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Quantification and Ecological Perspectives on Cyanophage and Aquatic Viruses

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To the Graduate Council:

I am submitting herewith a dissertation written by Audrey Renee Matteson entitled "Quantification and Ecological Perspectives on Cyanophage and Aquatic Viruses." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

Steven W. Wilhelm, Major Professor

We have read this dissertation and recommend its acceptance:

Alison Buchan, Erik R. Zinser, Mark A. Radosevich, George S. Bullerjahn

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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Quantification and Ecological Perspectives on Cyanophage and Aquatic Viruses

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

Audrey Renee Matteson
May 2011

DEDICATION

To my friends and family for their encouragement

and confidence when I needed it the most.

And Mike: you're awesome.

"There's a lot of things you need to get across this universe. Warp drive, wormhole refractors. You know the thing you need most of all? You need a hand to hold." –The Doctor

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I still cannot believe I am writing this! This whole process could not have been done on my own, and I would like to thank several people for their help throughout my years in Tennessee.

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ABSTRACT

The field of viral ecology is still relatively new and many processes by which viruses influence hosts are still widely unknown. One problem is that there are few standardized techniques in virus ecology, making comparisons of data very difficult. To better understand the methodology, we first set out to make a video showing the process for the viral production assay using the "dilution and reoccurrence" method, which has become the standard to analyze production rates in aquatic ecosystems. Using this method, we also determined the production rates of viruses during a seasonal pelagic phytoplankton bloom during a cruise off the coast of the north island of New Zealand in the subtropical Pacific Ocean. Other biotic and abiotic parameters were also compared throughout the bloom. Production rates were within normal ranges, but showed that viruses were very important for the remobilization of nutrients in the nitrogen-limited system.

It is well known that the cyanobacterial genera *Synechococcus* and *Prochlorococcus* thrive in the world's oceans with *Synechococcus* and other cyanobacterial species also succeeding in freshwater ecosystems. Cyanophages are viruses which infect cyanobacteria and many studies have investigated their diversity using the portal vertex *g20* gene in the *Cyanomyoviridae* family. Although we know that there is significant genetic richness in these phage in marine and freshwater environments, information on their numerical distributions is rare. Using quantitative PCR with the *g20* gene, we determined that cyanomyoviruses are ubiquitous and abundant in the Atlantic and Pacific Oceans as well as within Lake Erie. Using statistical analyses we were able to find correlations between cyanomyoviruses and other biotic and abiotic parameters: in the Sargasso Sea, cyanomyovirus abundance correlated well to biology, but in the other systems there was no significant correlation to biological abundances. This suggests that the constraints of this group of viruses may be different in different aquatic realms.

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List of Abbreviations

- CCGS, Canadian Coast Guard Ship
- chl *a*, chlorophyll *a*
- CTD, conductivity, temperature, density
- cyanos, cyanobacteria
- DGGE, denaturing gradient gel electrophoresis
- DOM, dissolved organic matter
- *pol*, DNA polymerase gene
- DNA, deoxyribonucleic acid
- DNA pol B, DNA polymerase I gene of the B family
- DRP, dissolved reactive phosphorus
- dsDNA, double stranded DNA
- Fe, iron
- FeCycleI, first cruise name in the southern Pacific Ocean
- FeCycleII, second cruise name in the southern Pacific Ocean
- HB, heterotrophic bacteria
- MCP, major capsid protein
- MPN, most probable number
- N, nitrogen
- NASB, North Atlantic Spring Bloom cruise
- P, phosphorus
- PEG, polyethylene glycol
- % MLV, percentage of mortality due to viral lysis
- PFGE, pulse field gel electrophoresis
- PCA, principal component analysis
- PCR, polymerase chain reaction

- qPCR, quantitative polymerase chain reaction
- RNA, ribonucleic acid
- RV, research vessel
- Se, selenium
- spp., species
- TEM, transmission electron microscopy
- TFF, tangential flow filtration
- TB, total bacteria
- VA, viral abundance
- VBR, virus to bacteria ratio

Chapter 1 Literature review

Viruses are the most abundant biological entities in the world's oceans. Globally there are estimated to be up to ~4 x 10^{30} viruses in the oceans (Suttle 2005), suggesting that within the marine microbial community there is 270 Mt of carbon just from viruses (Wilhelm and Suttle 1999). The study of the ecology of viruses, with a focus on marine bacteriophage, remains a relatively new field of study. It has only been during the past two decades that scientists have begun to address questions concerning on the importance of bacteriophage in aquatic ecosystems. Despite slow beginnings of this field, it has quickly demonstrated their sheer abundance and role in the microbial food web and biogeochemical cycling of nutrients makes viruses key players in global scale processes (Wilhelm and Matteson 2008).

First Identification of Viruses

In the late 1800s, the first reports of an ultramicroscopic virus named the tobacco mosaic virus, which infected the tobacco plant, were described by Dimitri Ivanowski (Ivanowski 1892a; Ivanowski 1892b), but his contributions were questioned since he was unsure if he had a new agent for infection. Martin Beijerinck was the man who received the recognition as the first scientist to describe this "soluble" agent that could be diluted and passed on to other plants (Beijerinck 1899). It was also around this time that work was done studying the cattle disease foot-and-mouth disease (Loeffler and Frosch 1898). All of these efforts laid the foundation for virology. To understand and believe such an agent existed at the time was very forward thinking. It is remarkable that this work was done only thirty years after Pasteur's experiments disproving spontaneous generation (Schwartz 2001). Until the early 1900s, there had been no publications describing a viral agent capable of lysing a bacterium.

In 1915, Frederick Twort published a paper which described a possible ultramicroscopic virus that infected a *Micrococcus* species isolated from calf vaccinia lymph (Twort and Lond 1915). He believed there were pathogenic and nonpathogenic viruses, and that only pathogenic ones had been isolated previously. He used a wide variety of different media types and samples, but never came across a virus able to grow without a host. He did, however, publish the first scientific data on bacteriophage. In the end, he was ultimately unsure of what he had. Since

there were accounts of viruses that infected plants and animals, he admitted what he had come across may be a virus which infects bacteria. He also believed it could be an enzyme produced by the bacterium. Unfortunately he did not have the funds to pursue the research, and his work was not acknowledged by his contemporaries.

The French-Canadian Felix d'Herelle was the first to name these viruses "bacteriophage", or a virus "devouring bacteria," (d'Herelle 1917). He was able to isolate bacteriophage effective against dysentery which he obtained from people recovering from the disease (Duckworth 1976). Unlike Twort, d'Herelle received significant attention for his work (perhaps due to its proposed use as a therapeutic agent against specific diseases). He also did not cite any references in his paper, leaving it to appear he was the sole discoverer of a bacteriophage. A few years later, Twort's paper was uncovered and there was much controversy on the true discoverer of the first bacteriophage (Duckworth 1976).

Discovery of Phage Infecting Marine Bacteria

The study of marine bacteriophage lagged behind those for human infectious diseases up until the 1950s (Wiebe and Liston 1968). During the years of 1943-1950, 101 of the 157 papers on phage dealt with *Escherichia coli* T series of phage (Baer and Krueger 1952). Nonetheless, phages isolated from the Black Sea by Kriss and Rukina in the 1940s were able to infect various species of terrestrial bacteria (Spencer 1959). Since no attempt was made to culture these viruses, it must be stated that perhaps these were not viral plaques but antibiotic effects or protozoan grazing (Wiebe and Liston 1968). Even more work was done during the 1950s to isolate new phage, as well as to try new methods to estimate virus production and burst size in cultured isolates. One system extensively studied was on vibriophage, phage that infect the *Gamma Proteobacteria Vibrio*, by Smith and Krueger (Smith and Krueger 1952, 1954).

Perhaps one of the first true marine bacteriophage was documented by Spencer during the late 1950s and early 1960s (Spencer 1955, 1959, 1963). Marine bacteria were often isolated from fish to screen bacteriophage. He was able to isolate species of *Pseudomonas*, *Photobacterium*, and *Cytophaga*. He concluded that these were true marine bacteriophage

because they were only effective in media mimicking seawater and had lower inactivation temperatures than terrestrial phage (Spencer 1955).

Through the 1950s and 1960s, most research focused on isolating new marine bacteriophage. The potential importance of viruses in the food web, their effects on community structure and their potential role in horizontal gene transfer were not suggested until 1968 (Wiebe and Liston 1968). What was known about the food web at the time did not include viruses or the viral shunt (Pomeroy 1974). This, in part, was due to the idea that bacteriophage were ecologically and numerically unimportant (Wiggins and Alexander 1985). Unfortunately, it was not until transmission electron microscopy (TEM) was first used to screen seawater which showed that viruses persist in higher abundances (upwards of 10^4 mL^{-1}) than previously thought (Torrella and Morita 1979).

In 1989, the abundance of bacteriophage in the ocean was rediscovered, with estimates of up to 10^8 bacteriophage mL^{-1} from various aquatic sources (Bergh et al. 1989). Building on this other research quickly confirmed the abundant distribution of viruses and determined that many ecologically important cyanobacterial and heterotrophic bacterial species were visibly infected (approximately 5-7%) (Proctor and Fuhrman 1990; Suttle et al. 1990). They went on to claim that infection by viruses reduced primary productivity in species such as diatoms and cyanobacteria (Suttle et al. 1990). These papers showing much higher abundances sparked new interest in studying the effects of phage in terms of distribution, activity, diversity of both host (by the "Kill the Winner" method (Thingstad and Lignell 1997)) and phage, and its impact on biogeochemical cycling in aquatic ecosystems.

Methods to Enumerate Viruses

Up to mid-1990s, the most common method to enumerate viruses was by TEM analysis (Torrella and Morita 1979; Bergh et al. 1989; Proctor and Fuhrman 1990). TEM was first used to visualize viruses in 1959 (Field 1982; Goyal 1987). This method has an advantage in that morphological characteristics (*e.g.*, estimates of capsid and tail size) can be determined which is often what is used to group phage into different families. TEM is also often used to determine

the minimum burst sizes. Minimum burst sizes are calculated by identifying the number of virus particles found in an infected cell (Bratbak et al. 1992; Weinbauer and Peduzzi 1994) and can be used in determining the virus-inferred bacterial lysis in the system. In terms of enumerating viruses, TEM has often proven to provide an underestimate of true abundance compared to other techniques used today (Hara et al. 1991; Suttle 1993; Noble and Fuhrman 1998).

Historically, to determine the abundance of phage able to infect a particular host scientists have used the most probable number (MPN) assay. In this method, a specific host (for cyanophages, a cyanobacterium) is propagated with dilutions of viral samples and lysis is investigated (Suttle and Chan 1993). This method allows you to study the population that is actively infectious (specific host-infecting), but it contains inherent problems. Often only laboratory strains of cyanobacteria are used for the MPN assays and very crude estimates of cyanophages are determined since cyanophages often only infect a small portion of hosts (Weinbauer 2004). A second method used is the plaque assay where the host is plated onto a Petri dish with a diluted natural sample. After a few weeks plaques of viral lysis are enumerated and phage titers are estimated by the dilution used and the quantity of plaques found. This method contains the same downfalls as the MPN assay, but also the problem that some strains of bacteria are difficult to grow on plates making the plaque assay unusable in some instances.

Most recently epifluorescence microscopy, using fluorescent dyes such as SYBR Green, SYBR Gold, DAPI, or YOPRO-1, has been used in estimating virus abundance (Suttle et al. 1990; Hennes and Suttle 1995; Noble and Fuhrman 1998; Weinbauer 2004; Wen et al. 2004; Sandaa and Larsen 2006). All of these dyes have been used to estimate abundance, but there have been many debates as to which yield the best results. Attributes such as low background fluorescence, stability of the dye compound, and adequate brightness to count the viruses are desirable (Wommack and Colwell 2000). Like most quantitative techniques, there are pitfalls which arise from excluding large genome viruses from counts based on their large size, leading to a decreased viral enumeration (Sommaruga et al. 1995). There are also issues of counting DNA bound to colloids which would also inaccurately influence viral abundance (Kepner and Pratt 1994).

Viral abundance has also been estimated by flow cytometry (Marie et al. 1999; Chen et al. 2001; Brussaard 2004). Some of the same dyes used in epifluorescence microscopy are used to sort phage from samples in this method (*i.e.,* SYBR Green). Sample preservation and consistency of analysis have been problematic in the past, but this technique has shown promise in analyzing viral abundances in natural environments (Brussaard 2004). Some of the drawbacks of using flow cytometry to enumerate viruses are similar to those for epifluorescence microscopy as well as the sensitivity of the flurometer used (Weinbauer 2004). A review of all the strengths and weaknesses of these techniques can be found in a review by Weinbauer (2004).

Quantitative PCR (qPCR) has been used to enumerate various groups of microorganisms in both terrestrial and aquatic environments. This topic has been reviewed previously (Zhang and Fang 2006). Despite the application to microorganisms, work quantifying specific groups of phage has lagged behind. Most work analyzing viral abundance in the environment using qPCR has been on viruses infecting humans and viruses in wastewater treatment plants (Lamothe et al. 2003; Laverick et al. 2004; Biofill-Mas et al. 2006; Carducci et al. 2008; Meleg et al. 2008). Analyses on the ecologically important bacteriophage and cyanophage have been sparse, most likely due to high diversity. The higher the diversity, the harder it is to make specific primers for qPCR. There has been one report of analyzing cyanomyoviruses in a Norwegian coastal water station using PCR primers specific for the *g20* gene (method described below) (Sandaa and Larsen 2006). QPCR has also been used to study cyanophages specific for the bloom-forming cyanobacterium *Microcystis aeruginosa* (Takashima et al. 2007; Yoshida et al. 2010). There has also been work done with qPCR on algal viruses with only one report on the freshwater Laurentian Great Lake Ontario (Short and Short 2009). As the pool of viral sequence information continues to grow, this technique will likely be utilized in the near future to tease apart abundances and production rates of specific phages in various aquatic ecosystems.

Cyanobacteria and Cyanophage

One of the most diverse and ecologically important groups of prokaryotes in aquatic ecosystems is the oxygenic phototrophic cyanobacteria (also called blue-green algae). They are photosynthetic bacteria that require light, carbon dioxide and inorganic substrates to survive in

aquatic systems (Mur et al. 1999). They exist as planktonic cells (*e.g. Prochlorococcus* and *Synechococcus* species (spp.)) or in colonial forms (*e.g. Microcystis aeruginosa* and *Trichodesmium* spp.). They reside at the base of the microbial food web and play a vital role in the primary production in the world's oceans and lakes (Callieri and Stockner 2002). Within the extensive research done on marine carbon fixation, it was found that the marine *Synechococcus* and *Prochlorococcus* species are able to fix up to 80% of the carbon in the oligotrophic oceans (Goericke 1993; Li 1995; Liu and Campbell 1997; Rocap et al. 2002). Their contribution to primary production in freshwater ecosystems, including the Great Lakes has yet to be determined, but with their high abundance in these systems (~40% (Carrick and Schelske 1997)), it is also likely to be high. Their importance in primary production does not eclipse their evolutionary impact on molding the primordial earth into its current oxygenated form. Cyanobacteria are found in wide variety of ecosystems and research on cyanobacteria inhabiting diverse marine environments has been abundant (Schmidt et al. 1991; Urbach et al. 1998; Partensky et al. 1999; Moore et al. 2002; Paerl 2002; Scanlan and West 2002; Zinser et al. 2006). The photosynthetic picoplankton is a major group of cyanobacteria (as well as picoeukaryotes) with sizes ranging from 0.2 to 2 µm (Callieri, 2002). Two genera, *Prochlorococcus* and *Synechococcus*, often dominate the world's oceans, and contribute greatly to primary production (Waterbury et al. 1986; Partensky et al. 1999). Despite co-occuring in marine waters, their spatial distributions (Moore et al. 1998; Scanlan and West 2002), light-harvesting apparatus (Goericke and Repeta 1992; Urbach et al. 1998; Ting et al. 2002), cell size (Johnson and Sieburth 1979; Chisholm et al. 1988) and nitrogen assimilation strategies (López et al. 2002; Moore et al. 2002) differ.

Viruses are obligate parasites and have RNA or DNA genomes and a protein capsid coat. They are often species-specific, and the types which target cyanobacteria are known as cyanophage. The first cyanophage was discovered in 1963; it infected the filamentous cyanobacteria *Plectonema*, *Phormidium* and *Lyngbya* (Safferman and Morris 1963). At the time it was believed that these cyanophage would be able to act as biological control agents against harmful cyanobacterial blooms that often persist in many freshwater ecosystems (Martin and Benson 1988; Suttle 2000). It was later shown that this is unlikely to be the case since many cyanobacteria have the ability to become resistant to infection and phages alone cannot inhibit

the formation and duration of a bloom event (Waterbury and Valois 1993; Suttle 2000). It was many years later that the first reports of cyanophage in a marine ecosystem were discovered in coastal waters of the Black Sea (Moisa et al. 1981; Suttle 2000).

Morphologically there are three different families of double-stranded tailed DNA cyanophage within the order *Caudoviridae*: *Myoviridae*, *Siphoviridae*, and *Podoviridae*. Their morphologically different tails help characterize them into their respective families. The *Myoviridae* are T4-like phage and have tails that contract with a neck separating the tail from the icosahedral capsid top. The *Siphoviridae* are λ-like phage and have long non-contractile tails, while the *Podoviridae* are T7-like phage and have short non-contractile tails (Suttle 2000). The myoviruses and podoviruses tend to be lytic phages while siphoviruses are able to undergo lysogeny (insertion of phage DNA into the host genome) and survive as a prophage in their host (Suttle 2005). To date, more work has been done studying the diversity and abundances of viruses in marine ecosystems (Fuhrman 1999; Wilhelm and Suttle 1999) compared to freshwater. In marine waters, the most common marine viruses are the Cyanomyoviruses which are in the *Myoviridae* family (Hambly et al. 2001) and have also been the most commonly isolated cyanophage family. The host ranges are also different among the different families. Myoviruses tend to have the broadest host ranges and are able to infect across genera and can infect both *Prochlorococcus* and *Synechococcus* (Suttle and Chan 1993; Waterbury and Valois 1993) while podoviruses have very narrow host ranges which suggests a different host-phage relationship between the two phage families (Wang and Chen 2008).

Phage Diversity

It has been proposed that marine viruses provide the largest reservoir of genetic diversity on Earth (Hambly and Suttle 2005). However, most studies have been inhibited by the fact that phage are only able to be propagated if a host has been cultured. Only around 0.1% of marine bacteria are able to be cultured so, on a whole, very little is known about marine bacteriophage or their true diversity (Kogure et al. 1980). Furthermore, there is no equivalent to the 16S or 18S rRNA gene in viruses to infer evolutionary relationships or diversity (Paul et al. 2002). Other problems such as insufficient DNA concentrations, DNA restriction enzyme resistance and the

inability to properly clone viral genes due to deleterious effects have hindered some molecular work (Paul et al. 2002). With the help of several whole genome sequencing efforts, several conserved genes have been identified within various families of phages and eukaryotic viruses that can help elucidate the natural diversity of unculturable phage. Currently (as of November 2010) the genomes of 595 phages have been sequenced, 21 of these being cyanophages, and have been deposited into the NCBI database. Of the 21 sequenced cyanophage genomes, 17 of these were myoviruses, 3 were podoviruses, and only 1 siphovirus. One of the first polymerase chain reaction (PCR) primers developed for marine viruses was developed in 1996 (Chen and Suttle 1996; Chen et al. 1996) and targeted the DNA polymerase gene (*pol*) in eukaryotic algal viruses. This quickly led to other bacteriophage-specific biomarkers to analyze phage diversity.

One marker that has been widely used is the *g20* gene which is a homologue of the coliphage T4 capsid portal protein (Fuller et al. 1998; Wilson et al. 1999). This gene is important in DNA packaging, viral capsid assembly and head tail junctions. The degenerate primer set CPS1 and CPS2 was developed to target the 165-bp gene specific for the *Myoviridae* family of cyanophage. These primers have been used for denaturing gradient gel electrophoresis (DGGE), pulse field gel electrophoresis (PFGE), clone libraries, and qPCR analysis, but the amplicon is too small for phylogenetic analysis. A second primer was developed to increase the product size to 592-bp using the same CPS1 forward primer (Zhong et al. 2002). Many studies of marine (Zhong et al. 2002; Muhling et al. 2005; Short and Suttle 2005; Sandaa and Larsen 2006), brackish (Marston and Sallee 2003; Wang and Chen 2004), and freshwater (Dorigo et al. 2004; Short and Suttle 2005; Wilhelm et al. 2006) viral communities have been investigated using both sets of these primers, and have shown much uncharacterized diversity. Despite high diversity found in these environments, similar isolates and environmental sequences have been found globally, which suggests the movement of viruses across different biomes (Chen and Suttle 1996; Short and Suttle 2005). An exhaustive list of published articles using the *g20* gene and the primer sets used can be found in Table 1.1. It has been proposed that some of the diversity exhibited may come from bacteriophage, instead of strictly cyanophages as once thought since several clades of environmental sequences have no cultured representatives. This reinforces the fact that more molecular tools need to be developed to infer true diversity of marine phage. Recently a new *g20* PCR primer set has been developed and claims to target a

Table 1.1: List of journal articles using the *g20* gene marker for phylogenetic analysis. Samples collected from marine ecosystems are bolded while freshwater ecosystems are not.

***g20* Gene Primer Set**	**Reference**
CPS1/CPS2 (165 bps) **CPS1-**GTAGWATTTTCTACATTGAYGTTGG **CPS2-**GGTARCCAGAAATCYTCMAGCAT	**(Marston and Sallee 2003)** **(Fuller et al. 1998)** **(Sandaa and Larsen 2006)** **(Wilson et al. 2000)** **(Frederickson et al. 2003)** **(Okunishi et al. 2002)** **(Sullivan et al. 2008)** **(Zhong et al. 2002)** **(McDaniel et al. 2006)** This Study
CPS3/CPS4 (850 bps) **CPS3-**TGGTAYGTYGATGGMAGA **CPS4-**CATWTCWTCCCAHTCTTC	**(Zhong et al. 2002)**
CPS1/CPS8 (592 bps) **CPS1-**GTAGWATTTTCTACATTGAYGTTGG **CPS8-**AAATAYTTDCCAACAWATGGA	**(Marston and Sallee 2003)** **(Wang and Chen 2004)** **(Zhong et al. 2002)** **(Short and Suttle 2005)** **(Sullivan et al. 2008)** **(Desbonnet et al. 2008)** (Dorigo et al. 2004) (Wilhelm et al. 2006) (Wang et al. 2010) (Short and Suttle 2005)
CPS1.1/CPS8.1 (585 bps) **CPS1.1-**GTAGWATWTTYTAYATTGAYGTWGG **CPS8.1-**ARTAYTTDCCDAYRWAWGGWTC	**(Sullivan et al. 2008)** **(John et al. 2010)** **This Study**

broader range of cyanomyoviruses based on information they gained on cultured isolates (Sullivan et al. 2008).

While many use the *g20* gene, others have used the major capsid protein (MCP) *gp23* gene to study the diversity of myoviruses, including the cyanomyoviruses. This work has shown that the T-4 like phages is a large and divergent superfamily that can be found in the world's oceans (Filée et al. 2005; Comeau and Krisch 2008). MCP-specific primers for freshwater filamentous cyanobacteria *Nostoc* and *Anabaena* have also been made and used to analyze the diversity of freshwater myoviruses. This type of freshwater cyanomyovirus was found to be genetically very different than the phages infecting unicellular cyanobacteria (*i.e. Synechococcus* and *Prochlorococcus*) in the world's oceans (Baker et al. 2006).

Recently primers specific for the DNA polymerase gene (*pol*) in the *Podoviridae* family of cyanophages (Wang and Chen 2008) were made available. The conservation of this gene among the podoviruses demonstrated how vital it is for the replication of its genome during lytic infections. This primer set is degenerate and was constructed from sequences from nine isolated podoviruses and four environmental sequences (Wang and Chen 2008). These primers have been used across several marine regimes (both coastal and pelagic) and have shown that podoviruses, like myoviruses, are ubiquitous in the world's oceans and are very diverse (Huang et al. 2010). Currently no PCR primers have been constructed for the *Siphoviridae*.

Two genes encoding the D1 and D2 proteins (*psbA* and *psbD*) important in Photosystem II core reaction center have been identified in many marine cyanophage isolates and from environmental samples (Mann et al. 2003; Millard et al. 2004; Paul and Sullivan 2005; Zeidner et al. 2005; Sullivan et al. 2006; Chenard and Suttle 2008; Wilhelm and Matteson 2008). More recently it was found that this gene was not limited to just marine cyanophages, but other freshwater cyanophages infecting unicellular cyanobacteria (Chenard and Suttle 2008; Wilhelm and Matteson 2008). These genes were found after whole genome sequencing methods of bacteriophage S-PM2 which infects *Synechococcus* spp. (Mann et al. 2003). This provides an interesting link of lateral gene transfer from host to virus. It has been shown that *psbA* is in fact expressed during cyanophage infection which is believed to increase cyanophage fitness by prolonging photosynthesis (Lindell et al. 2004; Lindell et al. 2005). These genes were shown to

be co-transcribed with other vital capsid genes in the phage (Lindell et al. 2005). The prolonged photosynthesis may increase the number of phage produced, leading to higher burst sizes from increased energy production.

Many PCR primers have also been developed to analyze the diversity and richness of eukaryotic algal viruses. Traditionally the DNA polymerase I gene of the B family (DNA *polB*) has been used for *Phycodnaviridae* (large double-stranded DNA viruses) (Chen and Suttle 1995; Chen et al. 1996), but more recently it was suggested that the MCP gene may also be a useful biomarker for studying all eukaryotic viruses except for *Emiliania huxleyi* based on increased sequence information (Schroeder et al. 2002; Larsen et al. 2008).

Since studying only conserved genes does not demonstrate the true diversity of a sample, viral metagenomic work has also been done to study diversity and distribution of viruses in various ecosystems. In 2002, seawater from coastal California was used to make a metagenomic library (Breitbart et al. 2002) and showed much uncharacterized diversity that included many of the double-stranded DNA viruses including algal viruses. In 2006, four geographically distinct oceanic regions were analyzed and found to have high global diversity (Angly et al. 2006). Other ecosystems such as the Chesapeake Bay (Bench et al. 2007), Yellowstone hot springs (Schoenfeld et al. 2008), Florida reclaimed water (Rosario et al. 2009) and the British Columbia (Culley et al. 2006) have been used for viral metagenomics. High diversity is often exhibited with most sequences not found in sequence databases. Although most often the results of these experiments leads to unique and unidentified genes, as more work is done, others can come back to this information later and use it in their analyses.

