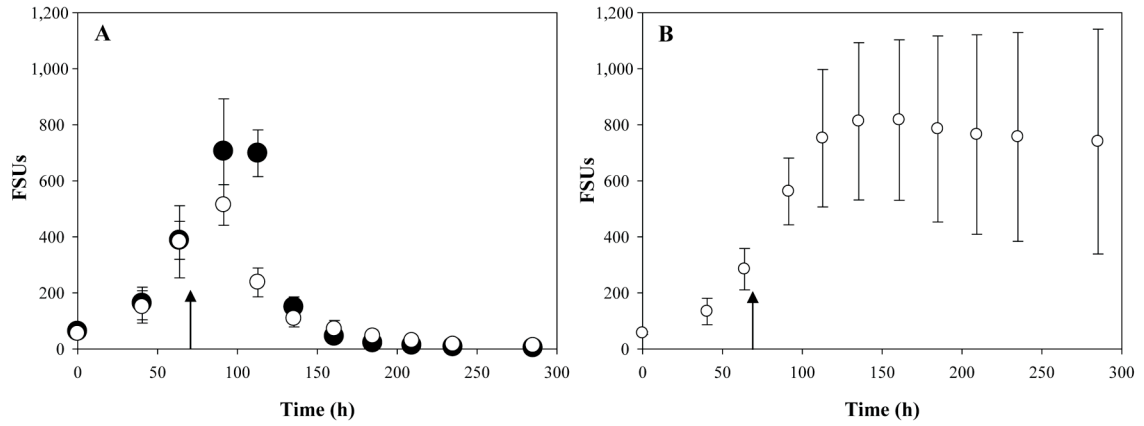


Figure 2.1. Growth curves of virus lysate-infected *Aureococcus anophagefferens* cultures. (A) *A. anophagefferens* exposed to unfiltered (open circles) and 0.2 μm filtered (dark circles) water samples containing the putative lytic agent ($\pm\text{SD}$, $n = 3$ per treatment). (B) Control growth of *A. anophagefferens* cells ($\pm\text{SD}$, $n = 9$). FSU = fluorescence spectral units. Arrows indicate the addition of 100 μL of virus lysate (A) or 100 μL of sterile medium (B).

with an $r^2 = 0.970$, indicating a strong correlation between cell number and fluorescence and eliminating concerns over the effects of infection on the use of this biomass measurement. Figure 1 shows the corresponding fluorescence measurements over time of cultures exposed to filtered or unfiltered lysates (Fig. 2.1A) or controls to which no lysate was added (Fig. 2.1B). In all experiments, the control tubes, whether they received no viral lysate or killed lysate, were unhindered in their growth. All cultures that received active lysate and were allowed to progress unhindered (*i.e.*, not sacrificed) were eventually completely lysed without recovery. We noted that lysis consistently began between 60 and 72 h postinoculation. Subsequent transfers of these virus lysates (after 0.2 μ m filtration) to naïve cell populations have consistently lead to lysis during the past 3 years (more than 20 generations).

TEM of free virus and infected cells

To examine the morphology of the infectious agent, a sample of lysate was prepared as described above (negative stain) and examined using TEM. Although the grids were not densely crowded, likely due to allowing the particles to attach randomly to the film via Brownian motion rather than by being spun down with centrifugal force, VLPs could be seen. The particles viewed in this manner were large (slightly <200 nm in diameter), and those that could be clearly seen displayed an icosahedral or nearly icosahedral shape (the latter likely explained as particles that collapsed without being completely flat, *i.e.*, folded over on themselves). Figure 2.2 shows a representative particle. No tail-like structures were observed attached to any of the VLPs viewed. Examination of *A. anophagefferens* cells infected with lysate as described above (thin section) via TEM revealed large (~ 140 nm) VLPs of icosahedral shape packed within the walls of the algal cells (Fig. 2.3). These viruses were consistent with both the free viruses observed by TEM (above) as well as VLPs observed within cells from natural populations (Gastrich et al. 2004).

SEM of surface attachment

In the samples analyzed via SEM, large (>100 nm in diameter) icosahedral-shaped VLPs were found only in the treatment sample and not the control (Fig. 2.4). Very small,

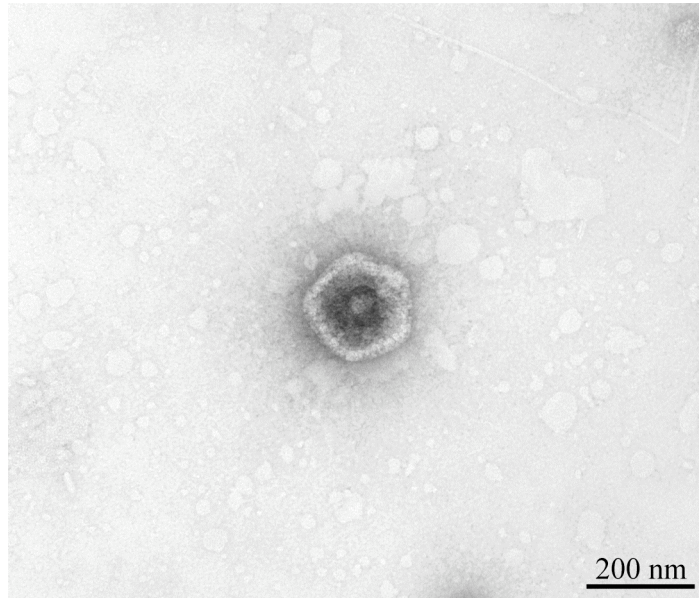


Figure 2.2. Negatively stained image of a virus-like particle associated with lysates of *Aureococcus anophagefferens*.



Figure 2.3. Thin-section image of *Aureococcus anophagefferens* cell exposed to 0.2 μm filtered lysates. Virus-like particles in cells are similar to those seen in field populations (Gastrich et al. 2004). Scale bar, 1,000 nm.

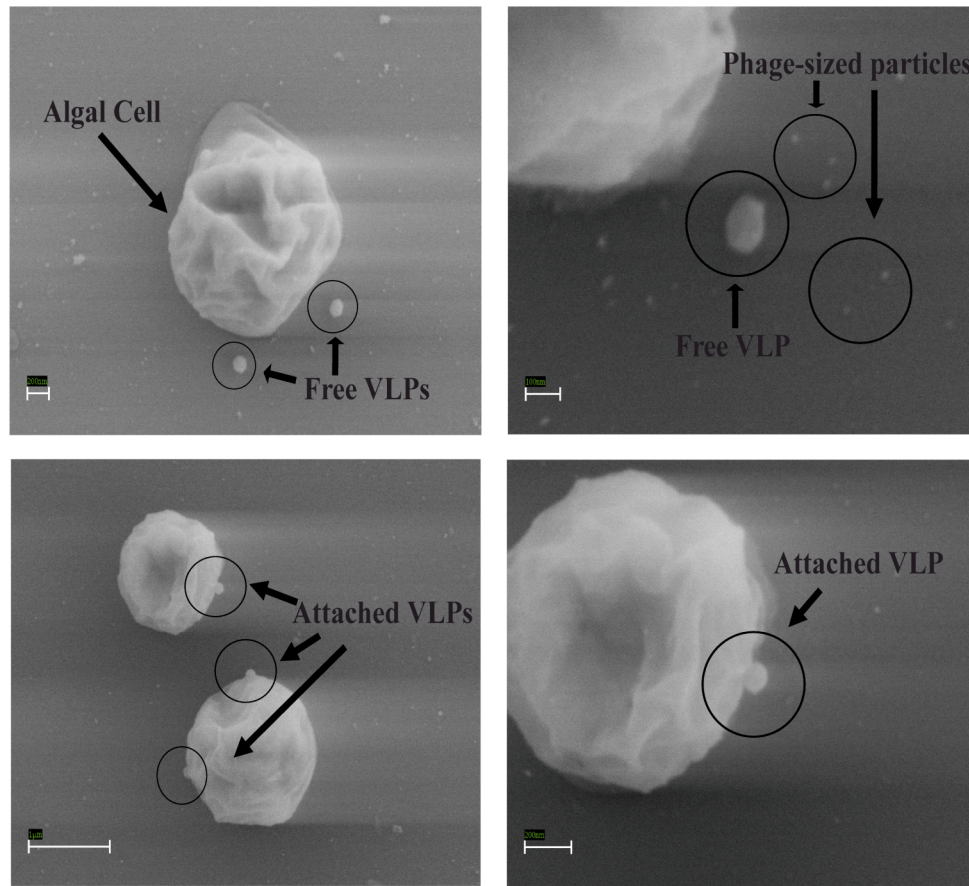


Figure 2.4. Scanning electron micrographs of naïve *Aureococcus* cultures exposed to virus-mediated lysates. After 30 min of exposure, the majority of the virus particles appear associated with *A. anophagefferens* cell surfaces (upper left: scale bar, 200 nm; upper right: scale bar, 1,000 nm; lower left: scale bar, 1,000 nm; lower right: scale bar, 200 nm). VLP, virus-like particle.

potentially phage-like particles were observed in both control and treatment samples, but in neither case could any be seen clearly attached to *A. anophagefferens* cells. Within the treatment group, 66% of the *A. anophagefferens* cells examined had at least one icosahedral-shaped VLP (~140 nm) attached to their surface after only 30 min. Free VLPs matching the same description were also observed in the treatment group, but again, none were found in the control group.

DISCUSSION

Interest in the ecological implications of virus infection in aquatic environments has grown significantly in the past few decades as realizations of the potential role of viruses on system geochemistry (Poorvin et al. 2004; Wilhelm and Suttle 1999) as well as diversity and lateral gene transfer (Suttle 2005; Weinbauer 2004) have become more clear. Moreover, the advent of molecular tools has made the correct identification of viruses infecting cells important, as the construction and validation of these tools requires appropriate positive controls (*i.e.*, the isolated virus of interest), which must first be accurately identified.

Koch's postulates, the standard that most microbial isolations must meet, were originally developed for confirming disease-causing agents in the medical field (Koch 1884). While they have been well applied in the medical field, they are more difficult to apply to the field of environmental microbiology. Here, to the best of our abilities, we attempted to apply Koch's postulates in confirming the identity and some basic characteristics of a naturally occurring virus infectious to *A. anophagefferens* CCMP 1784. We have in this study been able to show that the virus is present in many cases of cell death, but note that other agents can also cause death in this culture (e.g., bacteria as per Frazier et al. 2007). Furthermore, although the virus can be separated from the host, we have been unable to purify it away from contaminating phage. The virus size-class can, however, subsequently lead to death when new cells are exposed to the isolated agent, and the agent can be repeatedly isolated away from the host and used to reinfect future cell cultures (with appropriate controls). As such, while it is not possible to strictly satisfy these Koch's postulates as originally written, we feel this approach has built

sufficiently upon previous studies (Gastrich et al. 2002; Gastrich et al. 2004) and that it has satisfied the postulates *in sensu*.

First, we established that a particle susceptible to autoclaving, but not to dilution, caused nonrecoverable cell death of *Aureococcus* cultures, suggesting a living particle and not a toxin. We then used TEM to reveal a potential agent of cell lysis (negative stain described above) and used the morphology of these particles as criteria to confirm the presence of these same particles in later experiments. Because the smaller phage-like particles were present in both diseased and healthy cultures, they were eliminated from the pool of potentially infectious agents based on Koch's first postulate. The larger VLPs were the only particles we observed to be present in the diseased cultures, and the introduction of VLPs to healthy cultures repeatedly led to infected cells and lysis (collective experiments). Particles of similar size and shape were found only in treatment cultures—free and attached to the algal cell surface in freshly inoculated cultures (SEM described above) and packaged together and often densely within cells at later stages of infection (TEM thin section described above). The reisolation of these disease / lysis causing agents was demonstrated through their appearance in separate experiments, passed on by use of the filtered lysate of the previous experiment. This infection and reisolation has been completed at least 20 times during the past 3 years, with lysates used to infect new naïve cells (data not shown). Finally, EM also allowed us to confirm the viral nature of the pathogen, instead of relying on assumptions based on its filterability. To our knowledge, this is the first report of the use of SEM to demonstrate that a VLP specifically associates itself with *A. anophagefferens*.

Systematic observations of *Aureococcus* cells collected from natural bloom events over the past decade have consistently demonstrated that viruses play a potentially important role in the termination of blooms (Gastrich et al. 2002; Gastrich et al. 2004). These experiments collectively point to a virus-host system in which the VLP that associates with the host is large (~140 nm) and icosahedrally shaped. It remained somewhat surprising, however, that the isolated particles described in previous publications (Garry et al. 1998; Milligan and Cosper 1994) were so different from the particles observed inside cells. Not only were there variations in morphology (the

observation of icosahedral particles inside cells vs. putative isolated particles that were tailed and bacteriophage-like), but there was also a great discrepancy in capsid size (~140 nm size particles within infected cells vs. ~50–55 nm size capsid heads in isolated viruses). The isolation of an axenic culture of *A. anophagefferens* (Berg et al. 2002) should allow for algal viral interactions of this species to be examined without bacteria in the future and thus eliminate the propagation of any incidental bacteriophage.

The isolation of this virus in parallel with the isolation of bacteria capable of lysing *A. anophagefferens* (Frazier et al. 2007) provides a cautionary tale of the difficulties associated with identifying algal pathogens and conducting research with nonaxenic algal cultures. Significant replication as well as the application of companion techniques within our current work allowed us to distinguish between these different agents of mortality. Moreover, these results support the contention that the bacteriophage-like particles identified in previous work may have been microbial hitchhikers—viruses infecting companion or algicidal bacteria that persisted in low abundance in the cultures used in those studies.

The results of this study also provide some clarity in terms of the potential role of viruses in the termination of blooms of *A. anophagefferens*, as they confirm the presence of lytic agents in natural systems that are responsible for the formation of virus particles seen in previous studies (Gastrich et al. 2002; Gastrich et al. 2004). It remains doubtful, though, that such pathogens will ever be applicable to the control of bloom events. This contention is a function of the Red Queen Theory (Valen 1973) as it applies to virus ecology; host cell populations continue to evolve toward resistant states, even while the viruses continue to evolve toward new infectious states. Our results may help in addressing this as they will allow for the development of better tools (e.g., molecular markers) for monitoring the ontology of brown tides as well as the mortality mechanisms associated with their control and demise. Already the molecular characterization of this virus is underway. Our preliminary PCR results (data not shown), using the AVS1, AVS2, and POL 1 primers described by Chen and Suttle (1995) with the basic protocol described by Short and Suttle (2002), did not indicate that this virus was a member of the Phycodnaviridae family. However, because of its morphology and activity against a

eukaryotic alga, we believe that our preliminary results cannot exclude it from such a classification.

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SECTION III

CONSTRAINTS ON VIRAL PRODUCTION IN THE SARGASSO SEA AND NORTH ATLANTIC

This section is a version of an article that first appeared in Aquatic Microbial Ecology under the same title by Janet M. Rowe, Matthew A. Saxton, Matthew T. Cottrell, Jennifer M. DeBruyn, G. Mine Berg, David L. Kirchman, David A. Hutchins, and Steven W. Wilhelm.

Rowe, J. M. and others 2008. Constraints on Virus Production in the Sargasso Sea and North Atlantic. Aquatic Microbial Ecology 52: 233-244.

My contribution to this paper was participating in sample collection and onboard experiments during the NASB2005 cruise, processing viral and bacterial abundances, virus production rates, and chlorophyll *a* data, compiling data from other cruise participants and colleagues for statistical analysis, analyzing results of statistical analysis with help from Jennifer DeBruyn, and most of the background information gathering and writing.

ABSTRACT

During the spring of 2005, the production rate of virus-like particles as well as microbial and biogeochemical parameters in the Sargasso Sea and North Atlantic Ocean were investigated as part of a multifaceted North Atlantic Spring Bloom (NASB2005) survey. Here we present data on the spatial variations in viral production rate, abundance, and infection of microbial host populations, in an attempt to understand the constraint(s) on the rates of viral production and abundance. As expected, virus-like particle densities increased from the oligotrophic waters of the Sargasso Sea to the more productive northern Atlantic waters. Additionally, viral production rates increased across the Sargasso Sea transect and correlated with trophic status (chl *a* concentrations) and secondary (bacterial) productivity in this region. However, no clear pattern of viral production rates could be discerned in waters hosting the NASB. Results of both parametric and non-parametric analyses demonstrate that viral production rates and abundances only correlate well for our observations within the constant ‘stability’ of the Sargasso Sea, but suggest that more complex relationships including a threshold effect, may guide viral parameters in the North Atlantic. Overall, this study highlights the spatial and potentially temporal complexity of constraints on viral production and ultimately on viral function in surface marine waters.

INTRODUCTION

Although viruses have been recognized as agents of bacterial mortality for nearly a century (Duckworth 1976), their role in the environment has come under study only recently. Present evidence suggests that viruses play a critical role in the microbial loop of both freshwater (DeBruyn et al. 2004) and marine systems (Suttle 2005; Wilhelm and Suttle 1999). As obligate pathogens, viruses release previously fixed carbon in forms which are bioavailable to co-existing heterotrophs (Fuhrman 1992). Moreover, nutrients like N, P, Se and Fe (Gobler et al. 1997; Mioni et al. 2005; Poorvin et al. 2004), and even amino acids (Middelboe and Jorgensen 2006) can be made bioavailable to microbial community members after viral lysis of host cells. Aquatic viruses are also thought to influence the structure of microbial populations through the ‘kill the winner’ mechanism,

i.e. by controlling abundances of dominant community members, allowing less competitive members to survive (Thingstad and Lignell 1997). As such, it is apparent that viruses may influence community diversity during the establishment, proliferation and decline phases of any microbial population (Brussaard et al. 2008).

Much of our current knowledge concerning virus–host interactions and distributions in marine environments is derived from studies conducted on local spatial scales (~10s of km). Few studies have focused on temporal changes and fewer still have examined viral parameters on oceanic scales (1000s of km). To answer questions concerning the global impact of viruses on marine ecosystems and identify constraints on viral activity, we must first examine virus-associated parameters over these larger spatial regions. Viral abundances and viral decay rates have previously been examined over a ~5000 km transect along the western coast of South America (Wilhelm et al. 2003). That study distinguished the effects of both latitudinal gradients in light and localized ocean physics (e.g. upwelling) on communities. However, factors such as viral production rate and burst size (the abundance of viruses released per lytic event) have yet to be measured over such large distances.

No detailed process study on the NASB has been conducted since the end of the JGOFS North Atlantic Bloom Experiment in 1989/1990, despite the generally recognized biogeochemical importance of this occurrence. Early studies focused primarily on phytoplankton and parameters of photosynthesis (Balch et al. 1996; Sieracki et al. 1993). Typically, early blooms of diatoms are succeeded by coccolithophorids towards the later stages of the bloom, likely due to factors such as the changing availability of nutrients. These changes in the structure of the pelagic food chain can alter the diversity of higher trophic levels.

During a 2005 cruise to the spring bloom, we estimated parameters associated with viral infection and production rates as we transited from the oligotrophic Sargasso Sea to the more eutrophic North Atlantic. A study bridging these 2 contrasting regimes provides a unique opportunity to see how biotic and abiotic parameters influence viral distributions, production rates, and estimates of lytic burst sizes. The results provide

insights into processes across large spatial scales, and through natural transitions between 2 regimes of contrasting stability and trophic status.

MATERIALS AND METHODS

Cruise logistics

Samples were collected while on board the RV ‘Seward Johnson’ at 15 underway stations in the Sargasso Sea, and at 54 ‘process’ stations in the North Atlantic. On the Sargasso transit leg between Florida and the Azores (a non-stop ‘deadhead’ transit), daily samples were taken underway from the ship’s clean surface pump (~5 m) as well as from a single depth profile. On the North Atlantic transect from the Azores to Iceland, station locations were chosen based on satellite images indicating coccolithophorid blooms. On this leg, daily water column profiles were taken in the morning from 2 CTD casts; one shallow (with samples collected from 5 to 50 m) and one deep (with samples collected from 70 to 200 m). Salinity, water temperature, *in situ* fluorescence (a proxy for chl *a*), pressure, density, conductivity, oxygen, oxygen saturation, and SPAR (surface PAR) were all measured using the CTD package mounted on the rosette sampler. Data presented herein are from surface (5 m) water samples, and have been limited to those that correspond with 32 viral production assays that were carried out (discussed below). Fig. 3.1 shows the locations where assays were done.

Chlorophyll a

Size-fractionated chl *a* concentrations were determined on replicate samples collected on 0.22, 2.0, and 20.0 μm nominal pore size, 47 mm diameter polycarbonate filters (Osmonics). Samples were extracted in 90% acetone for 24 h at 4°C and quantified using a solid-standard normalized 10-AU field fluorometer (Turner Designs) using the nonacidification protocol (Welschmeyer 1994).

Viral abundance

The abundance of viral particles was determined for whole water samples which were preserved in 2% final glutaraldehyde and then processed immediately at sea (Wen et al. 2004). Slide preparation involved staining 850 μl of sample with SYBR Green (Noble

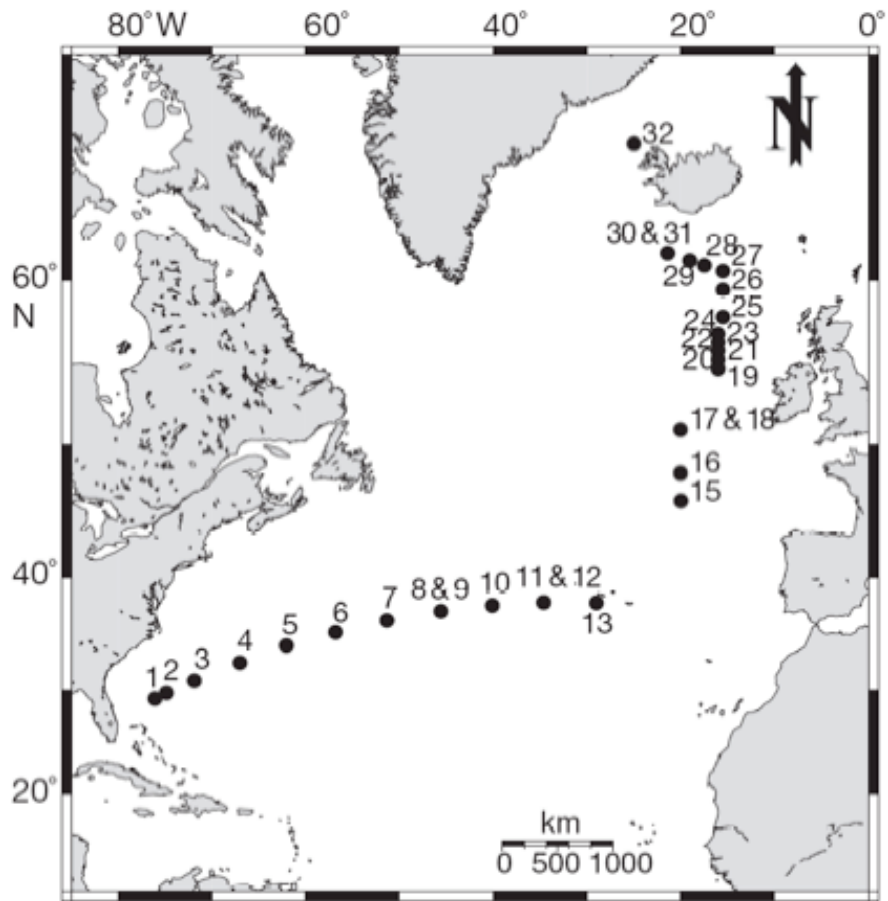


Figure 3.1. Locations where viral production estimates and corresponding measurements were completed. Numbers 1 to 13 correspond to stations within the Sargasso Sea; Numbers 15 to 32 correspond to stations within the North Atlantic.

and Fuhrman 1998). All slides were stored at -20°C until virus-like particles could be enumerated via epifluorescence microscopy using a Leica DMRXA with a 'wide blue' filter set ($\lambda_{\text{Ex}} = 450$ to 490 nm, $\lambda_{\text{Em}} = 510$ nm, with suppression filter at $\lambda = 510$ nm). For each slide, >20 independent fields were viewed with the total of each grid noted to ensure particles were evenly distributed across individual filters. Standard deviations were calculated from the range of counts per field per slide.