Virus Abundance

As stated previously, viruses are the most abundant organisms in the oceans and are pervasive in all aquatic ecosystems. The abundance of viruses across ecosystems is quite variable and often ranges from below 10^4 to over 10^8 mL^{-1} (Wommack and Colwell 2000) but are generally found between 10^5-10^7 mL^{-1}. Abundances have been determined in a vast array of locations such as oceans, lakes, sediments, hot springs, and soils and a review of these

abundances can be found in Weinbauer (2004). A subset of the abundances found in freshwater and marine ecosystems can be found in Table 1.2. Although abundant in both environments, viruses in freshwater systems tend to be higher, especially in more mesotrophic or eutrophic ecosystems with higher bacterial and phytoplankton biomass (Maranger and Bird 1996; DeBruyn et al. 2004; Filippini and Middelboe 2007).

Virus Production

Virus production rates and turnover have been analyzed in multiple environments and are important in quantifying the bacterial community that is infected with a virus. Many techniques have been used to study virus production rates, all with their own advantages and disadvantages which have led to additional problems with analyzing the datasets across the different types. Unfortunately no technique is perfect and no reliable method has been proposed to date (Suttle 2005). A few recent articles have highlighted these techniques and the issues with each (Thingstad et al. 2008; Weinbauer et al. 2010). The first method developed detected the decay rates of viruses after the addition of a chemical to stop virus production compared with untreated controls (Heldal and Bratbak 1991; Fuhrman 1999). A second popular technique is by radiolabeling virus particles with ^{3}H, ^{32}P or ^{14}C to determine the production rate of viral DNA produced over time which is similar to the method by estimating bacterial production rates (Steward et al. 1992a; Steward et al. 1992b). The method that has become the gold standard for virus production assays is the virus reduction (or dilution) method by which free viruses are reduced and the virally infected bacterial community is concentrated and the production of viruses are analyzed microscopically over time (Wilhelm et al. 2002). Methods to concentrate the bacterial communities using dead end and tangential flow filtration (TFF) have been compared and no significant differences were found (Weinbauer et al. 2010). Very similar to this technique is a method by which virus-free water is added to a natural sample to decrease the contact rate between host and virus to determine the production rate (Evans et al. 2003). Other methods have been employed, but intrinsic problems have decreased their usage in determining the production rate in natural samples.

Table 1.2: Estimates of virus abundances in several aquatic ecosystems.

Reference	Sampling Location	Virus Abundance (mL^{-1})
Lakes:		
Leff et al. 1999	Lake Erie	1.0×10^6-3.4×10^7
DeBruyn et al. 2004	Lake Erie	3.0×10^7-4.1×10^8
Wilhelm et al. 2006	Lake Erie	3.0×10^6-4.9×10^8 (surface)
Filippini et al. 2008	Lake Hallwil, Switzerland	1.9-9.7 x 10^7 (surface)
Weinbauer and Hofle 1998	Lake Plußsee, Germany	1.4×10^7 (surface)
Hennes et al. 1995	Lake Constance, Germany	1.0-4.0 x 10^7
Klut and Stockner 1990	Sproat Lake, BC, Canada	1.5-2 x 10^6
Tapper and Hicks 1998	Lake Superior	7.0×10^5-9.2×10^6 (surface)
Gouvea et al. 2006	Lake Ontario	1.7-7.1 x 10^7
Dorigo et al. 2004	Lake Bourjet, France	5.8×10^7- 2.6×10^8
Brum et al. 2005	Mono Lake, CA	1.4×10^8-1.9×10^9
Lymer et al. 2008	Lake Erken, Sweden	1.2-3.7 x 10^8 (surface)
Lymer et al. 2008	Lake Fyrsjön, Sweden	1.8-2.9 x 10^8 (surface)
Lymer et al. 2008)	Lake Klocktjön, Sweden	5.0-6.0 x 10^7 (surface)
Bettarel et al. 2006	Lake Guiers, Senegal	8.9×10^6-1.2×10^8
Vanucci et al. 2005	Lake Ganzirri, Italy	5.0×10^4-7.5×10^8
Nakayama et al. 2007	Japanese Paddy Field	5.6×10^6-1.2×10^9
Madan et al. 2005	Antarctic Lakes	8.9×10^6-1.2×10^8 (whole water column)
Säwström et al. 2007	Antarctic Lakes, Ultra-oligotrophic	2.0×10^5- 1.6×10^6
Marine:		
Parada et al. 2007	Subtropical North Atlantic Ocean	1.4×10^6 (100m)
Manini et al. 2008	Mediterranean and Pacific Hydrothermal Vents	1.9-8.8 x 10^5 (23-75m)
Hennes and Suttle 1995	Western Gulf of Mexico	4.0×10^7 (offshore)
Taylor et al. 2003	Cariaco Basin, Caribbean Sea	8.1×10^6-6.3×10^7 (many depths)
Baudoux et al. 2007	North Atlantic Ocean	1.6-2.5 x 10^7
Wilhelm et al. 1998	Gulf of Mexico	3.0×10^5-6.5×10^6
Wommack et al. 1992	Chesapeake Bay, USA	2.6×10^6-1.4×10^8
Rowe et al. 2008	Sargasso Sea	2.0×10^5-1.9×10^6 (surface)
Wilhelm et al. 2003	Southeastern Pacific Ocean	2.0×10^6-1.6×10^7
Clasen et al. 2008	Coastal Pacific Ocean	4.0×10^6-3.9×10^8
Noble and Fuhrman 1998	Santa Monica Bay, USA	1.5×10^7
Bettarel et al. 2002	Mediterranean Sea	6.0×10^6-1.1×10^7
Winter et al. 2005	North Sea	2.1×10^6-1.8×10^8
Ortmann and Suttle 2005	Hydrothermal Vents	9.2×10^6-8.9×10^7
Marie et al. 1999	Equatorial Pacific	5.3×10^6
Marie et al. 1999	Mediterranean Sea	2.3×10^6
Hara et al. 1991	Japanese coastal and offshore waters	1.2×10^6-3.5×10^7
Weinbauer and Peduzzi 1995	Northern Adratic Sea	1.0×10^6-6.0×10^7
Jiang and Paul 1994	Tampa Bay Coastal, USA seasonal	4.8×10^6-2.0×10^7
Cochlan et al. 1993	Southern California Bight, USA	~1.1×10^6-1.2×10^7 (surface)
Bratbak et al. 1996	Raunefjorden, Norway	2.0-5.0 x 10^7
Bratbak et al. 1996	Osterfjorden, Norway	2.0-8.0 x 10^7
Hwang and Cho 2003	East Sea, Korea	1.0×10^6-1.0×10^7

Biogeochemical Cycling of Nutrients

Viruses have shown to have an influence on bacterial communities by the regeneration of carbon and nutrients through the lysis of bacterial hosts. Approximately 10-40% of the total bacterial population is lysed daily and this process can release a high proportion of these bioavailable nutrients (Suttle and Chan 1994; Suttle 2005). The remainder of the bacterial community is grazed on by protists (Fuhrman and Noble 1995). The release of dissolved organic matter (DOM) through the lysis of hosts can be utilized by other bacteria in the system and is known as the viral shunt since it transfers carbon away from the classic grazer-driven microbial food web (Wilhelm and Suttle 1999) (Figure 1.1). An increase in bacterial production through the release of DOM from viruses has been shown previously (Gobler et al. 1997). Often these bacterial cells get consumed by grazers such as ciliates and flagellates which, in turn, get consumed by other larger grazers which moves the energy up the food chain in the process known as the 'microbial loop' (Pomeroy 1974; Azam et al. 1983). Several reviews have been written to show the importance of viruses in the global carbon cycle (Fuhrman 1999; Wilhelm and Suttle 1999; Suttle 2005).

Viruses may also may a role in the remobilization of nutrients from the lysis of bacterial hosts. This remobilization may be of importance in ecosystems that are nutrient limited (most often P in freshwater systems and Fe or N in marine systems) (Falkowski 1994). The first quantitative measurement of virus-mediated release of bioavailable nutrients was from a bloom of *Aureococcus anaphageffrens* which showed that C, N, P, Fe and Se were released and remobilized in different quantities after the bloom termination (Gobler et al. 1997). Others have focused their work on Fe since Fe released upon viral lysis is bioavailable for other prokaryotes in the system and may support a phytoplankton community (Poorvin et al. 2004; Mioni et al. 2005). Overall, little is known about the viral effect on biogeochemical cycling in freshwater ecosystems. There has been one report of the release of bioavailable P from viruses in Lake Erie and showed up to 122 - 1080 nM of phosphorus was recycled per day which reveals that viruses are an important part of the biogeochemical cycling of P in the Laurentian Great Lakes (Dean et

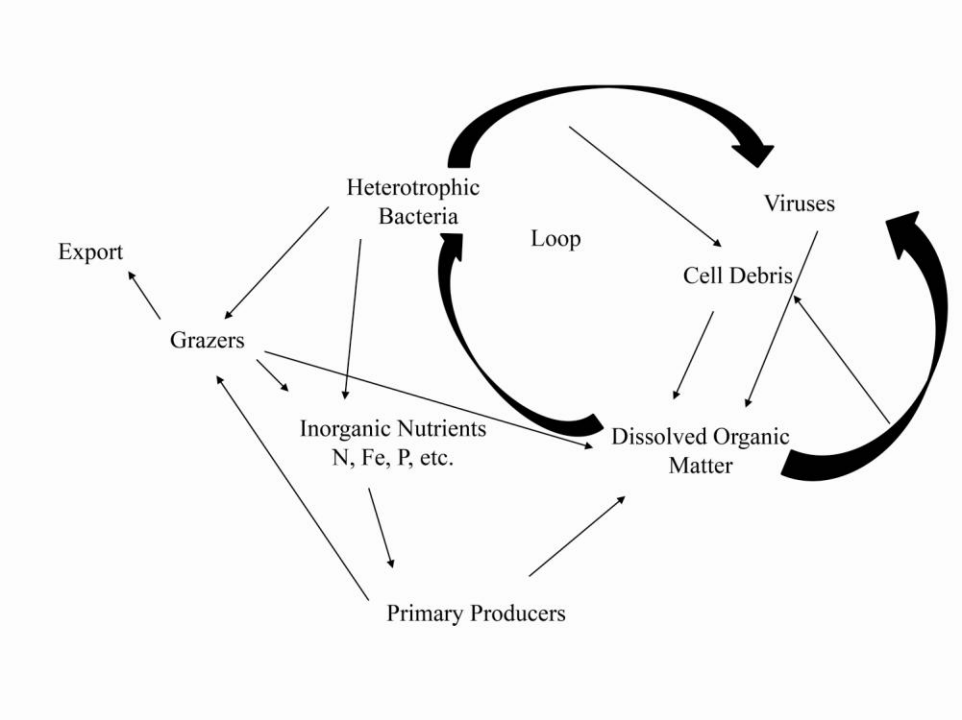

Figure 1.1: The microbial loop as adapted by Fuhrman (1999). Adapted by permission from Macmillan Publishers Ltd: Nature Fuhrman, copyright 1999.

al. 2008; Wilhelm and Matteson 2008). As phosphorus is often a limiting nutrient in Lake Erie, this recycling of P provides a significant proportion of this nutrient back to the pool.

Horizontal Gene Transfer

Viruses are able to move and shuffle DNA through the process of conjugation, transformation and transduction (Weinbauer 2004). They serve as reservoirs of genetic material that are important for the movement of genes through horizontal gene transfer from viruses to hosts or viruses to viruses. This shuffling of genetic material is important for evolutionary change and leads to increased diversity in both viruses and bacteria in aquatic ecosystems (Hendrix et al. 1999; Fraser et al. 2007). This increase in diversity may also lead to an increase in fitness for the host or virus. Viral metagenomic data from many different marine environments has shown a high number of bacterial-encoded genes which suggests horizontal gene transfer between viruses and their hosts (Breitbart et al. 2002; Angly et al. 2006; Dinsdale et al. 2008; Williamson et al. 2008).

From whole genome sequencing of several cyanophages, it was found that a few cyanobacterial-derived genes were found in the cyanophage genomes which suggest horizontal gene transfer. Comparative genome analysis showed that these genes likely were transferred from the host to the phage several times (Lindell et al. 2004). The *psbA* and *psbD* genes which encode for the D1 and D2 proteins for Photosystem II were found the genomes of several phages (Sullivan et al. 2005; Zeidner et al. 2005) and are functional during phage infection (Lindell et al. 2005). Recently a comprehensive analysis of the genomes of 16 cyanomyoviruses were compared and found a large number of genes originating from cyanobacterial hosts important in photosynthesis, carbon metabolism and phosphate stress among others (Sullivan et al. 2010). The abundance of host genes found in cyanophage genomes suggests that this phenomenon occurs often in aquatic ecosystems and may increase the fitness of phages.

Other sources of DNA for gene transfer occur from the viral release of host DNA into water by a lytic event (Weinbauer 2004). Studies have shown that 17-32% of all free dissolved DNA found in aquatic viruses is from lytic events (Jiang and Paul 1995). Upon bacterial lysis, host and viral DNA are released and can be taken up by other bacteria and viruses. Work on

laboratory cultures has shown that DNA released from a lytic event can be taken up by bacteriophages (Wikner et al. 1993).

Viral Control of Bacteria

It is well established that viruses can control the abundance and diversity of its bacterial hosts. As a particular bacterium becomes more densely populated, the contact rates between viruses and potential hosts increases which may lead to increases in lytic events in what is known as the "killing the winner" theory (Thingstad and Lignell 1997). In this, the most dominant members of a community will give rise to an increase in viruses able to infect it, allowing the less abundant (and also resistant) members to survive. This, in turn, will increase diversity in the system.

Viruses are also important in the control of phytoplankton blooms. Blooms are defined as quick increases in the population of phytoplankton and are found in many different ecosystems. These blooms are often associated with toxin production which can have detrimental effects on animals and humans. Work has shown that viruses are often able to terminate blooms by infecting the dominant species in marine systems (Bratbak et al. 1993; Waterbury and Valois 1993; Nagasaki et al. 1994; Brussaard et al. 1996; Castberg et al. 2001; Jacquet et al. 2002) as well as freshwater systems (Manage et al. 2001; Honjo et al. 2006; Wu et al. 2009).

Hypotheses and Objectives of this Study

The overall goal of this study was to better understand the abundance and production of cyanophages (specifically *cyanomyoviridae*) and total viruses on spatial and temporal scales. Currently the diversity and richness of phages has been at the forefront of aquatic viral ecology, but the evenness of specific families of phages has lagged behind. We aimed to use molecular biology techniques such as qPCR to quantify cyanomyoviruses. Overall, several null hypotheses were created for this dissertation and are as follows:

Chapter 3: Production of Viruses during a Spring Phytoplankton Bloom in the South Pacific Ocean

i. The production rate and abundance of viruses is higher in the FeCycleII bloom than the FeCycle bloom.
ii. The virus production rate correlates to host and ecological parameters.

Chapter 4: Quantification of Cyanophage in Marine Ecosystems using a Quantitative PCR Approach

i. Cyanomyoviruses are abundant in marine environments.
ii. The *g20* gene primers used amplify only cyanomyoviruses.
iii. Cyanomyovirus abundance correlates to host and ecological parameters.
iv. There is no difference in cyanomyovirus diversity throughout the FeCycleII bloom event.

Chapter 5: Seasonal Changes in the abundance of bacteria, viruses and cyanophage in a Laurentian Great Lake

i. Cyanomyoviruses are abundant in freshwater environments.
ii. There are significant temporal changes in the bacterial and viral communities in Lake Erie during the summer and winter months.
iii. Cyanomyovirus abundance correlates to host and ecological parameters.

To test these hypotheses, we propose several objectives from which to start. For Chapter 2, we aimed to provide a methodology in visual form to alleviate any problems with the technique and supply a standardized method by which to calculate virus production rates in aquatic ecosystems. Chapter 3 features samples collected in the FeCycleII cruise to determine the production rate of viruses during a natural phytoplankton bloom. We wanted to compare the communities from the

first FeCycle bloom cruise with the FeCycleII cruise and determine the effect viruses have on the lysis of the bacterial community.

For Chapter 4, we wanted to develop a method to quantify cyanomyoviruses using the *g20* gene in aquatic environments. A qPCR technique was optimized and used to compare samples collected from the Sargasso Sea and the southern Pacific Ocean during a phytoplankton bloom. Statistical methods were used to analyze the correlations between putative cyanomyoviruses and other biotic and abiotic parameters in the system to determine the possible hosts for the amplicons produced. Chapter 4 also aims to analyze the diversity of cyanomyoviruses using PCR primers *ca.* 585 bps in length to detect any shifts in the community throughout the phytoplankton bloom during the FeCycleII cruise. Chapter 5 uses the techniques optimized in Chapter 4 for use in freshwater ecosystems to quantify the abundance of cyanomyoviruses. Samples were collected in Lake Erie during the summer and winter months to temporally compare the bacterial and viral communities. Again, Pearson correlation statistics were used to analyze the effects of the environment on the cyanomyovirus community and to further determine possible hosts.

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Chapter 2 Estimating virus production rates in aquatic systems

This section is a version of a video produced in conjunction with a video journal article made through the Journal of Visualized Experiments.

Matteson AR, Budinoff CR, Campbell CE, Buchan A, Wilhelm SW (2010). Estimating Virus Production Rates in Aquatic Systems. J Vis Exp. 43.
http://www.jove.com/index/details.stp?id=2196, doi: 10.3791/2196

My contribution to the published video was the assistance of writing the script, setting up all equipment to be used in the video and showing a large portion of the techniques used in the video itself. My contribution to the written article produced was help with the background information, protocol, and all figures and tables made.

Short Abstract

The turnover rate of viruses in marine and freshwater systems can be estimated by a reduction and reoccurrence technique. The data allow researchers to infer rates of virus-mediated microbial mortality in aquatic systems.

Long Abstract

Viruses are pervasive components of marine and freshwater systems, and are known to be significant agents of microbial mortality. Developing quantitative estimates of this process is critical as we can then develop better models of microbial community structure and function as well as advance our understanding of how viruses work to alter aquatic biogeochemical cycles. The virus reduction technique allows researchers to estimate the rate at which virus particles are released from the endemic microbial community. In brief, the abundance of free (extracellular) viruses is reduced in a sample while the microbial community is maintained at near ambient concentration. The microbial community is then incubated in the absence of free viruses and the rate at which viruses reoccur in the sample (through the lysis of already infected members of the community) can be quantified by epifluorescence microscopy or, in the case of specific viruses, quantitative PCR. These rates can then be used to estimate the rate of microbial mortality due to virus-mediated cell lysis.

Protocol Text (see Video 2.1, Figure 2.1 and Table 2.1)

1 Ultrafiltration of seawater to generate "virus-free" water (Wilhelm and Poorvin 2001)

1.1 Approximately 20L of seawater / lakewater is collected as aseptically as possible.

1.2 Water is serially prefiltered through 142- mm diameter polycarbonate 0.8 μm filters that can be kept at -20 °C for community analysis. Larger pore size filters may be used before this step for very productive systems.

1.3 To obtain ultrafiltered water from an Amicon M12 system (Millipore) a 30 kDa-cutoff spiral cartridge is used to exclude all viruses, even small RNA viruses.

1.4 The samples are processed and concentrated at ~ 25% speed with ~15-16 kPa of backpressure.

1.5 The remaining sample of ~500 mL of water will contain a concentrated virus community (which can be saved for other experiments) while the remaining 19.5 L of virus-free water will be used for viral production assays.

1.6 After each day of use, the Amicon M12 system must be cleaned to prevent damage to the membrane of the filter cartridge.

1.7 If you are working with seawater, rinse the membrane out with at least 6 L of Milli-Q water followed by a washing with 0.1N NaOH solution for 30-45 minutes.

1.8 Again rinse the cartridge with at least 6 L of Milli-Q water.

1.9 When finished using the M12 system, the spiral cartridge should be stored in a 0.05M H_3PO_4 solution at 4°C.

2 Virus reduction method for viral production (Wilhelm et al. 2002)

2.1 Up to 500 mL of the seawater / lakewater sample with both host and viruses is obtained and placed in a sterifilter unit with a 0.2- μm nominal pore-size low protein-binding filter (e.g., Durapore)™ placed on it.

2.2 The sample is gently vacuum pressured at <200 mmHg while continually resuspending the sample using a sterile transfer pipette to inhibit bacterial cells from concentrating on the filter.

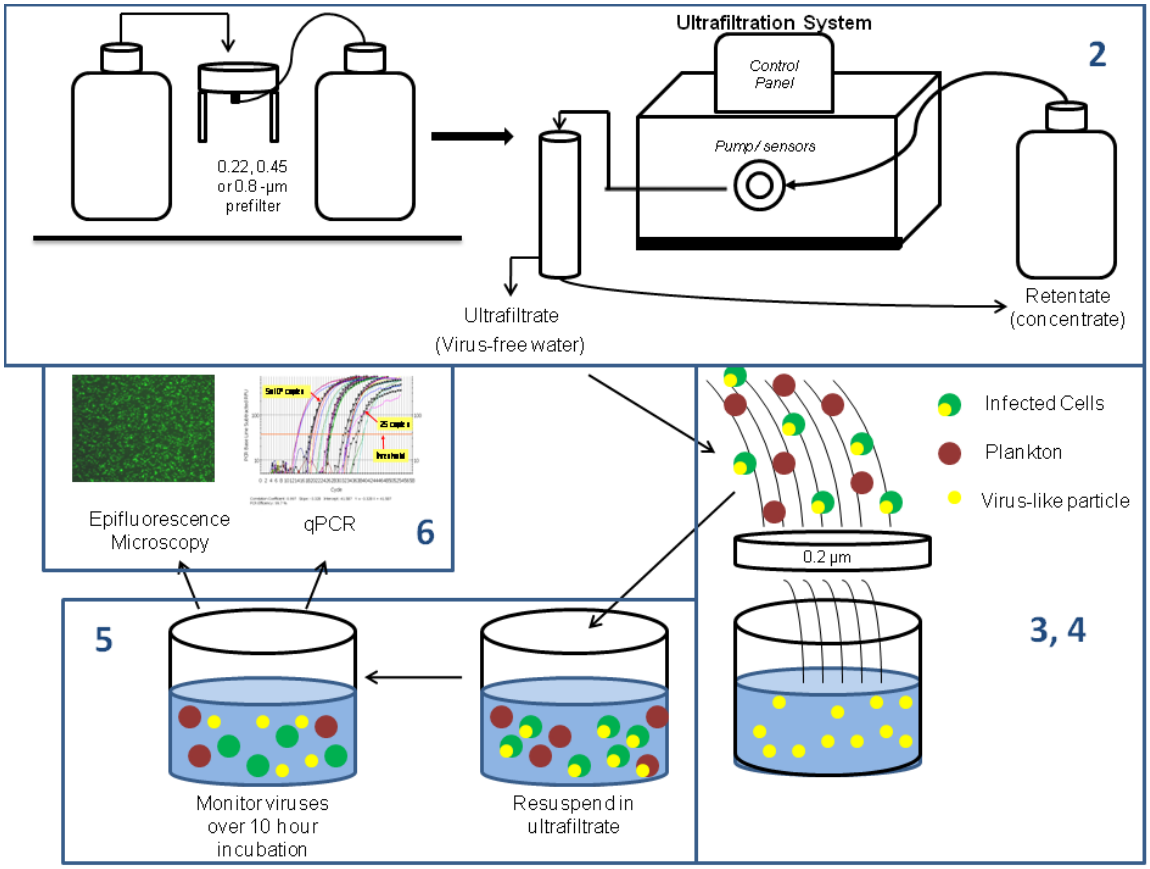

Figure 2.1: Schematic diagram of the work flow process for assaying virus production. The process starts with the ultrafiltration of sample water to generate virus-free water. This is completed using an ultrafiltration system. In parallel water samples are collected from the same site and the free viruses passed through a filter while the microbial community (containing a mixture of infected and non-infected cells) is retained. This community is then resuspended in the virus free water and incubated under *in situ* conditions. The reoccurrence rate of viruses is then monitored for the next 10 hours to determine rates of virus production.

Table 2.1: Table of specific reagents and equipment used. All reagents and equipment are those used by the Wilhelm and Buchan labs at the University of Tennessee. Other appropriate products may be substituted for the purpose of this work.

Name of the reagent	Company	Catalogue number	Comments (optional)
2.5% *p*-phenylenediamine	Acros	130575000	Stock for Antifade
Amicon Proflux M12 system	Millipore	N/A	Any ultrafiltration device may be used for this step
SYBR Green I nucleic acid gel stain	Invitrogen	S-7563	
Helicon S10 30kDa Filter	Millipore	CDUF010LT	
Pelicon XL filters 0.22 µm	Fisher	PXGVPPC50	
GE 0.2 µm PCTE membrane filters (47mm)	Fisher	09-732-35	
Millipore Labscale Tangential Flow Filtration System	Millipore	XX42LSS11	Other TFF systems may be used for this step
0.45 µm Micronstep, Cellulosic, white plain filters (25mm)	Fisher	E04WP02500	
GE Whatman 0.02 µm Anodisc filters (25mm)	Fisher	68-09-6002	
Leica DMRXA microscope			Any epifluorescence microscope with a blue filter set may be used
20L polycarbonate carboys	Fisher		
Glycerol	Fisher	BP229-4	
PBS (0.05 M Na_2HPO_4, 0.85% NaCl, pH 7.5)	Fisher	BP329-500, S640-500	
50% Glutaraldehyde	Fisher	G151-1	
Corning 2 mL Cryovials-External Thread polypropylene	Fisher	09-761-71	Any cryovials may be used
Corning 5 mL Cryovials-External Thread polypropylene	Fisher	09-761-74	Any cryovials may be used
85% H_3PO_4	Fisher	A242-212	Spiral Membrane storage
NaOH pellets	Fisher	S318-3	M12 cleaning
Graduated Cylinders			
Isopore 0.8-µm pore-size membrane filter (142mm)	Millipore	ATTP14250	
Millipore Stainless Steel Pressure Filter Holder (142mm)	Fisher	YY30 090 00	

2.3 Slowly three volumes of ultrafiltrate are added to the bacterial suspension to significantly reduce the number of free viruses in the sample.

2.4 The bacterial fraction is diluted back to 500 mL with virus-free water and divided into three replicates of 150 mL each and are placed in clear 250 ml polycarbonate bottles.

3 Tangential flow filtration (TFF) method for viral production (Weinbauer et al. 2002; Winget et al. 2005). (* step 3 this represents an alternative approach to step 2)

3.1 Approximately 500 mL of natural sample is collected as described above.

3.2 This sample is concentrated using a 0.2-µm nominal pore-size tangential flow filtration system.

3.3 When the bacterial fraction is reduced to approximately 10-15 mL, ultrafiltered, virus-free water is added and distributed as above.

3.4 The replicate bottles are incubated at in situ conditions using environmental chambers.

3.4.1 Light levels are altered to surface conditions by using blue-tinted acrylic or clear acrylic with screening net to decrease light intensity.

3.4.2 Ambient surface temperatures are often obtained by using a flowing seawater deck incubator.

3.5 Samples for bacterial and viral abundance estimates are taken at time 0 with a final concentration of 2.0-2.5% sterile glutaraldehyde added into cryovials. These samples are immediately flash frozen with liquid nitrogen and stored frozen until processed.

3.5.1 If liquid nitrogen is not available, microscopy slides may be prepared and processed immediately (see procedure below)

3.3 Subsamples are collected every 2.5 hours for at least 10 hours by the method described above.

3.3.1 At this time water may be collected for quantitative PCR analysis. Up to 5 mL of the sample may be added to a cryovial

with no fixative agent with immediate flash freezing in liquid nitrogen.

4 Viral Production Microscopy (Noble and Fuhrman 1998; Wen et al. 2004)

4.1 Frozen samples to be 0.02-µm filtered for microscopy should be thawed on ice.

4.2 Prepare a stock solution of SYBR Green by diluting the stock solution 1:10 with sterile water. Next, from the stock solution, prepare a working solution by adding 1 mL of the stock solution to 39 mL of sterile water. A 50% glycerol, 50% phosphate buffered saline solutions (PBS, 0.05 M Na_2HPO_4, 0.85% NaCl, pH 7.5) and fresh 2.5% stock solution of p-phenylenediamine should also be prepared before beginning. Keep the 50% glycerol/50% PBS solution at 4°C and the p-phenylenediamine stock at -20°C in the dark until starting. Right before filtering add the p-phenylenediamine to the 50% glycerol/50% PBS to a final concentration of 0.1% to be used as the Antifade solution.

4.3 Place a 25 mm 0.02-µm Anodisc filter on top of a 0.45-µm MicronStep, cellulosic backing filter. Add 850 µL of the fixed sample to the top of the Anodisc and vacuum at 20 pKa until completely dried. Place 100 µL of SYBR Green working solution to a sterile Petri dish and, with the vacuum still on, carefully remove the Anodisc from the filter tower and place on the SYBR Green. Incubate the samples in the dark at room temperature for 20 minutes. Carefully remove the filter from the SYBR Green solution and wick the back of the filter with a Kimwipe to remove all residual dye. If desired, return the filter to the tower and pass up to 800 µL of 0.02-µm filtered water or sterile media through the filter to rinse off excess stain.

4.4 Add a small drop of antifade solution to a microscope slide and place a cover slip on top. Remove the cover slip and add the dried filter to the microscope slide wet with the antifade solution. Again, add a small amount of antifade solution to the cover slip and slowly place it on top of the filter, making sure to get rid of any bubbles that may form.

4.5 Immediately freeze the slides at -20°C until needed (these should be used within a few months to prevent fading and lowered virus counts)

4.6 Viruses are enumerated using fluorescence microscopy (in our case a Leica DMRXA microscope) with a wide blue filter set (λ_{Ex}= 450 to 490 nm, λ_{Em}= 510 nm with a suppression filter at λ= 510 nm). Each filter will have at least 20 fields of view counted, making sure to quantify total viruses from each field grid to ensure even distribution of viruses across the filter membrane.

4.7 Averaged rates of virus reoccurrence from the three independent replicates are then calculated and a standard deviation is determined from the production rates.

Representative Results

The raw data collected by the researcher requires minimal mathematical processing to generate reoccurrence rates of virus abundance. The primary data set resulting from this study is the reoccurrence rates of virus abundance in the subsamples from the incubations. These results form independent regressions of virus abundance vs. time for each of the samples. For each sample the individual incubations act as one treatment, so by completing three replicate-incubations the researcher can calculate rates as well as an estimate of variation (*e.g.*, standard deviation) (see Figure 2.2)

One caveat of this process is that the reduction in virus abundance invariable leads to a reduction in the host cells in the sample that are carrying the virus burden. To offset this loss, enumeration of bacterial abundance from both the source (unfiltered seawater or lake water) and the T = 0 incubation sample are necessary. This information can be used to account for the percentage of cells lost: assuming that the process of reducing virus abundance is not selective for or against any members of the microbial community, this factor can then be used to estimate the *in situ* production rate of viruses.

Discussion

A critical component in the understanding how viruses influence marine microbial communities is to determine the rate at which virus particle are produced. Given that abundances are (more or

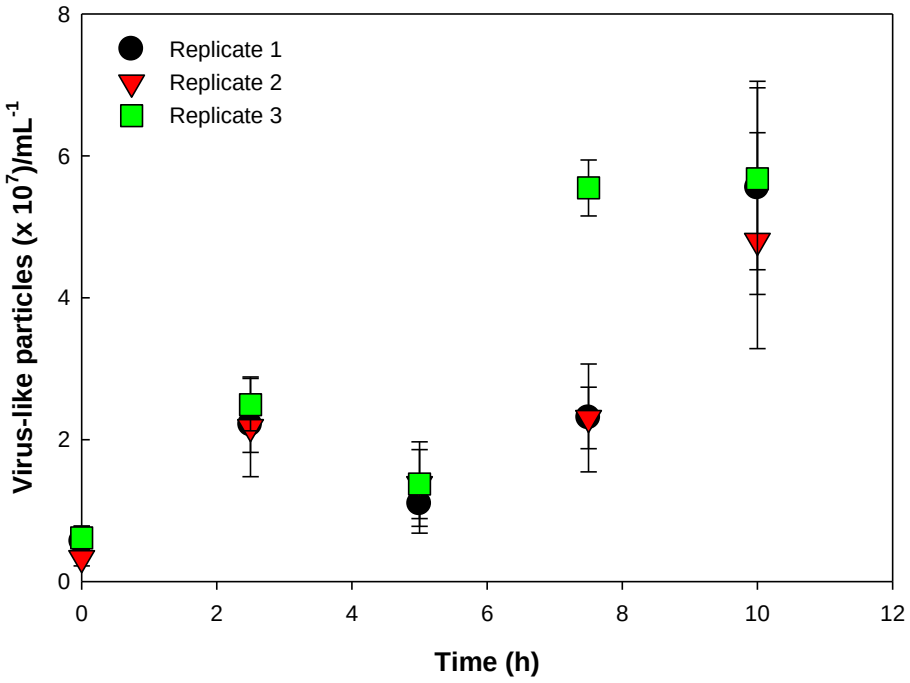

Figure 2.2: The production of virus-like particles over a 10 hour incubation *at in situ* conditions using epifluorescence microscopy. Samples were collected during a phytoplankton bloom off the coast of New Zealand in September of 2008.

less) static in most systems (Wilhelm and Suttle 1999; Weinbauer 2004), and that viruses are removed or rendered non-infectious quickly in aquatic systems (Wilhelm et al. 1998), then production rates must be relative rapid to replace lost particles.

Estimating the mortality viruses cause to the microbial community requires a knowledge of how many viruses are produced every time a virus lyses a cell (the "burst size"). Virus burst sizes in natural samples can vary greatly. Burst sizes can be determined directly by transmission electron microscopy (e.g., Weinbauer and Peduzzi 1994), but this is often beyond the capabilities of a given laboratory or not always practical. In situations where they cannot be empirically determined, literature values of 24 viruses per lytic event may be used for marine systems and 34 for freshwater systems (Parada et al. 2006). If the rate of virus production is divided by this number, the result is the abundance of cells per volume destroyed by viruses on a daily basis. The microbes lysed value can then be divided by the standing stock of bacterial abundance resulting in the virus induced mortality for the system in question: existing estimates range from few percent to nearly the entire population and are often dependent on other factors in the system in question (Wilhelm and Matteson 2008). To determine the percentage of total mortality this number is often multiplied by two (working from the assumption that 50% of the cells go on to reproduce and 50% of the cells are lost, Weinbauer 2004).

Given that nutrient and trace element bioavailability (*e.g.*, N, P, Fe) can limit the rate of primary productivity, and as such carbon flux, through aquatic systems, and understanding of the role of virus-driven microbial mortality in this process has become of interest to marine geochemists. Several estimates now exist that suggest viruses release a significant concentrations of nutrient elements back to the water column on a daily basis (Rowe et al. 2008; Higgins et al. 2009) and that these elements are rapidly assimilated by the microbial community (Poorvin et al. 2004; Mioni et al. 2005). The rate of nutrient flux to the environment can be determined by multiplying the number of cells destroyed by the amount of nutrient per cell (denoted "quotas"). This information can provide a critical component to our understanding of how microbial food webs function across aquatic systems.

Ongoing developments

Current efforts by a series of research groups involve adapting the above strategy to enumerate specific viruses within the community and, as such, to determine how specific organisms are influenced by virus activity. To do this researchers use the quantitative polymerase chain reaction (qPCR) to estimate the abundance of specific viruses groups or families in parallel to the estimates of the total virus community. The results are then directly applied to provide estimates of virus mortality, nutrient turnover, etc. for specific plankton groups. This powerful new approach will allow researchers in the coming years to dig much more deeply into processes associated with the ecology of viruses and, for the first time, to quantify the interactions of specific virus-host communities beyond the constraints of laboratory systems.

Acknowledgments

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Chapter 3 Production of Viruses during a Spring Phytoplankton Bloom in the South Pacific Ocean

This chapter is being prepared for the Aquatic Microbial Ecology journal with the same title and authors Audrey R. Matteson, Star N. Loar, Phillip R. Boyd and Steven W. Wilhelm.

My contribution to this chapter was the sample collection and production assays performed during the FeCycleII cruise, all virus abundance microscopy, data collection from other scientists, and all figures and written portion of the manuscript.

Abstract

The temporal abundance and production rate of viruses and bacteria were investigated during a natural spring phytoplankton bloom in the southern Pacific Ocean. Daily samples were collected in a mesoscale eddy from September 18th to October 4th of 2008. The bloom was productive despite often being N-limited, with picoplankton and nanoplankton dominating the system with chlorophyll *a* concentrations up to 2.43 $\mu g/L^{-1}$. Bacterial abundances were steady and ranged from 5.83 x 10^5-1.49 x 10^6 mL^{-1}, with a small *Synechococcus* bloom occurring during our observations. Virus abundances were variable and fluctuated *ca* one order-of-magnitude (2.11 x 10^7-1.59 x 10^8 mL^{-1}) over a 16 day period. Virus production rates were relatively high compared to those reported in other systems, with rates ranging from 1.36 x 10^{10}-2.06 x 10^{11} L^{-1} d^{-1} which was much higher than rates found during the first Fe-limited FeCycle cruise in 2003. Viruses played an important part of bacterial mortality through the bloom with between 19 and 216% of the bacterial standing-stock being lysed per day. This mortality released a large proportion of bioavailable nutrients such as C, N and Fe and likely helped sustain the bloom through the sampling period. To detect constraints on viral production, parametric analyses were performed and found significant correlations with both biotic (*i.e.* potential host abundances) and abiotic parameters (*i.e.* nutrient concentrations) in the southern Pacific Ocean.

Introduction

Over the past two decades it has been shown that viruses are numerically abundant in the world's oceans (Bergh et al. 1989; Fuhrman and Suttle 1993; Fuhrman 1999) and freshwater ecosystems (Maranger and Bird 1995; Wilhelm and Smith 2000; Wilhelm and Matteson 2008) and are important players in bacterial mortality, with ~20-40% of the bacterial standing stock being lysed daily from viruses (Suttle and Chan 1994). These mortality rates can often meet or exceed mortality rates due to grazing by larger flagellates or ciliates. Viruses are able to infect all trophic levels, and are important in the biogeochemical cycling of nutrients such as C, Fe, P and N and release bioavailable nutrients from the virus-induced lysis of hosts that can be utilized by other organisms in the system (Gobler et al. 1997; Poorvin et al. 2004; Mioni et al. 2005). Viruses can also release DNA and amino acids through the lysis of hosts which are rich in P and N and can also be utilized by bacteria in the system (Wilhelm and Suttle 1999). The effect of viruses on the global carbon pool has been reviewed previously (Fuhrman 1999; Wilhelm and Suttle 1999). Aquatic viruses are also able to control the community and diversity of hosts by killing the most abundant members in a system by the "killing the winner" method which allows the resistant and less abundant members to survive (Thingstad and Lignell 1997), potentially explaining G.E. Hutchinson's *Paradox of the Plankton* (Hutchinson 1961).

Several different methods have been used to determine the production of viruses in aquatic ecosystems with the "dilution and reoccurrence" technique (Wilhelm et al. 2002) becoming the most commonly used assay (Weinbauer et al. 2010). Production rates can vary in both marine and freshwater ecosystems, but generally range from 10^8-10^{11} viruses L^{-1} d^{-1} (Wilhelm and Matteson 2008) with an average of approximately 10^9 viruses L^{-1} d^{-1}. From these rates researchers can determine the mortality from viruses that the microbial community experiences every day and can estimate nutrient regeneration rates.

A large portion of the world's oceans are high nutrient low chlorophyll (HNLC) due to iron limitation (Martin and Fitzwater 1988; Martin et al. 1990). Many studies have focused on determining if bacteria and phytoplankton are limited by Fe (Amon and Benner 1996; Tortell et al. 1996; Boyd et al. 2000; Kirchman et al. 2000; Strzepek et al. 2005) or other nutrients (Kirchman et al. 2000), in both natural systems and by fertilization studies, while other studies

have focused on the role viruses play in the regeneration of these nutrients in nutrient-limited regions of the oceans through the biogeochemical cycling of nutrients such as C, Fe, N and P (Strzepek et al. 2005). Iron fertilization studies determining production rates of viruses have also been performed and have seen increases in virus production rates in these patches (Higgins et al. 2009; Weinbauer et al. 2009).

The first FeCycle cruise in 2003 analyzed grazing and virus-mediated regeneration of Fe during a natural Fe-limited bloom by radiolabel tracking with SF_6 (Boyd et al. 2005; Strzepek et al. 2005). Grazing in this system released enough bioavailable Fe to support 30 to >100% of the Fe needed for algal and bacterial communities. Viruses were quite variable and accounted for up to 28 pmol L^{-1} d^{-1} of iron and released 1-64% of Fe compared to those released by grazing. In 2008, the second FeCycle cruise set off to examine the ontogeny of the annual spring bloom event in subtropical waters off the coast of northern New Zealand. During this period we observed a population that started in N-limited, Fe-replete waters that was perturbed by a mixing event that reintroduced N (but not Fe). The goal of this study was to examine the bacterial and viral communities throughout this pelagic bloom, determining how changes in this event influenced virus turnover and, in turn, to determine the mineralization of bioavailable nutrients released from virally-infected bacteria. Overall, this work demonstrates that the virus production rates were rather high throughout the bloom, and were important players in bacterial mortality with viruses accounting for over 100% of bacterial mortality on certain days. In comparison to the first FeCycle cruise, virus production rates and remobilization of nutrients were also much higher in the system examined.

Materials and Methods

Study Area and Sample Collection

Samples were collected during morning casts while onboard the *RV Tangaroa* during the FeCycleII cruise. Daily sampling occurred off the northern coast of New Zealand from September 19 to October 4, 2008 during the seasonal spring phytoplankton bloom in a mesoscale eddy. Virus concentrates were generated in a similar manner as described by others previously

(Chen et al. 1996; Wilhelm and Poorvin 2001). Figure 3.1 shows the locations from which all samples were generated. 20 L of whole water samples were collected from a trace metal pump lowered to ~5 m and prefiltered through a 0.8-μm nominal pore-size, 142-mm diameter polycarbonate filter (Millipore). The virus-size fraction was then concentrated to ~500 mL using a Millipore M12 ProFlux tangential flow filtration unit with a 30kDa cutoff filter cartridge (Millipore) to make the virus concentrate and produce virus-free ultrafiltered seawater for virus production assays. Virus concentrates were stored in the dark at 4° C until needed.

Chlorophyll *a* and Nutrient Concentrations

Size fractionated chlorophyll *a* (Chl *a*) concentrations were determined from duplicate samples from ~5m for picophytoplankton (0.2-2 μm), nanophytoplankton (2-20 μm) and microphytoplankton (>20 μm) by passing samples through 47mm diameter polycarbonate filters (GE Osmonics). Chl *a* was extracted from the filters in 90% acetone at 4°C in the dark overnight. Concentrations were quantified with the non-acidification protocol (Welschmeyer 1994) using a 10-AU field fluorometer (Turner Designs) that was normalized with a solid-standard. All dissolved nutrients (N, P, Si) were determined by shipboard automated analysis (Frew et al. 2001).

Bacterial Abundance and Production

Bacteria, picocyanobacteria and picoeukaryote abundances were estimated by flow cytometry with a FACSCalibur instrument (Becton Dickinson, Mountain View, Calif.) using CellQuest Vers. 3.1f software as previously described (Hall et al. 2006). Bacterial production rates were determined using leucine incorporation in triplicate samples following standard procedures (Kirchman 2001; Rowe et al. 2008).

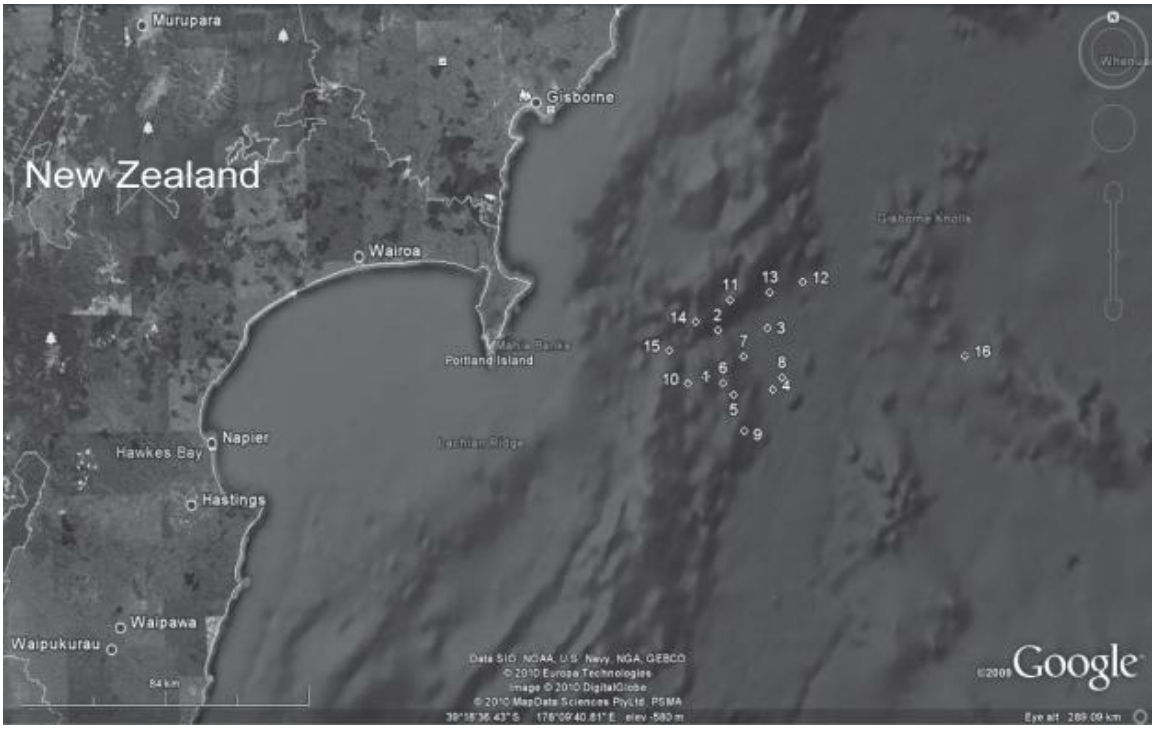

Figure 3.1: Sampling locations off the northern coast of New Zealand where all virus production assays were performed.

Virus Production

Virus productions were performed using the dilution and reoccurrence technique (Wilhelm et al. 2002). Water (500 mL) was collected during early morning casts and was continually resuspended over a 0.22-μm nominal-pore size, 47-mm diameter polycarbonate filter (Osmontics Inc.) on a plastic Vacuum Filter Flask (Nalgene) unit to reduce free virus particles and concentrate the virally-infected bacterial community. Several volumes of virus-free seawater (see virus concentration above) were added to the bacterial fraction to reduce the abundance of free viruses. The bacteria-containing retentate was reduced to ~10-15 mL prior to the addition of ultrafiltered seawater to return the volume to 500 mL. These samples were divided into triplicate 150 mL samples and placed into 250 mL clear polycarbonate bottles. Bottles were then placed in on board incubators continually flushed with surface water and covered in blue-colored acrylic for shading to simulate *in situ* temperatures and light. Subsamples were taken at time 0 and every 2.5 h for 10 h and placed in a final concentration of 2.5% sterile glutaraldehyde and flash frozen in liquid nitrogen. Samples were returned to the laboratory and stored at -80° C until processed for epifluorescence microscopy (see below). The average rate of virus reoccurrence (and also standard deviation of the rate) was determined from the independent triplicate samples and corrected for any bacterial loss from filtration as determined by total bacterial abundance.

Virus Enumeration

Viral abundance was estimated using epifluorescence microscopy of SYBR Green stained viruses. 850 μL of fixed sample was stained with SYBR Green for 20 minutes on 0.02-μm Anodisc filters (Whatman GE Healthcare, Buckinghamshire, UK) following the protocol of Noble and Fuhrman (Noble and Fuhrman 1998). Prepared slides were kept at -20°C (< 2 weeks) and processed using a Leica *DMRXA* microscope with a wide blue filter set (λ_{Ex}= 450 to 490 nm, λ_{Em}= 510 nm with a suppression filter at λ= 510 nm). Each slide had at least 20 fields of view counted with each grid being enumerated to ensure viruses were evenly distributed across the filter membrane. Averages and standard deviations of the ranges were calculated using Excel 2007 (Microsoft, Redmond, WA)

Calculating MLV, % Cells Lysed, Turnover Rates and Remobilized Nutrients

The host mortality from viral lysis (MLV) was calculated by dividing the rate of viral production by the minimum burst size. Since the burst size was not determined in this study, an estimated burst size of 29.8, previously determined in the region (Strzepek et al. 2005), was used to establish the virus inferred bacterial lysis. The percentage of cells lysed was determined by the equation:

$$\%\ Cells\ Infected = \frac{Total\ Bacteria}{MLV} * 100 \quad (1)$$

Virus turnover rates were determined by dividing the production rate ($L^{-1}d^{-1}$) by the virus abundance (L^{-1}). To estimate the remobilization of nutrients from viral lysis of the bacterial community, the MLV was multiplied by previously determined cell quotas of 1.1 ag $cell^{-1}$ of Fe (Tortell et al. 1996), 23.3 fg $cell^{-1}$ of C (Simon and Azam 1989) and 5.6 fg $cell^{-1}$ for N (Lee and Fuhrman 1987).

Statistical Methods

To determine the normality of the datasets, linear regressions of untransformed data points were analyzed in SigmaPlot ver. 10.0. Since the assumptions of normality were met, Pearson correlations were calculated to determine the correlation between several biotic and abiotic parameters to virus production rates. These correlations were calculated using the Pearson pairwise statistics from NCSS Statistical & Power Analysis Software. Significant correlations were determined by P-values <0.05 with high Pearson correlation coefficients.

Results

Daily samples were collected during a spring phytoplankton bloom in the southern Pacific Ocean during a 23 day period from September to October of 2008. Nutrient concentrations were

determined with ammonium found in very low concentrations throughout the bloom ranging from 0.00-0.15 nmol L^{-1} and nitrate levels from 0.83-3.46 nmol L^{-1} (Figure 3.2). The N:P ratio was well below Redfield ratio at all stations examined suggesting nitrogen limitation in the system. The bloom was quite productive with high chlorophyll *a* concentrations for a marine system with the highest concentrations found at the beginning of sampling (up to 2.43 μg L^{-1}) with decreasing concentrations toward the end of the cruise (Figure 3.3). Overall, picoplankton and nanoplankton fractions dominated the system and concentrations ranged from 0.94-2.43 μg L^{-1} in the surface samples. Total bacterial abundances were relatively stable through the bloom and ranged between 5.83 x 10^5-1.49 x 10^6 mL^{-1}, but slight decreases were found from September 27-October 1. Picocyanobacerial and picoeukaryote abundances were lower and remained around 10^3-10^4 and followed a similar trend to total bacterial abundances (Figure 3.4C). A small *Synechococcus* bloom formed from September 25-27 and reached concentrations of 3.38 x 10^4 mL^{-1}, but subsequently crashed following September 28 and remained relatively low until the end of the cruise (Figure 3.4C). This bloom corresponds to the increase in picophytoplankton chl *a* concentration on September 27 (Figure 3.3). Bacterial production rates followed a similar trend with the highest uptakes of leucine from September 24-27 followed by significant decreases (Figure 3.4D).