Viral production

Viral production was estimated based on the virus reduction assay (Wilhelm et al. 2002) with abundances for each time point enumerated as described above. Four of the dilutions were prepared using a Millipore lab scale tangential flow filtration system with $0.22\ \mu\text{m}$ filter cartridges (*vis-a-vis* Winget et al. 2005), although we have subsequently seen no difference between these 2 approaches (dead-end vs. tangential flow filtration, not shown). Briefly, 500 ml of sample water was reduced over a $0.22\ \mu\text{m}$ filter (either dead-end or tangential flow filtration), reducing free viral particles and briefly concentrating the cellular community. Cells were resuspended in 500 ml of ultrafiltrate (free of viral particles) and divided into three 150 ml samples within 250 ml clear bottles. Samples for viral particle and cell enumeration were taken every 2.5 h for a period of 10 h. Bottles were incubated in on-deck flow seawater incubators under ambient daylight attenuated through blue-tinted acrylic to simulate natural temperatures and light penetration through surface waters. Ultrafiltrate was generated from surface water from the same station where each viral production sample was taken. Standard deviations of production rate were calculated from the averaged slopes of independent triplicate samples (data plotted as virus-like particles over time).

Burst size and percentage of infected cells

A 50 ml sample of the whole water used for viral production estimation was preserved immediately with 2% glutaraldehyde (final) and stored at 4°C until processing (~ 2 mo post-cruise). Approximately 30 ml of sample was centrifuged onto carbon-coated collodian films on top of 400 mesh copper grids and each sample-laden grid then stained with 0.75% uranyl formate. Grids were examined using 100KeV on a Hitachi H-800

transmission electron microscope under magnifications of 15 000 to 20 000 $\times$ to scan over samples, 30 000 to 40 000 $\times$ to examine cells, and 50 000 $\times$ to photograph images. Burst size and abundance of visibly infected cells were determined by visual examination (Weinbauer and Suttle 1996). All burst sizes were considered minimal estimates as more particles may have been produced before lysis. Except for a few samples with very low cell abundance, a minimum of 1000 cells was examined for each sample. Because some of the samples from the Sargasso Sea had too few cells to allow complete examination, a subset of the results from several stations were combined and assumed to be constant for all of the Sargasso Sea.

Calculation of % MVL and % IC

The host mortality due to viral lysis (% MVL) was calculated by dividing the rate of viral production by the corresponding minimum burst size to determine the abundance of hosts lysed, and then dividing the quotient by the bacterial standing stock. Percentage of infected cells (% IC) was simply calculated from the percentage of visibly infected cells (VIC) as previously described (Binder 1999) using the following equation:

$$\% \text{ IC} = (7.1) \times (\text{VIC}) - (22.5 \times \text{VIC}^2)$$

Bacterial abundance and production

Bacterial abundance was determined using paraformaldehyde-fixed samples that were filtered onto 0.2 μm pore-size black polycarbonate filters (Porter and Feig 1980) and then stained with a solution containing 1 $\mu\text{g ml}^{-1}$ 4',6-diamidino-2-phenylindole (DAPI) in a phosphate buffer containing 0.5 M NaCl. The filter was mounted on a glass slide with a cover slip and total bacteria (including Archaea and picoplanktonic Cyanobacteria) were enumerated by epifluorescence microscopy using semi-automated image analysis. Bacterial production was estimated from leucine incorporation rates measured by standard methods (Kirchman 2001). Triplicate samples with ^3H -leucine (20 nmol l^{-1}) were incubated for 2 h at the *in situ* temperature. The incorporated ^3H -leucine was precipitated by trichloroacetic acid (TCA), collected by centrifugation and then rinsed with TCA and ethanol. The samples were then dried and radioassayed to determine ^3H -

leucine uptake. Killed controls were poisoned with TCA. A conversion factor of 1.5 kg C mol⁻¹ of incorporated leucine was used to calculate biomass production.

Bacterial biomass, cell size, and cell volume

Bacterial biomass was estimated from cell abundance data determined by epifluorescence microscopy and semiautomated image analysis of samples stained with 0.5 ng µl⁻¹ of DAPI. Cell sizes were estimated from the DAPI image as described previously (Cottrell and Kirchman 2003). Bacterial cell volumes were measured from solids of revolution constructed by digital integration (Sieracki et al. 1989).

Nutrient concentration and regeneration

Concentrations of nitrate + nitrite, ammonium, phosphate and dissolved silicate were determined colorimetrically (Grasshoff et al. 1999) by a standard autoanalyzer (Futura continuous flow analyzer, Alliance Instruments) as soon as the samples were collected at each process station. To provide an idea of virus-driven nutrient regeneration through both transects, estimates were calculated based on previously determined quotas of C (23.3 fg cell⁻¹) (Simon and Azam 1989), N (5.6 fg cell⁻¹) (Lee and Fuhrman 1987), and Fe (1.1 ag cell⁻¹) (Tortell et al. 1996) released as a result of daily viral lysis within a community.

Statistical correlations

For selected parameters in the Sargasso Sea and North Atlantic, linear regression of untransformed data was done using software associated with SigmaPlot (ver. 9.0). Since assumptions of normality were not met in distributions of all variables, the nonparametric Spearman Rank Correlation coefficient was calculated as a measure of correlation between all possible pairs of variables. Statistical analysis was done using the NCSS statistical analysis software package. Correlations warranting $p < 0.05$ were considered significant. Multivariate examinations of data were carried out using Canoco (ver. 4.5, Plant Research International). Canonical correspondence analysis (CCA) was performed to determine overall multivariate patterns in the data. Viral production and abundance, bacterial production and abundance, burst size, and cell volume were used as the

response (biological) variables, and latitude, total chl *a*, micro-, nano-, pico-chl *a*, salinity, and temperature as the predictor (environmental) variables. Monte Carlo permutation tests confirmed the significance of both canonical correspondence axes.

RESULTS

Size-fractionated chl *a* concentrations along the entire transect reflected an expected trend. Chl *a* was dominated by the picophytoplankton size fraction in the Sargasso Sea, while that in the North Atlantic had variable size distribution and higher concentrations (Fig. 3.2). Likewise, the abundance of virus-like particles followed a similar trend, with higher viral abundances in North Atlantic waters (Figs. 3.3A & 3.4A). Viral abundance in the Sargasso Sea showed a clear increase as the sampling headed northeast. However, the much higher viral abundances in the North Atlantic were highly variable and without a clear trend. Virus-like particle production rates in the Sargasso Sea and North Atlantic (Figs. 3.3B & 3.4B) did not follow the same trends as chl *a* concentrations or viral abundance. Production rates in the Sargasso Sea varied from $\sim 0.2 \times 10^8$ to 3×10^8 particles $L^{-1} h^{-1}$, showing a slight increase at stations in the middle of the transect but then decreasing down to the rate which predominated in the North Atlantic. With only one exception, the rates in the North Atlantic remained consistent and at a median level when compared to those of the Sargasso Sea.

The average minimal burst size for the Sargasso Sea ($n = 3$) was estimated at 11.67 virus-like particles per cell. For the North Atlantic samples, average minimal burst sizes were slightly higher (Fig. 3.5A) and were not significantly different between stations ($p > 0.05$). The percentage of visibly infected cells, however, showed no clear pattern for the North Atlantic samples (Fig. 3.5B).

To examine how viral production data related to environmental and other biological parameters, we performed several statistical analyses. Linear comparisons of viral production rate and chl *a* concentration (Fig. 3.6A), viral production rate and viral abundance (Fig. 3.6B), and viral abundance and chl *a* concentration (Fig. 3.7) demonstrated no overall correlations between these parameters across both regimes, but allowed for a clearer look at each separate regime. Results from the Sargasso Sea suggested a strong positive relationship between viral abundance and chl *a* concentrations

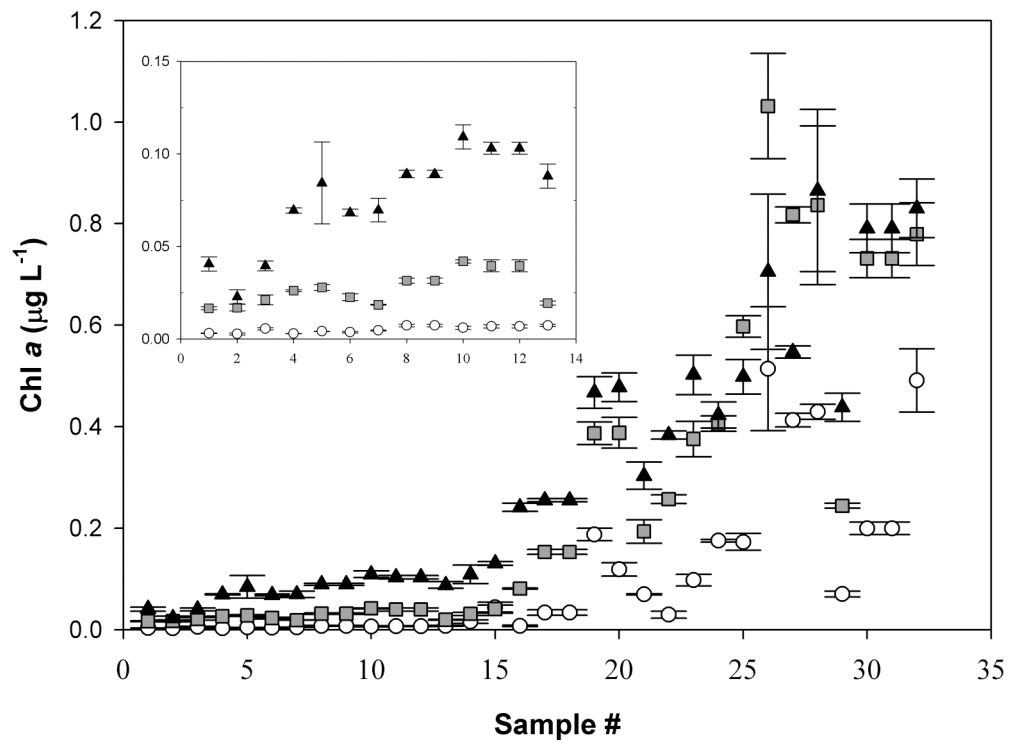


Figure 3.2. Size-fractionated chl *a* concentrations in surface water samples (taken along the cruise track) from which viral production assays were performed. Inset is a detailed view of the Sargasso Sea samples. (white circles) >20.0 μm , (grey squares) 2.0 to 20 μm , and (black triangles) 0.22 to 2.0 μm nominal pore size. Error bars are ranges of duplicate samples.

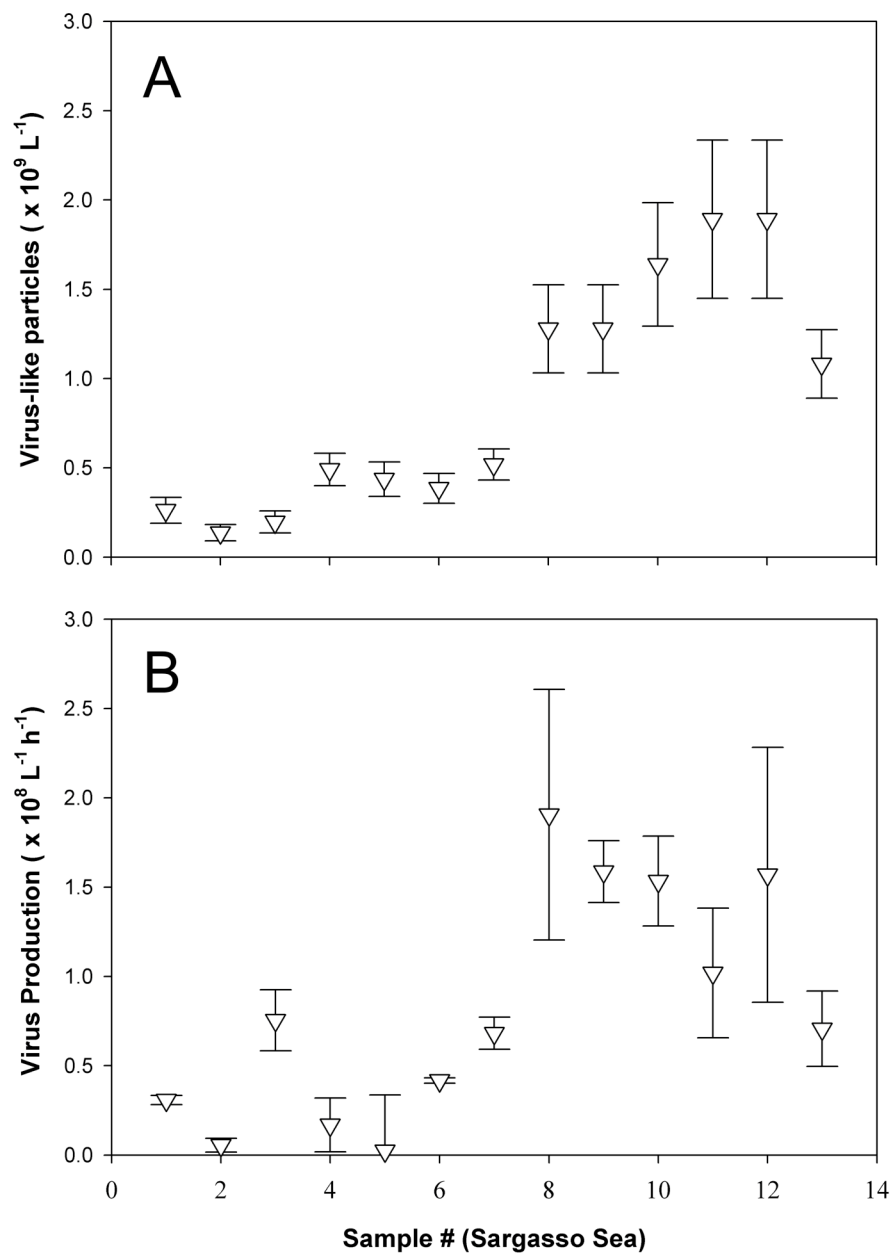


Figure 3.3. (A) Virus-like particle abundances in surface water samples taken along the Sargasso Sea transect, from which viral production assays were started. Error bars are variations in virus-like particle counts per field of view after 31 fields were viewed per sample. (B) Viral production rates of surface waters in the Sargasso Sea. Re-appearance of virus-like particles was enumerated over time after free particles were removed. Error bars are SDs of triplicate samples as described in ‘Materials and Methods.’

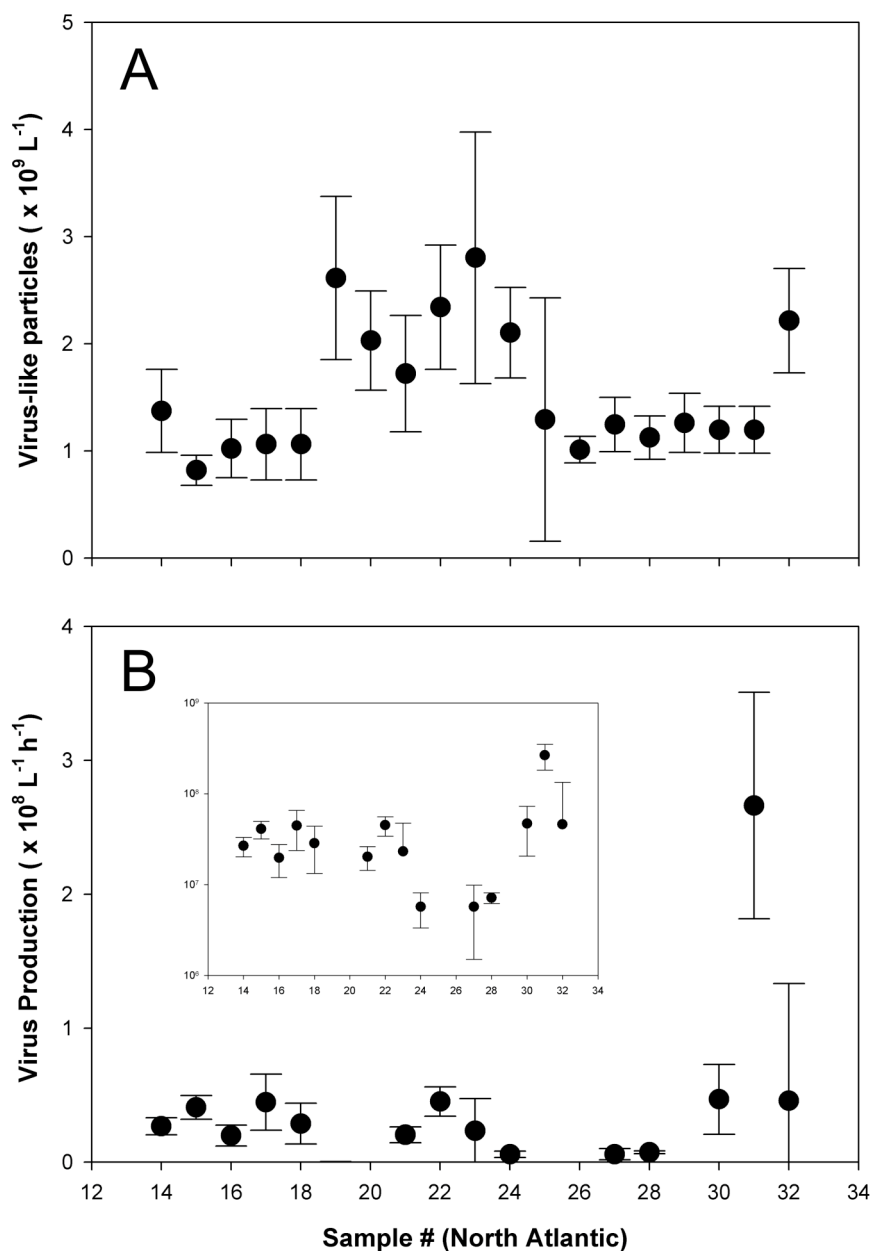


Figure 3.4. (A) Virus-like particle abundances in surface water samples taken along the North Atlantic transect, from which viral production assays were started. Error bars are variations in virus-like particle counts per field of view after 31 fields were viewed per sample. (B) Viral production rates of surface waters in the North Atlantic. Re-appearance of virus-like particles was enumerated over time after free particles were removed. Error bars are SDs of triplicate samples as described in ‘Materials and Methods.’ Inset is data plotted on a logarithmic scale.

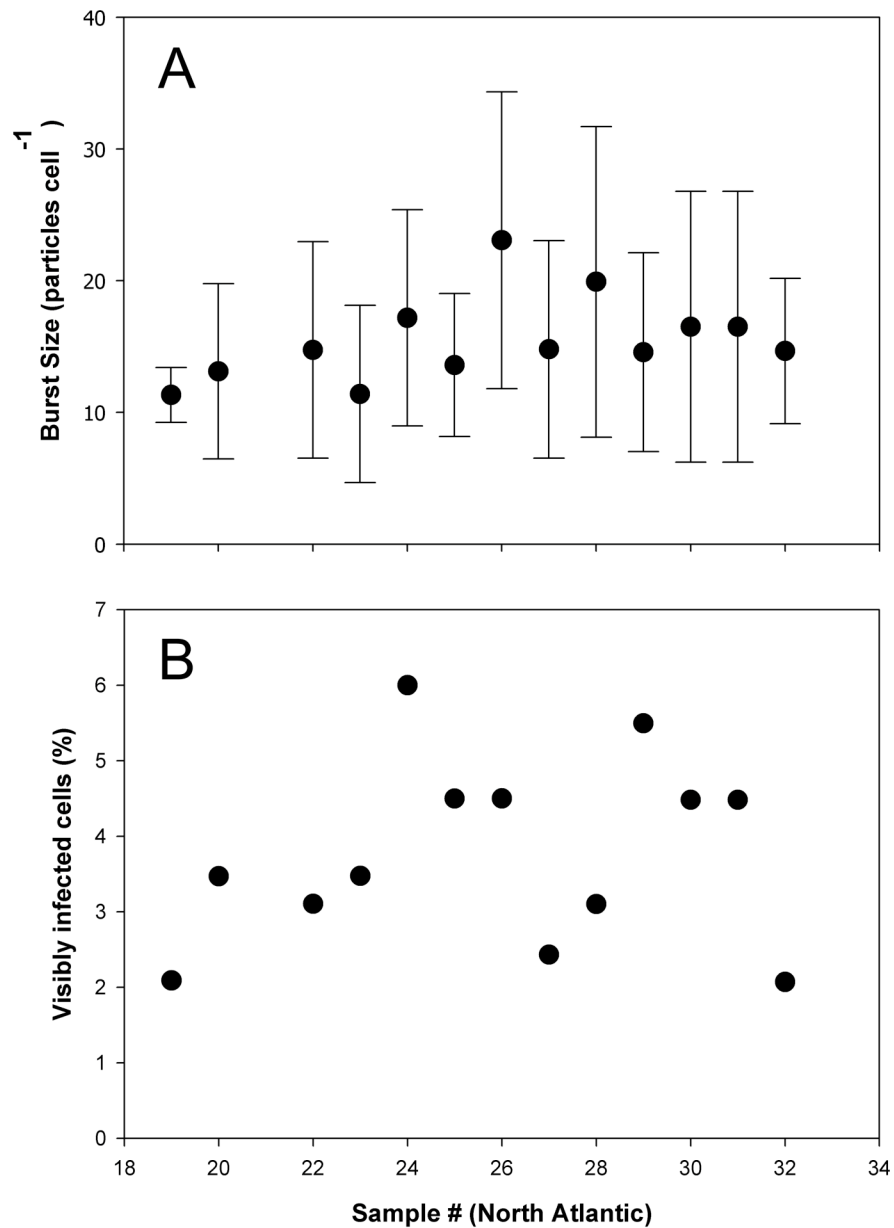


Figure 3.5. (A) Minimum burst sizes of infected cells in surface water samples from the North Atlantic. Error bars are variances of virus-like particles per cell within a sample. Sample 21 was lost during processing. (B) Percentage of visibly infected cells taken from surface water samples (North Atlantic) from which viral production assays were performed. Samples were viewed under transmission electron microscopy and analyzed for a minimum of 1000 cells. No variance estimates were generated, as only one sample was analyzed per station. Data from sample 21 not included.