Virus abundances were high during the bloom and ranged from 2.11 x 10^7-1.59 x 10^8 mL^{-1}. Decreasing trends were found from the beginning of the cruise until September 29 with the exception of September 24 and 28 followed by increasing concentrations until the end of the cruise (Figure 3.4A). Virus to bacteria ratios (VBR) were calculated throughout the bloom and ranged widely (25-165) with an average of 70 (data not shown). Virus production rates were determined in samples with ambient light concentrations and these rates peaked in the middle of observations, with lower rates at the beginning and the end of the cruise with total observations found from 1.36 x 10^{10}-2.06 x 10^{11} L^{-1} d^{-1} (Figure 3.4B). With the production rate data, we determined the virus-inferred bacterial lysis rate which ranged from 4.56 x 10^8- 6.92 x 10^9 L^{-1} d^{-1} with the highest rates found during the middle of the cruise followed by a significant decrease from October 2-4 (Figure 3.5). The percentage of bacterial cells lysed was variable and ranged from ~ 19-216% (average 63.77%) of the total standing stock, with higher percentages observed at the end of the cruise (Figure 3.5). The turnover rate of viruses was also quite variable and

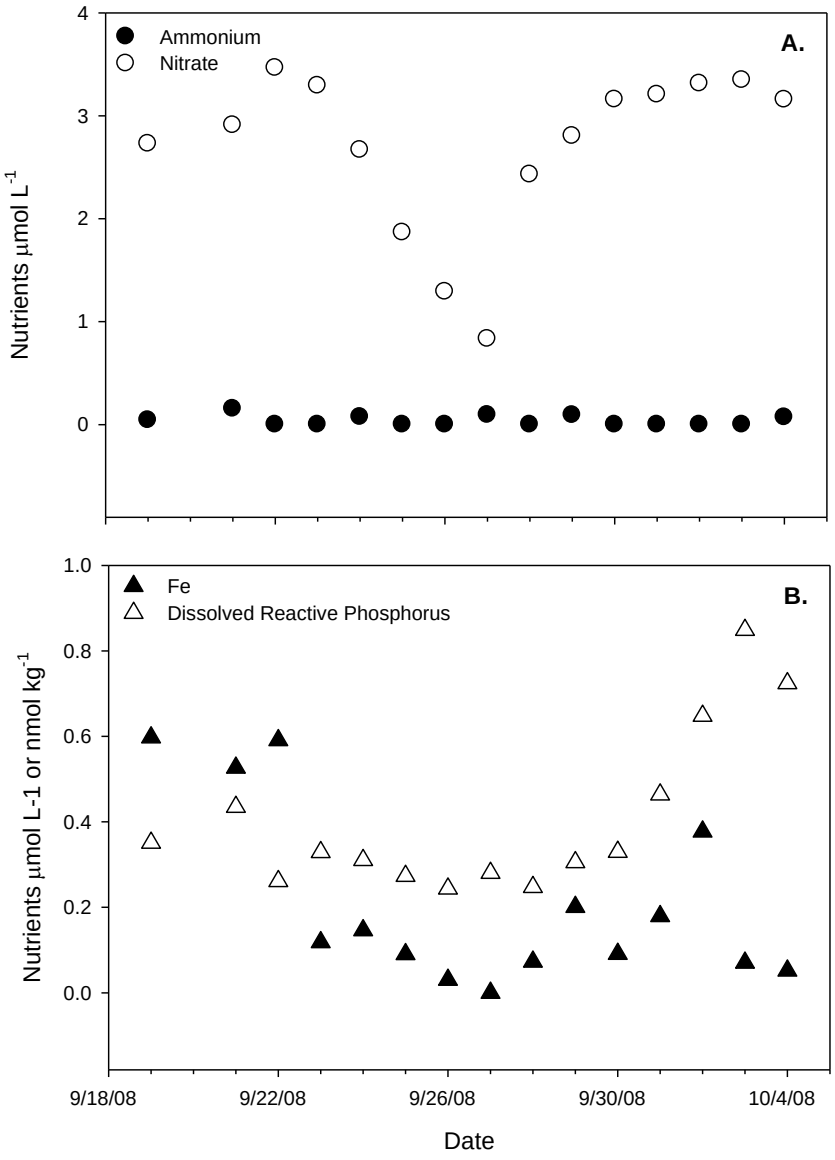

Figure 3.2: Nutrient concentrations collected through the bloom during the FeCycleII cruise from surface samples. A. Nitrogen concentrations calculated from the nutrients ammonium (black circles) and nitrate (white circles). B. Iron (black triangles) and dissolved reactive phosphorus (white triangles) concentrations. Samples were not collected on September 20.

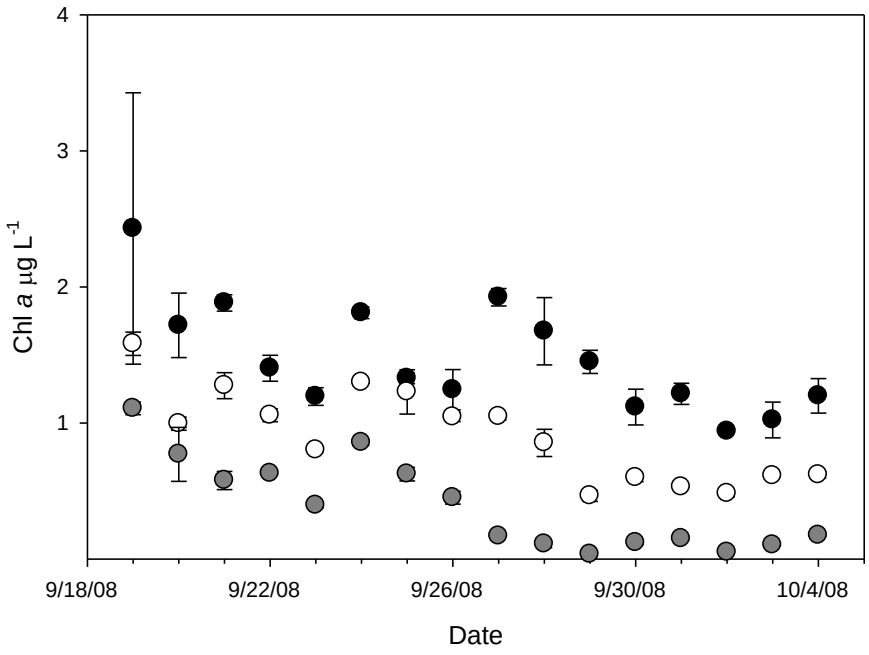

Figure 3.3: Size-fractionated chlorophyll *a* concentrations for all surface samples used for virus production assays. Picoplankton (0.2-2 µm, black circles), nanoplankton (2-20 µm, white circles) and microplankton (<20 µm, gray circles) were estimated. Error bars are ranges for standard deviations between the duplicate samples.

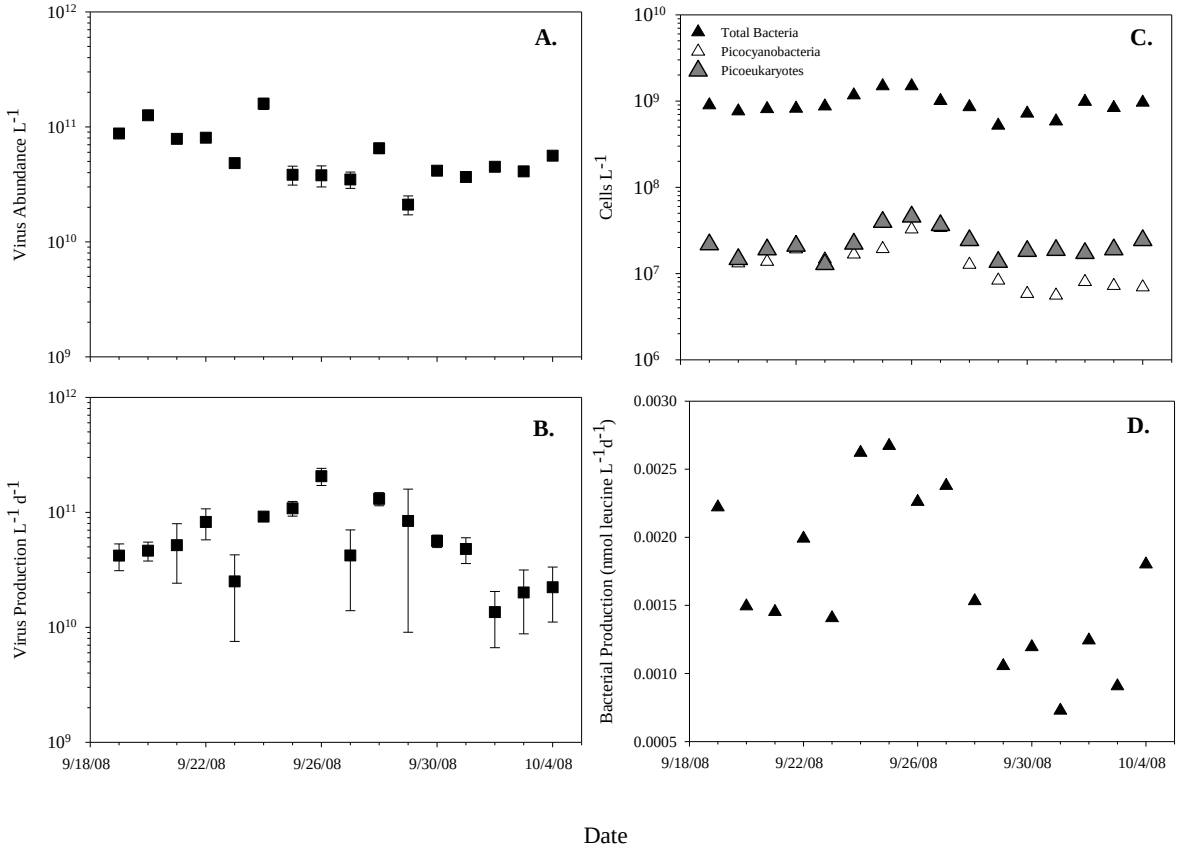

Figure 3.4: (A) Viral abundances estimated in surface waters during the FeCycleII cruise. Error bars were determined from the variability of viruses across the filter membranes from 20 grid views. (B) Virus production rates calculated during the FeCycleII cruise. Error bars were calculated from the standard deviations of triplicate production assays. (C) Bacterial (black triangles), picocyanobacterial (white triangles) and picoeukaryote abundances from surface waters using flow cytometry. (D) Bacterial biomass production rates (black triangles) determined.

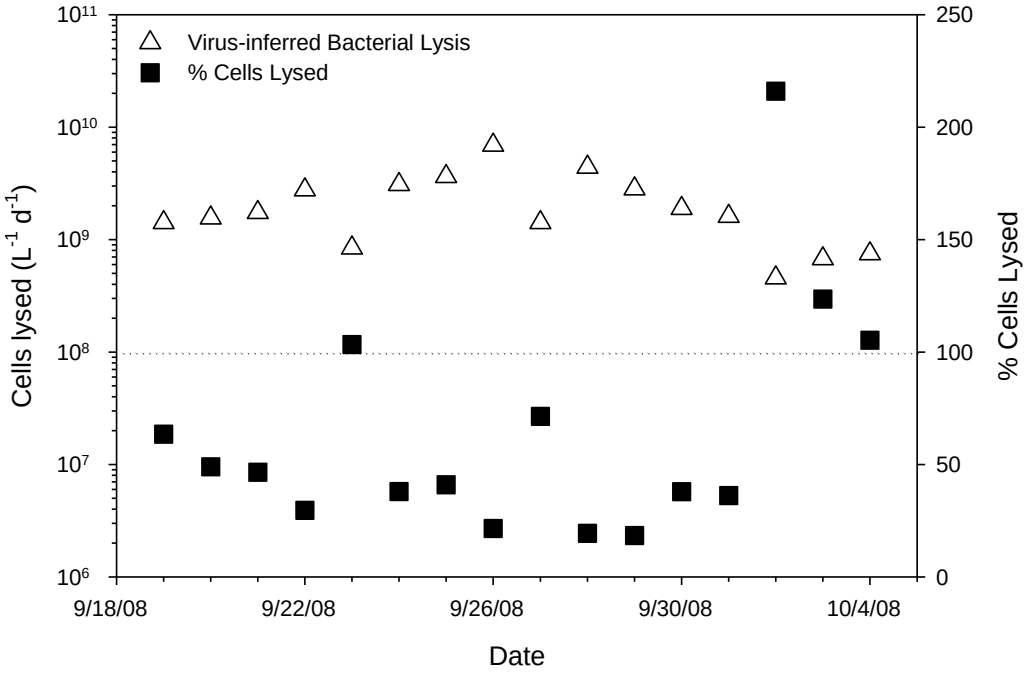

Figure 3.5: Virus-mediated bacterial lysis (Δ) plots from surface samples collected for virus production assays. The percentage of the bacterial population lysed by viruses can be found on the right axis (■).

followed no real trend with rates from 0.0-5.44 d^{-1} with an average of 1.48 (Table 3.1). The remobilization of nutrients such as carbon, iron and nitrogen were determined from the virus-inferred bacterial lysis rate for all stations and can be found in Table 3.1. Viruses recycled a substantial portion of nutrients into the system with the rates following the same trend as the virus-inferred bacterial lysis rate. The bacterial lysis produced approximately 0.10-18.68% of the total Fe demand and 0.23-8.93% of the total N demand in the system per day (Table 3.1).

Based on scatterplots of untransformed data, our data were determined to be normally distributed, so to better understand the relationships between virus production rates and other environmental parameters, parametric Pearson correlations were employed. Several biotic and abiotic parameters were compared and a subset of these data can be found in Table 3.2. Virus production rates were found to be significantly ($P<0.05$) and strongly correlated to several parameters such as temperature ($r_p=0.82$, $P=0.00$), nitrate ($r_p=-0.56$, $P=0.03$), dissolved reactive phosphorus ($r_p=-0.63$, $P=0.01$), total net primary production ($r_p=0.78$, $P=0.04$), total bacteria ($r_p=0.54$, $P=0.03$), picoautotrophs ($r_p=0.54$, $P=0.03$) and picoeukaryotes ($r_p=0.65$, $P=0.01$).

Discussion

The temporal production rate and abundance of viruses and bacteria were found during a natural annual phytoplankton bloom in the southern Pacific Ocean in 2008. The goal of this study was to analyze the abundance and production of viruses and bacteria during the onset and demise of a bloom. During this time, the system transitioned from one that was N-limited, to one that was likely Fe-limited after a significant upwelling event (PW Boyd and others, unpublished data). This 2008 spring bloom was productive in that high concentrations of chlorophyll, bacteria and viruses were observed. Virus production rates were within ranges observed in other systems and were responsible for a large proportion of remobilized nutrients during the bloom, but the system was not in a steady state. We discussed these observations within the background of significant correlations we observed between virus production and biotic and abiotic parameters in the bloom in an effort to better understand the ecological processes which constrain virus production rates.

Table 3.1: Estimated mobilization of nutrients by virus activity. Cellular quotas for bacterial were taken from Simon and Azam (1989) for carbon, Tortell (1996) for iron, and Lee and Fuhrman (1987) for nitrogen. The release of elements was determined from the turnover rate normalized by direct *in situ* measures. The turnover rate of viruses was estimated from production rates normalized for *in situ* virus abundance estimates. ND = Not Determined

Date	Carbon ($\mu g\ L^{-1}\ d^{-1}$)	Fe ($ng\ L^{-1}\ d^{-1}$)	N ($\mu g\ L^{-1}\ d^{-1}$)	Nutrient release relative to *in situ* concentrations		Virus Turnover (d^{-1})
				Fe%	N%	
9/19/2008	32.91	1.55	7.91	0.19	0.84	0.48
9/20/2008	36.25	1.71	8.71	ND	ND	0.37
9/21/2008	40.52	1.91	9.74	0.27	0.90	0.66
9/22/2008	64.43	3.04	15.48	0.38	1.33	1.02
9/23/2008	19.60	0.93	4.71	0.59	0.43	0.52
9/24/2008	71.85	3.39	17.27	1.73	1.83	0.58
9/25/2008	84.75	4.00	20.37	3.30	3.25	2.83
9/26/2008	161.17	7.61	38.74	18.68	8.93	5.44
9/27/2008	32.95	1.56	7.92	ND	2.31	1.21
9/28/2008	102.63	4.84	24.67	4.96	3.02	2.01
9/29/2008	65.84	3.11	15.82	1.16	1.58	3.69
9/30/2008	44.07	2.08	10.59	1.71	1.00	1.35
10/1/2008	37.54	1.77	9.02	0.74	0.84	1.31
10/2/2008	10.62	0.50	2.55	0.10	0.23	0.30
10/3/2008	15.73	0.74	3.78	0.79	0.34	0.49
10/4/2008	17.42	0.82	4.19	1.18	0.38	0.40

Table 3.2: Pearson correlation coefficients of virus production assays against various biotic and abiotic parameters. Significant correlations (<0.05) are given in bold face. r_p=correlation coefficient

Parameter	r_p	P
Viral Abundance	-0.31	0.24
Chl *a* (0.2-2 µm)	0.18	0.50
Chl *a* (2-20 µm)	0.06	0.81
Chl *a* (>20 µm)	-0.20	0.47
Total Bacterial Abundance	0.54	**0.03**
Bacterial Production	0.43	0.10
Picoautotroph Abundance	0.54	**0.03**
Picoeukaryote Abundance	0.65	**0.01**
Salinity	0.42	0.11
Temperature	0.83	**0.00**
Ammonium	-0.17	0.54
Nitrate	-0.56	**0.03**
Dissolved Reactive Phosphorus	-0.63	**0.01**
Silicate	-0.43	0.11
Iron	-0.27	0.34

Many studies have examined the production rates of viruses in aquatic ecosystems, but few have focused on the temporal dynamics of the formation and deterioration of a bloom in a natural system. There have been many reports on microcosms of blooms formed from the addition of nutrients and have shown increasing production rates upon bloom formation (Guixa-Boixereu et al. 1999; Almeida et al. 2001; Evans et al. 2003) as well as fertilization studies to which nutrients were added to a natural system to examine changes in the community structure (Higgins et al. 2009; Weinbauer et al. 2009). While these studies are important, examining natural blooms as they form and decline is important in understanding the true interactions between viruses and their hosts.

Virus production rates are often correlated to bacterial biomass and the trophic status of the system (Steward et al. 1992; Williamson and Paul 2004; Motegi and Nagata 2007). In the present study, sampling took place in a mesotrophic environment during a phytoplankton bloom, with high virus production rates observed; many samples were at the upper limit of normal rates determined in other aquatic systems (Rowe et al. 2008; Wilhelm and Matteson 2008; Higgins et al. 2009). For the production rates in the current study, we carried out production rates at *in situ* conditions to include phototrophic bacteria as well as heterotrophic bacterial rates as this system was often dominated by phytoplankton (Weinbauer et al. 2010). The high virus production rates observed here resulted in elevated percentages of the bacterial community lysed by viruses (average 63.77%) with observations from several stations showing percentages above 100%. Similarly high observations have been made in systems that were perceived to not be in steady-state (Wilhelm et al. 2002; Winter et al. 2004; Higgins et al. 2009). On October 2 it was found to have 215% of the bacterial community lysed: while we cannot rule out errors in any scientific measurement, the results suggest that a significant lytic event was occurring during this period and that viruses played an important role in community mortality during this phytoplankton bloom. Indeed it is plausible that many of the viruses observed resulted from the infection of the prevalent eukaryotic phytoplankton and diatoms in the system (which tend to have high burst sizes (Dunigan et al. 2006)) somewhat skewing our estimates of bacterial mortality.

To determine correlations between ecological variables and virus productions, we first performed tests to determine the normality of data and proceeded to use the Pearson correlation

coefficient to analyze the datasets. Correlation analysis identified several significant correlations between virus production and biotic and abiotic factors. Surprisingly, negative Pearson correlations were found between virus production rates and nutrients such as nitrate (r_p= -0.56, P=0.03) and DRP (r_p= -0.63, P=0.01). As nitrogen was a limiting nutrient, it would be expected that increases in N would cause an increase in lytic production rate from the increase in bacterial biomass and growth rate as it has been shown in other systems (Williamson and Paul 2004). Furthermore, increasing phosphorus concentrations have lead to increased production rates in other systems (Wilson et al. 1996; Williamson and Paul 2004; Motegi and Nagata 2007) while others have also seen negative correlations or no change in virus production or abundance (Tuomi et al. 1995; McDaniel and Paul 2005; Holmfeldt et al. 2010) demonstrating a complex relationship between nutrients and virus production likely due to the shifting nutrient concentrations through the bloom and upwelling events. Also, no significant correlation was found between production and silicate which is surprising as the bloom was rich in diatoms and significant correlations have been exhibited previously (Rowe et al. 2008). Production was significantly correlated to biology (and potential hosts) of the system with positive correlations to total bacteria, picoautotrophs and picoeukaryotes and shows that the production rates calculated were due to a wide range of potential hosts. This is not unexpected as production rates often depend on host density and trophic status of the system (Steward et al. 1992; Steward et al. 1996). While production was correlated to photosynthetic picoautotroph and picoeukaryote abundance, no correlation was found to any size fraction of chl *a* which is unpredicted and may be due to temporal disconnects or that chl *a* is only used as a proxy for photosynthetic biomass which does not always necessarily correlate to phytoplankton abundance. Therefore, changes in chl *a* may not be a change in abundance of phytoplankton, but due to species composition changes in a given sample (Cochlan et al. 1993).

In 2003, the first FeCycle cruise analyzed a natural bloom in subtropical Pacific Ocean waters southeast of New Zealand where primary productivity was Fe-limited (Boyd et al. 2005; Strzepek et al. 2005). The goal of FeCycleII was to compare this data to more Fe-replete regions to get a full understanding of Fe input on C cycling in the oceans, so that the plasticity in the cellular Fe:C ratio of plankton could be determined. Comparing the two cruises has shown that the bloom during the 2008 cruise was more productive than that of the 2003, with the FeCycleII

having chl *a* concentrations of up to 2.43 μg L^{-1} and the FeCycleI having concentrations up to 0.6 μg L^{-1} (McKay et al. 2005). Furthermore, viruses played a larger role in the lysis of hosts (from both the production and abundance standpoint) in the 2008 observations. In the FeCycle cruise, virus production rates were quite variable and ranged from 4.3 x 10^7- 2.3 x 10^9 L^{-1} d^{-1} while the stations with lowest rates were up to four orders of magnitude lower than the highest rates found in the FeCycleII cruise. Comparing these cruises, virus abundances were similar to each with FeCycle ranging from 4.0 x 10^6-8.4 x 10^8 mL^{-1} while FeCycleII ranged from 2.11 x 10^7- 1.59 x 10^8 mL^{-1}. The higher trophic status observed during the FeCycleII cruise may have contributed to increased contact rates between hosts and viruses, high virus production rate and fast turnover rates. Furthermore, this suggests that Fe may, in part, play a larger role in the production and turnover of phytoplankton in the world's oceans as seen by the lower productivity (and also virus productivity) in that system.

The potential importance of viruses in biogeochemical cycling of nutrients has been hypothesized for almost two decades (Fuhrman 1992). Since bacteria contain nutrient rich proteins, DNA and other organic N and P compounds (Bratbak et al. 1994), high virus-inferred bacterial lysis rates caused a large proportion of bioavailable nutrients to be released back into the nutrient pool to potentially sustain the phytoplankton bloom (Wilhelm and Suttle 1999). It has previously been estimated that over 70% of marine productivity is due to the recycling of nutrients in which viruses play an important role (Raven and Falkowski 1999). To determine the effect viruses had on nutrient cycles, we analyzed the release of C as well as trace elements N and Fe and found up to 161 μg L^{-1} d^{-1} of C, 7.61 ng L^{-1} d^{-1} of Fe and 38.74 μg L^{-1} d^{-1} of N in the southern Pacific Ocean, which is approximately three times higher than concentrations found during the North Atlantic Spring Bloom cruise in 2005 (Rowe et al. 2008) and also higher than other studies of marine systems (Poorvin et al. 2004; Strzepek et al. 2005; Higgins et al. 2009; Weinbauer et al. 2009) demonstrating the importance of viruses in recycling of nutrients (especially N in this N-limited system) during this bloom. Of the dissolved total N and Fe pools, viral lysis of cells on a daily basis mobilized 0.23-8.93% of the total N and 0.10-18.68% of the total Fe pools, demonstrating that virus activity could count account for significant fluxes of nutrients during this Pacific Ocean bloom.

Overall, this work identified viruses as important agents of bacterial mortality during a nutrient limited phytoplankton bloom. This mortality, in turn, released copious amounts of nutrients back to the nutrient pool which may have allowed for the sustained bloom progression. Whether or not these nutrients are indeed bioavailable are unknown, and more empirical data needs to be obtained to determine the role viruses play in the biogeochemical cycling of nutrients in freshwater and marine environments.

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Chapter 4 Quantification of Cyanophage in Marine Ecosystems using a Quantitative PCR Approach

This section is a version of a paper that will be submitted to the ISME Journal with the same title and the authors Audrey R. Matteson, Janet R. Rowe, Phillip R. Boyd and Steven W. Wilhelm.

My contribution to the paper was to collect samples while on board the FeCycleII cruise in 2008, optimize the qPCR reactions, run all qPCR reactions from the Sargasso Sea and FeCycleII, quantify total viruses in the FeCycleII, performed all PCR and cloning reactions, analyzed sequence data and made all phylogenetic trees and figures. Furthermore, I gathered data from other groups and contributed to most of the data analysis and manuscript preparation.

Abstract

The distribution of marine *Cyanomyoviridae* was estimated using a quantitative PCR approach. Samples were collected spatially through the Sargasso Sea in 2005 and temporally during a phytoplankton bloom off the coast of northern New Zealand in the southern Pacific Ocean in 2008. PCR primers for the *g20* gene were used as a proxy for cyanomyovirus abundance in these samples. Abundances were generally lower in the Sargasso Sea and varied with station with 2.75×10^3-5.15×10^4 mL^{-1} copies of the *g20* gene and correlated well with the potential host abundance of *Prochlorococcus* and *Synechococcus* in the system. Abundances in the Pacific Ocean were steady and followed the trend of *Synechococcus* hosts in the system. Following the demise of a *Synechococcus* bloom in the Pacific Ocean, the *g20* gene copies dropped from 4.3×10^5 to 9.6×10^3 mL^{-1}. Specific contact rates between cyanomyoviruses and cyanobacterial hosts were higher in the southern Pacific Ocean compared to the Sargasso Sea with ranges from 0.003-0.12 $cell^{-1}d^{-1}$ in the Sargasso Sea and 0.010-2.27 $cell^{-1}d^{-1}$ in the southern Pacific Ocean. Statistical correlations between *g20* gene copy numbers and biotic and abiotic parameters were performed and found strong correlations with biology in the Sargasso Sea, especially the abundance of cyanobacteria. In the southern Pacific Ocean, correlations between abiotic factors such as salinity and virus abundances were found, reasserting questions regarding the products of the PCR primers as well as highlighting the potential for temporal disconnects between virus and host abundance.

Introduction

During the last few decades, research has shown high abundances (up to 10^8 ml^{-1}) of virus-like particles in marine systems (Bergh et al. 1989; Bratbak et al. 1990; Proctor and Fuhrman 1990) with even higher values exhibited in lake systems (Wilhelm and Matteson 2008) and sediments (Manuela and Mathias 2007; Danovaro et al. 2008). The importance of viruses in many global processes has now been documented in both freshwater (DeBruyn et al. 2004; Madan et al. 2005) and marine (Wilhelm and Suttle 1999; Suttle 2005) environments. Viruses influence these systems by releasing bioavailable carbon and nutrients such as carbon, phosphorus, nitrogen, iron and selenium and have been shown to be released upon viral lysis and can be directly utilized by other bacterial species in the system (Gobler et al. 1997; Poorvin et al. 2004; Brussaard et al. 2008). Viruses are also able to control the community structure by constraining the most successful microorganisms *via* the 'kill the winner' method (Bratbak et al. 1990; Thingstad et al. 1993; Thingstad and Lignell 1997) by which viruses control dominant species in an environment. Through this mechanism, viruses are able to control the community structure (Hennes et al. 1995; Muhling et al. 2005; Rodriguez-Brito et al. 2010) and the genetic diversity (Weinbauer and Rassoulzadegan 2004; Sandaa et al. 2009) of hosts.

Perhaps the most studied aquatic phage-host system has involved the ecologically important cyanobacteria *Prochlorococcus* and *Synechococcus*. These picophytoplankton dominate the world's oceans and significantly contribute to primary production (Partensky et al. 1999). Cyanophage infecting these cells are readily identified (and isolated) in both freshwater and marine ecosystems (Suttle and Chan 1994; Garza and Suttle 1998; Frederickson et al. 2003; Marston and Sallee 2003) and are thought to reduce primary production by increasing cyanobacterial mortality (Proctor and Fuhrman 1990; Suttle et al. 1990). The known cyanophage can be divided into three morphologically different families of tailed, dsDNA containing phage: the *Cyanomyoviridae*, *Cyanopodoviridae* and *Cyanosiphoviridae* (Suttle 2000). Despite not having the equivalent to the 16S rDNA gene in bacterial species (Paul et al. 2002), genes that are conserved in specific phage families have been identified and used for constructing molecular probes (Fuller et al. 1998). For example, PCR primers targeting the conserved DNA polymerase (DNA *pol*) gene have been recently used to study the genetic richness of podoviruses in the open ocean (Huang et al. 2010) and the Chesapeake Bay (Chen et

al. 2009). Molecular analyses using the *g20* gene, which encodes for the portal vertex protein in myoviruses, has perhaps been the most widely used marker for the genetic richness of the cyanomyophage (Fuller et al. 1998; Zhong et al. 2002; Frederickson et al. 2003; Dorigo et al. 2004; Wang and Chen 2004; Muhling et al. 2005; Wilhelm et al. 2006; Wang et al. 2010) in marine, estuarine and freshwater environments. In recent years the specificity of these *g20* primers for only cyanomyoviruses and not other bacteriophage has been in question (Sullivan et al. 2003; Short and Suttle 2005; Wilhelm and Matteson 2008). To date, there has been no direct resolution and the primers are still used for phylogenetic analysis.

In the laboratory the cyanomyoviruses are the most commonly isolated and have the broadest host range of the three viral families, spanning across *Synechococcus* and *Prochlorococcus* (Suttle and Chan 1993; Waterbury and Valois 1993; Wilson et al. 1993; Lu et al. 2001; Sullivan et al. 2003; Weigele et al. 2007). Despite evidence of high total viral titers (Garza and Suttle 1998) and significant genetic richness in *Cyanomyoviridae* in marine and freshwater environments (see above references), there has been only one report (constrained to a Norwegian coastal water station) that has used quantitative molecular tools to quantify cyanomyovirus genomes in aquatic ecosystems (Sandaa and Larsen 2006). Given that a major component of diversity is abundance, we felt it was important to determine how abundant these well-characterized phage were on a global scale. Within this study we first optimized the conditions for quantitative PCR (qPCR) to enumerate the capsid protein gene, *g20* in cyanomyoviruses. Abundances were subsequently estimated from two different locations: spatially across the oligotrophic Sargasso Sea, and temporally through a spring bloom event off the coast of northern New Zealand. Overall, the data demonstrate that cyanomyoviruses are highly abundant across all these regions yet undergo temporal changes as the microbial community responds to changing environmental conditions. Furthermore, using statistical analysis, our data indicate that during the FeCycleII cruise, *g20* abundance did not correlate with cyanobacteria or heterotrophic bacteria, suggesting that other potential hosts may exist or that a temporal disconnect between virus and host abundance exists. We present this data in the context of further defining the relevance of cyanomyoviruses in marine microbial processes.

Materials and Methods

Sampling Collection

Water samples were collected from May 23 to June 1 through the Sargasso Sea while on board the *RV Seward Johnson* at 16 stations in 2005. Samples were collected each day from the ship's clean surface pump at a depth of ~5 m on a transit from Florida to the Azores. Salinity, water temperature, *in situ* fluorescence (a proxy for chlorophyll *a*), pressure, density, conductivity, oxygen, oxygen saturation, and SPAR (surface PAR) were all measured using the CTD package mounted on the rosette sampler (Rowe et al. 2008).

Whole water was also collected from the southern Pacific Ocean during the FeCycleII / Spring Bloom cruise on board the *RV Tangaroa* (September 19 to October 5, 2008). Daily samples (~ 0300 local time) were collected from surface water (~5 m) during the bloom from Niskin bottles mounted on the ships rosette system. Water temperature, salinity, *in situ* fluorescence (a proxy for chl *a*), oxygen, conductivity, pressure, and density were measured by the ship's CTD package attached to the rosette sampler.

Chlorophyll *a*.

Size-fractionated chl *a* concentrations for both cruises were determined on replicate samples collected on 0.22 (pico-), 2.0 (nano-), and 20.0 μm (microphytoplankton) nominal pore-size, 47 mm diameter polycarbonate filters (GE Osmonics). Samples were subsequently extracted following the methods of Rowe et al. (2008) and quantified using the nonacidification protocol (Welschmeyer 1994).

Virus abundance

The abundance of virus particles was determined for whole water samples which were preserved in 2 or 2.5% final sterile glutaraldehyde. The samples collected during the NASB cruise (Rowe et al. 2008) were processed immediately at sea as suggested by Wen et al. whereas the FeCycleII cruise abundance tubes were flash frozen and kept at -80°C until processed in the lab. Slide preparation involved staining 850 μL of the thawed sample with SYBR Green as described

previously (Noble 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 (λ_{Ex} = 450 to 490 nm, λ_{Ex} = 510 nm, with suppression filter at λ_{Ex} = 510 nm). At least 20 independent fields were viewed for each slide with the total of each grid noted to ensure particles were evenly distributed across individual filters.

Bacterial and Cyanobacterial Abundance

For the Sargasso NASB cruise, total bacteria, *Prochlorococcus* and *Synechococcus* were enumerated as previously described (Michelou et al. 2007). For samples collected during the FeCycleII cruise, the abundance of bacteria was measured using flow cytometry on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA.) with CellQuest Vers. 3.1f software as previously described (Hall et al. 2006).

Extraction of Viral DNA

To optimize qPCR analysis, several DNA extraction methods were compared. A phenol-chloroform extraction protocol (Wilson et al. 1993) was followed except that RNase, DNase, and precipitation steps were omitted. A hot-cold extraction was performed (Chen et al. 1996) by chilling the sample on ice for 2 min, followed by a boiling step for 2 min before another 2 min ice step. The Invitrogen Genomic DNA kit (Invitrogen, Carlsbad, CA) was used with the addition of a polyethylene glycol precipitation step by adding 1 volume of 20% PEG 8000/2.5M NaCl to 5 volumes of the phage solution for 15 min before centrifuging at 12,000 x *g* for 10 min. The AquaRNA solution (MultiTarget Pharmaceuticals) was also used following manufacturer's instructions. The final method used was to alter the salt concentration in the sample by dialysis. A 0.025µm pore-size mixed cellulose ester membrane filter (Millipore) was placed on several drops of sterile Milli-Q water. Approximately 20 µL of sample was placed on the filter for 15 min before removal for analysis. Samples were either used immediately or frozen at -20°C until needed.

Quantitative PCR

Samples for qPCR analysis were collected in 5 mL cryovials and immediately flash frozen in liquid nitrogen or placed at -30 °C until samples were brought back to the lab where they were kept at -80°C. To quantify cyanomyoviruses, the 165-bp primer pair CPS1 and CPS2 was used to estimate *g20* gene copies (Fuller et al. 1998). Plasmid standards were prepared using *Synechococcus* phage S-PWM1 and primer pair CPS1 and CPS8 which produces a 592-bp product that contains the 165-bp fragment from CPS1 and CPS2. PCR with S-PWM1 phage was done with PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) using 0.2 μM each primer, 1 μL of filtered S-PWM1 phage stock and sterile water with reaction conditions previously reported (Zhong et al. 2002). The DNA fragment was cloned into the PCR 2.1 TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Plasmid DNA was purified using the Qiagen Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA) and cloned inserts were confirmed using an EcoRI digestion at 37°C for two hours followed by electrophoresis with 1.5% agarose gels. Plasmid DNA was sequenced by the University of Tennessee Sequencing Facility for confirmation. The DNA concentration (A_{260}) of the plasmid was determined spectrophotometrically using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Surrey, UK). The molecular weight of the double-stranded plasmid and product was calculated using the Oligocalc Calculator version 3.23 (Kibbe 2007). Copy numbers of *g20* per μL were determined using Avogadro's number, 6.022×10^{23} plasmid copies mol^{-1}. Each plasmid contained one copy of the target gene and was freshly diluted and used within 24 hours with at least four different duplicate dilutions from 10^6-10^1 copies to make a linear standard curve for the analyses. Plasmid standards were validated against known quantities of diluted S-PWM1 phage. Each 25 μL qPCR reaction contained 12.5 μL Thermo Scientific ABsolute SYBR Green QPCR mix (Thermo Scientific, Surrey, UK), 280 ng μL^{-1} bovine serum albumin (Fisher Scientific), 0.30 μM cps1 forward primer and 0.60 μM cps2 reverse primer and 2 μL viral sample or standard. Primers used were purified using HPLC to ensure decreased truncated oligonucleotide sequences (Eurofins MWG Operon, Huntsville, AL). Each sample was subjected to ultracentrifugation at 60,000-81,000 x *g* (Beckman TL-100) for 3 hours at 4°C and kept at -20°C before running each reaction. Samples were run in triplicate on a 96-well plate

(Bio-Rad, Hercules, CA) sealed with optically clear flat cap strips (Bio-Rad, Hercules, CA) and assayed with up to three different dilutions. A single spiked sample of known concentration of *Synechococcus* phage S-PWM1 was run with each dilution to estimate PCR inhibition. The conditions for the primers consisted of an initial denaturation step at 95°C for 15 min (due to the hot start master mixed used) followed by thirty-five cycles of 95°C for 10 sec, 53°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 10 min. Following the program, a melting curve was read every 1°C from 40-95°C to check specificity of primers and formation of primer dimers. These dimers were sometimes evident from the melting curve with low copy numbers, but a second plate read at 77°C was used to eliminate the quantification of fluorescence of any unspecific binding (Morrison et al. 1998). To further deter unspecific binding, all reactions were prepared on a 4°C CoolSafe system (Diversified Biotech, MA) for PCR setup. Negative controls were conducted along with samples using sterile water and no DNA template. Positive controls with lysate from *Synechococcus* phage S-PWM1 were used. Quantities of phage for positive controls were estimated by epifluorescence microscopy following the protocol as stated above for viral abundances. Plates were centrifuged at 100 x *g* for 2 min prior to running each reaction. Detection limits for the qPCR were calculated to be *ca* 10^1 copies per reaction.

Virus Host Contact Rates

Contact rates (using *g20* gene copies as virus particle estimates) to both heterotrophic bacteria and cyanobacteria were calculated according to Murray and Jackson (1992). The diameter of a marine bacterium of 0.45 x 10^{-4} cm was used for heterotrophic bacteria (Lee and Fuhrman 1987), while the diameter of *Synechococcus* WH7803 at 1.0 x 10^{-4} cm was used for *Synechococcus* contact rates (Waterbury et al. 1986) and 0.5 x 10^{-4} cm was used for *Prochlorococcus* species (Morel et al. 1993). As well, the populations were assumed to be ~ 10% motile, providing a Sherwood number of 1.06 (Wilhelm et al. 1998).

Phylogenetic Diversity of *g20* gene sequences

Virus concentrates were generated in a similar manner as described by Chen, et al. (1996) and Wilhelm and Poorvin (2001). Concentrates were collected at all stations and were used for phylogenetic diversity at days September 20, 22, 26 and 30 as well as October 1 and 3 during the FeCycleII cruise. 20 L of whole water samples were collected from a trace metal pump lowered to ~5 m and prefiltered through a 0.8-µm nominal pore-size, 142-mm diameter polycarbonate filter (Millipore). The virus-size fraction was then concentrated to ~500 mL using a Millipore M12 ProFlux tangential flow filtration unit with a 30kDa cutoff filter cartridge (Millipore) to make the virus concentrate. Virus concentrates were stored in the dark at 4°C until needed for molecular analyses. Viral concentrates were further concentrated for PCR amplification using a PEG centrifugation method similar to that of Chen, et al. (2009). Powder PEG 8000 (Fisherbrand) was added to 1.5 mL of concentrate to a final concentration of 10% w/v. Samples were briefly vortexed and stored in the dark at 4°C overnight to precipitate viral particles followed by centrifugation at 15,000 x *g* for 1 hour. The pellets formed were resuspended in 35-50 µL of sterile SM buffer (10 mM NaCl, 50 mM Tris, 10mM $MgSO_4$ and 0.1% gelatin) and was immediately heated to 95°C for 15 min to release DNA from the protein capsid. Oligonucleotide primers CPS1.1 5′-GTAGWATWTTYTAYATTGAYGTWGG-3′ and CPS8.1 5′-ARTAYTTDCCDAYRWAWGGWTC-3′ were used to analyze diversity of the *g20* gene (Sullivan et al. 2008). Reactions were performed in 50 µL volumes using Easystart tubes (Molecular BioProducts) with 1 unit of *Taq* DNA Polymerase (Promega, Madison, WI), 1.2 µM each primer and up to 10 µL boiled viral fraction. Easystart tubes contain 50 mM $MgCl_2$, 10X PCR buffer, and 2.5 mM dNTP mix. Prior to running the samples, the optimum annealing temperature was found to be higher than that previously published (Sullivan et al. 2008). The reactions were performed with an initial 3 min denaturation at 95°C followed by 35 cycles of denaturation at 94 °C, annealing at 45°C, and elongation at 73°C for 1 min each with a final 4 min incubation at 73°C. PCR products were visualized on 1% agarose gels using the Fotodyne Investigator digital imager with ethidium bromide filter using Foto/Analyst® PC Image v. 9.0.4 software. Despite optimizing the PCR reaction, two bands were generally found in all reactions and the band of the correct size was cut and purified using the Qiagen QIAquick PCR purification kit (Qiagen, Valencia, CA). Purified products were immediately inserted into the TOPO pCR 2.1-TOPO cloning vector and transformed into electrocompentent *Escherichia coli*

using the TOPO TA kit (Invitrogen, Carlsbad, CA). Cloning efficiency was analyzed using colony PCR with EconoTaq PLUS 2X GREEN master mix (Lucigen, Middleton, WI), 3 μL of bacterial cells, and 0.16 μM M13 Forward (-20) (5´-GTAAAACGACGGCCAG-3´) and M13 Reverse (5´-CAGGAAACAGCTATGAC-3') plasmid-targeting primers. PCR products were visualized as stated above. Clones to be sequenced were grown overnight as suggested by the manufacturer. Plasmid purification and sequencing was subsequently performed at Clemson University Genomics where all clones were forward sequenced using the M13 Forward primer.

Sequences were first manually inspected to ensure quality sequencing before removing primers with the help of FinchTV ver. 1.4.0. Identical sequences were identified using the 454 Replicate Filter program (Gomez-Alvarez et al. 2009) and DNA sequences were aligned using ClustalW (Larkin et al. 2007). Blastn searches of the sequences were performed to find closely related phage sequences (Altschul et al. 1990) as well as all reference sequences used. Neighbor-joining trees of sequences were constructed using MEGA 4 with 1000 replicates (Tamura et al. 2007) with the Jukes Cantor parameter. Maximum likelihood trees were constructed by first aligning the sequences with MUSCLE using phylogeny.fr (Dereeper et al. 2008). The outgroup for phylogenetic analyses came from coliphage T4 gp23 gene. Sequences were further analyzed using Unifrac distance metric statistical tools to compare libraries (Lozupone and Knight 2005) and can be found at http://bmf.colorado.edu/unifrac/.

Statistical Analyses

Threshold cycle (C_t) calculations for each qPCR assay were completed using the MJ Opticon Monitor Analysis software (ver 3.1) with the Global Minimum setting. The threshold for all reactions was manually adjusted to obtain the highest correlation coefficient (r^2) for standard curves produced. Standard curves (from the gene copy number vs. C_t) were analyzed for each qPCR assay to help determine the total gene copies in the samples. All averages, standard deviations and paired two-tailed *t*-tests were calculated using Microsoft Excel 2007. Percent recoveries of spiked samples were analyzed by the equation: percent recovery = (measured copies in water sample spiked with phage genomic DNA – average of measured copies in unspiked water samples)/ (viral genomic DNA added) x 100. Figures were constructed using

SigmaPlot ver. 10.0. The relationships between biological parameters and *g20* gene copies were compared using regression and correlation data on samples that were untransformed or log transformed using the Pearson pairwise statistics (NCSS Statistical & Power Analysis Software).

Results

DNA Extraction Efficiencies

Optimizing DNA extraction protocols is necessary to obtain the best DNA recovery in a sample for qPCR analysis. The most efficient recoveries were for the unconcentrated untreated and dialysis treatments. Preconcentration of the sample resulted in an order of magnitude loss in recovery within the comparison. However, due to very low titers in some samples, the ultracentrifugation step was performed prior to running all reactions so that results could be uniformly obtained. Figure 4.1 compares all extraction protocols, and demonstrates that ultracentrifugation was significantly higher than the Invitrogen Genomic DNA Extraction kit ($p=0.001$) and the AquaRNA Viral Extraction kits ($p=0.002$), but significantly lower than the dialyzed treatment sample ($p=0.014$). Other treatments were not significantly different than the ultracentrifuged samples ($p>0.05$) which was ultimately used for the analyses. When low copy numbers were detected, unspecific binding was exhibited in the melt curve analysis. To inhibit the SYBR Green from binding to these products, a second fluorescence read was performed at 77°C (Morrison et al. 1998).

Abundance of cyanomyoviruses was compared from two distinct aquatic ecosystems and can be found in Table 4.1. Samples were collected from the oligotrophic Sargasso Sea in 2005 and the mesotrophic South Pacific Ocean during a seasonal phytoplankton bloom. To infer the proportion of myoviruses in each environment, the total viral abundances were estimated using epifluorescence microscopy to identify the percentage of myoviruses from the total population.

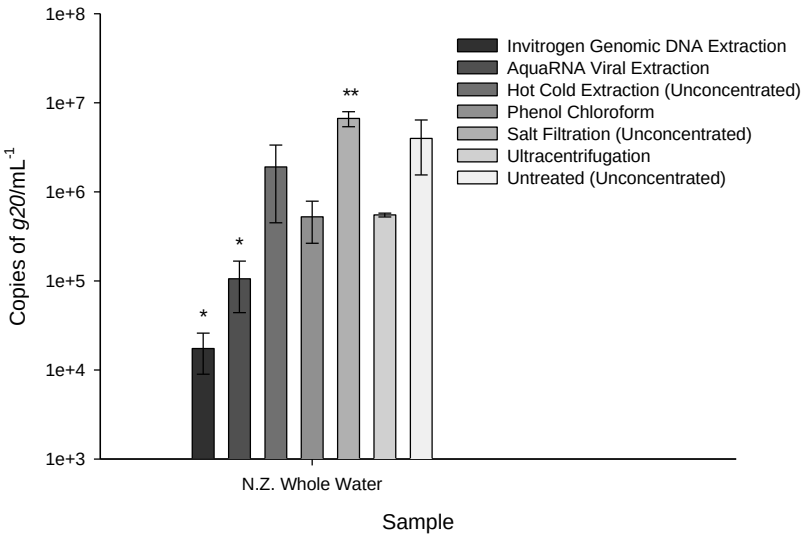

Figure 4.1: Comparison of DNA extraction protocols to identify extraction efficiency. New Zealand whole water was taken in October 2008 and was used with various extraction methods and compared. All qPCR conditions were followed as described here. Samples that were significantly lower than the ultracentrifugation method was designated with one asterisk (*) while the one extraction that was significantly higher was labeled with two asterisks (**).

Table 4.1: Sample stations on the NASB 2005 and FeCycle II cruise. All samples were collected at ~ 5 m unless noted. * collected at 10 m. **collected at 12 m.

Cruise/ Station	Date Sampled	Latitude	Longitude	Salinity	Temperature (°C)
NASB 1	5/23/2005	29°16'31.0”N	76°5'49.2"W	36.53	24.3
NASB 2	5/24/2005	30°55'31.7"N	71°51'56.5"W	36.78	22.4
NASB 3	5/24/2005	31°40'47.3"N	69°45'1.2"W	36.72	21.9
NASB 4	5/25/2005	32°34'46.6"N	67°0'40.9"W	36.51	20.7
NASB 5	5/25/2005	33°15'2.9"N	64°49'30.5"W	36.61	20.9
NASB 6	5/26/2005	34°8'29.7"N	62°3'52.6"W	36.69	21.4
NASB 7	5/26/2005	34°45'15.0"N	59°40'53.7"W	36.64	20.8
NASB 8	5/27/2005	35°17'48.2"N	56°47'24.8"W	36.62	20.3
NASB 9	5/27/2005	35°48'33.6"N	54°16'59.3"W	36.45	20.6
NASB 10	5/28/2005	36°20'33.8"N	51°18'52.9"W	36.51	20.0
NASB 11	5/28/2005	36°44'17.4"N	48°43'7.6"W	36.52	20.1
NASB 12	5/29/2005	37°8'40.4"N	45°35'5.3"W	36.54	19.8
NASB 13	5/29/2005	37°21'24.9"N	42°55'31.8"W	36.37	19.4
NASB 14	5/30/2005	37°38'50.4"N	40°6'8.6"W	36.54	20.0
NASB 15	5/30/2005	37°47'15.0"N	37°26'23.9"W	36.49	19.6
NASB 16	5/31/2005	37°52'48.8"N	34°36'31.6"W	36.34	19.3
FeCycleII 1	9/19/2008	39°20'43.8"S	178°35'9.6"E	35.37	13.8
FeCycleII 2	9/20/2008	39°13'59.3"S	178°38'2.3"E	35.37	13.8
FeCycleII 3	9/21/2008	39°13'28.7"S	178°48'16.1"E	35.37	13.8
FeCycleII 4	9/22/2008	39°22'34.7"S	178°49'26.04"E	35.36	13.8
FeCycleII 5	9/23/2008	39°23'11.9"S	178°41'31.2"E	35.36	13.8
FeCycleII 6	9/24/2008	39°21'4.1"S	178°39'18"E	35.37	13.9
FeCycleII 7	9/25/2008	39°17'46.1"S	178°43'33.5"E	35.37	14.1
FeCycleII 8	9/26/2008	39°20'55.1"S	178°51'12.5"E	35.36	14.1
FeCycleII 9	9/27/2008	39°28'4.8"S	178°43'47.9"E	35.36	14.0
FeCycleII 10	9/28/2008	39°21'44.3"S	178°32'0.6"E	35.35	14.0
FeCycleII 11*	9/29/2008	39°9'14.9"S	178°40'51.6"E	35.34	13.8
FeCycleII 12**	9/30/2008	39°6'48.6"S	178°55'3.7"E	35.34	13.8
FeCycleII 13	10/1/2008	39°8'0.5"S	179°9'47.5"E	35.34	13.8
FeCycleII 14	10/2/2008	39°12'37.1"S	179°22'58.8"E	35.33	13.8
FeCycleII 15	10/3/2008	39°16'58.8"S	179°28'24.2"E	35.33	13.7
FeCycleII 16	10/4/2008	39°17'18.5"S	179°28'36.8"E	35.33	13.9

Spatial Variation of Virioplankton and Bacterioplankton in the Sargasso Sea

Overall, the abundance of myoviruses varied with and within each individual ecosystem. The oligotrophic Sargasso Sea system from the NASB cruise in 2005 contained low abundances of total viruses ranging from only 2.02 x 10^5-1.94 x 10^6 ml^{-1} with numbers increasing the farther northeast samples were taken. Unsurprisingly, cyanobacteria were prevalent throughout the sampling locations and contained from 7.66 x 10^2-1.20 x 10^5 cells mL^{-1} of *Prochlorococcus* with the highest found at Station 16. *Synechococcus* abundances were generally very similar to *Prochlorococcus* until later stations where *Prochlorococcus* often outnumbered *Synechococcus* cells. *Synechococcus* numbers ranged from 8.54 x 10^3-5.90 x 10^4 mL^{-1} with Station 11 containing the highest concentration of cells (Figure 4.2 C). Total heterotrophic counts and chlorophyll *a* concentrations were analyzed and followed a similar trend to total virus concentrations. Chlorophyll *a* levels were extremely low in all size fractions, but were clearly dominated by picophytoplankton throughout the Sargasso Sea (Figure 4.2A) with the highest concentration of 0.11 μg L^{-1} in the 0.2 μm size fraction. Approximately 4.61 x 10^5-1.15 x 10^6 mL^{-1} of heterotrophic bacteria were enumerated and contained higher abundances farther northeast into the Sargasso Sea (Figure 4.2C). Total *g20* gene copies showed no true trend and varied with each station and contained from 2.75 x 10^3-5.15 x 10^4 mL^{-1} (Figure 4.2E). The numbers exhibited correspond well to their host abundances throughout the cruise. To analyze the percent recovery of spiked qPCR samples, spiked samples of known quantities of *Synechococcus* phage S-PWM1 were added to each sample and varied greatly from 42-102% recovery of all gene copies. The Sargasso Sea composed the highest percentages of putative cyanomyoviruses from the total viral population with 0.21-25.5% (Figure 4.2G). The highest percentages of myoviruses came from the samples closest to the Florida coastline, with a decreasing trend with the higher viral abundances found in the last stations examined. These estimates were significantly higher than those found in the FeCycleII samples.