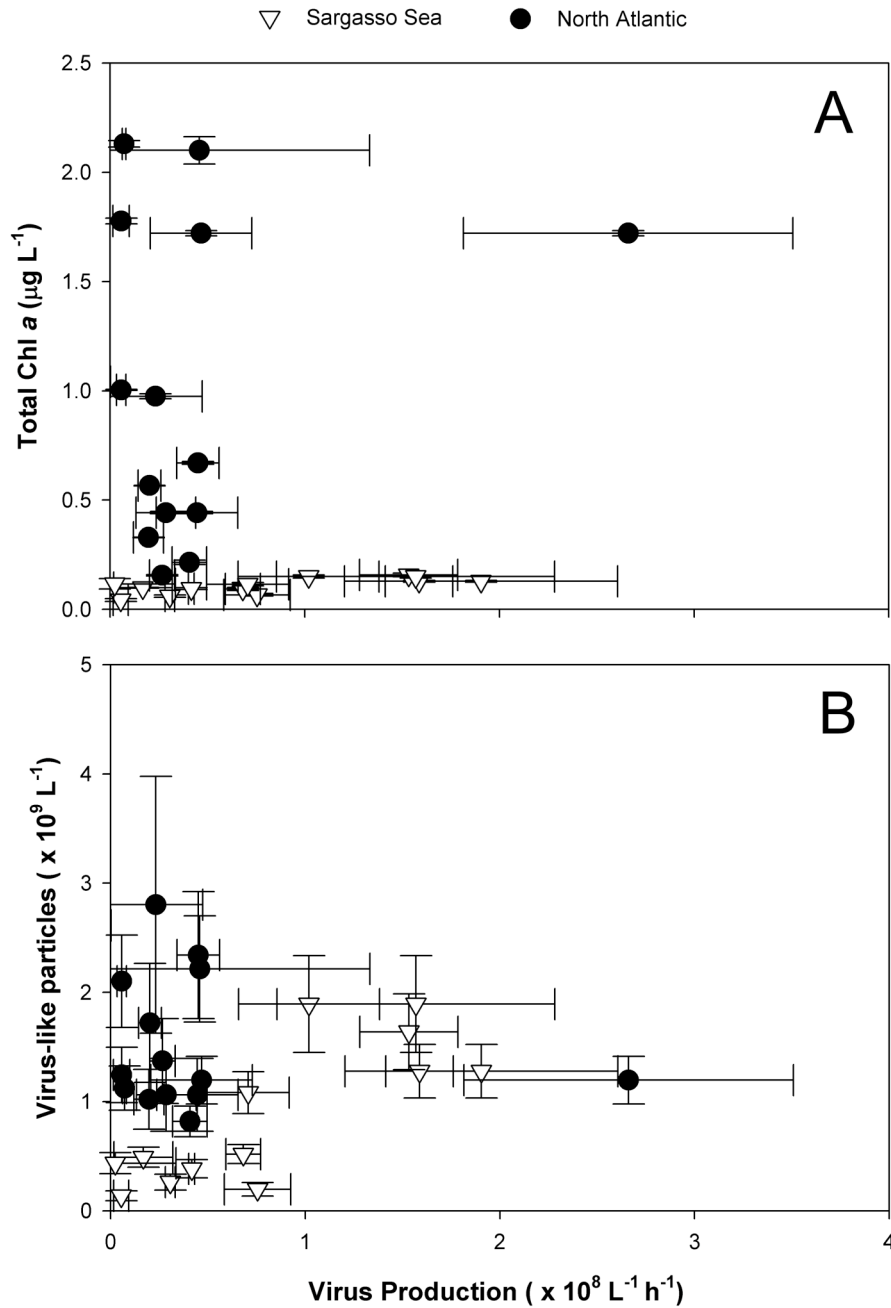


Figure 3.6. (A) Virus-like particle production rates vs. total chl *a* concentrations and (B) virus-like particle production rates vs. virus-like particle abundances for the Sargasso Sea and the North Atlantic. For both (A) and (B), all North Atlantic samples (except for sample no. 31) group together and separately from Sargasso Sea samples, which also group together. The linear relationship of the Sargasso Sea samples has an r^2 of 0.630 ($p = 0.0012$).

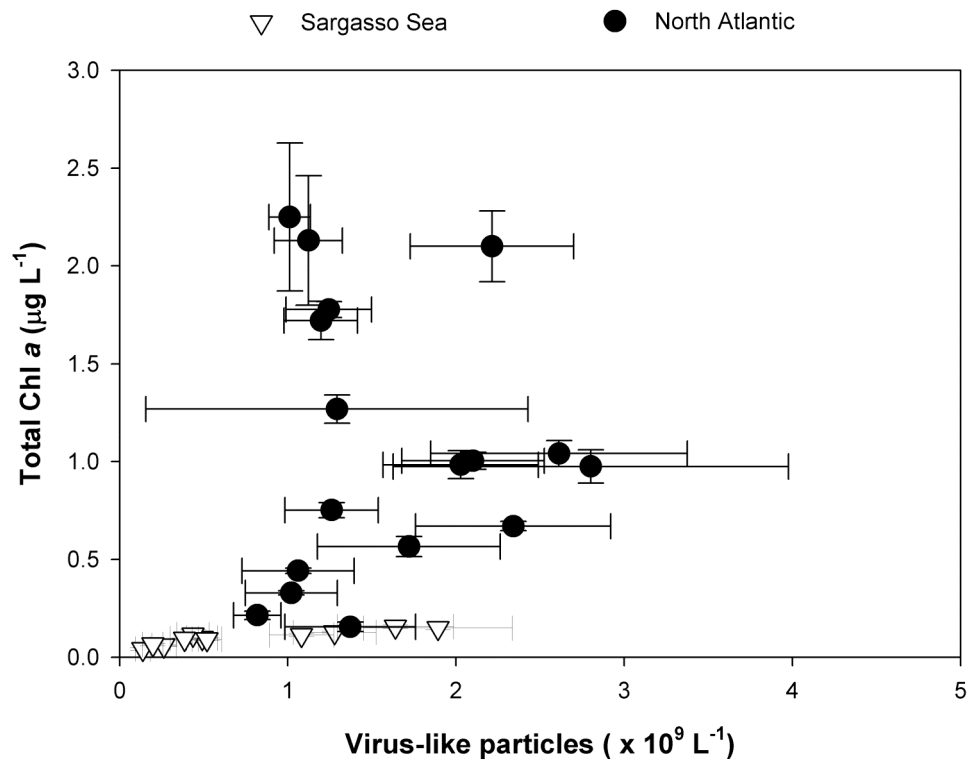


Figure 3.7. Virus-like particle abundances vs. total chl *a* concentrations for the Sargasso Sea and the North Atlantic. Linear regression of Sargasso Sea samples has an r^2 of 0.832 ($p < 0.001$).

(Fig. 3.7) ($r^2 = 0.832$, $p < 0.001$), and to a lesser extent between production rate and abundance (Fig. 3.6B) ($r^2 = 0.630$, $p = 0.0012$). Although no significant linear relationships in the same parameters were evident in the North Atlantic results, 2 trends were made evident in Fig. 3.6; a constant narrow range of viral production rate during large changes in chl *a* and in viral abundance. Additionally, we examined the ratio of viral to bacterial abundances for the Sargasso Sea (Fig. 3.8A) and the North Atlantic (Fig. 3.8B). Again, a statistically significant linear relationship was evident in the Sargasso Sea ($r^2 = 0.830$, $p < 0.001$), but not in the North Atlantic.

Because only a subset of the data analyzed is being discussed, an abbreviated set of the Spearman Rank Correlation analyses is given in Table 3.1. To simplify this massive dataset, relationships where no statistical significance was observed are not shown. Although a strong correlation between silicate and virus-like particle abundance was observed, no relationship was noted between viral abundance and the other nutrients measured. The large number of significant relationships ($p \leq 0.05$) between viral parameters and other biological estimates are discussed below.

Viruses were estimated to be responsible for the daily regeneration of ~9 to 761 nM of organic C, 1.9 to 157 nM of organic N, and 0.1 to 8 pM of organic F within the Sargasso Sea, with similar levels of activity in the North Atlantic (Table 3.2). Results shown are for each station where viral production rates were also determined.

CCA performed to determine overall multivariate patterns in the data, and Monte Carlo permutation tests that confirmed the significance of both canonical correspondence axes revealed the data to be in 2 clusters; one for the Sargasso Sea, and one for the North Atlantic, indicating that these are biologically different systems (Fig. 3.9). Redundancy analysis was also done using the same biological and environmental variables as for the CCA. This linear model also revealed a separation of Sargasso Sea and North Atlantic data (Fig. 3.10), supporting the CCA results.

DISCUSSION

During the NASB 2005 project, we had the opportunity to collect samples from 54 characterized stations in the North Atlantic. As part of this study, we were also able to collect a subset of data during the transit run from Florida to the Azores. While the latter

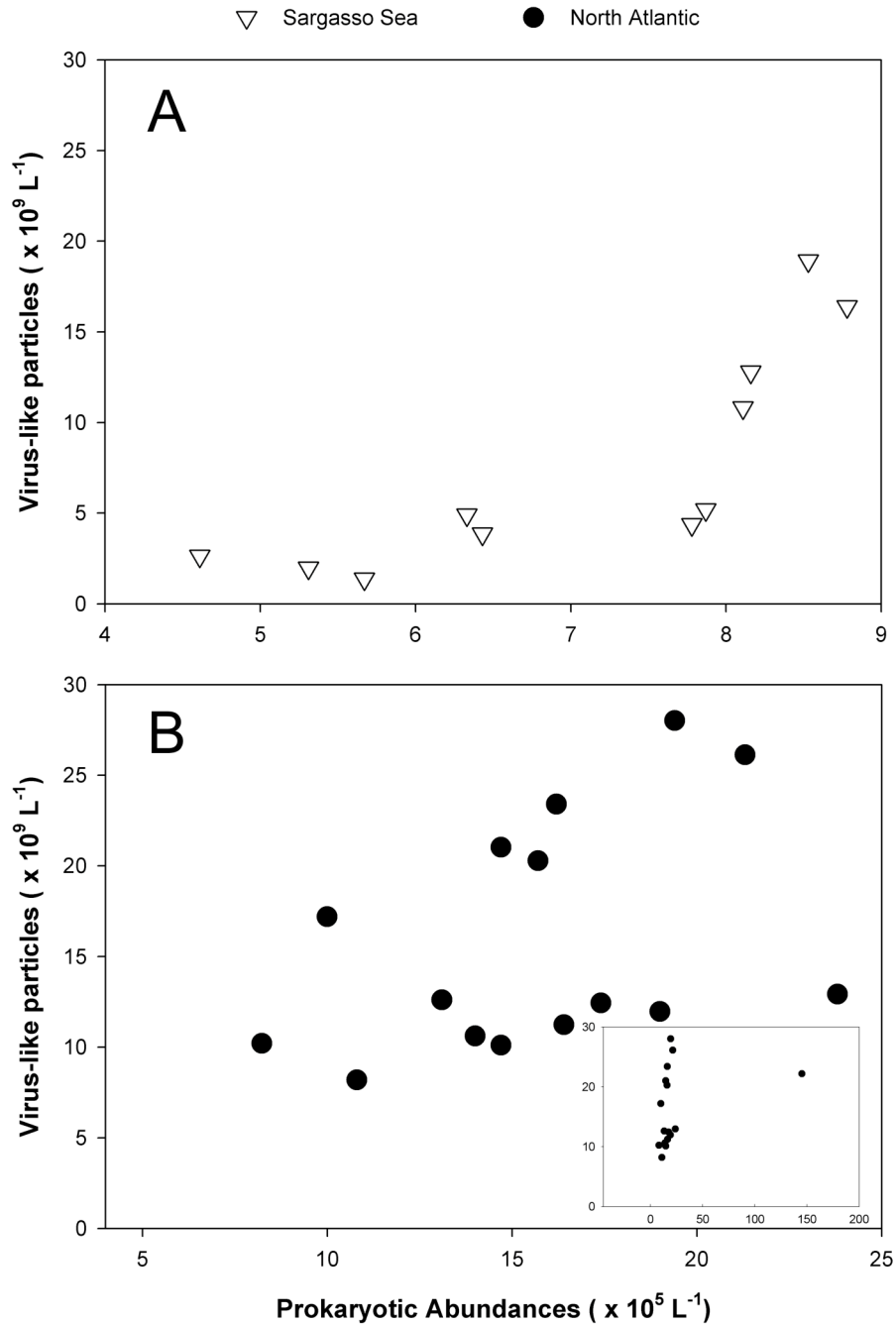


Figure 3.8. Viral vs. bacterial abundance in stations along the (A) Sargasso Sea, and (B) North Atlantic transects. Results show that bacterial abundance is a better predictor of viral abundance in the Sargasso Sea ($r^2 = 0.690$, $p < 0.001$) than in the North Atlantic ($r^2 = 0.137$, $p > 0.05$). North Atlantic data were analyzed without one outlier (sample 32, included in inset).

Table 3.1. Spearman rank correlation analysis contrasting viral production rates (VP) and viral abundance (VA) with biotic and abiotic parameters. Significant relationships ($p < 0.05$) are given in bold. ND = not determined, r_s = correlation coefficient, VIC = visibly infected cells.

Parameter	ENTIRE TRANSECT				SARGASSO ONLY				NORTH ATLANTIC ONLY			
	Virus Production		Virus Abundance		Virus Production		Virus Abundance		Virus Production		Virus Abundance	
	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p	r_s	p
Virus Abundance	0.028	0.882	x	x	0.725	0.005	x	x	-0.052	0.842	x	x
<u>Chlorophyll</u>												
total (> 0.2 μm)	-0.438	0.014	0.533	0.002	0.653	0.016	0.923	<0.001	-0.288	0.262	0.156	0.536
0.2 – 2.0 μm	-0.391	0.030	0.553	< 0.001	0.686	<0.001	0.967	<0.001	-0.132	0.615	0.225	0.371
> 20 μm	-0.383	0.033	0.499	0.004	0.852	<0.001	0.730	0.005	-0.276	0.284	0.088	0.728
2.0 – 20.0 μm	-0.417	0.030	0.524	0.002	0.625	0.022	0.818	<0.001	-0.273	0.289	0.142	0.575
Bacterial Abundance	-0.389	0.037	0.664	< 0.001	0.702	0.007	0.945	<0.001	-0.191	0.478	0.516	0.034
Bacterial Production	-0.392	0.036	0.409	0.025	0.572	0.052	0.789	0.002	0.087	0.740	-0.111	0.662
Cell volume	-0.382	0.037	0.257	0.163	-0.267	0.377	0.006	0.986	-0.276	0.284	0.382	0.118
Burst Size	-0.263	0.152	-0.013	0.946	0.000	1.000	0.000	1.000	0.233	0.367	-0.646	0.004
Salinity	0.448	0.028	-0.455	0.022	-0.117	0.748	-0.511	0.131	0.155	0.597	0.163	0.562
<u>North Atlantic only</u>												
% VIC	-	-	-	-	ND	ND	ND	ND	-0.283	0.373	-0.278	0.357
Ammonium	-	-	-	-	ND	ND	ND	ND	0.334	0.190	0.305	0.218
Nitrate/Nitrite	-	-	-	-	ND	ND	ND	ND	-0.298	0.245	0.229	0.362
Phosphate	-	-	-	-	ND	ND	ND	ND	-0.158	0.545	0.331	0.180
Silicate	-	-	-	-	ND	ND	ND	ND	0.072	0.783	0.808	<0.001

Table 3.2. Estimated regeneration of nutrients from the biological pool to the POM/DOM pool. Nutrient regeneration rates were determined from estimates of virus-induced mortality of bacterioplankton and estimated quotas for the following: C, 23.3 fg cell⁻¹ (Simon and Azam 1989); N, 5.6 fg cell⁻¹ (Lee and Fuhrman 1987); and Fe, 1.1 ag cell⁻¹ (Tortell et al. 1996).

	Virus Production Estimate Carbon (nM d ⁻¹)	N (nM d ⁻¹)	Fe (pM d ⁻¹)
1	122.7	25.3	1.3
2	21.9	4.5	0.2
3	301.5	62.1	3.1
4	67.3	13.9	0.7
5	9.3	1.9	0.1
6	166.6	34.3	1.7
7	272.5	56.1	2.8
8	761.1	156.7	7.8
9	634.0	130.6	6.5
10	612.4	126.1	6.2
11	407.2	83.9	4.1
12	626.7	129.0	6.4
13	282.4	58.2	2.9
14	80.2	16.5	0.8
15	122.7	25.3	1.2
16	59.3	12.2	0.6
17	134.2	27.6	1.4
18	86.1	17.7	0.9
21	60.8	12.5	0.6
22	142.5	29.3	1.5
23	95.0	19.6	1.0
24	15.5	3.2	0.2
27	17.9	3.7	0.2
28	16.8	3.5	0.2
31	751.7	154.8	7.7
32	145.5	30.0	1.5

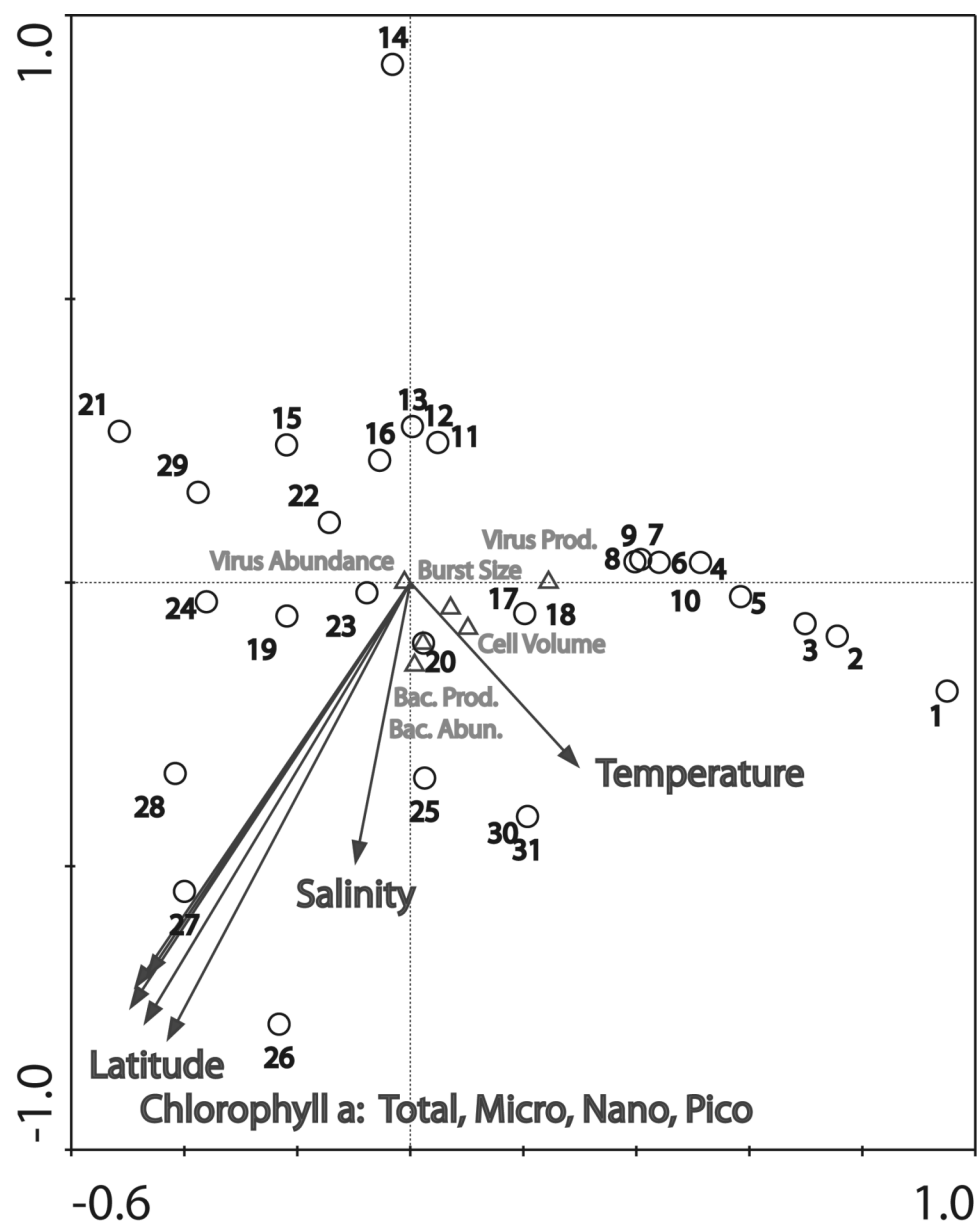


Figure 3.9. Plot of canonical correspondence analysis, a unimodal and constrained statistical model. Response (biological) variables = viral production (VP) and abundance (VA), bacterial production (BP) and abundance (BA), burst size (BS), and bacterial cell volume (CV). Predictor (environmental) variables = latitude, total chl *a*, micro-, nano-, pico-chl *a*, salinity, and temperature. Black circles: Sargasso Sea samples (1–14), white circles: North Atlantic samples (15–31), and white triangles: response variables. Outlier sample 32 was removed.