Using the Pearson pairwise statistical test, *g20* gene copies significantly correlated to biotic factors in the system (Table 4.2). The strongest negative correlations were with potential cyanobacterial hosts of *Prochlorococcus* (-0.78, p=0.001) and *Synechococcus* (-0.60, p=0.024). Samples were also significantly correlated to heterotrophic bacteria (-0.51, p=0.044), but the

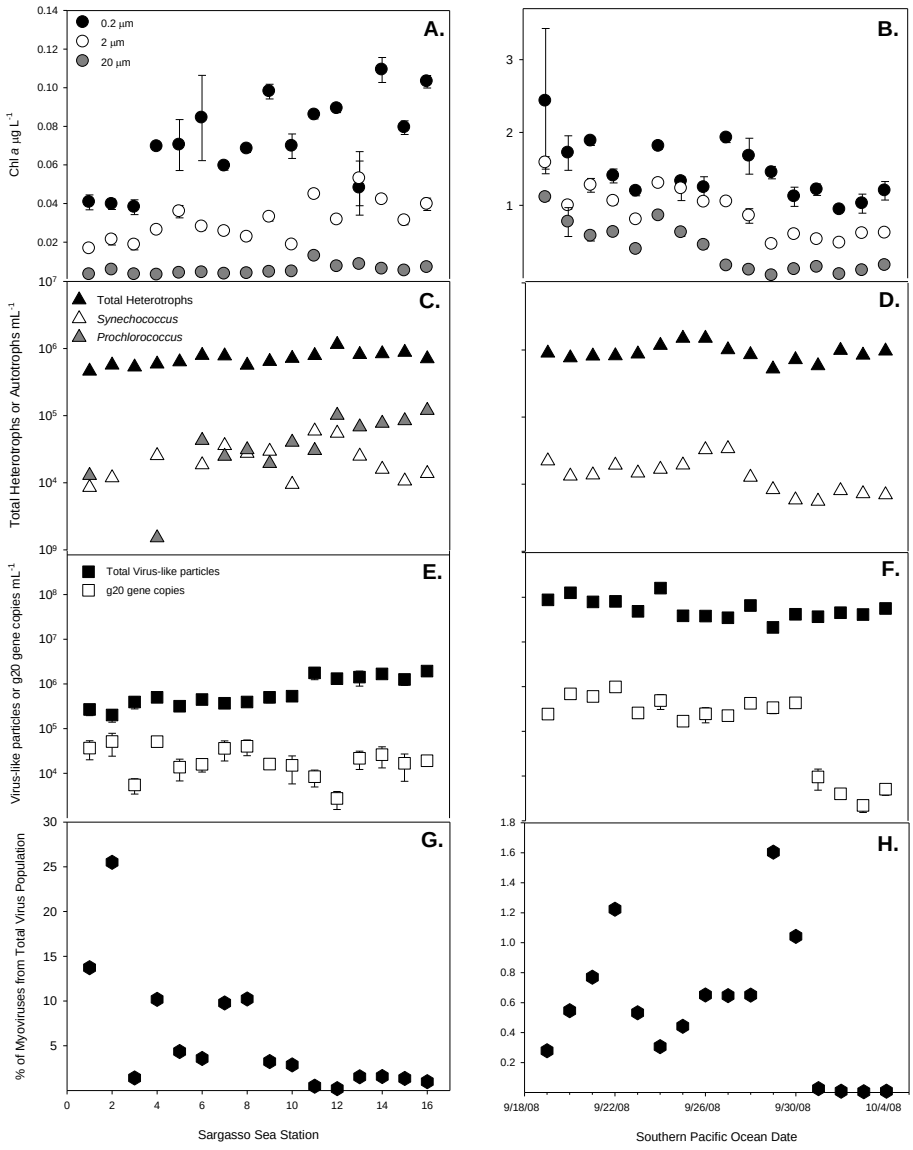

Figure 4.2: (A & B) Size-fractionated chlorophyll *a* concentrations in the 0.2-2 (black circles), 2-20 (white circles), and >20 μm (gray circles) fractions. (C & D) Bacterial cell abundances analyzing the total heterotrophic bacteria (black triangles), *Prochlorococcus* (gray triangles) and *Synechococcus* (white triangles) abundances. (E & F) Total virus abundances (black squares) and *g20* gene copy numbers (white squares) from both ecosystems. (G & H) The percentage of putative cyanomyovirus *g20* gene copies from the total virus population.

Table 4.2: Pearson correlation matrix from the *g20* gene copies to biological parameters from two independent cruise transects along with the combined datasets. Those significantly correlated ($p<0.05$) are in bold face. NA is designated for those samples not available.

Cruise	NASB 2005	FeCycleII	Total
Temperature	0.36 p=0.167	-0.12 p=0.657	**-0.59** **p=0.001**
Salinity	0.22 p=0.424	**0.56** **p=0.024**	**-0.60** **p=0.001**
Virus Abundance	-0.41 p=0.117	**0.54** **p=0.031**	**0.74** **p=0.001**
Chl *a* 0.2 µm	-0.36 p=0.172	0.37 p=0.16	**0.68** **p=0.001**
Chl *a* 2.0 µm	-0.33 p=0.217	0.41 p=0.118	**0.69** **p=0.001**
Chl *a* 20.0 µm	-0.39 p=0.139	0.46 p=0.75	**0.67** **p=0.001**
Synechococcus	**-0.60** **p=0.024**	0.18 p=0.506	-0.17 p=0.378
Prochlorococcus	**-0.78** **p=0.001**	NA	NA
Heterotrophic Bacteria	**-0.51** **p=0.044**	-0.173 P=0.522	0.134 p=0.463
Total Bacteria	**-0.74** **p=0.003**	-0.16 p=0.5443	0.023 p=0.904
Bacterial Production	**-0.51** **p=0.04**	0.199 p=0.458	**-0.56** **p=0.001**

correlation was not as strong. Furthermore, there was correlation to total bacteria, which would be expected since this fraction would include cyanobacteria as well.

Contact rates of total viruses and putative cyanomyoviruses with cyanobacteria and heterotrophic bacteria in the Sargasso Sea were calculated and can be found in Table 4.3. The rates between *Prochlorococcus* and *Synechococcus* with putative cyanomyoviruses were relatively low throughout the Sargasso Sea, likely due to the oligotrophic status of the system. For *Synechococcus*, the rates ranged between 0.01-0.12 contacts $cell^{-1}$ day^{-1} while for *Prochlorococcus*, it was between 0.003-0.06 contacts $cell^{-1}$ day^{-1} (Table 4.3). Putative cyanomyoviruses contacted heterotrophic bacteria between 0.003-0.05 contacts $cell^{-1}$ day^{-1}. Other contact rates between total viruses and bacteria can be found in Table 4.3 and were overall significantly lower than rates determined for the southern Pacific Ocean samples.

Temporal Changes during the South Pacific Spring Bloom

Samples were collected from a seasonal phytoplankton bloom off the coast of the northern island of New Zealand during the FeCycleII cruise in 2008. Chlorophyll *a* concentrations in this marine system were significantly higher in all size fractions than those found in the Sargasso Sea (Figure 4.2B) due to the bloom event and mesotrophic status of the system. The picophytoplankton fraction was dominant through all stations, especially at the start of the cruise and around September 27 when there was a small *Synechococcus* bloom as confirmed from flow cytometry data (Figure 4.2B). *Synechococcus* counts remained steady with approximately 1 x 10^4 cells mL^{-1} through the first week of the cruise, followed by a rapid increase in cell abundance to 3 x 10^4 mL^{-1} by September 26 and 27 (Figure 4.2D). Following September 28, the bloom crashed and remained at cell concentrations of 10^3 until the end of the cruise. *Synechococcus* was the abundant cyanobacterium as *Prochlorococcus* abundance was not detected through the bloom (O. Ulloa, pers. com.). Cyanomyovirus *g20* gene copy numbers closely resembled the host abundances having a stable number of myoviruses with an order of magnitude higher virus density of approximately 10^5 gene copies mL^{-1}. On October 1 there was a significant drop in abundance to around 10^3 mL^{-1}

Table 4.3: Contact rates were calculated in the Sargasso Sea using the Jackson and Murray (1992) equation. Contact rates and specific contact rates of total viruses and *g20* genes (as a proxy for cyanomyovirus abundance) to HB=Heterotrophic Bacteria *Syn.=Synechococcus Pro.=Prochlorococcus*. NA is designated for those samples not available.

	Contact Rate (mL^{-1} hr^{-1})						**Specific Contact Rate ($cell^{-1}$ d^{-1})**					
	Total Viruses vs.			**g20 genes vs.**			**Total Viruses vs.**			**g20 genes vs.**		
Station/Cruise	**HB ($x10^5$)**	***Syn.* ($x10^4$)**	***Pro.* ($x10^4$)**	**HB ($x10^3$)**	***Syn.* ($x10^2$)**	***Pro.* ($x10^2$)**	**HB**	***Syn.***	***Pro.***	**HB**	***Syn.***	***Pro.***
NASB 1	1.28	0.53	0.40	17.6	7.25	5.46	0.28	0.62	0.31	0.04	0.08	0.04
NASB 2	1.19	0.55	0.02	30.3	14.1	0.45	0.21	0.47	0.23	0.05	0.12	0.06
NASB 3	2.17	NA	NA	30.4	NA	NA	0.41	NA	NA	0.01	NA	NA
NASB 4	3.06	2.94	0.09	31.2	30.0	0.09	0.52	1.16	0.58	0.05	0.12	0.06
NASB 5	2.08	NA	NA	9.04	NA	NA	0.33	NA	NA	0.01	NA	NA
NASB 6	3.67	1.91	2.20	13.1	6.83	7.85	0.46	1.03	0.51	0.02	0.04	0.02
NASB 7	2.99	3.06	1.06	29.2	30.0	10.4	0.38	0.85	0.43	0.04	0.08	0.04
NASB 8	2.31	2.50	1.42	23.6	25.6	14.5	0.41	0.91	0.45	0.04	0.09	0.05
NASB 9	3.33	3.41	1.12	10.8	11.0	3.62	0.52	1.15	0.58	0.02	0.04	0.02
NASB 10	3.93	1.16	2.46	11.2	3.32	7.03	0.55	1.22	0.61	0.02	0.03	0.02
NASB 11	14.4	24.0	6.18	6.86	11.4	2.95	1.83	4.06	2.03	0.01	0.02	0.01

Table 4.3 Continued

Station/Cruise	Contact Rate (mL^{-1} hr^{-1})						Specific Contact Rate ($cell^{-1}$ d^{-1})					
	Total Viruses vs.			*g20* genes vs.			Total Viruses vs.			*g20* genes vs.		
	HB ($x10^5$)	*Syn.* ($x10^4$)	*Pro.* ($x10^4$)	HB ($x10^3$)	*Syn.* ($x10^2$)	*Pro.* ($x10^2$)	HB	*Syn.*	*Pro.*	HB	*Syn.*	*Pro.*
NASB 12	15.6	16.5	15.3	3.27	3.47	3.21	1.36	3.01	1.51	0.003	0.01	0.003
NASB 13	12.0	8.16	11.1	18.4	12.5	17.1	1.47	3.27	1.63	0.02	0.05	0.03
NASB 14	14.6	6.14	14.9	22.8	9.61	23.3	1.74	3.86	1.93	0.03	0.06	0.03
NASB 15	11.5	3.09	12.2	15.3	4.14	16.3	1.30	2.90	1.45	0.02	0.04	0.02
NASB 16	14.2	6.14	26.7	14.0	6.09	26.5	2.01	4.46	2.23	0.02	0.04	0.02

(Figure 4.2F). The decrease in cyanomyovirus gene copies came just three days after the demise of the *Synechococcus* bloom on September 29. Spiked samples recovered from 48-134% of *g20* genes added to the reactions. Although the *g20* gene copies were significantly higher in this system than the Sargasso Sea, the putative cyanomyoviruses only consisted from 0.0054-1.6% of the total virus population (Figure 4.2H). Despite the drastic changes in *g20* gene abundances later in the bloom, total virus concentrations maintained levels from 2.11×10^{7}-1.59×10^{8} mL^{-1} with the highest quantities found around the beginning of the bloom (Figure 4.2F).

Very different statistical correlations were evident from the two systems examined. The *g20* gene copy numbers were only weakly correlated to two parameters, salinity (0.56, p=0.024) and virus abundance (0.54, p=0.31) unlike the Sargasso Sea which was strongly correlated to biology in the system. This disconnect we see may be due to the fact that in the Sargasso Sea spatial differences were examined, while in the South Pacific Ocean we were looking at temporal differences in gene copy number. Moreover, conditions in the Sargasso Sea were in a steady state whereas conditions during the southern Pacific Ocean were continually fluctuating likely causing these differences.

Contact rates between *Synechococcus* cells and putative cyanomyoviruses were higher than those found in the Sargasso Sea and often followed a similar trend to the bloom formation around September 25. The highest contact rates were toward the beginning of the cruise and reached up to 2.27 contacts $cell^{-1}$ day^{-1} on September 22 (Table 4.4). During the bloom formation, rates decreased to ~0.5 until September 28 where there was another increase in the contact rate up to 1.0 contact $cell^{-1}$ day^{-1} due to the decreasing abundance of *Synechococcus* cells. During the last 4 days of the cruise the contact rate again dropped, likely due to the similar drop in *g20* gene copy numbers. Rates between putative cyanomyoviruses and heterotrophic bacteria were slightly lower than those for *Synechococcus* and reached rates from 0.002-1.02 $cell^{-1}$ day^{-1}. All other contact rates for the FeCycleII cruise can be found in Table 4.4.

g20 Gene Diversity in the Pacific Ocean

Table 4.4: Contact rates were calculated in the southern Pacific Ocean during the FeCycleII cruise using the Jackson and Murray (1992) equation. Contact rates and specific contact rates of total viruses and *g20* genes (as a proxy for cyanomyovirus abundance) to HB=Heterotrophic Bacteria and *Syn.=Synechococcus.*

	Contact Rate (mL^{-1} hr^{-1})				**Specific Contact Rate ($cell^{-1}$ d^{-1})**			
	Total Viruses vs.		***g20* genes vs.**		**Total Viruses vs.**		***g20* genes vs.**	
Date During Bloom	**HB ($x10^7$)**	***Syn.* ($x10^6$)**	**HB ($x10^4$)**	***Syn.* ($x10^3$)**	**HB**	***Syn.***	**HB**	***Syn.***
9/19/2008	7.92	44.7	22.1	12.5	90.58	201.28	0.25	0.56
9/20/2008	9.77	38.2	53.4	20.9	130.39	289.75	0.71	1.58
9/21/2008	6.48	24.8	49.9	19.1	81.37	180.81	0.63	1.39
9/22/2008	6.66	35.5	81.5	43.5	83.36	185.24	1.02	2.27
9/23/2008	4.28	16.3	22.8	8.69	50.15	111.44	0.27	0.59
9/24/2008	19.0	61.2	58.1	18.8	164.94	366.54	0.51	1.12
9/25/2008	5.83	17.0	25.8	7.52	39.63	88.07	0.18	0.39
9/26/2008	5.73	28.4	37.3	18.5	39.23	87.18	0.26	0.57
9/27/2008	3.51	27.1	22.7	17.5	36.06	80.13	0.23	0.52
9/28/2008	5.67	19.0	36.9	12.4	67.50	150.00	0.44	0.98
9/29/2008	1.12	4.03	17.9	6.46	21.85	48.55	0.35	0.78
9/30/2008	3.06	5.58	31.9	5.81	43.08	95.72	0.45	1.00
10/1/2008	2.19	4.69	0.57	0.12	37.96	84.36	0.01	0.02
10/2/2008	2.54	8.27	0.40	0.07	46.56	103.46	0.004	0.01
10/3/2008	3.51	6.79	0.19	0.04	42.42	94.27	0.002	0.01
10/4/2008	5.54	8.96	0.50	0.08	58.12	129.16	0.01	0.01

To detect changes in the cyanophage community through the bloom, sequence analysis was performed on the *g20* gene. Samples were collected on September 20, 22, 26 and 30 as well as October 1 and 3 and correspond to different time points during the *Synechococcus* bloom. A total of 216 sequences were collected from the 6 time points and were used for phylogenetic analysis. Neighbor-joining and maximum likelihood phylogenetic trees of sequences had similar topologies and can be found in Figure 4.3. Sequences clustered with both environmental clones and other cyanophage isolates and no subcluster arose independently of the other times during the bloom. Six subclusters of FeCycleII clones formed and had moderate to high bootstrap support from 74-100%. A large subcluster of 109 sequences were obtained and clustered with Atlantic Ocean coastal shelf cyanophage S-SM2 with 69% bootstrap support. A second subcluster (#5) contained 9 sequences from all different points during the bloom and was similar to cyanophage isolate P-RSM5 from the Red Sea with low bootstrap support (59%) that originally infected *Prochlorococcus* NATL1A. Most other sequences were similar to environmental sequences obtained from the Atlantic Ocean (Jameson, unpublished data).

Unifrac distance metric statistical analysis was used to compare the sequences at the different points during the *Synechococcus* bloom (Figure 4.4). From the Principal Component Analysis (PCA), two main clusters arose from the Unifrac data and showed that the samples collected from before the bloom and after the bloom are more similar to each other than to the samples collected during the bloom. The single sample collected during the bloom was distinct and clustered away from the other samples. This shows that there was likely a community shift based on the distribution pattern of samples taken from the bloom. The libraries after the bloom are positioned between the samples taken during and before the bloom, which suggests that the community is shifting back toward the community found prior to the bloom (Figure 4.4).

Discussion

In recent years there has been significant interest in the diversity of marine and freshwater phages. Research on cyanophage has historically focused more on marine systems, but freshwater systems have recently been explored and compared with marine work (Clasen et al. 2008; Wilhelm and Matteson 2008). One concern remains is that within almost all of these

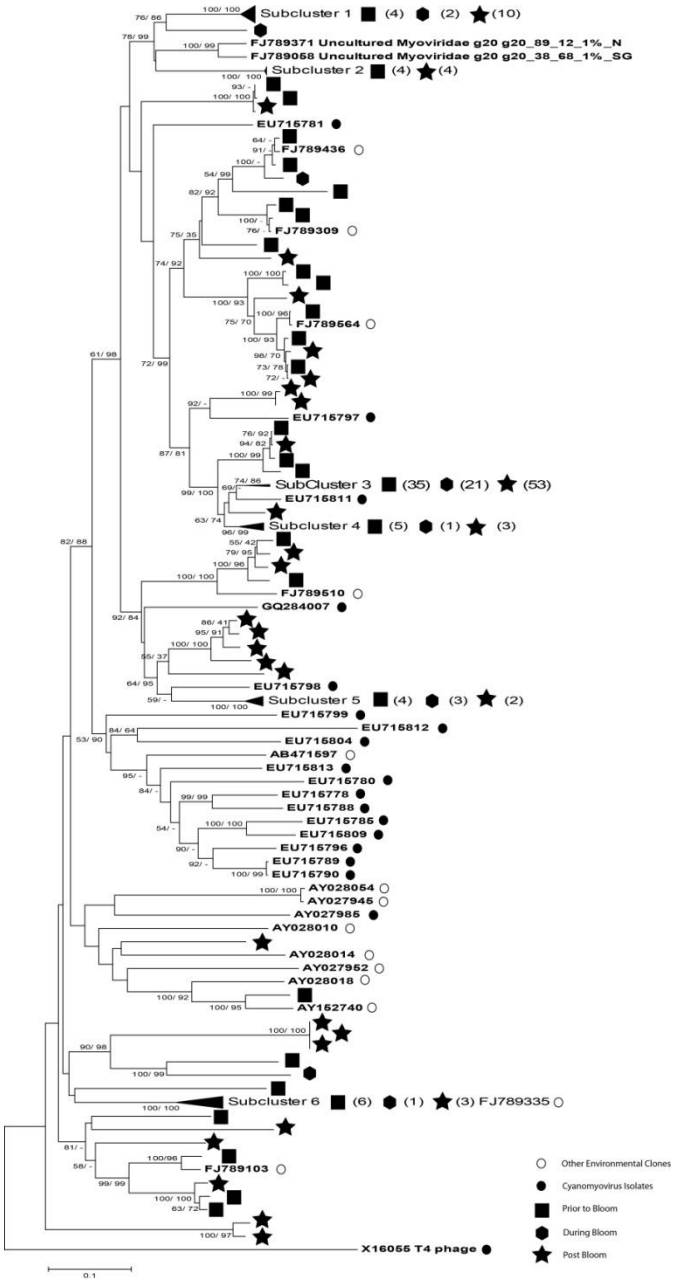

Figure 4.3: DNA neighbor-joining tree using the Cantor Jukes parameter on *g20* gene environmental sequences collected from the FeCycleII bloom. Bootstrap support is only shown for those >50%. Bootstrap values from maximum likelihood trees are the second values found at each node. Accession numbers of reference sequences from environmental clones (○) and isolates (●) are noted. Sequences from libraries before the bloom can be found in black squares (■), sequences during the bloom are in black hexagon (⬢) and sequences after the bloom are black stars (★).

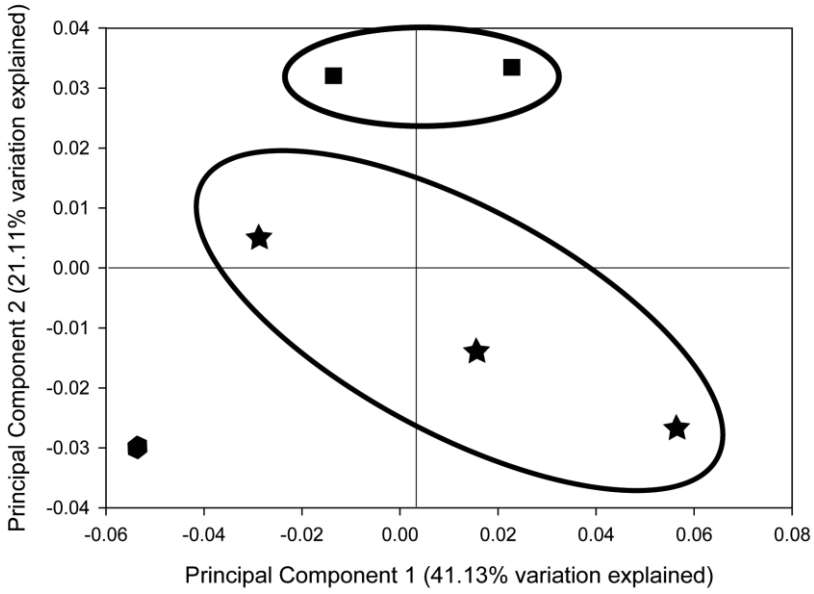

Figure 4.4: Principal coordinate plot of Unifrac coordinate analysis of *g20* gene sequences collected in the southern Pacific Ocean. Samples from before the *Synechococcus* bloom (black squares), during the bloom (black hexagon) and after the bloom (black stars) are shown.

studies, estimates of the abundance of cyanophage has been missing. This is in part due to the lack of a permissive host for all members of this group (making most-probably number or plaque assay estimates prohibitive). Here, we have investigated the abundance of cyanomyoviruses in multiple environments using qPCR and the dominant molecular marker used in the study of this group - the *g20* portal capsid gene. We have examined this data in light of estimates of the total abundances of virus particles and available metadata for each of the environments. From this work, it has been shown that putative cyanomyoviruses are ubiquitous and abundant in marine ecosystems and are a significant proportion of the total virus communities in both oligotrophic and mesotrophic ecosystems. Statistical analysis suggests relationships constraining cyanomyoviruses, including direct comparisons with potential hosts (cyanobacteria and bacteria) are different in the Sargasso Sea and southern Pacific Ocean.

Extraction Method and qPCR Efficiency

As part of this study we first validated our DNA extraction methods. Here we show that the highest gene yields were from samples subjected to no treatment steps. Others have also suggested that cyanophage do not need a DNA extraction protocol to obtain PCR products (Fuller et al. 1998; Wilson et al. 2000; Sandaa and Larsen 2006). Our results followed this trend with highest abundances in samples with no extraction protocol used. The CPS1/CPS2 primer set has been shown to be specific for ~80% of cultured cyanomyoviruses, suggesting that estimates made here are likely underestimates of true abundance. Despite this, the primer set we employed to amplify *g20* genes targets more cyanophage than two previously used primer sets CPS1 and CPS8 (Zhong et al. 2002) and CPS1 and CPS4 (Wilson et al. 2000; Marston and Sallee 2003). Moreover, our 165-bp fragment is the proper size for qPCR (although likely too small for any phylogenetic analysis, Zhong et al. 2002). For these reasons phylogenetic analysis was performed in the Pacific using the *ca*. 585 bp fragment obtained from the newest *g20* primer set (Sullivan et al. 2008). To further determine the efficiency of reactions, we performed a single spike test with genomic DNA from *Synechococcus* phage S-PWM1 and often found inhibition of samples with recoveries of less than 100%, further suggesting that the estimates made here are underestimates.

g20 gene Abundance and Contact Rates

It is well established that cyanobacteria, especially *Prochlorococcus* and *Synechococcus*, are prevalent in the Sargasso Sea system (DuRand et al. 2001; Sullivan et al. 2003) which was confirmed from the high abundances found in the current study (Michelou et al. 2007). Previous studies have suggested that *Synechococcus*, and, to a lesser extent, low-light adapted *Prochlorococcus* spp. are infected by cyanomyoviruses more often than from cyanopodoviruses or cyanosiphoviruses (Lu et al. 2001; Sullivan et al. 2003) and these are likely the hosts in the Sargasso Sea. This is surprising since the samples were collected from surface samples which would select for more high-light adapted *Prochlorococcus*. Since coastal waters are more biologically productive and contain more picoplankton per volume than pelagic waters of the Sargasso Sea, it is not surprising that the highest percentages of cyanomyoviruses in the Sargasso Sea were found at the stations nearest the Florida coastline. Viral metagenomics of Sargasso Sea samples have shown a high number of cyanophage genes related to cyanomyoviruses (*i.e.*, *Prochlorococcus marinus* phage P-SSM2 and P-SSM4, and *Synechococcus* phage S-PM2) (Angly et al. 2006), although the domination of search databases by cyanophage (relative to other viruses) may be tempering these results. Regardless, it is not surprising that stations dominated by cyanobacteria contained a very high percentage of *g20* genes from potential cyanomyoviruses out of the total virus population, making this gene target a very important group of phage to study.

The FeCycleII cruise was a follow-up to the FeCycleI cruise and aimed to study the dynamics of the spring phytoplankton bloom event off eastern New Zealand; FeCycleI, carried out to the south, focused primarily on developing a model for iron cycling in an unperturbed system (Boyd et al. 2004). Viral abundance estimates during the FeCycleII cruise were similar to those collected during the first FeCycle cruise conducted in 2003 with 4×10^7-8.4×10^8 mL^{-1} (Strzepek et al. 2005). Temporal variations in virus abundances during phytoplankton blooms have been analyzed previously in only a few ecosystems, often mesocosm experiments (Guixa-Boixereu et al. 1999; Yager et al. 2001; Jacquet et al. 2002; Larsen et al. 2004). It has been long suggested that viruses can play a role in the demise of phytoplankton blooms (Suttle 2000). Even under non-bloom conditions, ~ 10% of the total *Synechococcus* standing stock is removed

daily by viral mortality (Waterbury and Valois 1993; Suttle and Chan 1994; Garza and Suttle 1998). In the FeCycleII samples, the demise of a *Synechococcus* bloom from September 25-27 did not appear to be from cyanomyoviruses as there was no significant increase in the *g20* gene copy number after the bloom event. Other cyanophages, including *cyanopodoviridae*, were also present in the water column (Huang et al. 2010) and may have had a role in the decline. However, it is likely that a significant wind driven mixing event experienced during this period sufficiently changed the water column chemistry (*i.e.*, it specifically introduced upwelled-N) such that *Synechococcus* populations were no longer competitive (PW Boyd et al., unpublished).