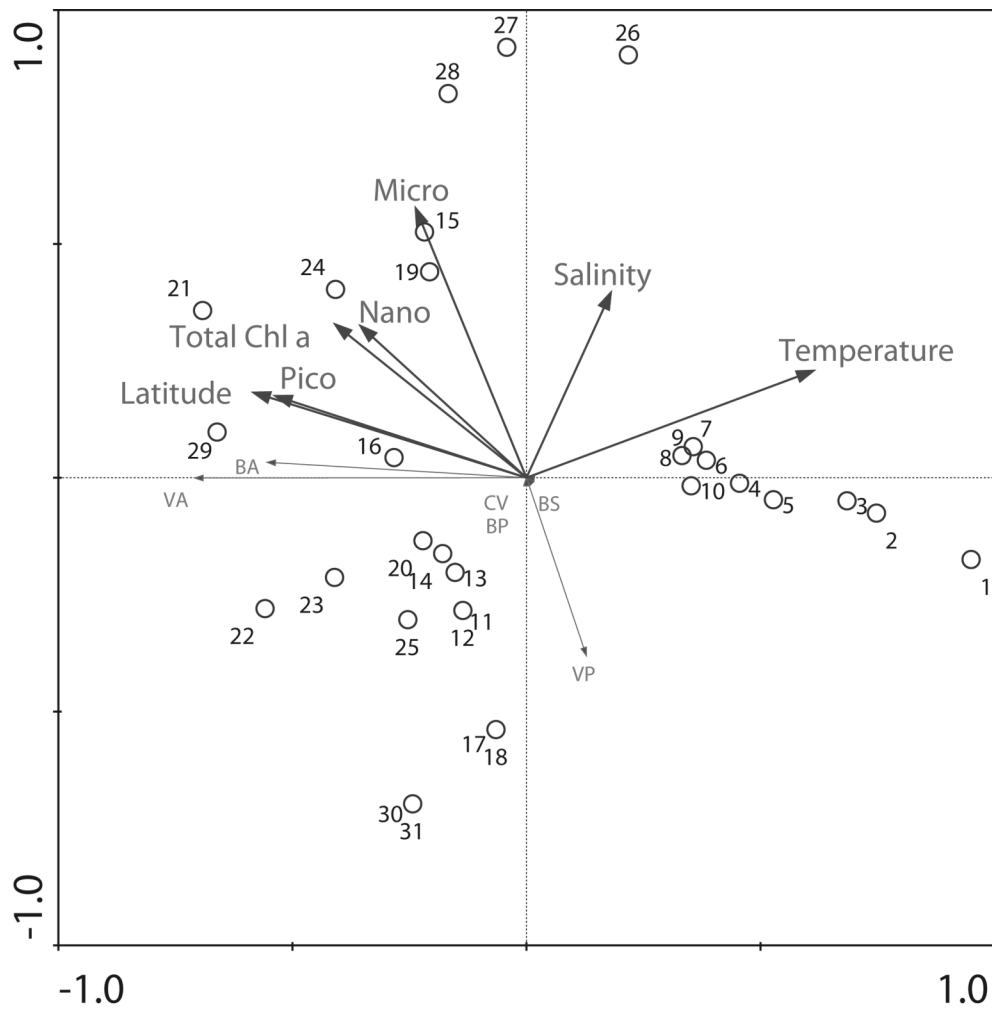


Figure 3.10. Plot of redundancy analysis, a linear counterpart to canonical correspondence analysis. Response (biological) variables = viral production and abundance, bacterial production and abundance, burst size, and cell volume. Predictor (environmental) variables = latitude, total chl *a*, micro-, nano-, pico-chl *a*, salinity, and temperature. Black circles: Sargasso Sea samples (1– 14), with 11–14 transitioning into the North Atlantic; white circles: North Atlantic samples (15–31). Outlier sample 32 was removed.

data was less complete than the data set for the North Atlantic, the results of both surveys are included in this paper in an attempt to shed some light on differences in the production, abundance, and estimated effects of viruses in these well-defined and trophically different systems.

Although interest in viral ecology has grown significantly in recent years, the factors which constrain the rates of viral production in aquatic systems have remained elusive (Brussaard et al. 2008; Suttle 2005; Wilhelm and Matteson 2008). Our data demonstrate that viral production rates are better predicted by common oceanographic parameters (e.g. chl *a*, viral abundance) in the Sargasso Sea than in the North Atlantic. The constraints on viral production within the less dynamic Sargasso appear to be a function of the relatively constant and comparably low abundance and diversity of the potential host population that contain both heterotrophic and phototrophic cells (Ducklow et al. 1993; Harrison et al. 1993; Steinberg et al. 2001, this study). In the more dynamic North Atlantic, with its episodic and transitory phytoplankton blooms, viral production rates appear to hold a more complex relationship with the coexisting biotic and abiotic factors. This relationship suggests a possible threshold effect of lesser known constraints on viral production and abundance that act/become more significant after thresholds have been reached by factors of greater impact. These situations render pair-wise analyses less effective and emphasize the need for methods and statistical analyses which can tease apart the true multi-factor relationships, including those involving specific virus-host populations.

Chl *a* concentrations measured over the length of the cruise reflect the disparate biomass of primary producers in these 2 oceanic systems. The oligotrophic Sargasso Sea was predictably dominated by picophytoplankton (Li et al. 1992) and the nutrient-rich North Atlantic was dominated by a series of events collectively termed the NASB (Martin et al. 1993). Abundance of viruses in both regimes reflected the expected trends based on previous examinations of oligotrophic and mesotrophic marine waters (Wommack and Colwell 2000). Abundance increased in more productive waters, but reached a plateau at the most productive stations, suggesting that a stable dynamic between the factors influencing virus like particle appearance and decay had been

reached (Weinbauer et al. 1999; Wilhelm et al. 1998a). Although total virus-like particle abundances remained within a narrow range in the North Atlantic samples, this does not necessarily indicate an unchanging viral community as turnover rates were high. It is likely that a change in host community structure would be mirrored by a change in the viral community structure, although this was outside the scope of the current study.

In this study, viral production assays were always carried out under *in situ* light conditions to reduce artificially imposed limits and to ensure accurate inclusion of viruses infecting phototrophic organisms. As such, most of the rates observed were within the ranges seen in other studies (Poorvin et al. 2004; Weinbauer et al. 2002; Wilhelm et al. 2002; Winter et al. 2004), although our highest estimate (from Stn 31) was an order of magnitude greater than rates observed in other systems, suggesting that this may be an outlier. Viral abundance and production rates in the Sargasso Sea transect increased as sampling progressed from the gyre into more productive waters. However, as we moved into North Atlantic waters, both abundance and production rates leveled off. Although it was not surprising to see an increase in viral production in waters of increased system productivity, that viral abundance and production remained constant during sampling across different algal bloom stages and across changes in community structure was not expected. These results suggest that the effects of system productivity on viral proliferation may have reached an upper limit (a threshold effect), leaving traditionally less influential factors responsible for minor fluctuations. Although this resulted in an apparent lack of relationship between system productivity and viral parameters, monitoring of changes in system productivity and viral parameters over such a large scale (2 distinct oceanic regimes) has provided us with a far more complete picture than would have been obtained from examining the North Atlantic system alone. It is not logical to assume that the relationships between system productivity and viral parameters would become insignificant or even suddenly cease to exist without some other factor or set of factors exerting influence.

Burst size estimates for lytic events in both the Sargasso Sea and North Atlantic were low in this study, ranging from ~11 to 25. Historically, estimates of burst size used in calculations of virus-inferred mortality have been high (up to ~500), and were based

on estimates taken from observations of primarily coastal populations (Weinbauer 2004; Wommack and Colwell 2000). While burst sizes were relatively invariant in the North Atlantic, the percentage of visibly infected cells fluctuated between 2 and 6% with no apparent pattern. These results indicate that there are factors that influence the rates of infection and lysis. Infection rate is most affected by contact rates and host density, while burst size depends on initial cell size and/or host metabolism, as well as viral particle size (Weinbauer 2004).

Cells in the Sargasso Sea samples were too sparse to allow estimation of the percentage of visibly infected cells at each station, but a collectively estimated burst size was clearly lower than those determined for the North Atlantic. The lower production of the Sargasso Sea could explain the lower burst size. Host cells may not have sufficient energy or nutrients to package as many viral particles as their counterparts in more productive waters. That bacterial burst sizes in the North Atlantic were higher, yet fairly constant, could be an indicator that host metabolism in this system had reached influential maxima. It has been accepted that system productivity via host metabolism is a significant factor limiting burst size (Weinbauer 2004). The fluctuating percentages of visibly infected cells in the North Atlantic could have been due to changes in specific host cell density and/or diversity.

Regulators of viral production

To examine how viral parameters relate to one another as well as to environmental variables, pair-wise correlations were first examined for all data followed by a more in-depth examination using Spearman Rank Correlation analyses. This analysis does not make assumptions on how the data are distributed (e.g. normal as in parametric analyses) and is therefore more suitable for analyzing results from natural samples. The multivariate approach (CCA) used to assess the grouping of North Atlantic and Sargasso Sea data (those common in both transects) was chosen because it is unimodal (*i.e.* does not assume linear relationships, allowing for data to find an optimum along a gradient) and constrained (*i.e.* allows axes to be made from the known environmental parameters to better show relationships). In short, CCA provided the statistical backing to differentiate the North Atlantic oceanic regime from that of the Sargasso Sea, allowing us to separate

these transects for parametric and nonparametric analyses discussed below. Redundancy analysis, which uses a linear species distribution model, also showed that the data fell in 2 distinct clusters. This analysis was performed to see any differences between unimodal and linear models of data representation. Both analyses, however, did not yield any multivariate relationships within or between the biological and environmental parameters examined.

Within the parametric analyses, viral production rate vs. total chl *a*, viral abundance vs. chl *a*, and viral production rate vs. viral abundance were of sufficient interest to present and discuss. From these 3 comparisons, we see different trends in the data depending on sample location. Correlations between viral production rates and chl *a* have been suggested to be due to the linkage between primary production rates and overall ecosystem energetics (Maranger and Bird 1995). In our study, viral production was positively correlated to chl *a* for the Sargasso Sea data set. In the North Atlantic, rates of viral production were relatively constant, and as such appeared independent of changes in phytoplankton biomass (chl *a*). Many new hypotheses arise from these observations, with the relative simplicity and stability of the system in question potentially being of great importance. The Sargasso Sea, with sparse host population abundance, minimal water movement (*i.e.* no interior currents), and persistent sunlight which can induce UV damage in viral particles (Wilhelm et al. 1998b), is an environment where viral production rates are most likely to be closely dependent on system energetics. In contrast, the North Atlantic is an environment where biological processes are more dynamic; periods of low productivity are contrasted by the significant bloom events of the NASB, and multiple currents provide mixing and turbulence as well as importation of nutrients (Ducklow and Harris 1993; Faure and Speer 2005; Gregg 2002; Limeburner et al. 2005). Factors controlling viral production within this region may thus be more complicated and intertwined. Transient changes in host populations along with changes in water column chemistry and mixing undoubtedly lead to rapid seasonal cycling of viral populations ('boom and bust'), somewhat contrasting with Thingstad & Lignell's (1997) 'kill the winner' hypothesis. Other factors such as the transient nature of water column clarity (due to biological fluctuations and mixing) in this region may influence processes

such as UV-mediated decay. The decay rate of viral particles was not examined in this study.

In contrast to previous efforts, our study included rates of production of virus-like particles infectious to both algae and bacteria (as estimates were made at *in situ* light levels and not in the dark). Weinbauer (2004) has previously suggested that correlations between viral and bacterial abundance exist primarily because most viruses are believed to infect bacteria. In our study, analysis of virus to bacterium abundance ratios suggests that a relationship exists for the Sargasso Sea (Fig. 3.8A) ($r^2 = 0.690$) but not for the North Atlantic (Fig. 3.8B) ($r^2 = 0.137$). However, again the grouping of data points across both regimes suggests that a threshold effect comes into play when bacterial abundance reaches 8×10^5 , perhaps explaining why no apparent relationship exists between these 2 parameters when looking solely at the North Atlantic data. It thus appears that viral production in the Sargasso is a function of bacterial activity and abundance. However, the strong correlation between viral abundance and chl *a* (Fig. 3.7) indicates that viral production is also a function of primary production and biomass, and this is supported by the significant correlation between viral abundance and viral production (Table 3.1) for the Sargasso Sea data. The same correlations may exist for the North Atlantic, but the higher primary and heterotrophic production rates may have masked the correlation of viral parameters to bacterial parameters and vice versa. This does not reject the possibility of a threshold effect as discussed earlier, which may be another complicating factor to this mixture of viral and system parameters. Overall differences between the 2 data sets clearly show that relationship patterns are regime dependent, and that analyses must examine data separately as well as collectively.

For the entire data set for both regimes, all of the biological parameters except burst size correlate significantly with viral production rates. Correlations with viral abundance are similarly significant except for cell volume. Further examination of the data, however, suggests that the combined observations are driven by the Sargasso Sea data, with only bacterial and viral abundance correlating significantly in the North Atlantic samples (although not under normal distribution as discussed previously). In all, these observations support the contention that viral abundance and production in the

constant Sargasso Sea environment are more predictable than in the dynamic North Atlantic system.

Also notable are several observations of weak, yet significant correlations that cannot be adequately explained due to limited sample size and range (Table 3.1). In the entire data set, but not for either of the individual transects, bacterial cell volume was negatively related to viral production, but not to abundance. Burst size displayed a negative relationship to viral abundance in the North Atlantic samples. It remains surprising that burst size and the percentage of visibly infected cells did not correlate with virus-like particle production rate or with abundance. One possibility is that burst size only influences viral abundance and production within certain limits (*i.e.* to the degree that host diversity and trophic status control the abundance of viruses produced). As suggested for marine phytoplankton, the available nucleotide resources may be of more direct influence on burst size (Brown et al. 2006), but genome sizes were not analyzed here. Cell size could be an indicator of the cell's ability to produce virus-like particles, with larger cells potentially having more energy stores to utilize and/or more machinery. This is also supported by the significant relationship between bacterial production and virus-like particle production rates and abundances. More studies focused on this area would be of value in learning the exact nature of the relationship between cell size and viral production. Indeed, given the constancy of our observations across these 2 regimes, our results indicate that viral production rate and abundance may be largely independent of the number of particles released during cell lysis.

Viral interactions with system geochemistry

In the combined data set, analyses demonstrate that viral abundance and production are tightly coupled to the biological parameters examined, yet are only significantly correlated to one geochemical parameter (silicic acid). However, estimates from this study suggest that viruses in both regimes regenerate significant concentrations of dissolved organic C as well as trace elements (as shown for N and Fe). These results support the growing evidence that viruses are major drivers of the nutrient regeneration that supports >70% of marine productivity (Raven and Falkowski 1999), and that viral

productivity is tied directly to biological rather than biogeochemical processes (Poorvin et al. 2004).

Dissolved silicate concentrations in the North Atlantic had a strongly significant positive correlation with viral abundance but not with production ($r_s = 0.808$, $p < 0.001$) in the Spearman analysis. We hypothesize that nutrient usage by primary producers would likely lead to increased cellular metabolism, and subsequently increase viral production rates and abundance. However, the lack of relationship between the viral parameters and nearly all of the nutrients suggests a disconnect between viral production rates and abundance from the trophic status of the North Atlantic. In part, this may be explained by nutrient concentrations that are subject to the episodic bloom events described above. In the case of the relationship between viral abundance and silicate, these results suggest that correlations between viral abundance and diatom proliferation may be strong and negative (floristic data is not currently available). Viral infection of diatoms in marine waters has rarely been documented, and viruses have only recently been characterized for a few diatoms (Nagasaki et al. 2004; Nagasaki et al. 2005). However, for other algae in this ecosystem (e.g. *Emiliania huxleyi*), viral infection has been very well studied and is thought to be common (Evans et al. 2007; Schroeder et al. 2003). As such, the correlation between high dissolved silicate and high viral abundance and production rates is probably a reflection of the role of viruses in the regulation of populations of algae other than diatoms when diatom densities are too low to draw down dissolved silicate. While system geochemistry appears to influence viral abundance and production, it is well known that viral activity also influences system geochemistry. In this study, viruses were estimated to be responsible for the daily regeneration of ~9 to 761 nM of organic C, 1.9 to 157 nM of organic N and 0.1 to 8 pM of organic Fe within the Sargasso Sea, with similar levels of activity in the North Atlantic (Table 3.2). While the amount of organic C (dissolved and particulate) recycled by viral activity is small compared to the 68 to 78 μM DOC pool in the Sargasso Sea (Carlson and Ducklow 1996), this pool should include a labile fraction which is highly bioavailable to the microbial community (Middelboe and Jorgensen 2006).

Perhaps more significant are the concentrations of N and Fe which are recycled back into the water column during the lysis of host cells. Previous studies have demonstrated that the vast majority of Fe released during virus-mediated cell lysis is organically bound (Poorvin et al. 2004). It can be anticipated that most of the N released is organic as well. Viral activity may thus drive biogeochemical pathways that favor organic rather than inorganic nutrient cycles, ultimately influencing community structure and diversity. Fe released by virus-mediated cell lysis (and other mechanisms such as grazing) has been shown to be highly bioavailable to various marine plankton (Gobler et al. 1997; Mioni et al. 2005; Poorvin et al. 2004). Therefore, it may be rapidly recycled even though it represents only 1 to 3% of the total dissolved Fe concentrations that have been previously measured in this system (Wu and Boyle 2002).

In summary, this study has provided insights on how virus-host interactions are affected by environmental parameters, and how the constraints on viral abundance and production may be a function of the stability of the regime being considered (constant vs. dynamic). The ability of viruses to respond to their environment highlights the adaptability of these obligate pathogens. Indeed, if we were to readdress previous efforts to understand how different plankton communities are structured (Hutchinson 1961), it becomes apparent that regardless of the trophic status or temporal stability, viruses must play a significant role in the outcome. The adoption of novel molecular tools which can more specifically address processes and functions associated with individual viral groups will, in the future, address many of the other questions raised over the course of this investigation.

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SECTION IV

VIRAL AND BACTERIAL PRODUCTION ACROSS THE
THERMAL GRADIENT OF THE WESTERN PACIFIC WARM
POOL: A MODEL FOR CLIMATE CHANGE STUDIES

This section is a version of an article that has been submitted to Aquatic Microbial Ecology under the same title by Janet M. Rowe, Jennifer M. DeBruyn, Leo Poorvin, Eric A. Webb, Zackary I. Johnson, Erik R. Zinser, and Steven W. Wilhelm.

Rowe, J. M. and others Submitted. Viral and Bacterial Production Across the Thermal Gradient of the Western Pacific Warm Pool: a Model for Climate Change Studies. Aquatic Microbial Ecology.

My contribution to this paper was participating in sample collection and onboard experiments during the WP2 cruise, processing viral and bacterial T= 0 abundances, virus production rates, chlorophyll *a*, burst size, and % of visibly infected cells data, compiling data from other cruise participants and colleagues for statistical analysis, analyzing results of statistical analysis with help from Jennifer DeBruyn, and most of the background information gathering and writing.

ABSTRACT

With growing concerns over current trends in climate, it is important to identify and investigate systems adapted to higher temperatures as well as those systems where transitions may be occurring. We recently completed a transect through the Western Pacific Ocean sampling across the natural temperature gradient through the Western Pacific Warm Pool to examine how temperature changes may influence microbial and viral abundances and processes. Although some trends appeared, temperature was not directly correlated to viral or host abundances or activities. Comparisons to two other large scale oceanic transects (the Sargasso Sea and North Atlantic, >10,000 km of open ocean in total) indicate however that there are regime-dependent correlations between viral metrics and environmental variables, as well as trends that become more apparent when all 3 data sets are combined. These observations highlight how variations between oceanic realms may influence viral and microbial activity.

INTRODUCTION

The combined activities of marine viruses represent a diverse range of significant contributions to the aquatic microbial community. As agents of mortality (Thingstad and Lignell 1997; Weinbauer 2004; Wommack and Colwell 2000), nutrient recycling and regeneration (Gobler et al. 1997; Middelboe and Jorgensen 2006; Poorvin et al. 2004; Wilhelm and Suttle 1999), and potentially horizontal gene transfer (Lindell et al. 2004), viruses are integral members of all aquatic ecosystems (Brussaard et al. 2008b; Wilhelm and Matteson 2008). Given the global importance of their microbial prey in global biogeochemical cycling (Field et al. 1998; Fuhrman 1992), viruses also have a global influence as predators of these key players: a top-down effect (Wilhelm and Suttle 1999).

Projected increases in sea surface temperatures, particularly at the polar regions (Solomon et al. 2007), have led researchers to query how alterations in temperature will affect the microbial community of the World's Ocean. Studies have been performed where natural communities are presented with proposed future temperature and/or CO₂ incubation conditions (Feng et al. 2008; Hoppe et al. 2008). While this work has indicated that there are likely to be shifts in community structure, it is in part confounded

by the need to use communities that are adapted to temperatures over experimental time scales (days) vs. realistic ones (years).

In the case of viruses, knowledge concerning the effect of temperature on their activity is limited with respect to *in situ* studies. We do know that temperature should affect virus-host interactions by altering host biochemistry and physiology, including rates of photosynthesis (Seaton et al. 1995) as well as microbial and algal growth (Eppley 1972; Ingram and Marr 1996; Shiah and Ducklow 1997b). Increased temperatures have been shown to induce virus infection of the zooxanthellae of *Anemonia viridis*, indicating a potential linkage to temperature-induced coral and anemone bleaching (Wilson et al. 2001). Other insights on how climate change may affect virus-host interactions come from higher plant systems with a range of results (increases to decreases in infectivity, to no effect observed) (Boland et al. 2004).

Research on the effects of temperature on bacterial growth has provided a preliminary insight into the effects on marine communities. It is likely that increasing temperature increases the ability of the cell to assimilate dissolved organic material (DOM), providing a link between temperature and bacterial production (Kirchman and Rich 1997; Kirchman et al. 1995; Pomeroy and Wiebe 2001). Studies using ^3H -leucine and ^3H -thymidine tracer methods together suggest that warmer, or more favorable environments, trigger DNA replication over protein synthesis to promote cell replication, while cooler, less favorable environments, trigger the opposite to maximize survival (Shiah and Ducklow 1997a). Muren et al. (2005) showed that increases from 5-10° C result in increases in the ratio of heterotrophic to autotrophic biomass and decreases in the carbon fixation to respiration ratio. Overall, there are indications that in temperate environments, climate change may affect ecosystem function by altering the balance between heterotrophy and autotrophy. More recently, analysis of the microbial community in the tropical Atlantic showed changes in bacterial and archaeal community composition were related to changes in temperature (Winter et al. 2008).

While viral and bacterial activity during climate change mesocosm experiments and bacterial activity *in situ* could provide interesting insights, a more valuable approach may be to examine communities (both hosts and viruses) along natural temperature

gradients where surface transport mimics longer-term adaptations to higher temperatures. While these studies preclude the associated effects of ocean acidification (Doney 2006), they do allow for examination of not only direct temperature effects, but also the effects of change in temperature on water column structure.

Our goals within this study were two-fold. Firstly we wanted to collect data from across a large oceanic range to determine how parameters that may constrain the activity of marine viruses varied in relation to virus production and abundance. Traditionally, information on marine viruses has been collected from coastal or short open ocean transects (Brussaard et al. 2008a; Hewson and Fuhrman 2007; Middelboe et al. 2006; Wilhelm et al. 2003; Winter et al. 2008; Wommack et al. 1992). However, a recent research cruise to the North Atlantic through the Sargasso Sea allowed researchers a chance to examine these parameters and relationships over > 8,000 km of open ocean (Rowe et al. 2008). Data from this previous Sargasso Sea transect demonstrated that sea surface temperature significantly correlated ($p < 0.05$) with virus abundance, virus production rate, size-fractionations of chl *a* (> 20.0 μm , 2.0 - 20.0 μm , and 0.22 - 2.0 μm), cell abundance, and bacterial biomass production rate (Rowe and Wilhelm, unpublished; this study, see Table 4.1). These observations indicated that increasing temperatures may have a significant influence on viruses and their activity.

As such, within this project we wanted to also determine how a naturally occurring temperature gradient may influence viral and bacterial production and abundance, and their relationship with other environmental metrics. The Western Pacific provided an area of open ocean with a natural temperature gradient from ~21 to 30.5° C inside the Western Pacific Warm Pool. This region is not only the origin of the El Niño/Southern Oscillation event, which makes it of interest for general climate studies (Yan et al. 1992), but it is also an ideal area for studies on microbial communities that are naturally acclimated to temperatures that are higher than anywhere else in the open ocean. The Western Pacific Warm Pool transect (> 7,000 km) provided the chance to observe changes in microbial dynamics and oceanic parameters over a temperature gradient that could mimic future climate change temperature scenarios.

Table 4.1. Spearman rank correlation analysis contrasting virus production rates and viral abundance with biotic and abiotic parameters. Data from the Sargasso Sea and North Atlantic are reprinted from Rowe et al. 2008. Relationships considered significant in this study ($p < 0.05$) are given in bold. ND – not determined, r_s – correlation coefficient, p – probability that association is due to chance.

	ALL TRANSECTS				WESTERN PACIFIC				SARGASSO SEA				NORTH ATLANTIC					
Parameter	Production		Abundance		Production		Abundance		Production		Abundance		Production		Abundance			
	r _s	p	r _s	p	r _s	p	r _s	p	r _s	p	r _s	p	r _s	p	r _s	p		
Virus Abundance	0.049	0.775	x	x	-	0.517	0.154	x	x	0.725	0.005	x	x	-	0.052	0.842	x	x
<u>Chlorophyll</u>																		
total (> 0.22 μm)	-	0.245	0.423	0.001	0.152	0.676	0.291	0.167	0.653	0.016	0.923	<0.001	-	0.288	0.262	0.156	0.536	
> 20 μm	-	0.478	0.226	0.098	0.127	0.726	-	0.143	0.852	<0.001	0.730	0.005	-	0.276	0.284	0.088	0.728	
2.0 – 20.0 μm	-	0.398	0.319	0.018	0.079	0.829	-	0.845	0.625	0.022	0.818	<0.001	-	0.273	0.289	0.142	0.575	
0.22 – 2.0 μm	-	0.370	0.453	0.001	0.164	0.651	0.312	0.138	0.686	<0.001	0.967	<0.001	-	0.132	0.615	0.225	0.371	
Cell Abundance	-	0.774	0.066	0.650	0.552	0.098	-	0.417	0.702	0.007	0.945	<0.001	-	0.191	0.478	0.516	0.034	
Bacterial Production	-	0.874	0.272	0.056	-	0.855	-	0.397	0.572	0.052	0.789	0.002	0.087	0.740	-	0.111	0.662	
Contact Rate	-	0.487	0.727	<0.001	-	0.668	0.854	<0.001	0.588	0.074	0.993	<0.001	0.042	0.878	0.911	<0.001		
Burst Size	0.119	0.509	0.046	0.791	0.200	0.704	-	0.285	0.000	1.000	0.000	1.000	0.233	0.367	-	0.646	0.004	
Salinity	0.011	0.951	-	0.003	-	0.603	-	0.630	-	0.748	-	0.131	0.155	0.597	0.163	0.562		
Temperature	0.206	0.251	0.087	0.550	-	0.987	-	0.440	-	0.050	-	0.001	0.004	0.990	0.072	0.791		
PO ₄	---	---	---	---	-	0.627	0.038	0.865	N/D	N/D	N/D	N/D	-	0.158	0.545	0.331	0.180	
Nitrate + Nitrite	---	---	---	---	0.103	0.777	-	0.211	N/D	N/D	N/D	N/D	-	0.298	0.245	0.229	0.362	
Silicate	---	---	---	---	-	0.907	-	0.911	N/D	N/D	N/D	N/D	0.072	0.783	0.808	<0.001		
NO ₂	---	---	---	---	0.527	0.117	-	0.218	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	N/D	

MATERIALS AND METHODS

Western Pacific Cruise Transect

Sampling was completed onboard the RV 'Kilo Moana' during the WP2 (Western Pacific Warm Pool) cruise (Jan. 3-Feb. 11, 2007). Daily CTD data and water samples were collected while traveling through the Western Pacific Warm Pool (9° 15' 1.018" S, 169° 59' 58.621" E, see figure 4.1 for sampling locations). For this study, all samples were collected at 5 m during morning (8 A.M.) casts. Water temperature, salinity, *in situ* fluorescence (a proxy for chlorophyll *a*), oxygen, conductivity, pressure, and density were measured by the ship's CTD package attached to the rosette sampler.

Nutrient Analysis

Surface water samples for nutrient analyses were collected from a trace metal clean rosette, 0.4- μ m filtered, and rapidly frozen at -20° C. Dissolved inorganic phosphorus (DIP) and nitrate+nitrite (DIN) concentrations were determined at Oregon State University (J. Jennings) using a hybrid analyzer that includes components from a Technicon AutoAnalyzer II (used for phosphate) and an Alpkem RFA 300 (used for the nitrate + nitrite). The detection limits for these analyses were $\sim 0.006 \mu\text{M}$ for phosphate and $\sim 0.05 \mu\text{M}$ for nitrate + nitrite.

*Discrete Chlorophyll *a**

Size-fractionated chlorophyll *a* (chl *a*) concentrations were performed in duplicate for pico- (0.22 – 2.0 μm), nano- (2.0 – 20.0 μm), and microphytoplankton (> 20.0 μm) size classes (parallel filtrations) of phytoplankton biomass, using the corresponding nominal pore-size, 47-mm diameter polycarbonate filters (Osmonics). Assessments were made using the non-acidification, standard method with extraction of chl *a* in 90% acetone during a 24 hour incubation at + 4° C (Welschmeyer 1994). Chl *a* concentrations were determined using a solid-standard normalized 10-AU field fluorometer (Turner Designs).

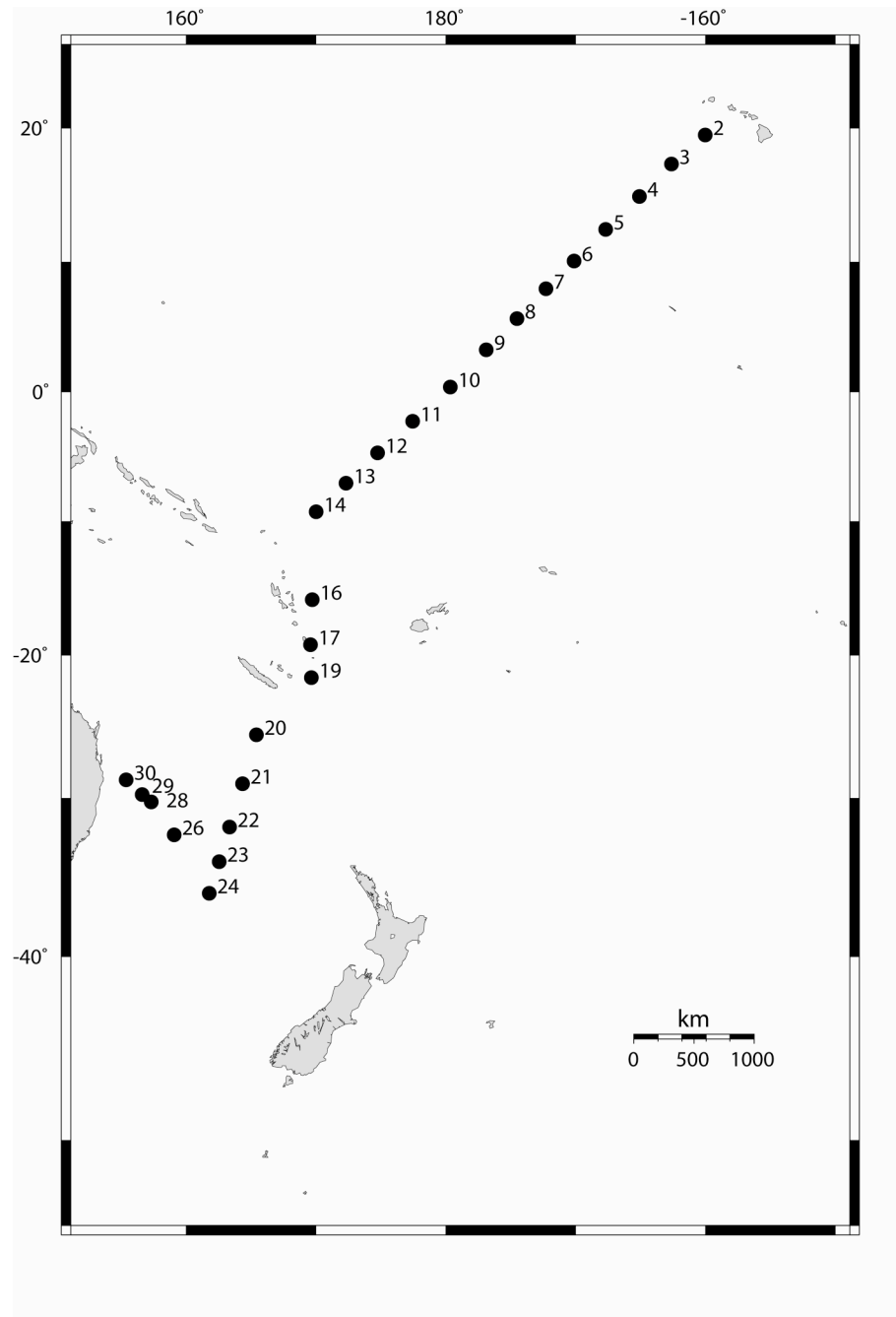


Figure 4.1. Data ranges for selected measurements in the Sargasso Sea, North Atlantic, and Western Pacific. Sargasso and North Atlantic data are further described elsewhere (Rowe et al. 2008).

Virus Abundance

Virus-like particle abundance was determined observing recommendations to prepare microscopic slides immediately after sample collection (Wen et al. 2004). Slides were stored at -20° C until particles could be enumerated. Briefly, virus-like particle abundance was examined with epifluorescence microscopy using SYBR Green I (Noble and Fuhrman 1998) on a Leica *DMRXA* microscope with a “wide blue” filter set. Only single microscopic slides were prepared for each *in situ* sample with the variation of particles over the > 20 fields of view examined, used to estimate analytical variation.

Virus Production

The virus reduction assay (Wilhelm et al. 2002) was used to measure virus-like particle production rates. For a 500 mL sample, viruses were reduced over a 0.22 µm filter and then cells (briefly concentrated) were resuspended in 500 mL of ultrafiltrate generated from water at the same depth and station. Triplicate 250 mL bottles with 150 mL each of the prepared sample were incubated under *in situ* temperatures and light so as to avoid any additional artifacts. Bottles were incubated for 10 h with samples for virus enumeration taken every 2.5 h. Time zero (T = 0) cell abundance was also determined with epifluorescence microscopy, using Acridine Orange stain (Hobbie et al. 1977). All microscopic slides were prepared immediately after sampling. Production rates were calculated as the average of virus reoccurrence rates in the three independent bottle incubations. Rates were corrected for host cell losses during processing by multiplying by the ratio of experimental total to experimental T = 0 cell abundance.

Water Column Bacterial Abundance, Production, and Viral and Bacterial Contact Rates

Total bacterial abundance for water samples at each station was determined by flow cytometry. Subsamples for flow cytometry were collected and stored in the dark until live processing (within 3 hours) for eukaryotic phytoplankton populations or frozen with 0.125% glutaraldehyde at -80° C following Vaultot et al. (1989) for later analysis for prokaryotic populations (*Prochlorococcus*, *Synechococcus*, and total bacteria). Samples for total bacteria (including Archaea) were stained with SYBR Green I prior to analysis,

following Marie et al. (1997). All flow cytometry samples were run on a Becton Dickinson FACSCalibur flow cytometer modified with a syringe pump for quantitative sample delivery. Particles were excited with 488 nm excitation (15 mW Ar laser) and forward ($< 15^\circ$) scatter, side (90°) scatter, green (530/30 nm) fluorescence, orange fluorescence (585/42 nm) and red fluorescence (> 670 nm) emissions were measured. *Prochlorococcus*, *Synechococcus*, total bacteria, pennate diatoms and other eukaryotic phytoplankton (all less than $< \sim 20 \mu\text{m}$) were classified based on their characteristic flow cytometric signatures relative to standard fluorescent microspheres (Polysciences) following Olson et al. (1985) for phytoplankton or Marie et al. (1997) for total bacteria.

Calculations for virus-bacterium contact rates were completed according to (Murray and Jackson 1992);

$$\text{contact rate} = (2S\pi\omega D_v)VB$$

where S is the dimensionless Sherwood number; estimated at 1.06 (Wilhelm et al. 1998) based on assumptions of 10% of cells being motile (Mitchell et al. 1995). ω is the estimated diameter of a marine bacterium (0.45×10^{-4} cm) based on work by Lee and Fuhrman (1987). D_v , the diffusivity of viruses, is estimated at $3.456 \times 10^{-3} \text{ cm}^2 \text{ d}^{-1}$. V and B are the abundances per mL of viruses and bacteria, respectively.

Heterotrophic bacterial biomass production rates were measured using the microcentrifuge method of determining ^3H -leucine incorporation described by (Kirchman 2001) using 2.0-ml screw cap copolymer microfuge tubes (Phenix) (Pace et al. 2004). Independent triplicate samples were corrected using killed (5% TCA) controls to provide average rates for each water sample.

Minimum Burst Size and Percentage of Visibly Infected Cells

Burst size and % of visibly infected cells were determined by transmission electron microscopy as described previously (Weinbauer and Suttle 1996). Samples were preserved with 2% (final concentration) glutaraldehyde and stored at 4°C until processing (~ 8 months). Centrifugation was used to collect ~ 30 mL of sample on top of 400-mesh copper grids covered with collodian film and coated with carbon. Samples on top of grids were stained with 0.75% uranyl formate. Prepared grids were then viewed

with a Hitachi H-800 transmission electron microscope under various magnifications (~15,000-20,000 for scanning and ~30,000-40,000 for examining cells).

Statistical Correlations

Linear regressions of select parameters were performed using SigmaPlot (ver. 10.0) on untransformed data. Spearman rank correlations (a non-parametric analysis) were performed using NCSS and SPSS to generate correlations between all pairs of variables. Significance was defined as $p < 0.05$. CANOCO (ver 4.5, Plant Research International) was used for Canonical Correspondence Analysis (CCA), with the significance of the axes determined by Monte Carlo permutation tests.

RESULTS

Figure 4.2 shows A) temperature, B) salinity, C) size-fractionated chl *a*, D) viral abundance, E) viral production rate, F) bacterial abundance, and G) bacterial biomass production rate at 5 m (depth) across the Western Pacific Warm Pool transect (see Fig. 4.1). Temperatures increased and then decreased across the transect, with the maximum temperature (30.5 °C) inside the Warm Pool at station 14 (9° 15' 1.018" S, 169° 59' 58.621" E). Discontinuities in salinity (Fig. 4.2B) demonstrate that these were in part due to changing water masses. Size-fractionated chl *a* concentrations show a consistent dominance of picophytoplankton (0.2-2.0 µm) with peak concentrations just before and just after the Warm Pool, while nano- (2.0-20.0 µm) and microphytoplankton (> 20.0 µm) remained at fairly low concentrations. Nanophytoplankton mirror the picophytoplankton trends with smaller, but still significant ($p < 0.05$) increases before and after the Warm Pool. Estimates of viral abundance also showed small, but significant ($p < 0.05$) fluctuations across the transect, although no significant overall trends were noted. Similarly, viral production rates demonstrated very few significant changes and no clear trend across this transect. Bacterial abundance fluctuated without any obvious pattern, which contrasts with bacterial biomass production rates, suggesting that bacterial production is not a simple function of bacterial abundance, or *vice-versa*. The trend of bacterial biomass production rate mirrors that of temperature suggesting a possible relationship.

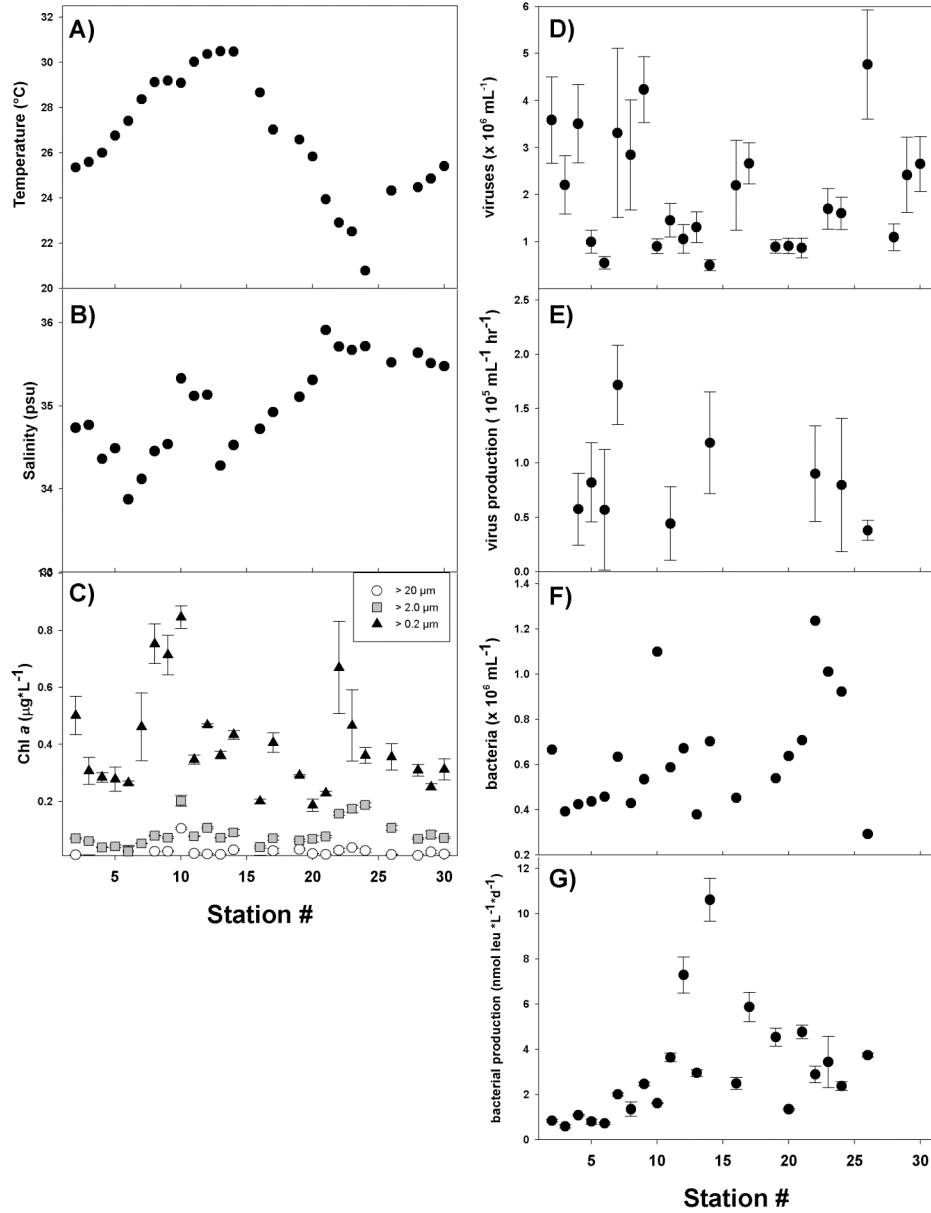


Figure 4.2. (A) Temperature. (B) Salinity. (C) Size-fractionated chlorophyll *a* concentrations: Circles (> 20.0 µm), Squares (2.0 - 20.0 µm), and Triangles (0.22 – 2.0 µm). Error bars indicate the range of duplicate samples. (D) Virus-like particle abundance measurements. Error bars indicate the variation found after 31 fields of view were counted. (E) Virus-like particle production rate measurements. Error bars indicate the standard deviation from triplicate samples. (F) Bacterial abundance. (G) Bacterial biomass production rate measurements. Error bars indicate the standard deviation of triplicate samples.

Due to the potential importance of temperature in the Western Pacific, pair-wise comparisons were generated to examine correlations among measured parameters (Fig. 4.3). Temperature was compared with A) viral abundance, B) viral production rate, C) bacterial abundance, and D) bacterial biomass production rate. Viral abundance and production were constant across the temperature range. With one exception, bacterial abundance above 24° C appeared to hit a maximum just below 0.7×10^6 mL, while samples below 24° C showed a higher average (although $n = 3$). While bacterial biomass production rate compared with temperature at first look presents a positive relationship, no significant correlations were observed.

Previously, viral and bacterial abundance and production rates were examined across the Sargasso Sea and North Atlantic, but their relationship to temperature was not analyzed in detail (Rowe et al. 2008). Figure 4.4 shows a comparison of A) viral abundance, B) viral production, C) bacterial abundance, and D) bacterial biomass production rate vs. temperature for the combined Atlantic and Pacific transect data. The North Atlantic viral abundances and production rates were comparable with the Sargasso Sea and Western Pacific. While the lowest viral abundances and some of the lowest production rates were observed in the Sargasso Sea, the abundance and production rates of viruses were consistent across all temperatures as well as the different regimes.

Bacterial abundance was highest in the North Atlantic, with the Sargasso Sea and Western Pacific, having comparable (lower) abundances. In contrast, the majority of bacterial production rates in the Western Pacific are significantly higher than the Sargasso Sea and even the highly productive North Atlantic. The highest bacterial production rate is nearly an order of magnitude higher than the lowest production rates observed in all 3 transects.