Since viruses in surface waters are destroyed rapidly by solar UV, they must be replaced rapidly (Wilhelm et al. 1998; Wilhelm et al. 2002). As such, regular infections of host cells need to occur. To better understand the frequency that bacteria are contacted by virus (necessary to maintain infection rates), contact rate kinetics was estimated for both cruise transects. When comparing total virus to heterotrophic bacterial contact rates, the Sargasso Sea had from 0.21-2.01 contacts $cell^{-1}$ day^{-1}, which is similar to total bacterial contacts found in the Gulf of Mexico (0.20-4 $cell^{-1}$ day^{-1}) (Wilhelm et al. 1998), but lower than those found in the FeCycleII or the eutrophic Masan Bay in Korea which ranged from 12-321 $cell^{-1}$ day^{-1} (Choi et al. 2003). Since the FeCycleII cruise sampled more mesotrophic waters, total virus to heterotrophic bacterial contact rates were higher and ranged from 21-164 $cell^{-1}day^{-1}$. Similar contact rates to total bacteria have been described in eutrophic freshwater systems such as Lake Pluβsee (49-180 $cell^{-1}day^{-1}$) (Weinbauer and Hofle 1998) or Alte Donau (91-230 $cell^{-1}$ day^{-1}) (Fischer and Velimirov 2002). Contact rates between *Synechococcus* cells and putative cyanomyoviruses and total viruses were higher than those to heterotrophic bacteria in both ecosystems due to their larger cell size. Their larger size allow for more potential contacts and a better chance of successful infections. Therefore, for any larger cell, fewer viruses are needed since there will be more contacts that may help sustain the virus community.

Wiggins and Alexander (1985) suggested that there was a minimum threshold of cell abundance at around 10^4 mL^{-1} that must be met before bacteriophage can replicate. This abundance was found to be lower for *Synechococcus* (Waterbury and Valois 1993) likely due to its slower growth rate. In both systems examined here, cyanobacterial hosts were above the

necessary threshold for infective and active cyanophage replication, implying there was sufficient activity to maintain the population of cyanophage (and providing further support that the *g20* amplicons were from cyanophage). The cyanomyovirus to cyanobacteria contact rate was found to be lower in the oligotrophic Sargasso Sea compared to the more productive southern Pacific Ocean which has been shown before (Waterbury and Valois 1993; Suttle and Chan 1994).

Statistical Correlations and Possible Hosts for Putative Cyanomyoviruses

This work is supported by statistical data using Pearson correlation data showing that the strongest correlations were with *Prochlorococcus* and *Synechococcus* in the Sargasso Sea system. This suggests that *g20* gene copies amplified from this environment are abundant despite the low total virus abundances (Rowe et al. 2008) and are likely cyanomyoviruses since there is a large standing stock of cyanobacterial hosts in this ecosystem. Previous studies (Short and Suttle 2005) have shown that only three out of ten clusters of *g20* amplicons contain isolated cyanophages (Sullivan et al. 2008) and the rest are not related to any known isolates, questioning whether these amplicons may be from other bacteriophage. Unlike the Sargasso Sea samples, samples from the Pacific Ocean did not significantly correlate to biology, but only weakly to virus abundances and salinity. These observations leave us with several contrasting possibilities: 1. that the *g20* gene primer set may not be specific just for cyanophages and that other bacteriophages were abundant, present and contained an amplifiable target or 2. there are temporal disconnects between host abundance and virus abundance that make standard statistical examinations unreliable. While no evidence exists (to date) for the first point, it is well known that viruses function through a kill-the-winner (Thingstad and Lignell 1997) approach: that increases in cell density is followed by an increase in contacts, and virus production correlated to the demise of a host. To this end it is highly likely that the cycling of viruses and hosts (*vis a vis* (Wommack and Colwell 2000) Figure 6) requires statistical models that allow for temporal offsets.

Only three clusters within the phylogenetic analyses of *g20* amplicons contain isolated viruses. There are several current gaps in our data that provide explanations for this discrepancy, including the use of only a limited range of hosts in lab work (most of the cultured isolates were obtained using *Synechococcus* WH7803) (Zhong et al. 2002; Dorigo et al. 2004; Short and Suttle 2005; Wilhelm et al. 2006; Sullivan et al. 2008), the amplification of markers from other bacteriophage (Sullivan et al. 2003; Short and Suttle 2005; Wilhelm and Matteson 2008), the chance that these amplicons are from phages that infect other ecologically important cyanobacteria such as diazotrophic *Crocosphaera* or *Trichodesmium* spp. (Capone 2001; Montoya et al. 2004) , or the amplification of altered forms of the gene that are nonfunctional. In spite of several efforts, no resolution to this problem has arisen and these primers remain in use for phylogenetic studies. Our work here suggests that the *g20* gene may be specific to cyanomyoviruses in particular environments, as was shown in the Sargasso Sea data. When looking at more complex environments, such as a seasonal phytoplankton bloom, significant correlations were not tied to cyanobacterial hosts, suggesting that these primers may amplify genes from other targets. To confirm this, more work to model the temporal disconnects in virus-host dynamics (and beyond the scope of the current study) is needed.

g20 Gene Diversity in Pacific Ocean

Although it is unlikely that the bloom crash in the southern Pacific Ocean was due to cyanomyoviruses, our observations show that *g20* diversity changed during and after the formation of the bloom, demonstrating the tight linkage between virus and host diversity. After the *Synechococcus* bloom crash, we observed a significant decrease in the abundance of *g20* genes which is likely due to the response of a decrease in host abundance and contact rates between viruses and potential hosts. A strong storm occurred around the same time as the bloom which mixed the water samples, possibly exposing some of the phage to UV radiation (Suttle and Chen 1992; Wilhelm et al. 1998), thus inactivating them or even destroying the virus particles. To detect specific changes in the community we sequenced clone libraries from several stations before and during the bloom and then from the subsequent crash. The sequencing effort showed typical diversity with sequences from both marine cyanophage isolates as well as from other

environmental clones from oceanic environments. Unifrac is a relatively new phylogenetic method that compares the phylogenetic distance between communities by measuring the branch length leading to descendents in the samples analyzed (Lozupone and Knight 2005). Using Unifrac and PCA cluster analysis, changes were detected in the samples during the bloom leading to two different clusters suggesting that the phage community was different during this time. Overall, this shows that cyanomyovirus community was indeed changing in both diversity and quantification throughout the bloom likely in response to host changes.

Constraints on virus abundance across ecosystems

Viruses in different ecosystems may be constrained by different ecological parameters (Rowe et al. 2008). To analyze this further, we examined the two locations used in this study to determine parameters correlated with the abundance of *g20* gene copy numbers. In the Sargasso Sea, we see many negative correlations with both biotic and abiotic parameters, which suggest a complex relationship between viruses and its environment. In the southern Pacific Ocean, there were both positive and negative correlations found in the system. When combining the two datasets, several significant correlations with abiotic parameters are evident (*i.e.* temperature and all size fractions of chl *a*) (Table 4.2), but when systems were analyzed separately, it can be found that these significant correlations are driven by the extreme differences in the two environments, not a true correlation. Any extreme values in the datasets can skew the regression line as to appear to be a strong correlation, but it is only due to these excessive values. This suggests that the two systems were ecologically different and need to be analyzed separately so that true relationships can be identified.

In this study we were able to capture quantitative information on a specific cyanophage family during both a bloom event as well as across a large spatial scale. Little work has been done to quantify specific ecologically important groups of phage in aquatic systems. Quantitative work on cyanophage has been limited to phages infecting the bloom-forming *Microcystis aeruginosa* (Takashima et al. 2007) and using the *g20* gene on one Norwegian station on a seasonal scale (Sandaa and Larsen 2006). To this end, our study demonstrates that

these *g20* genes are abundant in marine environments, and this opens the door for a deeper ecological (i.e., quantitative) study of this group of important phage. Information on their abundance and activity will no doubt contribute to a better understanding of the biogeochemical cycling of nutrients in ecologically different environments.

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Chapter 5 Seasonal Changes in the abundance of bacteria, viruses and cyanophage in a Laurentian Great Lake

This is a paper that will be submitted to the journal of Applied and Environmental Microbiology with the same title and the authors Audrey R. Matteson, Star N. Loar, Richard A. Bourboniere and Steven W. Wilhelm.

My contribution to this article was all qPCR analysis from winter and summer Lake Erie samples, all virus abundance estimates, most bacterial abundance estimates, data gathering from other scientists, all data analysis (with the help of Jennifer DeBruyn), all figures made and most of the written manuscript.

Abstract

Much work has been done to show that viruses and cyanobacteria are pervasive during the summer months in Lake Erie. Few studies have temporally analyzed freshwater systems and little is known about the bacterial and viral communities that coexist during the harsh winters of the Laurentian Great Lakes. Here, we compared the abundances of total bacteria and viruses across several stations in the western and central basins of Lake Erie during the summer and winter months. Furthermore, quantitative PCR was used to detect the abundances of cyanomyoviruses in this system, using the portal vertex *g20* gene as a proxy for *myoviridae* abundance. Lake Erie was productive during both seasons with high chlorophyll *a* concentrations in the summer (up to 10.3 $\mu g/L^{-1}$) and winter (up to 5.2 $\mu g/L^{-1}$). Both bacterial and viral abundances were significantly higher during the summer compared to the winter ($p<0.05$). Summer bacterial abundances ranged from 3.3×10^6-1.6×10^7 mL^{-1} while winter abundances were between 3.39×10^5-1.2×10^6 mL^{-1}. Viral abundances were high during both months with summer abundances significantly higher at most stations ranging from 6.52-8.88 x 10^7 mL^{-1} with winter abundances ranging from 3.4-6.6 x 10^7 mL^{-1}. Cyanomyoviruses were quite high at both months with up to 3.1×10^6 copies of *g20* genes mL^{-1} with no significant changes found between months at several stations and depths. This work shows that viruses, including putative cyanomyoviruses, are ubiquitous and temporally abundant in freshwater systems.

Introduction

Approximately 20% of the world's freshwater is contained within to the North American Great Lakes (Wetzel 2001). Lake Erie is the smallest (by volume), shallowest, and most productive of the Laurentian Great Lakes. Despite this, it is the 18th largest freshwater system in terms of volume (483 km^3). Lake Erie also has an extensive fishery that exceeds all other Great Lakes combined. The large anthropogenic influence of a population base of over 30 million people, in addition to agricultural and industrial influences on Lake Erie, has resulted in historically high biological productivity in this lake and subsequently ecosystem degradation (Munawar et al. 1999). Excessive primary productivity has been the highlight of research for decades in this system: most recently studies have focused on Lake Erie due to seasonal harmful algal blooms that form during the summer months (Brittain et al. 2000; Rinta-Kanto et al. 2005; Rinta-Kanto and Wilhelm 2006). Factors promoting or constraining production in the lake are thus of great interest not only to the academic community, but also to ecosystem managers.

Since the late 1980s interest in aquatic viruses has increased due to the sheer abundance (up to 10^8 ml^{-1}) of viruses in aquatic environments (Bergh et al. 1989; Proctor and Fuhrman 1990). Despite the wealth of information that has been produced in marine ecosystems, work published on freshwater environments remains sparse. To date researchers have shown that viruses in freshwater environments are ubiquitous and are often, although not always (Clasen et al. 2008) persist at higher densities than their marine counterparts (DeBruyn et al. 2004). Viruses have been linked to the biogeochemical cycling of nutrients (P, N, Fe) as well as carbon (Gobler et al. 1997; Poorvin et al. 2004; Mioni et al. 2005; Dean et al. 2008) but only limited work has been done to confirm this in freshwater systems (Dean et al. 2008; Wilhelm and Matteson 2008). Viruses have also been shown to be drivers of community diversity by the "kill the winner" model whereby viruses control dominant members in the system and allow less abundant, resistant members to thrive (Bratbak et al. 1990; Thingstad et al. 1993; Thingstad and Lignell 1997).

A body of literature now exists on seasonal changes of viruses in the community structure in marine ecosystems and has focused on coastal regions (Jiang and Paul 1994; Weinbauer et al. 1995; Sandaa and Larsen 2006). The same has been done in freshwater systems (Mathias et al.

1995; Bettarel et al. 2003; Goddard et al. 2005; Lymer et al. 2008), and has shown that there are stronger seasonal cycles that can alter the viral community structure in freshwater systems over their marine counterparts (Wilhelm and Matteson 2008). There seems to be a connection between the system productivity and the host abundance, as well as with other abiotic processes such as nutrient concentration and temperature that may alter the host abundance (Jiang and Paul 1994; Maranger and Bird 1995; Weinbauer et al. 1995). Unfortunately there has been no breakthrough in the true linkage between virus abundances and seasonal cycles, and more work needs to be done.

Cyanobacteria, including *Synechococcus* (Wilhelm et al. 2006b; Ivanikova et al. 2008), *Microcystis* (Rinta-Kanto et al. 2005; Ouellette et al. 2006) and *Planktothrix* (Rinta-Kanto and Wilhelm 2006) spp. have been shown to be prevalent in different parts of Lake Erie and thus are potential hosts for cyanophage infection. To this end, researchers have developed primers specific for conserved genes within groups of viruses, such as the *g20* gene used in the study of *cyanomyoviridae*. The *g20* gene encodes for the capsid assembly, DNA packaging and the head-tail junction on *cyanomyoviridae* and has been used for studying the phylogenetic diversity of the phage infecting *Synechococcus* in both marine (Marston and Sallee 2003; Muhling et al. 2005; Short and Suttle 2005; Sandaa and Larsen 2006) and freshwater (Dorigo et al. 2004; Short and Suttle 2005; Wilhelm et al. 2006a) ecosystems for many years (Fuller et al. 1998; Zhong et al. 2002) despite controversy over whether these primer sets are specific for cyanophages, or may amplify targets from other bacteriophages as well (Sullivan et al. 2003; Short and Suttle 2005; Wilhelm and Matteson 2008). Perhaps more importantly, while significant genetic richness in both marine and freshwater environments has been evident, little has been done to quantify these virus particles.

Cyanophage abundance has historically been estimated via infection assays (e.g., the most probable number method (Garza and Suttle 1998)). One problem with this approach is that this and other assays are constrained to viruses that infect laboratory isolates of cyanobacteria: the assays, as such, likely miss a significant proportion of cyanophage that may not infect (or may not infect under the chosen laboratory conditions) the cyanobacterium chosen. To better understand the role cyanomyoviruses infecting *Synechococcus* spp. may play in freshwater

ecosystems, we set out to develop quantitative estimates of their abundance by employing the *g20* gene as a molecular proxy for the *cyanomyoviridae*. Moreover, to begin to address seasonal alterations in virus density in the water column samples from Lake Erie were collected during both winter (February) and summer (August) expeditions in 2009. To our knowledge, only two papers have been published looking at the abundances of cyanophages using a qPCR approach (Sandaa and Larsen 2006; Takashima et al. 2007) with one focusing on a coastal Norwegian station (Sandaa and Larsen 2006) and the other second targeting viruses specific for *Microcystis* (Takashima et al. 2007). Our results demonstrate that this marker gene (and thus putative cyanomyoviruses) are ubiquitous and abundant across Lake Erie during both the summer and winter months and that there are temporal changes in both bacteria and viruses in this system. Moreover, this work helps our understanding of the biotic and abiotic factors that may affect the viral and bacterial communities in the Great Lakes.

Materials and Methods

Sampling

Winter water samples were collected during the daylight hours from Lake Erie using the icebreaker *CCGS Griffon* as a research platform from February 16-20 2009 (Figure 5.1). Where ice cover was evident, small patches of ice were cleared with the ship (and allowed to settle for 30 min). Samples were collected using a single Niskin bottle lowered into the water column. Summer samples were collected while aboard the *CCGS Limnos* from August 17-20, 2009. Samples were collected at surface and at depth at identical stations using Niskin bottles attached to the ships rosette system. Salinity, water temperature, *in situ* fluorescence (a proxy for chlorophyll *a*), pressure, density, conductivity, and oxygen concentrations were all measured using the ship's profiling instruments.

Measurement of Nutrients and Chlorophyll *a*

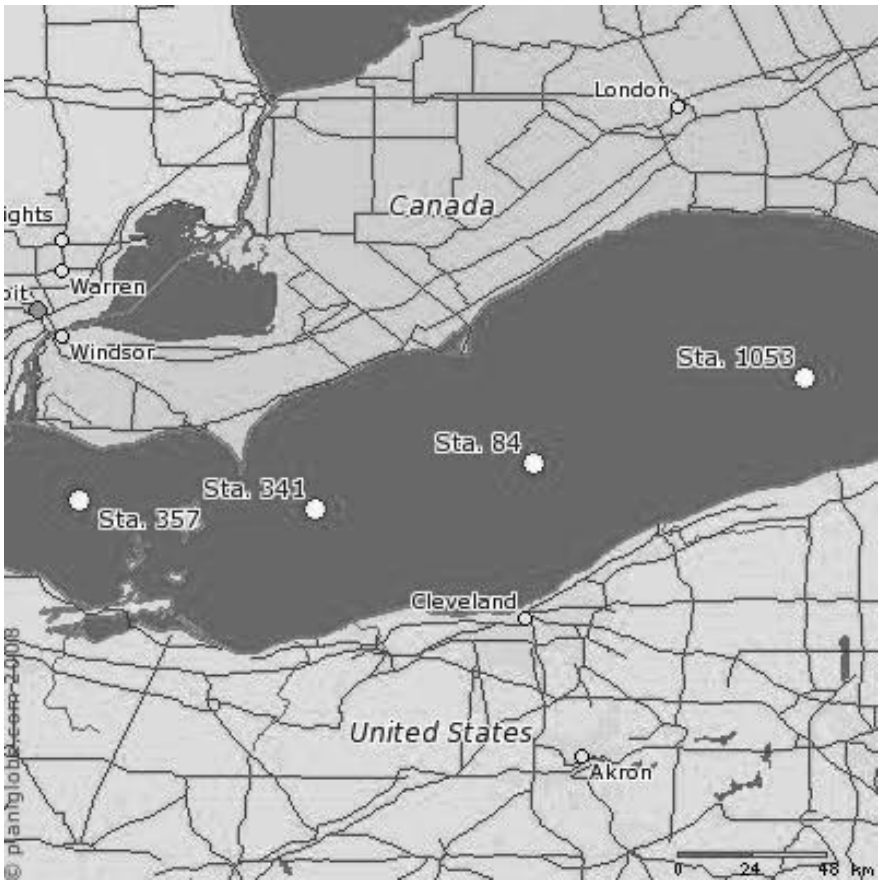

Figure 5.1: Four Lake Erie stations where samples were collected in February and August 2009 (Station 84: 41°54.55'N 81°40.38'W Station 1053: 42°10.52'N 80°52.36'W Station 357: 41°49.30'N 82°58.30'W Station 341: 41°47.30'N 82°17.00'W).

The concentration of nutrients (NO_3^-, NH_4^+, SIO_2, SRP, total dissolved nitrogen and total dissolved phosphorus) were measured from whole water samples. Concentrations were estimated at the National Laboratory for Environmental Testing (Environment Canada) using standardized techniques (NLET 1994). For dissolved metrics, sample filtration was completed while onboard and kept at 4° C until analysis could be completed.

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

Virus abundance

The abundance of virus particles was determined for whole water samples which were preserved in 2.5% final sterile glutaraldehyde. Abundances for the winter Lake Erie stations were preserved and stored at 4°C until processed (< 2 weeks) while summer samples were flash frozen in liquid nitrogen and stored at -20°C until needed. Slide preparation proceeded as previously described for 850 μL of thawed sample (Noble and Fuhrman 1998). All slides were stored at –20°C until virus-like particles could be enumerated *via* epifluorescence microscopy using a Leica DMRXA microscope with a 'wide blue' filter set (λ_{Ex} = 450 to 490 nm, λ_{Ex} = 510 nm, with suppression filter at λ_{Ex} = 510 nm). At least 20 independent fields or 200 particles were examined, with the total of each field of view noted to ensure even distribution of particles across individual filters, for each slide.

Bacterial and Cyanobacterial Abundance

Samples collected for bacterial abundance estimates during the Lake Erie cruises were suspended in 2.5% (final) sterile glutaraldehyde and kept at 4° C in the dark until processed in the laboratory. Fixed samples were stained for 3 minutes with a 1% working solution of acridine orange stain (Invitrogen, Carlsbad, CA). The stained cells were then collected on a 0.22-μm

nominal pore-size, 25 mm diameter black polycarbonate filter (GE Osmontics) and cells were counted as described above for viral abundances. *Synechococcus* and cyanobacterial abundances were estimated using the Texas Red filter set (λ_{Ex} 595 nm, λ_{Em} 610-615 nm) on the same slides as those used for total bacterial counts.

Quantitative PCR of *g20* gene

Samples for qPCR analysis were collected in 5 mL cryovials, immediately flash frozen in liquid N_2, and stored at -80°C until they were processed. The primer set CPS1/CPS2 was used to estimate *g20* gene copies (Fuller et al. 1998). Plasmid standards were prepared using *Synechococcus* phage S-PWM1: the *g20* gene was amplified using primer pair CPS1/CPS8 (Zhong et al. 2002), which produces a 592-bp product that contains the 165-bp fragment from CPS1 and CPS2. PCR with S-PWM1 phage was completed with PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK) using 0.2 μM each primer, 1 μL of filtered S-PWM1 phage stock, and sterile water and reaction conditions previously reported (Zhong et al. 2002). The DNA fragment was cloned into the PCR 2.1 TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Plasmid DNA was purified using the Qiagen Qiaprep Spin Miniprep kit (Qiagen, Valencia, CA) and cloned inserts were confirmed using an EcoRI digestion at 37°C for two hours followed by electrophoresis with 1.5% agarose gels. Plasmid DNA sequence was confirmed at the University of Tennessee Molecular Biology Resource Facility. The DNA concentration (A_{260}) of the plasmid was determined spectrophotometrically using a Nanodrop ND-1000 Spectrophotometer (Thermo Scientific, Surrey, UK). The molecular weight of the double-stranded plasmid and product was calculated using the Oligocalc Calculator version 3.23 (Kibbe 2007). Copy numbers of *g20* per μL were determined using Avogadro's number (6.022×10^{23} plasmid copies mol^{-1}). Each plasmid contained one copy of the target gene and was freshly diluted and used within 24 hours with at least four different duplicate dilutions from 10^6-10^1 copies to make a linear standard curve for the analyses. Approximately 1-1.5 mL of sample was ultracentrifuged at 60,000-81,000 x *g* (Beckman TL-100) for 3 hours at 4°C and was resuspended in sterile water and kept at -20°C prior to qPCR. Each 25 μL qPCR reaction contained 12.5 μL Thermo Scientific ABsolute

SYBR Green qPCR mix (Thermo Scientific, Surrey, UK), 280 ng μL^{-1} bovine serum albumin (Fisher Scientific), 0.30 μM CPS1 forward primer and 0.60 μM CPS2 reverse primer and 2 μL viral sample or standard. Samples were run in triplicate and assayed with up to three different dilutions. PCR conditions consisted of an initial denaturation step at 95°C for 15 min followed by thirty-five cycles of 95°C for 10 sec, 53°C for 30 sec, and 72°C for 30 sec, followed by 72°C for 10 min. A melting curve was produced following the program and was read every 1°C from 40-95°C to determine the specificity of primers and formation of primer dimers. Unspecific binding was exhibited at the lowest copy numbers so a second plate read at 77°C was performed after every cycle to inhibit the fluorescence quantification of primer dimers (Morrison et al. 1998). Negative controls contained sterile water and no DNA template while positive controls consisted of lysate from *Synechococcus* phage S-PWM1. To determine the effect of prefiltering samples before qPCR, whole water from the winter samples were passed through a 1 μm nominal pore-size 47mm polycarbonate filter (GE Osmontics) on a plastic Vacuum Filter Flask (Nalgene) and compared to unfiltered and untreated samples.

Virus contact rates

Contact rates of putative cyanomyoviruses (from *g20* gene copy numbers) with total bacterial populations and cyanobacteria were estimated following the equation from Murray and Jackson 1992:

$$(2 \bullet S \bullet \pi \bullet \omega \bullet D_v) \quad V \bullet B \tag{1}$$

using the dimensionless Sherwood number (S) of 1.06 for a 10% motile population (Wilhelm et al. 1998). The average diameter (ω) of a bacterial cell (1.0×10^{-4} cm) (Fischer and Velimirov 2002), and the diffusivity of viruses (D_v) 3.456×10^{-3} cm^2 d^{-1} were taken from previous sources (Murray and Jackson 1992). Viral abundance (V) and bacterial abundance (B) were both expressed per mL^{-1}. For contact rates between viruses and cyanobacterial cells, a diameter of 1.5×10^{-4} cm was used for *Synechococcus* cells (Suttle 2000). Specific contact rates of viruses per bacterium per day were determined by dividing the contact rates by the host abundance.

Statistical Analyses

Threshold cycle (C_t) calculations were made for each qPCR assay using the MJ Opticon Monitor Analysis software (ver 3.1) with the Global Minimum setting. The threshold was manually adjusted to give the highest correlation coefficient (r^2) for each standard curve. Gene copy abundance in each sample was calculated based on the standard curve (gene copy number vs. C_t). Averages, standard deviations, F-tests (for variance) and paired and unpaired two-tailed *t*-tests were determined using Microsoft Excel 2007. Pearson correlations for temperature, latitude, virus abundances, bacterial abundances, *g20* gene copies, cyanobacterial abundance and chl *a* were analyzed using the NCSS Statistical & Power Analysis Software.

Results

Water Conditions

Samples were collected in February and August of 2009 at several stations across Lake Erie. Lake Erie was isothermal during the winter with water temperatures below freezing at all stations and depths (Table 5.1). Temperatures ranged from 0.03-0.42° C with the highest temperatures at the snow and ice covered Stations 357 and 341. Summer temperatures in the lake were warmest at the surface (23.96-25.1°C) and decreased with depth (11.9-24.88°C). Stations analyzed during the summer were thermally stratified with the exception of station 357 due to its shallow depth.

Nutrient concentrations were generally higher during winter sampling compared to the summer (Table 5.1). No trends in nutrients comparing surface and deep samples. NO_3^- was the significant nitrogen source (relative to NH_4^+) at all depths with total N:P ratios (molar) were all above the canonical Redfield ratio (16:1). Silicate (SiO_2) concentrations were significantly higher in the winter (up to 4.46 mg L^{-1}) which likely supports the abundant diatom community that has been observed (Twiss, McKay and others reference, in review). Dissolved oxygen concentrations were supersaturated in the winter at all stations and depths (Table 5.1). Hypoxic conditions were detected in the hypolimnion of Stations 341 (0.58 mg L^{-1} at 15m) and Station 84 (data not shown) during the summer with normal concentrations found at the other stations.

Table 5.1: Water column characteristics during sample collection.