Parametric analyses are not always ecologically relevant statistical methods because basic assumptions (e.g. normal distribution) may not be met, and such is the case with this data set. Spearman rank correlation is a non-parametric means of assessing significant correlations (*i.e.* does not assume the data to be normally distributed, nor does it require data be transformed to interval scales) and is therefore better suited to the data we collected. For this reason, we undertook a Spearman rank correlation analysis. All

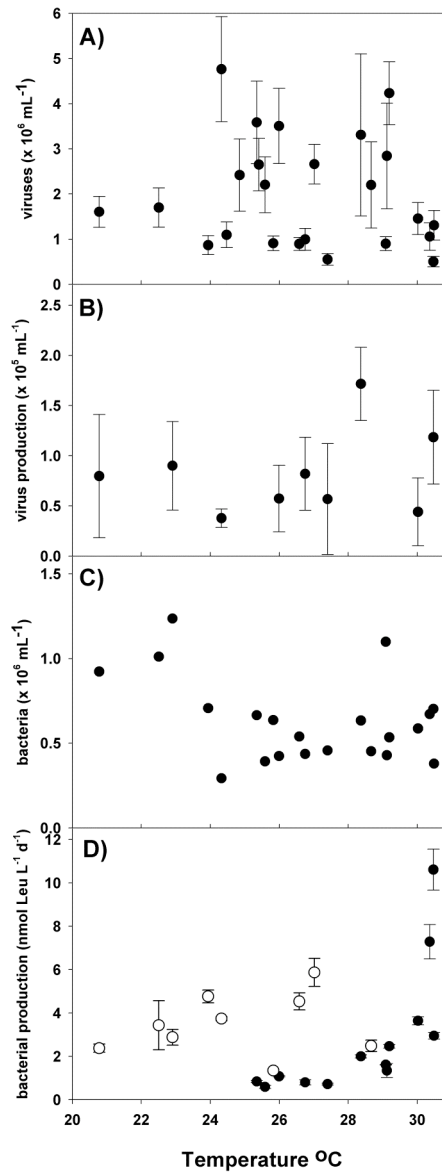


Figure 4.3. Virus-host parameters compared with temperature measured across the Western Pacific. Points presented in chronological order of sampling date. (A) Size-fractionated chlorophyll *a* concentrations: Circles ($> 20.0 \mu\text{m}$), Squares ($2.0 - 20.0 \mu\text{m}$), and Triangles ($0.22 - 2.0 \mu\text{m}$). Error bars indicate the range of duplicate samples. (B) Virus-like particle abundance measurements. Error bars indicate the variation found after 31 fields of view were counted. (C) Virus-like particle production rate measurements. Error bars indicate the standard deviation from triplicate samples. (D) Cell abundance. (E) Bacterial biomass production rate measurements: Black circles (Stations 1-14) and White circles (stations 15-30). Error bars indicate the standard deviation of triplicate samples.

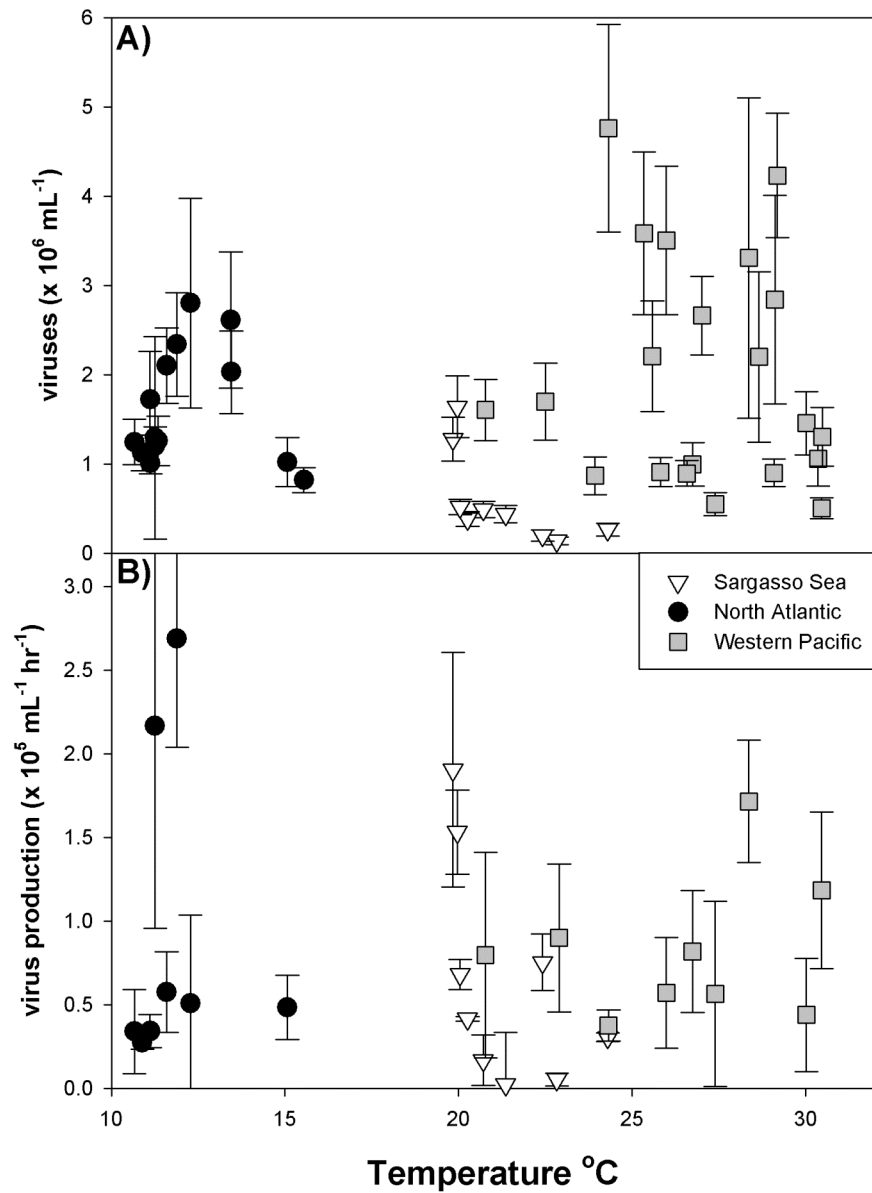


Figure 4.4. Viral and bacterial parameters compared with temperature across all 3 transects. (A) Viral abundance vs. temperature and (B) viral production rate vs. temperature. (C) Bacterial abundance vs. temperature and (D) bacterial production rate vs. temperature. Sargasso Sea = White Triangles, North Atlantic = Black Circles, and Western Pacific = Grey Squares.

possible pairs were examined for the Western Pacific, as well as with observations from the Sargasso Sea and North Atlantic. Abbreviated results are shown in Table 4.1 (see Appendix). Despite being oligotrophic (like the Sargasso Sea) there were surprisingly few significant correlations in the Western Pacific.

To better view the relationships among all measured parameters, Canonical Correspondence Analysis (CCA) was used to assess the similarity (shown by proximity) of data from the Western Pacific and the stations from all 3 transects. Virus-like particle abundance, virus-like particle production rate, cell abundance, bacterial biomass production rate, burst size, and contact rate were used as the biological, response variables, and temperature, salinity, and chlorophyll *a* were used as the environmental, predictor variables. While the axes of the Western Pacific analysis were not significant (determined by the Monte Carlo permutation test), those of the overall analysis (including data from all 3 transects). Table 4.2 shows an abbreviated list of the ranges of data commonly measured across all 3 transects. CCA was completed using all data (56 stations) from these 3 open ocean regimes (Fig. 4.5). As demonstrated by the clustering of station points, each transect is statistically distinct from the others. Several stations on the geographic boundary between the Sargasso Sea and North Atlantic (Fig. 4.5, ½-filled circles) clustered at the edge of the North Atlantic samples, implying that this area of transition is both real and potentially unique.

DISCUSSION

At the onset of this study we intended to test the hypothesis that changes in temperature across a naturally-occurring gradient would result in variations in viral and bacterial abundance and production. The transect through the Pacific Warm Pool provided such an opportunity and serves as a tractable model system for climate change studies. Analysis of results from the transect was further developed by a comparison to two other large scale oceanographic data sets gathered from the Sargasso Sea and North Atlantic Ocean. The overall picture that results from this study is one that highlights the complexity of temperature effects on microbial activity in different oceanic realms.

Table 4.2. Data ranges for selected measurements in the Sargasso Sea, North Atlantic, and Western Pacific. Sargasso and North Atlantic data are further described elsewhere (Rowe et al. 2008).

	<u>SARGASSO SEA</u>	<u>NORTH ATLANTIC</u>	<u>WESTERN PACIFIC</u>
Chl a ($\mu\text{g L}^{-1}$, > 0.2 μm)	0.043 - 0.16	0.16 - 2.25	0.27 - 1.15
Virus abundance ($\times 10^5 \text{ mL}^{-1}$)	1.38 - 18.92	8.19 - 28.02	5.02 - 47.64
Virus production ($\times 10^5 \text{ mL}^{-1} \text{ h}^{-1}$)	0.02 – 1.91	0 - 28.96	0 - 1.72
Bacterial abundance ($\times 10^5 \text{ mL}^{-1}$)	4.61 - 8.78	8.23 - 23.80	2.93 - 12.36
Bacterial production ($\text{nmol leu L}^{-1} \text{ d}^{-1}$)	0.20 - 0.54	0.44 – 1.88	0.59 – 10.61
Burst size (cell^{-1})	11.67	11-23	12-30
Temperature ($^{\circ}\text{C}$)	20 - 24	11 - 15.5	20.8 - 30.5
Salinity (psu)	36.51 - 36.78	35.20-35.87	33.87 - 35.91

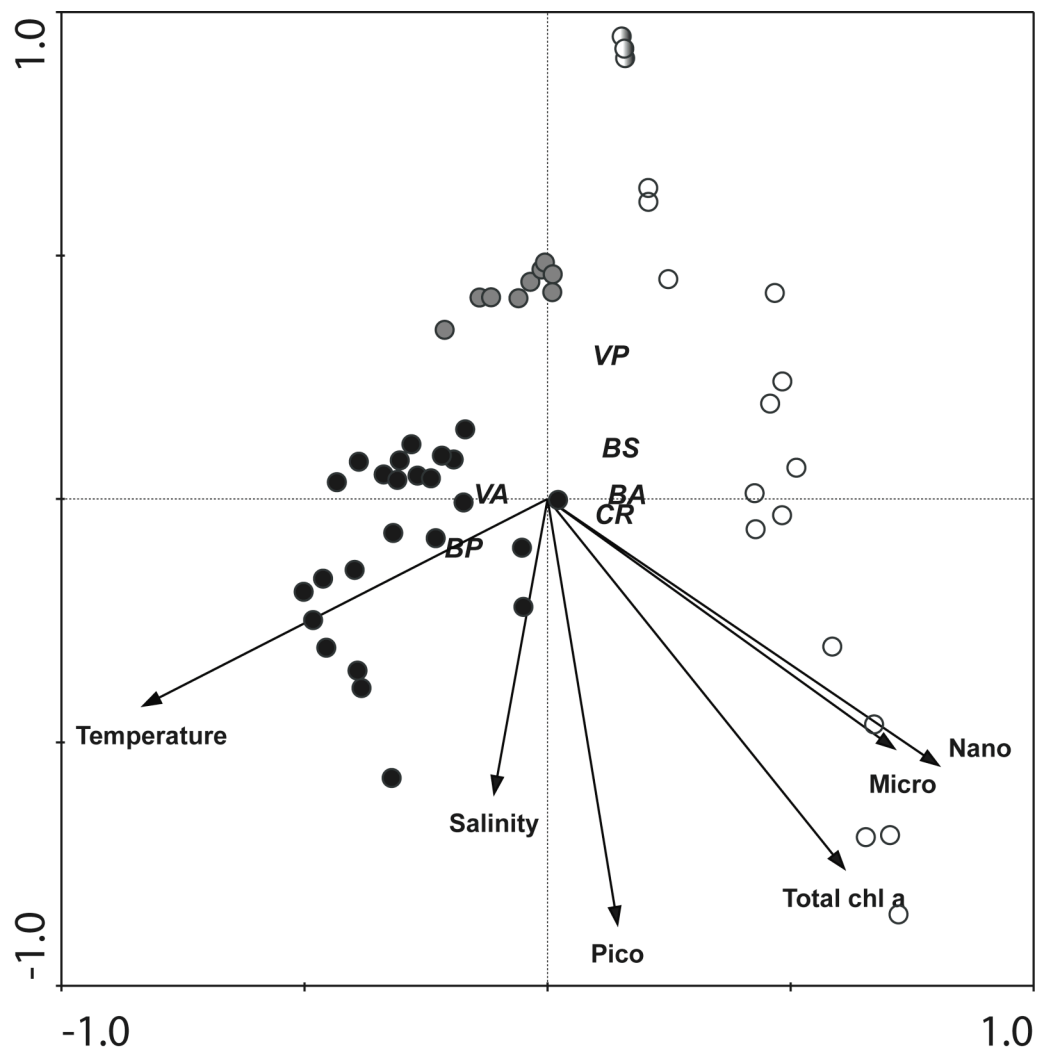


Figure 4.5. Canonical correspondence analysis of Sargasso Sea (grey), North Atlantic (white), and Western Pacific (black) stations, with three stations at the transition of the North Atlantic and Sargasso gradient (1/2-filled). Clustering of data points suggests that each transect is statistically distinct from the other two. Predictor (environmental) variables = total, micro-, nano-, and pico-size-fractionated chlorophyll *a*, salinity, and temperature. Response (biological) variables = VA, virus abundance; VP virus production rate; BA, bacterial abundance; BP bacterial production rate; BS, burst size; CR, contact rate, and are located at their optima.

Across the Western Pacific

During the transect through the Western Pacific Warm Pool, temperatures increased to 30.5° C and then decreased. Salinity confirms that sampling occurred through multiple water masses, which is important to note when questions concerning community structure arise (discussed later). While it is not unexpected that picophytoplankton, likely *Prochlorococcus* and *Synechococcus* (Blanchot et al. 2001; Matsumoto et al. 2004), would dominate the Western Pacific (based on size-fractionated chl a), it is interesting to note that the highest picophytoplankton concentrations were found just before and just after the Warm Pool, in contrast to the trend in temperature. To a lesser extent, this pattern is mirrored by the nanophytoplankton, but not the microphytoplankton. Though picophytoplankton concentrations decrease inside the warm pool, these concentrations are still within the range of those in other distant locations. As such there are certain changes in phytoplankton biomass across this transect, however, these observations are beyond the scope of the current study and will be addressed elsewhere (Johnson and Zinser, unpublished).

In contrast to phytoplankton, viral abundance and viral production remained constant across the transect. The ranges of viral abundance and production are similar to other open ocean data sets (Poore et al. 2004; Rowe et al. 2008; Weinbauer et al. 2002; Winter et al. 2004). However despite these similarities, it is likely the virus communities across these regions differ in composition and relative rates of production, given the changing phytoplankton and bacterial populations.

Bacterial abundance and biomass production provide contrasting results from those of viral metrics. Bacterial abundances over the transect displayed no clear trends, while bacterial production drastically and significantly peaked inside the Western Pacific Warm Pool. In our survey, bacterial production rates were higher than reported in other open oceanic regimes, that similarly have oligotrophic waters (Church et al. 2004; Noble and Fuhrman 2000; Rowe et al. 2008; Steinberg et al. 2001). It is possible that this regime is more conducive to higher production, though it is also possible that the higher rates were the result of more efficient detection (see Pace et al. 2004)

It has been noted that significant correlations between bacterial production and viral abundance are indications of the predominance of phages infectious to heterotrophic bacterioplankton (Weinbauer 2004). In our previous research, bacterial production correlated significantly to virus-like particle abundance in the Sargasso Sea (linear regression, $r^2 = 0.581$, $p = 0.004$, and spearman correlation, $r_s = 0.789$ $p = 0.002$) (Rowe and Wilhelm, unpublished; Rowe et al. 2008), but not in the North Atlantic or in this study of the Western Pacific. Although this finding does not necessarily disprove previous assumptions, it should be noted that the Sargasso Sea is dominated by prokaryotes (including photosynthetic ones). Due to the complexity of host communities and seawater chemistry in different marine environments, it is likely that while in certain regimes there is a predominance of bacteriophage, algal viruses or cyanophages may constitute a higher portion of the total virus populations in other regions. As molecular tools continue to develop, viral populations can be teased apart so as to gain a better understanding of their compositions, as well as how these compositions change over differing gradients and temporal scales. Such approaches could also tease apart the host and virus communities to elucidate any influential roles their compositions have.

Pair-wise comparisons between temperature and viral and bacterial parameters suggested no effects of temperature on virus production or abundance in the Western Pacific, implying that temperature is not a direct driver of *in situ* virus activity. It is equally surprising that bacterial abundance and temperature were not correlated, although community structure changes (not measured in the current study) may be significant. What was surprising was that bacterial production and temperature were not significantly correlated, in spite of observable differences between bacterial production in the Warm Pool relative to other stations. Because of this trend in biomass production rate over the course of the WP2 transect, surface current data (not shown) was used to group sampling sites by 'water mass' (defined as flowing within the same current). Correlations between bacterial biomass production and water mass were significant ($r_s = 0.654$ $p = 0.001$), suggesting differences in the microbial community in different water masses were a major influence.

It is somewhat surprising that temperature did not have a more noticeable effect on the biological parameters we measured in the Western Pacific. However, it is doubtful that this should serve as any indication that temperature will not have a dramatic effect on communities (such as in the polar regions) which are unaccustomed to such increases in temperature that are being predicted (Meehl et al. 2005). While production rates of populations may demonstrate no overall net change, production rates of individual members of the population may change.

Comparison with Other Regimes

Previously, we observed that biological parameters were “predictable” in the oligotrophic Sargasso Sea based on environmental measures, while in the North Atlantic, a threshold effect of environmental factors on viral production and abundance was reached (Rowe et al. 2008). Curiously in the Western Pacific Warm Pool we observed neither of these trends. Comparisons between temperature and viral parameters across all 3 transects, demonstrated no temperature effects. A recent study of viral metagenomic sequences from 4 different marine environments suggests that viral populations may be universally distributed on these scales, with regional selection pressures affecting relative abundances (Angly et al. 2006) and likely activity as well. Though the oceans are composed of spatially separate regions and water masses, collectively viral abundance and production remained within a constant range. Comparative ranges for other standard metrics were also overlapping (Table 4.2).

A closer look at bacterial abundance and production compared to temperature across all 3 transects revealed a dynamic relationship. Bacterial abundance in the cooler North Atlantic was higher than in the oligotrophic Sargasso Sea and Western Pacific, probably because of the high primary productivity associated with the North Atlantic Spring Bloom, which could support bacterial carbon demand. While it is logical that temperature could affect bacterial abundance and increase bacterial productivity, the actual standing stock is likely a more complicated function of various loss rates (e.g., viral lysis and grazing) that may also be temperature dependant. Though regression analysis of bacterial abundance and temperature did not yield significant results for the Western Pacific alone, the combined transects together yielded a significant second order

linear regression ($r^2 = 0.729$, $p < 0.001$), with Spearman rank correlation analysis yielding further support ($r_s = -0.785$, $p < 0.001$), suggesting that within the individual transects the range and number of observations may be too limited for trends to be observed.

Despite higher bacterial abundances and high primary productivity, the bacterial production rates in the North Atlantic were lower than in the Western Pacific. As with abundance, bacterial production was not significantly correlated to temperature in the Western Pacific, but for all 3 transects both regression ($r^2 = 0.365$, $p < 0.001$) and Spearman rank correlation ($r_s = 0.451$, $p = 0.002$) demonstrated positive relationships. It is interesting to note that within the Western Pacific transect, stations North of the Warm Pool center (1-14, Fig. 4.3D, black circles) did demonstrate a significant correlation between bacterial production and temperature ($r^2 = 0.645$, $p < 0.001$), while the stations South of the Warm Pool center (15-30, Fig. 4.3D, white circles) did not. Consequently when overall analyses were again performed, but with Western Pacific data shortened to either the first or second half (Sargasso Sea, North Atlantic, and Western Pacific stations 1-14 or 15-30), the strength of correlation was higher, $r^2 = 0.518$, $p < 0.001$ and $r^2 = 0.435$, $p < 0.001$, respectively, than with the full Western Pacific transect included. These results suggest that while our general assumption that the Warm Pool may be a good model for climate change effects studies is correct, that transect through the Western Pacific may be crossing two very distinct oceanic realms. Indeed, the Northern stations are all open ocean in nature, while the Southern stations are in waters that in many cases are probably influenced by regional land masses (see Fig. 4.1).

Taken altogether the results also indicate the difficulties in comparing entire oceanic regimes. Table 4.1 shows how an overall view of the data from all 3 transects tells a different story than each transect separately. For example, significant correlations observed in the combined analysis are not present in all 3 of the separate transects. This could be the result of a need for a larger sample size before a correlation reaches significance, as may be the case for salinity. Though another option is that the correlations found in one transect could be strong enough to be remain seen in the overall analysis, albeit as weaker correlations (e.g. viral abundance and Chl *a* measures from the Sargasso Sea transect). Because of these concerns, there was a need for multivariate

analyses to examine these measured parameters and to determine whether or not the data sets should be combined.

Canonical Correspondence Analysis (CCA), a unimodal and constrained multivariate analysis was chosen to compare all three complete data sets because of its ecological relevance (Fig. 4.5). The unimodal component of this analysis allows one to detect optima along gradients rather than assuming the data should be linear, an assumption discounted from earlier uni- and bivariate analyses. CCA for all three combined transects displayed distinct clusters corresponding to the different oceanic regions. Because sampling in the Sargasso Sea progressed into the North Atlantic it was not unexpected that a few of the boundary sampling sites of the Sargasso Sea clustered closer to the North Atlantic sites. Overall, while temperature effects on virus abundance and production showed no parametric variations between regimes, CCA demonstrates that these are clearly three statistically different open ocean regimes. This is interesting to note, as bivariate analyses presented earlier, could by themselves, have suggested otherwise. As such, the results point to the need for both increased spatial and temporal resolution of viral and bacterial turnover and abundance, as well as the need for a better understanding of virus and host richness in the development of models for controls of virus activity in the global ocean.

Future Directions

One current impetus for much of marine microbial research is to begin teasing apart virus and host communities to better understand factors that constrain these groups. Already, metagenomic studies have provided databases of host and viral sequences across marine environments (Angly et al. 2006; Delong 2005; Tringe et al. 2005; Venter et al. 2004; Williamson et al. 2008). With more genomic studies on marine viruses, molecular tools are being designed to focus on individual virus populations within microbial communities. So far, researchers have been able to detect Phycodnaviridae, a family of algae-infecting viruses, from marine samples (Chen and Suttle 1995; Chen et al. 1996; Larsen et al. 2008; Schroeder et al. 2002) and quantitative molecular approaches are just around the corner (e.g. Sandaa and Larsen 2006). Monitoring microbial communities and global scale effects will require a delicate balance between assessing an oceanic region as

its own environment and as part of a larger whole. Understanding how viruses interact with the environment is just the first step in our efforts to develop a clearer picture of global ocean ecology; a necessary component of all models (and management strategies) that focus on anthropogenic influences and climate change.

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SECTION V

**APPLICATION OF THE MAJOR CAPSID PROTEIN GENE AS A
MARKER OF PHYLOGENETIC DIVERSITY OF *EMILIANA*
HUXLEYI PHYCODNAVIRUSES IN THE NORTH ATLANTIC**

This section is a version of an article that is being prepared for submission to Applied Environmental Microbiology under the same title with current authors, Janet M. Rowe, Marie-Francoise Fabre, Daniel Gobena, and Steven W. Wilhelm.

My contribution to this paper was participating in sample collection while onboard the NASB2005 cruise. Performing most of the lysate generations, most of the target amplification and subsequent cloning, sequence editing, tree building, most of the data analysis, and most of the background information gathering and writing.

ABSTRACT

Studies on the diversity of Phycodnaviridae in aquatic systems have traditionally relied on the use of the DNA polymerase (*pol*) gene as a biomarker. However, recent investigations have suggested that the use of the Major Capsid Protein (MCP) gene may be a reliable phylogenetic biomarker for this group of viruses. Herein, we describe the use of MCP gene sequences gathered across the North Atlantic to assess the diversity of *Emiliana huxleyi*-infecting Phycodnaviridae. Nucleotide and amino acid sequences were examined across > 6,000 km of open ocean, with comparisons between concentrates of the virus-size fraction of seawater and of lysates generated by exposing host strains to these same seawater, virus concentrates. Analyses revealed that many sequences were only sampled once, while several were over-represented, suggesting a disproportional evenness. Rarefaction curves indicate that richness in the North Atlantic is likely greater than that which is represented in samples, however, phylogenetic analyses of nucleotide sequences reveal that there are sequences distinct from those of coastal isolates. The examination of virus-lysed cultures also revealed a new breadth in phylogeny, as MCP sequences that were previously unrepresented within the known collection of *Emiliana huxleyi* viruses were found associated with viruses lysing cultures in the laboratory. Overall, this investigation provides a wealth of new sequence data and an assessment on the use of MCP gene sequences to discern phylogenetic relationships.

INTRODUCTION

Phycodnaviridae are eukaryotic algal viruses which have large ~100-560 kb genomes, 100-220 nm diameter capsids, and have only been studied in recent decades (Brussaard 2004). The activities of marine Phycodnaviridae are particularly important when considering the potential top-down effects of their predation on their photosynthetic hosts (Wilhelm and Suttle 1999), which have dramatic global-scale influence through carbon and other biogeochemical cycling (Falkowski et al. 1998; Field et al. 1998; Fuhrman 1992; Redfield 1958). In general, aquatic viruses are known to play a variety of roles and are key members of aquatic ecosystems (Brussaard et al. 2008; Suttle 2007; Wilhelm and Matteson 2008).

Traditionally, researchers have focused on abundance and production rate of the entire community of marine viruses, while quantitative viral community composition has been largely (though not entirely) ignored. To a great extent, this is due to the need for more advanced molecular techniques to detect viruses from environmental samples and the need for more genomic investigations of these viruses to identify acceptable biomarkers. Issues have come into play with relatively low concentrations of viral DNA, inhibition of restriction enzymes by viral DNA, and difficulties in cloning sequences that encode products detrimental to the cloning hosts (Paul et al. 2002). However, researchers have still made advances in examining viral, and particularly phage, genetics. After examining virus metagenomes from 4 different ocean environments, Angly, et al., (2006) suggested that the viral population of the World's Ocean is universally distributed, but that regional selection factors control the overall composition. Additionally, RNA-virus population metagenomics (Culley et al. 2006) and viroplankton genetic diversity (Comeau et al. 2006) have been investigated as well. However, while several studies (Brussaard et al. 2004; Chen et al. 1996; Larsen et al. 2008; Schroeder et al. 2002; Short and Suttle 2002) describe the phylogenetics of Phycodnaviridae sampled from coastal and offshore waters, ocean-wide diversity of these eukaryotic algal viruses has yet to be examined.

So far, Phycodnaviridae have been characterized primarily on the basis of their DNA polymerase gene (*DNA pol*) sequence (Chen and Suttle 1995; Van Etten 2006). However, because *DNA pol* cannot always resolve phylogenetic needs (Sandaa et al. 2001; Schroeder et al. 2002), the major capsid protein (MCP) gene has recently been examined as a potential biomarker for Phycodnaviridae phylogeny. For *Emiliania huxleyi*-infecting Phycodnaviridae, or EhV (*Emiliania huxleyi* viruses), that were isolated from the Western English Channel, it was found that, based on the host-ranges of the individual isolates, MCP gene sequences better resolved the identification of virus isolates than *DNA pol* (Schroeder et al. 2002). It was proposed that MCP amplicons could be a useful second biomarker for resolving genotypes, especially since it appears to be more variable in EhV than *DNA pol* (Schroeder et al. 2002). Additionally, examinations of natural viral assemblages from Raunefjorden and Puddefjorden, and of

virus isolates of different non-EhV Phycodnaviridae members, further indicated that the MCP gene could be a valuable genetic marker for Phycodnaviridae (Larsen et al. 2008). Although the MCP gene from the *Micromonas pusilla* virus isolate, MpV-12T, could not be amplified, the phylogenetic analysis based on the MCP genes of other Phycodnaviridae isolates supported the distinction of the currently listed genera of Phycodnaviridae (Dunigan et al. 2006; Larsen et al. 2008). This however, also raised questions over the phylogenetic ancestry of Phycodnaviridae, suggesting that a resolution needs to be found between the different phylogenetic relationships proposed by the analysis of DNA *pol* and MCP genes (Larsen et al. 2008).

Our goals for the current study were to expand our knowledge of MCP gene diversity and assess the use of MCP as a biomarker for EhV in the open ocean. The diversity of EhV-MCP was examined in the North Atlantic to assess spatial changes across > 6,000 km of open ocean. This diversity was compared to previous coastal examinations (Schroeder et al. 2002) and to the diversity of MCP amplicons from lysates generated after exposing virus concentrates from the North Atlantic to host cultures in the laboratory.

MATERIALS AND METHODS

Sample Collection

Virus concentrates were generated while onboard the RV ‘Seward Johnson’ during NASB2005 (Rowe et al. 2008) in a similar manner as described by Chen, et al. (1996) and Wilhelm and Poorvin (2001). Figure 5.1 shows the locations from which these virus concentrates were generated. 20 L of water samples were prefiltered through 142 mm diameter GF/F filters (Whatman) and then through 0.45 and 0.22- μ m nominal pore-size, 142-mm diameter polycarbonate filters (Millipore). The virus-size fraction was then concentrated to ~500 mL using Millipore’s M12 ProFlux tangential flow filtration with a 30kDa cutoff filter cartridge (Millipore). Virus concentrates were stored in the dark at 4° C.

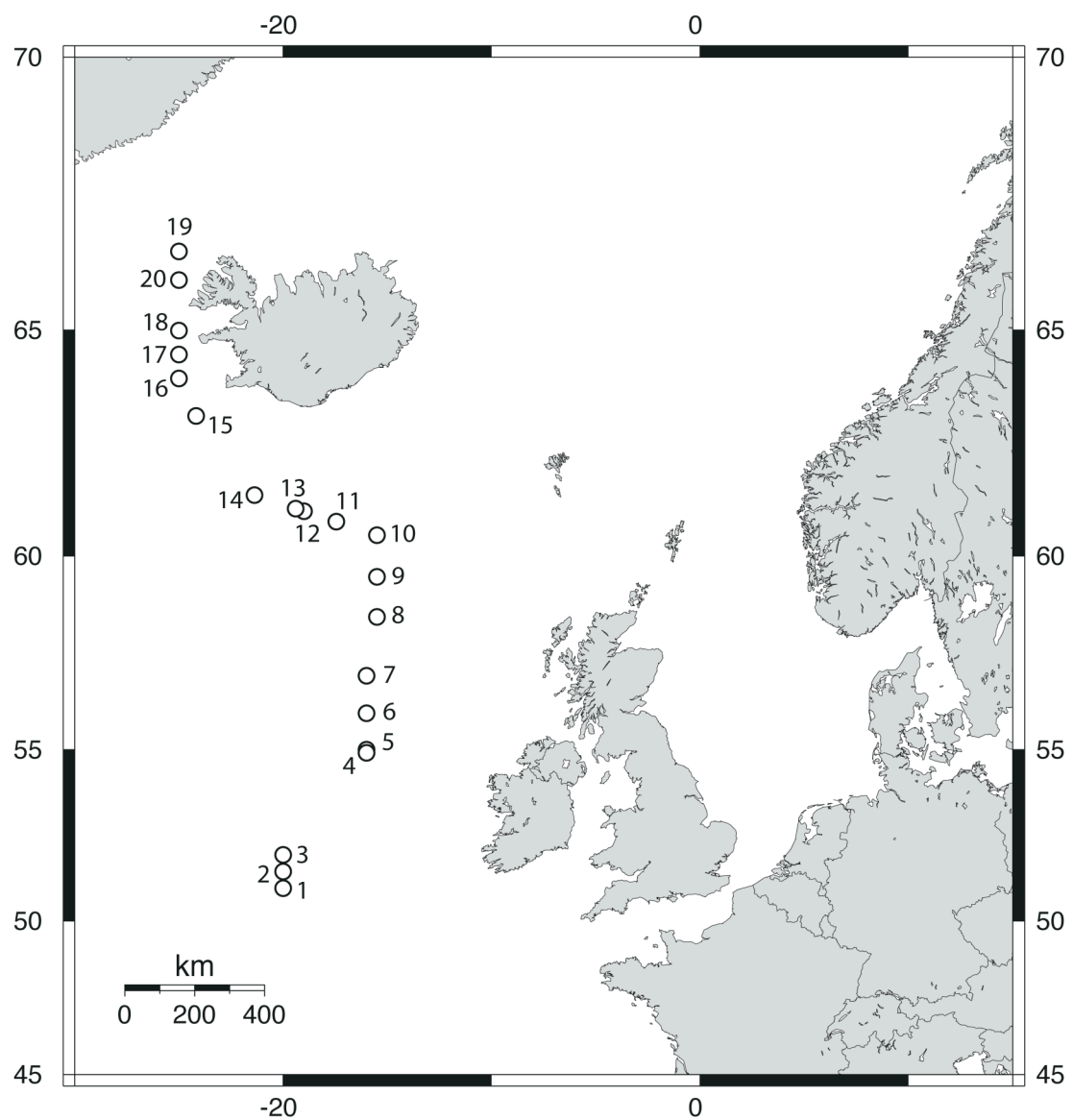


Figure 5.1. Station locations in the North Atlantic from where virus concentrates were generated.

Lysate Generation

Virus concentrates were screened for viruses infectious to host cultures, *Emiliania huxleyi* strains CCMP 373 and DWN 61/87/10. Cultures were grown in L1 medium (Guillard 1975; Guillard and Hargraves 1993; Guillard and Ryther 1962), and both strains were kept at ~21° C on a 12/12 day/night cycle. Cultures were inoculated with virus concentrate and *in vivo* fluorescence was monitored for a minimum of 14 days using a Turner Designs TD-700 fluorometer, which was equipped with an *in vivo* chl *a* filter set (ex. λ = 340–500 nm; em. λ = > 665 nm).

DNA Extraction

Viral DNA was extracted from concentrates and lysates following an adapted protocol by Smith and Burgoyne (2004). First, the salt concentration was reduced by placing sample on top of a pre-moistened 0.025- μ m nominal pore size, mixed cellulose filter (Millipore) and incubated at room temperature for 30-40 min. Sample was then spotted onto a FTA CloneSaver card (Whatman) and dried for 40 min at room temperature. ~2.0 mm diameter samples were punched from the sample dried on the FTA card and washed twice with 500 μ L 10mM NaOH for 15 min, then twice with 200 μ L of Tris-EDTA (pH 8) for 5 min, and finally with 500 μ L of 1:5, Tris-EDTA:Bovine Serum Albumin (1mg mL⁻¹) for 10 min. Punches were dried for 10-15 min at 56° C. DNA was eluted by submersing punches in 30 μ L of sterile water and incubating for 10 min in a water bath at 90° C. Samples were briefly centrifuged at high speed and then stored at -20° C. FTA cards were stored at room temperature, away from light, and with desiccant.

PCR

The MCP gene, was amplified using the primers MCP-F and MCP-R, as described by Schroeder, et al. (2002). Annealing temperature was optimized to 65° C for in house equipment (Eppendorf Mastercycler Gradient Thermal Cycler) and a final extension step of 74° C for 5 min was added to facilitate cloning. Reaction mixes were set up using PuRe Taq Ready To-Go PCR beads (GE Healthcare), with ~1-3 μ L of DNA,

20 pmol of each primer, and sterile water up to 25 µL. Reactions mixes minus template served as negative controls for all PCRs. All PCRs were performed on an Eppendorf Mastercycler Gradient thermocycler, with product stored at 4° C until examined via electrophoresis: 2% agarose gel in 1X TBE buffer at ~100-120 volts, with subsequent visualization and documentation on a Fotodyne Investigator digital imager with ethidium bromide filter using Foto/Analyst® PC Image v. 9.0.4 software.

Cloning and Sequencing

From electrophoresis gels, bands of interest were excised and DNA eluted using Promega's Wizard SV gel and PCR clean-up system. Eluted DNA was cloned using Invitrogen's TOPO TA cloning kit and resulting plasmids extracted and purified using the QIAprep Spin Miniprep Kit from QIAGEN. DNA was sequenced at Clemson University Genomic Institute using plasmid-targeting M13 primers.

Sequence and Phylogenetic Analyses

Sequences were first assessed for quality and clarification of read, and primers removed using 4Peaks. Sequences were initially aligned with ClustalX2.0 and Bayesian Inference trees (Huelsenbeck and Ronquist 2001) built using Geneious 4.0.4, with the MRBayes Plugin using the recommended default settings: HKY85 nucleotide substitution model, unconstrained branch lengths, a subsampling frequency of 200, and 1.1×10^6 generations. Known sequences were obtained from National Center for Biotechnology Information (NCBI). Rarefaction curves were generated with the use of the Analytical Rarefaction program provided by Steven Holland (University of Georgia, Athens), available at <http://www.uga.edu/~strata/software/>. Shannon-Wiener Diversity Indices and measures of evenness were calculated using software provided by Chang Biosciences, Inc. ©, available at <http://www.changbioscience.com/genetics/shannon.html>.

RESULTS

For all 20 virus concentrates and all lysates generated from inoculating host cultures with those same concentrates, the MCP genes were amplified and clone libraries

generated with 4-6 clones picked per sample for sequence analysis. Because of the limited number of colonies sequenced from individual libraries, rarefaction curves were generated, with all libraries grouped as one, to assess the sampling of the viral population across the North Atlantic: nucleotide sequences (Figure 5.2A) and amino acid sequences (Figure 5.2B), with sequences grouped by 99.5% and 98% identity, respectively. Both curves demonstrate a positive linear trend without ‘leveling off,’ indicating that sampling of richness was not reached. This trend reflects the sampling pattern observed wherein most sequences were detected only once, with a few detected < 5 times, and a few more detected multiple times (≥ 10). As an indicator of sample diversity and for comparison of diversity under two different qualifications, the Shannon-Wiener Diversity Index was calculated for total nucleotide sequences detected (both environmental and generated lysate samples), and for nucleotide sequences detected only in environmental (virus concentrate) samples. Though the former includes all sequences detected, a side effect of including sequences found in lysates is that the selection for these sequences can skew the measure of evenness. Shannon-Wiener Diversity Indices, for the total and for environmental sequences only, were 2.452 and 1.795, respectively. Comparing the two results indicates a greater diversity when all sample are considered. Because of skewing due to virus selection during host amplification, a more appropriate comparison can be made from the measure of evenness derived from the Shannon-Wiener Diversity Indices. Evenness is calculated as a number between 0-1, with 0 indicating no evenness determined for the population of interest and 1 indicating that all sampling units (species, strains, sequences, etc.) are present in the same amount. For total sequences, evenness was calculated to be 0.652, and for environmental sequences only, it was calculated to be 0.599. Both of these indicate moderate sample evenness.

To infer relationships among the detected sequences, Bayesian Inference based phylogenetic trees were utilized because of the model’s tree-searching, based on posterior probabilities, rather than tree-building method of analysis, as well as this heuristic model’s Metropolis-coupled Monte Carlo Markov Chain permutation, which allows the model to search for other “best” trees without commitment towards a particular direction (Hall 2004). Phylogenetic trees showing the relationships of the MCP sequences

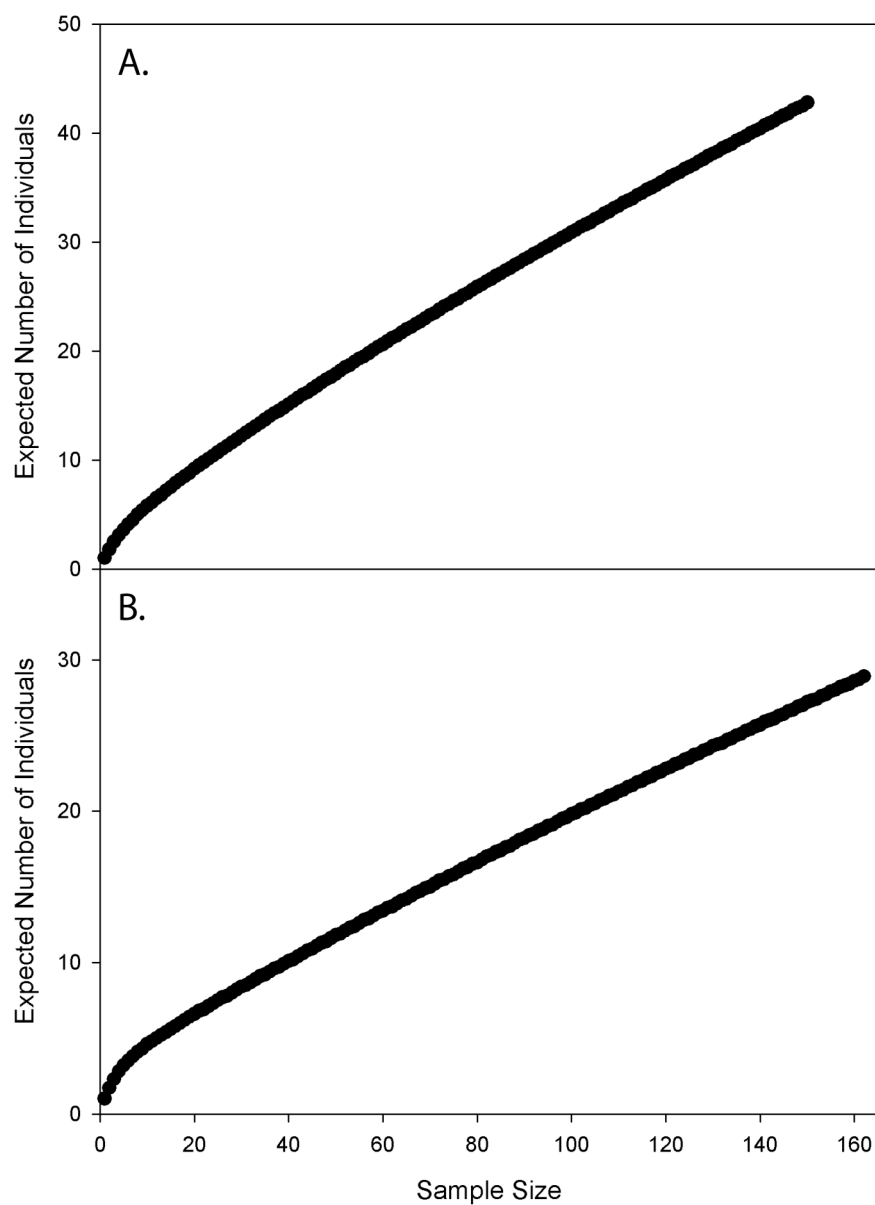


Figure 5.2. Rarefaction curves generated for (A) nucleotide sequences observed from total samples after sequences $\geq 99.5\%$ identity were grouped together, (B) amino acid sequences observed from total samples after sequences $\geq 98\%$ identity were grouped together.

gathered from this study are shown in Figures 5.3 (nucleotide) and 5.4 (amino acid). Trees include EhV MCP sequences, previously reported (Schroeder et al. 2002), and a non-EhV MCP-outgroup sequence (Larsen et al. 2008). Sequences from this study were uploaded to GenBank and received accession numbers FJ543391-FJ543465. Lastly, to accompany the sampling locations depicted in Figure 5.1, Table 5.1 presents an abbreviated list of data ranges for chlorophyll *a*, total viral abundance, salinity, and temperature (Rowe et al. 2008; Rowe and Wilhelm unpublished) observed at these same stations.

DISCUSSION

The current study has confirmed the utility of the MCP gene as a valuable phylogenetic marker to be used within a group of algal viruses infecting the cosmopolitan phytoplankton, *Emiliania huxleyi*. Our results suggest that the EhV community is dominated by several clones, represented by a high abundance of conserved sequences. Moreover, the results demonstrate that this community also contains numerous unique viral MCP sequences, implying that significant genetic richness exists. Finally, the identification of MCP sequences from viral lysates of lab cultures that reside in previously unrepresented clades suggests that the diversity of these viruses is much broader than seen in the original, more regional, study. In total, this work represents a validation of the use of the MCP gene marker for studies of EhV diversity and provides some new insight into the composition of a specific Phycodnavirus community on an oceanic scale.

Lab-scale studies of isolated virus-host systems have provided a valuable foundation for marine viral population studies. Additionally, oceanic-scale work examining *in situ* viral activities has provided a foundation for a global understanding of viruses' potential top-down influences. Current directions in molecular work now allow researchers to blend these two extremes of analysis and develop the necessary tools for teasing apart *in situ* viral populations. Though this study focused on EhV, non-EhV belonging to Phycodnaviridae could feasibly be assessed in a similar matter, and recently published work has shown success in amplifying MCP from many non-EhV

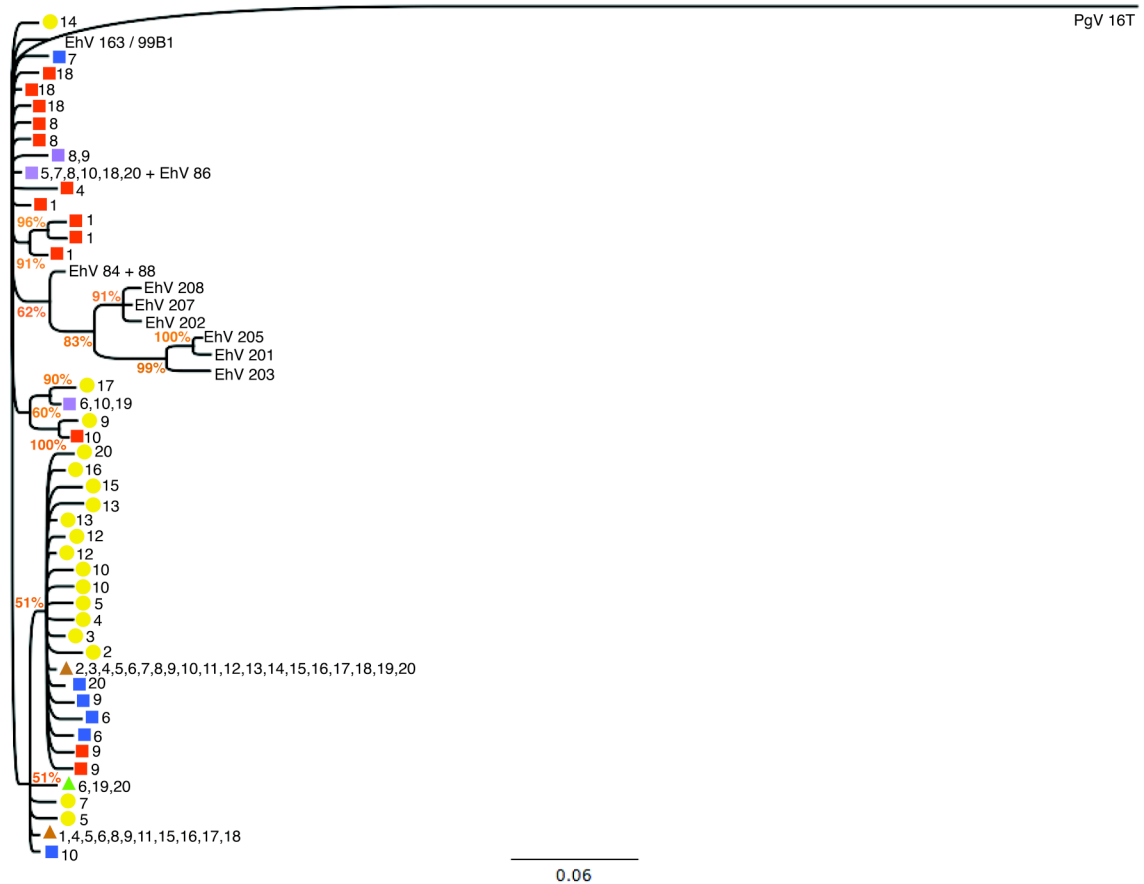


Figure 5.3. Bayesian Inference Tree of nucleotide sequences (those of 99.5% identity grouped together). EhV isolates from Schroeder, et al. (2002) and *Phaeocystis globosa* virus strain 16T (PgV 16T) from Larsen, et al. (2008) are included as known EhV-MCP sequences and MCP-outgroup, respectively. Orange Numbers = Clade Support Value (*i.e.* posterior probabilities), Yellow Circles = Sequences only detected in virus concentrates, Squares = Sequences only detected after host lysis (Red = *Emiliana huxleyi* host strain CCMP 373, Blue = *Emiliana huxleyi* host strain DWN 61/87/10, Purple = Both Strains), Triangles = Sequences detected in both virus concentrates and after host lysis (Green = Only *Emiliana huxleyi* host strain DWN 61/87/10 lysed, Brown = Both host strains lysed), Black Numbers = Sampling location from where original samples were collected, Scale Bar = Substitutions per site.

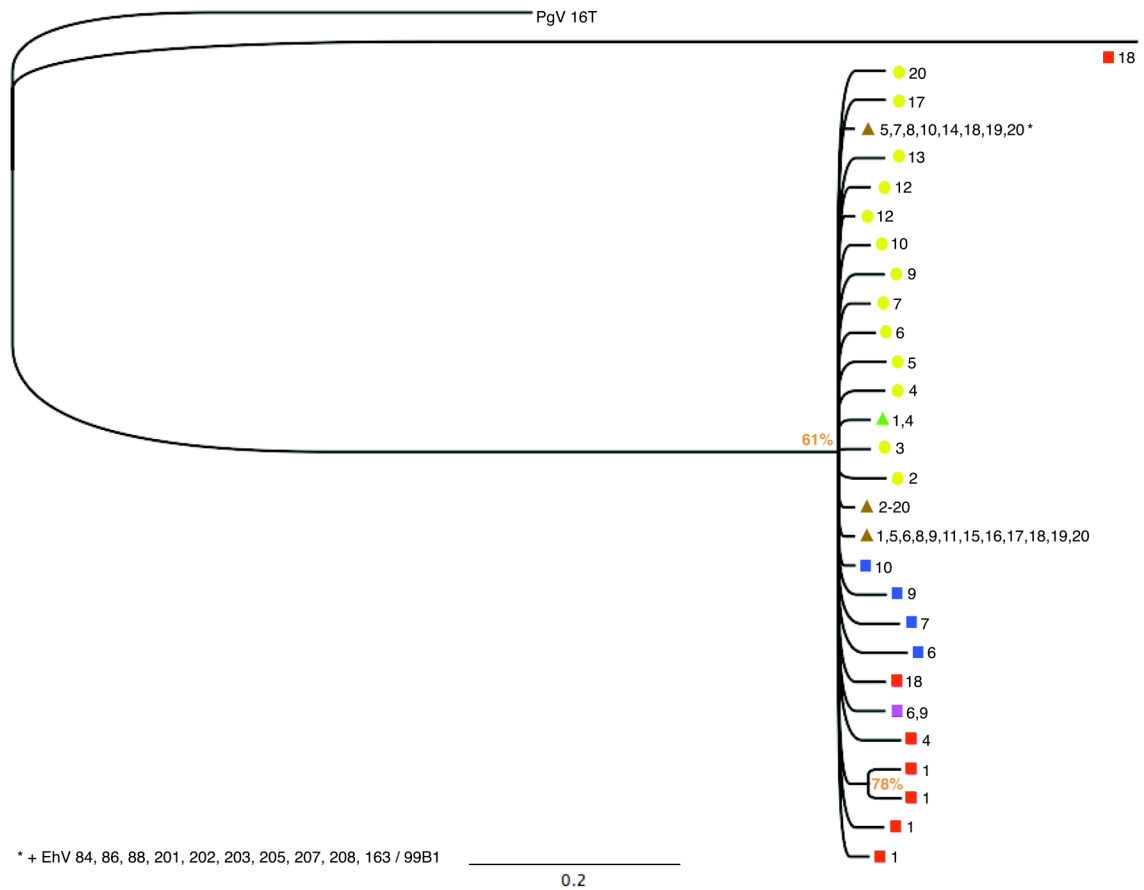


Figure 5.4. Bayesian Inference Tree of amino acid sequences (those of 98% identity grouped together). EhV isolates from Schroeder, et al. (2002) and *Phaeocystis globosa* virus strain 16T (PgV 16T) from Larsen, et al. (2008) are included as known EhV-MCP sequences and MCP-outgroup, respectively. Orange Numbers = Clade Support Value (*i.e.* posterior probabilities), Yellow Circles = Sequences only detected in virus concentrates, Squares = Sequences only detected after host lysis (Red = *Emiliana huxleyi* host strain CCMP 373, Blue = *Emiliana huxleyi* host strain DWN 61/87/10, Purple = Both Strains), Triangles = Sequences detected in both virus concentrates and after host lysis (Green = Only *Emiliana huxleyi* host strain DWN 61/87/10 lysed, Brown = Both host strains lysed), Black Numbers = Sampling location from where original samples were collected, Scale Bar = Substitutions per site.

Table 5.1. Measurements of total chlorophyll *a* (chl *a*) concentrations, total viral abundance, salinity, and temperature that were observed at each of the stations from where virus concentrates were generated. Virus concentrates were generated from surface (5 m) water samples, unless otherwise specified. VLP = Virus-like Particle, DCM = Deep Chlorophyll Max, N. D. = Not Determined.

Sampling Location	Total Chl <i>a</i> ($\mu\text{g L}^{-1}$)	Total Viral Abundance (VLP $\text{L}^{-1} \times 10^9$)	Salinity	Temperature ($^{\circ}\text{C}$)
1	0.442	1.1	N. D.	N. D.
2 (25 m, DCM)	N. D.	N. D.	N. D.	N. D.
3	0.556	1.7	35.35	13.34
4	0.843	N. D.	35.41	13.64
5 (10 m)	0.793	2.0	35.42	13.46
6	0.67	2.3	35.36	11.89
7	1.004	2.1	35.33	11.59
8	1.273	3.0	35.3	11.07
9	2.955	1.0	35.27	11.13
10	1.777	1.2	35.28	10.66
11	2.13	1.1	35.27	10.89
12	0.752	1.3	35.2	11.34
13	N. D.	N. D.	35.17	11.93
14	1.721	1.2	35.25	11.26
15	1.863	1.4	35.2	8.99
16	1.076	2.4	35.19	10.12
17	1.605	3.0	35.13	9.72
18	2.32	4.2	34.71	9.46
19	2.1	2.2	33.28	2.6
20	1.661	8.1	34.88	9.3

Phycodnaviridae, though not all (Larsen et al. 2008). Critical to improving the detection of Phycodnaviridae is the genomic sequencing of representative members so that primers for select biomarkers can be better designed. Presently there are 4 marine Phycodnaviridae genomes completely sequenced and available for analysis: EsV-1 (Delaroque et al. 2001), EhV-86 (Wilson et al. 2005), OsV5 (published as OtV5 in Derelle et al. 2008) and FsV1 (GenBank: EU916176). More isolations and subsequent genome sequencing will provide the means to refine present biomarker-targeting primers so that diversity can be more fully assessed.

In the current study we detected a total of 35 unique (only seen once) sequences, 17 of which appeared from direct amplification of environmental samples. In contrast, several MCP gene sequences were detected multiple times, with two sequences detected 10 and 37 times within the environmental samples, but a total of 31 and 56 times, respectively, when both environmental and culture-generated lysate samples are combined. That these viruses are now in culture demonstrates that they are functional, and their detection multiple times emphasizes how they are important representatives of the natural population. Such a disproportionate evenness, especially when considering that sampling occurred during the North Atlantic Spring Bloom event, suggests that these sequences were from viruses which may have recently infected resident host species, likely bloom-formers high in abundance, in a manner described as “kill the winner” (Thingstad and Lignell 1997). The distribution of sequences from both sets of samples (virus concentrates and lysates) suggests an agreement with previous observations about marine viral diversity: that specific environments within the ocean select from a universal “pool” of viruses (Angly et al. 2006). Rarefaction curves for both DNA and protein sequences grouped by 99.5% and 98% identity, respectively, demonstrate that our libraries did not fully cover the viral richness available, indicating that there is likely a greater diversity still to be sampled from this region.

It is also interesting to note the differences in viral diversity of water samples taken directly from the North Atlantic and of the lysates generated by host exposure to the same water samples. Only four sequences were common between the two sets of MCP gene sequences, indicating an agreement with the rarefaction curves, that true

richness in this region is higher than sampled. It is likely that sequences observed in lysates but not from the environmental samples themselves were in such low abundance that amplification via host infection was necessary for such detection. However, a second possibility is that the viral population was selected during host infection. Examinations of aquatic viral diversity have suggested that changes can occur rapidly (Bratbak et al. 1990; Marston and Sallee 2003; Steward et al. 2000). However, given discrepancies between natural populations and laboratory culture collections (Suttle 2005), the first possibility is perhaps more likely: it is worth noting that these two possibilities are not mutually exclusive. Lastly, that there were many sequences detected by direct amplification from virus concentrates, but not from the lysates, emphasizes that host ranges of EhV in the North Atlantic may not be fully represented by the range of *Emiliania huxleyi* strains in culture, highlighting the importance of increasing algal culture collections. This observation also highlights one important caveat: that PCR products from *in situ* seawater amplifications may be from virus particles that are non-infectious. It is not known how long these non-infectious particles last before decay, nor their percentage of the total population. Thus, it is important that researchers do not fully turn away from culture-based techniques as lysates generated in the laboratory can confirm the viability of at least a portion of the infection particle population.

In addition to assessing the diversity of EhV MCP gene sequences across the North Atlantic and between natural samples and lysates, comparisons were made between open and coastal ocean waters. MCP gene sequences from EhVs isolated in the English Channel (UK) and Raunefjorden (Norway) (Schroeder et al. 2002) were included in phylogenetic analyses. It is interesting to note that of these 10 coastal isolates, only one, EhV-86, was represented in the North Atlantic nucleotide sequences, only being found in the lysate samples that were generated. Placement of these coastal sequences on the phylogenetic tree indicates that while there may be some spatial differences in diversity, they are not very pronounced and are without clear trend. Analysis of the amino acid sequences reveals a different pattern. All 10 of the coastal isolates group together and with North Atlantic samples; in this analysis less diversity is depicted.

As part of this study we had to address whether DNA or inferred protein was a better choice to represent EhV diversity. Differences in DNA do not necessarily mean different amino acid sequences, as such making DNA a choice for phylogenetic modeling in systems where closely related populations are being studied. However, with sequences of a gene transcribing for a protein, it may seem logical to run phylogenetic analyses on amino acid sequence rather than nucleotide; and there is the additional concern with both methods as to where % identity demarcations should be placed. Certain sequences from known EhV (described in Schroeder et al. 2002), were grouped together when amino acid sequences were considered, and when the % identity demarcation was pushed below 100%, more sequences were grouped, including two based solely on nucleotide sequences. It may be tempting to assume the reason for this clustering is that the slight variation on the nucleotide level is the result of PCR not being 100% error proof and that these clustered isolates are actually the same virus, or otherwise too similar to distinguish. However, Schroeder et al. (2002) point out that the MCP nucleotide sequences reflect the differences in host range of the different isolates. This presents a caveat when working with aquatic viruses.

Species, strain, and ecotype demarcation in the microbial world has never been straightforward, but from collective phylogenetic and genomic studies, general guidelines have been established for members of the Bacteria domain (Cohan 2002; Konstantinidis et al. 2006). Demarcation for viruses, however, has required revision for individual families or groups of viruses (e.g. Flexiviridae (Adams et al. 2004), Geminiviridae (Fauquet et al. 2008; Fauquet and Stanley 2005), *Tombusvirus* within Tombusviridae (Koenig et al. 2004), and *Ilarvirus* within Bromoviridae (Maliogka et al. 2007)). Until more genomes have been sequenced and critical biomarkers identified with representative samples assessed, it may be too soon for analyses of families such as Phycodnaviridae to assign % identity guidelines; more members will need to be isolated as well. It is therefore important that as molecular studies are progressing researchers make note of the selected cutoff percentages of the operational taxonomic units used in their phylogenetic assessment so that in the future, proper guidelines may be more easily developed.

Overall, this research provides a foundation for future work by providing new

sequences of EhV-specific MCP, and for the first time, new sequences of EhV-specific MCP and crude isolates from an open ocean transect. That many of these sequences were detected from lysates after amplification via host infection is important, as now researchers have representatives of these sequences in culture. The infectivity of the viruses represented by these new sequences is not a question, due to their culturability. Moreover, from these crude isolates, clonal isolates can be attained and characterized (DNA *pol* sequences, host ranges, etc.) to bring about more resolution in the phylogenetics of EhV. Indeed, it may be possible to attain > 20 new clonal EhV representatives in culture, more than doubling what is presently available, while more representatives from the environmental samples may be detected using additional host strains.

Though it is not unexpected that several sequences detected from the open ocean are similar to those from coastal waters of the same ocean, it is very clear that the richness of EhV is higher than previously known. The evenness (admittedly a crude measure in this case) of these sequence libraries alone, and in consideration with the dynamic nature of the North Atlantic of that time (Rowe et al. 2008), suggest an ever-changing EhV population (Thingstad and Lignell 1997) that can be monitored with the MCP gene as a biomarker. Such analyses can be extended to other marine habitats and presumably to other Phycodnaviridae as well. Lastly, it may well be worth the effort for researchers to attempt to generate lysates, via host cultures, from the same natural samples from which they are looking to determine genetic variability of a target viral population. This can bring any new sequences into culture and demonstrate the viability of the viral population being assessed, which differs from previous studies wherein an isolate is obtained first, with its presence across an environment determined subsequently. Through the combination of culture-independent (first) and culture-dependent (second) methods described herein, the clonal isolates of interest, based on their numerous detections in environmental samples, can be selected for further study. The end result being the targeted isolation of environmentally relevant isolates for the continued assessment of their role(s).

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SECTION VI

CONCLUSIONS

This collection of experiments, observations, and analyses cover a broad range within the study of marine viral ecology. Aquatic viruses play integral roles in their microbial communities (Brussaard et al. 2008; Wilhelm and Matteson 2008): maintaining host diversity by killing “the winner” (Thingstad and Lignell 1997), initiating and perpetuating (Gobler et al. 2004; Gobler et al. 1997), as well as terminating (Gastrich et al. 2004; Nagasaki et al. 1994) algal blooms, releasing cellular components and nutrients that are bioavailable to surviving community members (Gobler et al. 1997; Middelboe and Jorgensen 2006; Poorvin et al. 2004; Wilhelm and Suttle 1999), releasing cellular dimethylsulphoniopropionate (DMSP), which could indirectly progress into cloud formation (Hill et al. 1998; Liss et al. 1997; Thornton 2002), and transferring genetic material between hosts (Canchaya et al. 2003; Lindell et al. 2004). These activities can have a significant impact on the global-scale influences of their hosts (Wilhelm and Suttle 1999) and therefore it is important to understand what constrains aquatic viruses. There is also a need for better methods to assess the relationships between virus and host and between virus and ecosystem. This dissertation has served in part to address these issues. A variety of tools were employed i) to examine a specific virus-host system fraught with inconsistencies, ii) to examine the constraints on marine virus parameters across three oceanic regimes, and iii) to examine the diversity within a family of marine eukaryotic algal viruses and provide a foundation for future quantitative studies. From such analyses, the following have been determined:

- 1) The filterable agent causing cell death of *Aureococcus anophagefferens* CCMP 1784 is a virus that resembles members of Phycodnaviridae: icosahedral shape, ~ 140 nm in diameter. Though PCR using AVS primers was performed, DNA polymerase was not amplified from viral DNA, making taxonomic classification inconclusive at this point.
- 2) Phage-like particles observed in both treatment and control groups of *A. anophagefferens* cultures are unlikely to be the cause of cell death for this algal host.

Moreover it stands to reason that these phage-like particles were bacteriophage, infectious to the heterotrophic bacterial contaminants. Previous reports of phage-like viruses of *A. anophagefferens* could have actually been examining similar bacteriophage infecting contaminants and host cell dead could have been caused by the introduction of pathogenic bacteria instead (Frazier et al. 2007). Overall, this emphasizes the need for proper isolation and identification techniques.

3) The activity of viruses in the Sargasso Sea, an oligotrophic, low nutrient, marine regime, correlates to environmental variables. This “predictability” is likely related to the system’s relative low productivity and the static nature of the marine system.

4) Viruses in the North Atlantic during an annual spring algal bloom, appear to respond to environmental queues up to a certain threshold. After major influential factors reach a maximum, factors not correlated to viral activity in other systems (e.g. the Sargasso Sea) may have an impact on viral activity. This observation was made possible due to the gradient in productivity between the Sargasso Sea and North Atlantic, demonstrating the influence of sampling schemes that cross the boundaries of neighboring regimes.

5) Viruses in the Western Pacific Ocean, including the Western Pacific Warm Pool (9° 15' 1.018" S, 169° 59' 58.621" E), demonstrated few correlations to environmental parameters.

6) The three oceanic realms studied: the Sargasso Sea, the North Atlantic, and the Western Pacific, are different regimes. Thus, we cannot generalize across them. While not surprising, an upshot of this study is the statistical demonstration of this with respect to viruses as well as other biological and environmental metrics. Though analyses of total (*i.e.* cross-realm) data are valuable for understanding overall trends, regime-dependents trends may be lost and the strength of overall correlations may be skewed. Additionally, measuring parameters across multiple regimes requires multivariate analyses of data sets.

7) The diversity of *Emiliana huxleyi* Phycodnaviruses based on nucleotide sequences of the major capsid protein gene demonstrates a new richness in the North Atlantic that is distinct from what has been previously examined in the English Channel and coastal waters of Norway. Spatial trends in diversity were not observed in phylogenetic tree structure. Rarefaction curves suggest a greater species richness exists in the North Atlantic than is represented by these new samples. That several sequences were overrepresented during coccolithophorid bloom progression suggests a peaking of viruses infectious towards the bloom-forming species, as described by the “kill the winner” hypothesis (Thingstad and Lignell 1997).

8) The major capsid protein gene as a biomarker for *Emiliana huxleyi* Phycodnaviruses is effective for detection and identification of such viruses. The high conservation of this gene, leading to primers without degeneracy, makes the use of this biomarker well-suited for environmental samples. However, this high conservation makes phylogenetic analyses difficult, suggesting that the use of an additional biomarker could provide better resolution.

Overall, it is clear that to understand virus-host dynamics and how these interactions affect higher trophic levels, research is needed at multiple levels; specific studies of virus-host systems need to be complemented by examinations of the total virus and host populations. The work done with the *A. anophagefferens* and infecting virus system also serves to highlight the importance of visualizing the pathogenic agent, especially in a field where molecular analyses are not yet able to cover the vast diversity available. Moreover, if adequate molecular tools are to be developed so that populations can be teased apart and diversity examined, proper isolation of target viruses must be a priority in order to eliminate future confusion.

It is from these single virus-host systems that researchers are able to develop tools to study the complex natural systems that possess multiple virus-host systems. These large-scale studies provide overall as well as regime-dependent trends that can further

elucidate those factors that influence and are influenced by viral activities. For a global assessment, the world's oceans cannot simply be treated as a whole. Statistical examinations of three, large spatial regions have shown that with an overall analysis, regime-dependent trends may be lost. Researchers must be prepared to tease apart marine ecosystems to understand how they work in sum. In much the same way, while a great deal can be learned about marine virus populations by examining parameters of the total population, it is important to one day be able to examine the individual components of this total *in situ* to understand more specifically, who is doing what, where and when.

Because viruses lack universal genes similar to 16S and 18S rRNA genes, it will take much more work to assess viral diversity, especially in aquatic systems for which, more sampling is an absolute necessity. In order for these large-scale viral populations to be teased apart into composite families, genera, or perhaps even strains, biomarkers must be found and tested that can serve not only to identify the population of interest, but allow for a measurement of diversity. From this work, as well as that done by others (Larsen et al. 2008; Schroeder et al. 2002), it is not unreasonable to suggest that the MCP gene may become a biomarker for Phycodnaviridae, though it is unlikely to completely replace DNA *pol* (Chen and Suttle 1995; Chen et al. 1996; Dunigan et al. 2006).

This research presented herein provides for several avenues for future work. For example, it may be possible that assessing DNA *pol* and MCP genes from clonal isolates could provide the necessary resolution to determine phylogenetic relationships among *Emiliania huxleyi* Phycodnaviruses. Moreover, the high conservation of MCP gene sequences suggests, and preliminary work also indicates, the feasibility of using this biomarker for quantitative work, such as *Emiliania huxleyi* Phycodnavirus-specific qPCR. The quantitation of these and other specific viruses from environmental samples can facilitate teasing apart the total viral abundances and production rates observed into relative abundances and production rates of the targeted viruses. From this, researchers can monitor how viruses of interest relate to the total population and how they change over spatial and temporal scales as well as over differing gradients. These observations could be combined with observations of potential host diversity changes over these same scales and gradients, such as presently being investigated over the Western Pacific

transect. Lastly, it is important for researchers to remember while taking the initial steps towards molecular work with new viruses, isolation and identification, that such steps are critical for effective work down the line. One avenue that will always be valuable is the exploration of more aquatic habitats and the collection and culturing of more viruses and hosts. Though more work lies ahead for research on viruses in aquatic microbial systems, the work described herein provides new and foundational information that can facilitate these future directions.

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