Station	Depth	Date	NO_3^- mg L^{-1}	NH_4^+ mg L^{-1}	SIO_2 mg L^{-1}	SRP mg L^{-1}	TN mg L^{-1}	TP mg L^{-1}	DO mg L^{-1}	Temp. °C	Chl *a* 0.2-2 µm	Chl *a* 2-20 µm	Chl *a* < 20 µm
84	1 m	2/19/2009	0.15	0.016	1.18	0.0098	0.376	0.0171	17.34	0.03	0.66 (0.15)	0.84 (0.03)	0.67 (0.01)
84	1 m	8/18/2009	0.02	0.009	0.3	0.0007	0.288	0.0076	9.13	24.63	5.08 (0.11)	3.15 (0.11)	0.65 (0.00)
84	20 m	2/19/2009	0.14	0.011	1.18	0.0085	0.396	0.0111	17.23	0.03	0.62 (0.07)	0.75 (0.09)	0.65 (0.04)
84	18 m	8/18/2009	0.11	0.022	0.2	0.0010	0.391	0.0070	7.44	22.46	0.72 (0.00)	0.32 (0.02)	0.19 (0.00)
1053	1 m	2/19/2009	0.17	0.007	0.81	0.0069	0.393	0.0111	15.54	0.06	2.21 (0.41)	2.55 (0.35)	1.97 (0.03)
1053	1 m	8/19/2009	0.09	0.006	0.21	0.0011	0.308	0.0067	9.69	25.1	2.84 (0.69)	2.14 (0.01)	0.19 (0.06)
1053	18 m	2/19/2009	0.16	0.011	0.84	0.0080	0.436	0.0106	15.46	0.04	2.17 (0.07)	2.61 (0.18)	2.02 (0.00)
1053	18 m	8/19/2009	0.09	0.007	0.41	0.0013	0.318	0.0068	8.39	21.75	0.73 (0.44)	0.64 (0.02)	0.23 (0.03)
357	1 m	2/17/2009	0.42	0.025	1.99	0.0043	0.665	0.0060	15.32	0.39	0.40 (0.02)	0.15 (0.02)	0.03 (0.01)
357	1 m	8/18/2009	0.07	0.009	0.82	0.0013	0.330	0.0074	7.93	24.9	6.96 (3.25)	6.44 (2.14)	6.08 (0.79)
357	8 m	2/17/2009	0.44	0.027	1.99	0.0041	0.612	0.0050	14.97	0.42	0.35 (0.02)	0.17 (0.00)	0.04 (0.00)
357	8 m	8/18/2009	0.07	0.006	0.83	0.0013	0.335	0.0074	9.08	24.88	10.31 (1.12)	6.08 (0.46)	4.85 (0.18)
341	1 m	2/17/2009	0.40	0.035	1.56	0.0081	0.634	0.0101	16.16	0.39	3.54 (0.00)	3.44 (0.00)	2.39 (0.18)
341	1 m	8/20/2009	0.09	0.029	1.15	0.0023	0.346	0.0094	7.35	23.96	5.26 (1.39)	4.22 (0.39)	1.94 (0.02)
341	16 m	2/17/2009	0.24	0.020	0.88	0.0055	0.456	0.0073	16.27	0.39	5.19 (0.38)	6.35 (0.00)	4.79 (0.55)
341	15 m	8/20/2009	0.10	0.020	4.46	0.0048	0.338	0.0178	0.58	11.9	1.48 (0.02)	1.10 (0.26)	0.54 (0.04)

The biomass of primary producers was relatively high during both sampling events. Chlorophyll *a* concentrations were generally higher during summer sampling times, although deep samples from two stations (Sta. 1053 and Sta. 341) contained chlorophyll *a* concentrations up to 10 μg L^{-1}. Picophytoplankton densities were also high during the summer (Table 5.1). *Synechococcus* abundances were estimated to be between 10^5-10^6 cells mL^{-1} at all stations with the highest quantities at central basin Station 84 at both the surface and at depth. Between the winter and summer, there was a community shift from a more diatom dominated system as reflected in the shift in chlorophyll size distribution as an increase in the nanophytoplankton (Table 5.1). Total bacterial abundance was significantly higher ($p<0.05$) in the summer compared to the winter, with up to one order of magnitude higher abundances of bacteria found at all stations and depths.

Although we observed shifts in phytoplankton and bacterial biomass, the abundance of viruses at each station remained relatively constant with slight increases in the summer compared to the winter (Figure 5.2A and B). Total virus particles ranged from 3.4-6.6 x 10^7 in the winter to 6.5-8.9 x 10^7 mL^{-1} in the summer. Statistical analysis showed significantly ($p < 0.05$) higher viral densities in the summer at all stations examined except for Station 1053 at 1 m ($p=0.053$). The total virus to bacteria ratio (VBR) in the summer ranged from 4.7 to 24.3 viruses per bacterium and from 28.0 to 131.8 in the winter (data not shown).

Quantitative PCR targeting the *g20* gene estimates for putative cyanomyovirus abundance varied between stations, depth and season across the range of 1.3 x 10^5-4.3 x 10^6 mL^{-1} (Figure 5.2A and B). Overall, summer abundances were significantly higher for stations 84 at 1m ($p=0.002$), 1053 at 1m ($p=0.001$), 357 at 1m ($p=0.02$) and 357 at 8m ($p=0.001$), but lower at depth with stations 84 at 18-20m ($p=0.03$) and 341 at 15-16m ($p=0.0002$). Differences in *g20* copies in summer *vs.* winter samples for stations 1053 at 18m ($p = 0.09$) and 341 at 1m ($p = 0.08$) were less significant. The *g20* to bacterium ratio was from 0.11-2.37 in the winter with the *g20* to cyanobacteria ratio from 0.26-9.02 in the summer sampling. Putative cyanomyoviruses composed from 0.39-2.42% of the total viral population in the winter and 0.20-4.64% in summer samples (Figure 5.3). To determine the effect of prefiltering the samples, winter samples were

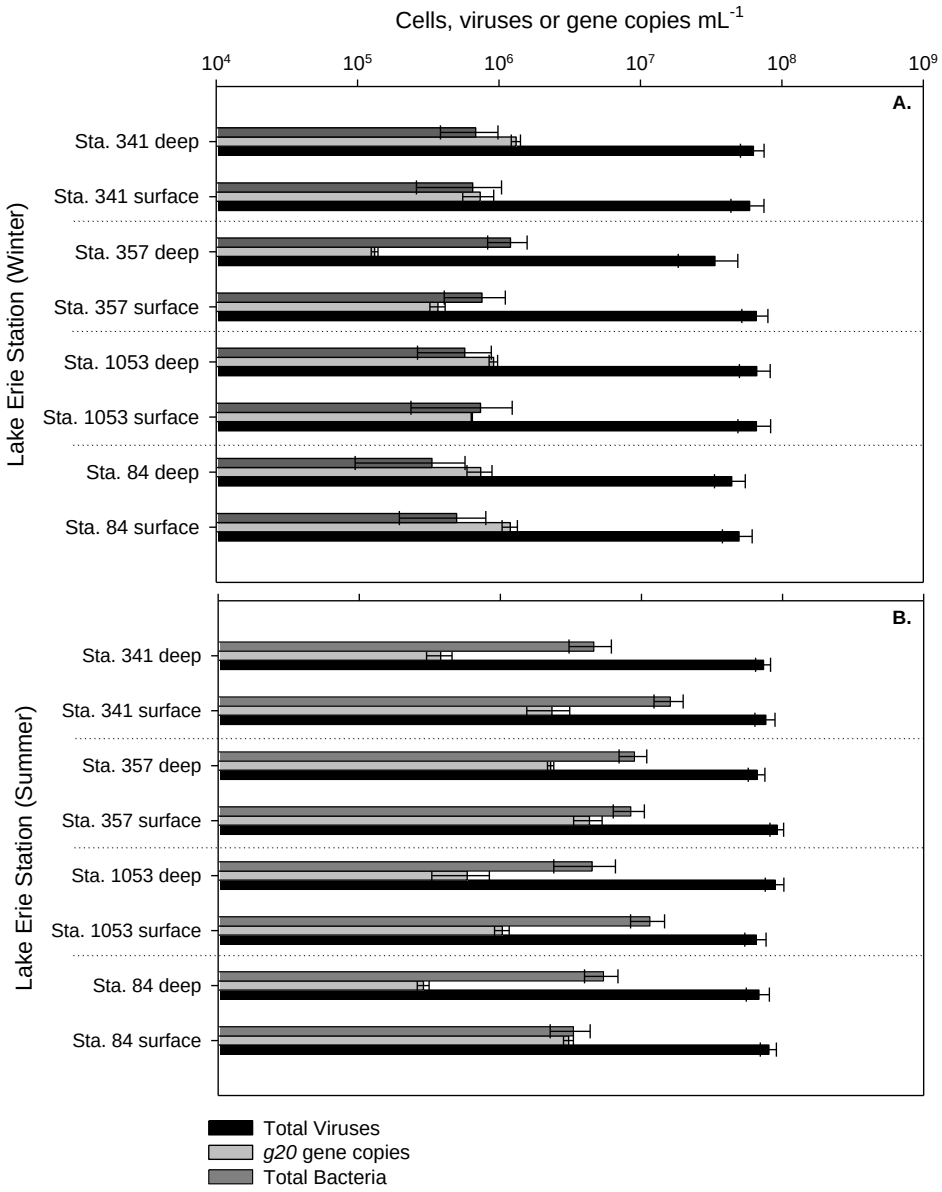

Figure 5.2: Abundances of total viruses (black), bacteria (dark gray) and *g20* genes (light gray) for each station and month sampled. (A) Abundances in the winter samples (B) Abundances in the summer samples. Error bars for virus and bacterial abundances were calculated from standard deviations from 20 grid views while *g20* gene abundance error bars were calculated from triplicate qPCR reactions.

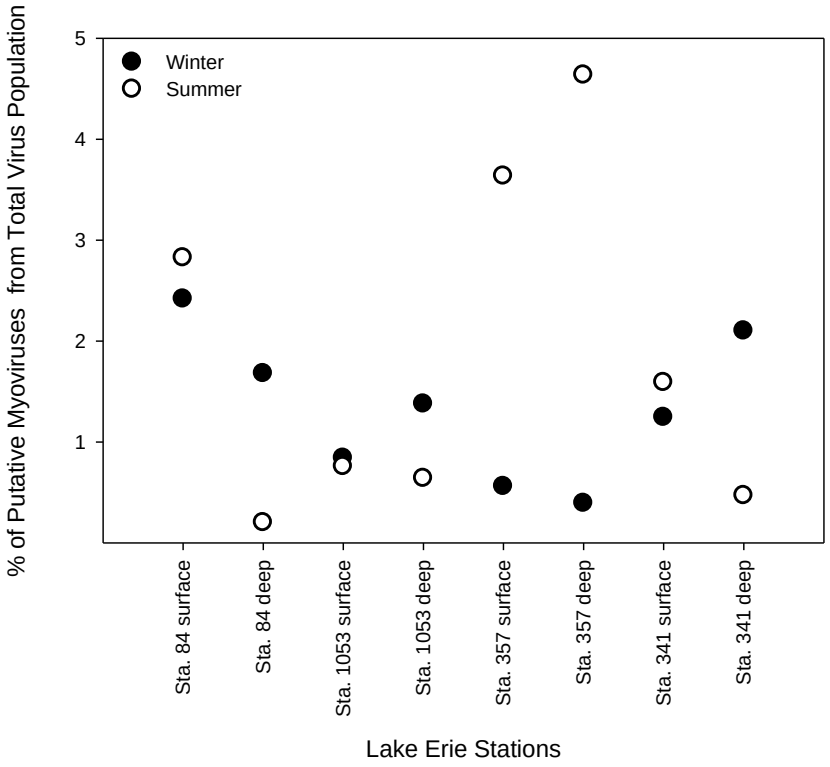

Figure 5.3: Percentage of the total virus population that is composed of putative cyanomyoviruses using *g20* gene copies as a proxy for myovirus abundance. Winter percentages are designated by black circles, while summer percentages are designated by white circles.

passed through a 1 μm nominal pore size filter and compared to the whole water samples. A lysate of *Synechococcus* phage S-PWM1 was also filtered and compared for the analysis. Significant decreases were found at all stations except for stations 1053 1m and 357 8m after filtering the samples, but no significant difference was detected after filtering the phage lysate (Figure 5.4).

At station 341, hypoxic conditions were detected during summer sample collection at 15m, with dissolved oxygen concentrations estimated at 0.58 mg L^{-1} while concentrations at the surface were 7.35 mg L^{-1}. Bacterial abundances were significantly higher in the oxic surface waters compared to its hypoxic counterpart ($p = 0.000$) although there was no significant change in virus abundance ($p = 0.41$) or *Synechococcus* abundance ($p = 0.82$). The density of *g20* gene copies were, however, significantly higher in the hypoxic waters at station 341 compared to the oxygenated surface waters at the same location ($p = 0.05$).

Contact Rates

Contact rates varied widely with season, station and depth (Table 5.2). Contact rates between putative cyanomyoviruses (using *g20* gene copies as virus particle estimates) and heterotrophic bacteria in the system were estimated and specific contact rates were higher in the summer (from 0.66-9.87 contacts with an average of 4.09 contacts $cell^{-1}$ d^{-1}) compared to the winter (0.30-3.0 contacts with an average of 1.74 contacts $cell^{-1}$ d^{-1}). Total contact rate trends of bacteria to *g20* genes followed that of specific contact rates with rates from 3.56×10^{6}-8.64×10^{7} contacts mL^{-1} hr^{-1} with an average of 3.52×10^{7}. Unfortunately no estimates of cyanobacterial abundance were made during winter months. During the summer the rates between cyanobacteria and cyanomyoviruses were also very high and ranged from 0.98-10.52 (average of 6.13) contacts $cell^{-1}$ d^{-1} with highest rates found in the surface waters at all stations. Total contact rates between cyanomyoviruses and total bacteria also varied with winter rates ranging from 3.69×10^{5} to 2.10×10^{6} contacts mL^{-1} hr^{-1} with an average of 1.07×10^{6} contacts mL^{-1} hr^{-1}. Total contacts in the summer between cyanobacteria and cyanomyoviruses were also made and ranged between 3.07×10^{5} and 1.44×10^{7} (average of 4.92×10^{6}) contacts mL^{-1} hr^{-1} . Cell-specific contact rates between total viruses and total bacteria ranged from 77.3-152.8 (average of 128.9) contacts $cell^{-1}$

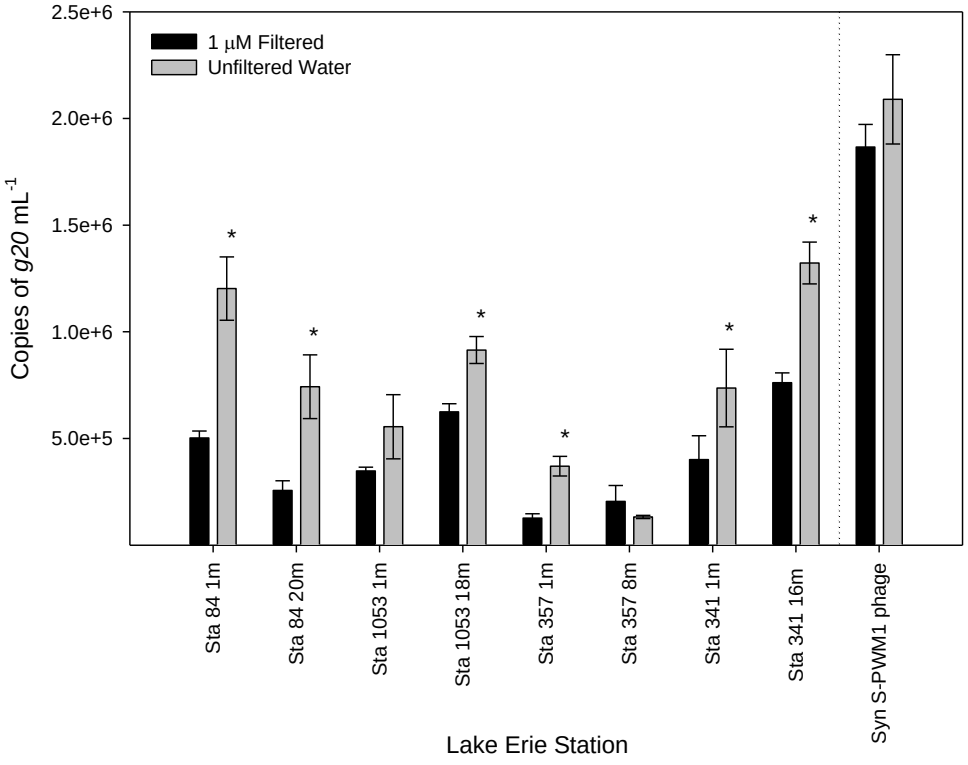

Figure 5.4: Comparison of Lake Erie winter cyanomyovirus abundance in whole water (black bars) and water that is 1 μm filtered (gray bars). This was compared to a lysate filtered and unfiltered using *Synechococcus* phage S-PWM1. Error bars represent the standard deviation of triplicate samples from qPCR analysis. Samples that were significantly different ($p<0.05$) are designated with an asterisk (*).

Table 5.2: Contact rates between putative cyanophage (*g20* copies) or total viruses and total bacteria (TB), heterotrophic bacteria (HB) or cyanobacteria (cyanos). Rates are given as both total contacts (*vis a vis* Murray and Jackson 1992) as well as per cell (*vis a vis* Wilhelm et al. 1998). NA - not available.

		Contact Rate (mL^{-1} hr^{-1})						**Specific Contact Rate ($cell^{-1}$ d^{-1})**					
		Total Viruses vs.			***g20* genes vs.**			**Total Viruses vs.**			***g20* genes vs.**		
Station	**Depth**	**TB ($x10^8$)**	**HB ($x10^8$)**	**cyanos (10^7)**	**TB ($x10^6$)**	**HB ($x10^6$)**	**cyanos ($x10^6$)**	**TB ($x10^2$)**	**HB ($x10^2$)**	**cyanos ($x10^2$)**	**TB**	**HB**	**cyanos**
Sta. 84 Winter	1m	0.58	NA	NA	1.40	NA	NA	1.15	NA	NA	2.76	NA	NA
	20m	0.35	NA	NA	0.58	NA	NA	1.02	NA	NA	1.71	NA	NA
Sta. 84 Summer	1m	6.13	3.75	35.6	23.3	14.3	13.6	1.84	1.84	2.76	7.01	7.01	10.52
	18m	8.50	6.82	25.3	3.56	2.86	1.06	1.56	1.56	2.35	0.66	0.66	0.98
Sta. 1053 Winter	1m	1.13	NA	NA	1.10	NA	NA	1.52	NA	NA	1.48	NA	NA
	18m	0.88	NA	NA	1.22	NA	NA	1.53	NA	NA	2.11	NA	NA
Sta. 1053 Summer	1m	17.4	15.7	24.7	27.6	25.0	3.92	1.50	1.50	2.25	2.38	2.38	3.57
	18m	9.21	8.87	5.21	6.04	5.81	0.34	2.04	2.04	3.06	1.34	1.34	2.01
Sta. 357 Winter	1m	1.16	NA	NA	0.65	NA	NA	1.52	NA	NA	0.85	NA	NA
	8m	0.94	NA	NA	0.37	NA	NA	0.77	NA	NA	0.30	NA	NA
Sta. 357 Summer	1m	14.0	12.4	24.0	83.7	74.2	14.4	1.65	1.65	2.53	9.87	9.87	14.80
	8m	13.7	12.9	11.0	47.1	44.5	3.78	1.52	1.52	2.47	5.22	5.22	7.83
Sta. 341 Winter	1m	0.90	NA	NA	1.11	NA	NA	1.36	NA	NA	1.69	NA	NA
	16m	1.00	NA	NA	2.10	NA	NA	1.45	NA	NA	3.04	NA	NA
Sta. 341 Summer	1m	28.3	27.9	6.77	86.4	85.1	2.06	1.75	1.75	2.63	5.34	5.34	8.02
	15m	7.83	7.43	5.96	4.03	3.83	0.31	1.69	1.69	2.53	0.87	0.87	1.30

d^{-1} in the winter and 150.1-204.3 (average of 169.4) contacts $cell^{-1}$ d^{-1} within the summer (Table 5.2).

Statistical correlations using the Pearson's correlation of *g20* gene copies to biotic and abiotic parameters were determined for both the summer and winter samples (Table 5.3). Estimated *g20* abundance in summer samples was significantly correlated to depth (-0.77, p=0.03) and all chlorophyll *a* size fractions ($r = 0.75$, $p = 0.03$ for picophytoplankton; $r = 0.87$, $p = 0.004$ for nanophytoplankton, $r = 0.77$, $p = 0.03$ for microphytoplankton) while the winter samples did not significantly correlate to any biotic or abiotic factors.

Discussion

The goal of the current study was to determine the abundance putative cyanomyoviruses in Lake Erie using the *g20* gene as a proxy for virus abundance, and to their distribution in the water column with respect to biotic and abiotic parameters. Significant seasonal changes were evident in the lake with respect to the abundance of both the bacteria and viruses. Lake Erie is a well-studied lake during the summer, with many papers focusing on seasonal toxic cyanobacterial blooms (Brittain et al. 2000; Ouellette et al. 2006) and its hypoxic central basin (Charlton 1980; Wilhelm et al. 2006a): studies of winter communities are much rarer (Twiss, McKay and others, in review). The abundance of virus particles estimated during this study were similar to those found in previous years in Lake Erie with abundances from 3.7×10^7-3.7×10^8 mL^{-1} in 2000 (Wilhelm and Smith 2000) and 2.0-4.1 x 10^7 mL^{-1} in 2001 (Wilhelm et al. 2003). These abundances are comparable to other freshwater lakes (Wilhelm and Matteson 2008), including Lake Ontario (Gouvea et al. 2006), although they are higher than virus abundances observed in oligotrophic Lake Superior (Tapper and Hicks 1998).

Seasonal changes in viral abundances are often exhibited in freshwater systems and are correlated to temperature and bacterial community changes (Lymer et al. 2008). Similar differences in abundance can be seen days apart (Hewson et al. 2006; Lymer et al. 2008) as can be seen seasonally (Riemann and Middelboe 2002). Despite having no cyanobacterial winter abundances, previous years provided evidence of sporadic and occasionally high densities (up to

Table 5.3: Pearson correlation coefficients and P values for biotic and abiotic factors affecting putative cyanomyoviruses in Lake Erie. Values considered significant (<0.05) are bolded.

	Winter 2009		**Summer 2009**	
Depth	-0.13	p=0.76	**-0.77**	**p=0.03**
Temperature	-0.36	p=0.38	0.55	p=0.16
Total Bacteria	-0.65	p=0.08	0.23	p=0.58
Total *Synechococcus*	N/D		0.32	p=0.43
Total Heterotrophic Bacteria	N/D		0.19	p=0.65
Total Virus Abundance	0.35	p=0.39	-0.03	p=0.95
Chl *a* (0.2-2 µm)	0.59	p=0.13	**0.75**	**p=0.03**
Chl *a* (2-20 µm)	0.65	p=0.08	**0.87**	**p=0.00**
Chl *a* (<20 µm)	0.68	p=0.06	**0.77**	**p=0.03**

2.5×10^4 mL^{-1}) of autotrophic picoplankton in winter waters; there were even higher abundances in ice patches (Twiss, McKay and others, in review). Other work has shown a high diversity of *Synechococcus* during the winter (Loar 2009), further suggesting that cyanobacteria are prevalent during the winter and may support this cyanomyovirus assemblage. Summer bacterial abundances were similar to those found in previous years in Lake Erie (Wilhelm and Smith 2000; DeBruyn et al. 2004) and to other freshwater systems (Tapper and Hicks 1998; Weinbauer and Hofle 1998) while cyanobacterial counts were slightly higher than previous years in Lake Erie (Wilhelm et al. 2006b).

Only recently has Lake Erie been the subject of significant biological research during winter months. Despite a reduced bacterial density relative to summer months, virus particles, including cyanomyoviruses, were abundant during our sample collection in February 2009. Virus particles in aquatic systems are sensitive to many factors that can destroy or remove them, such as the UV component of sunlight (Suttle and Chen 1992; Noble and Fuhrman 1997; Wilhelm et al. 1998), enzymes produced by co-occurring microorganisms, and even specific grazing by small flagellates (Suttle and Chen 1992). In summer months these removal rates are thought to be in balance with rapid production rates of viruses due to the infection and lysis of host cells. Observations made during sample collection for this study have demonstrated that significant primary production occurs in Lake Erie in both summer and winter months (Twiss, McKay and others reference, in review) but that bacterial activity, while high in summer months (DeBruyn et al. 2004) is ~ 1% of summer rates (Wilhelm and Bullerjahn, unpublished). To this end it appears that maintenance of the high abundance of total viruses, including cyanomyoviruses, observed during the winter months is likely due to decreased rates of particle removal. In the laboratory, it is well known that aquatic virus samples collected during research cruises remain stable for many years if stored in the dark at 4° C (Angly et al. 2006) (similar to conditions we observed in the lake). Solar radiation, which is a major cause of virus destruction in aquatic environments (Suttle and Chen 1992; Suttle and Chan 1994; Wilhelm et al. 1998) and may affect the infectivity of viruses (Suttle and Chan 1993; Wommack et al. 1996) is also reduced in the winter due to the shorter day length during the winter, as well as ice and snow cover. Indeed in a previous study of an alpine lake, decreases in viral abundance to undetectable

levels after ice breakage has been attributed to increased UV levels (Hofer and Sommaruga 2001).

High *g20* gene densities were found at all stations and in both seasons, with up to 3.1 x 10^6 mL^{-1}. In comparison to the only other quantitative studies of these viruses our estimates are slightly higher. The highest *g20* abundances were found at station 84 in the summer which may be due to the high *Synechococcus* hosts found in the central basin during the summer months and seasonal hypoxia (Wilhelm et al. 2006b). Takashima (2007) reported abundances of up to 9.7 x 10^5 mL^{-1} in Hirosawanoike Pond, but this was only detecting phages similar to cyanophage Ma-LMM01 that infect *Microcystis aeruginosa*, not an entire family of phage like the *g20* gene so it is not surprising that these abundances were often lower. Cyanomyoviruses were also temporally detected in a Norwegian coastal marine station with low *g20* gene copies of up to 7.2 x 10^3 mL^{-1} (Sandaa and Larsen 2006) with the lowest abundances during the winter months. Overall, it is not very surprising that we found such high cyanomyovirus abundances in Lake Erie since it is such a productive system all year long.

Often samples are prefiltered to eliminate bacterial biomass in biologically productive systems. Furthermore, ultrafiltration steps to concentrate viruses using spiral wound cartridges at 30 kDa are often used and may lead to lower viral titers by adhering to the filters. To determine if a prefiltration step decreases cyanophage abundance, we compared copy numbers between whole water and samples passed through a 1 μm polycarbonate filter. Significant decreases in abundances were exhibited in several samples, suggesting that these phage may adhere to the filters themselves, or those virally infected bacteria may not pass through the filter which may cause this decrease. Chen, et al. (2001) has previously shown that samples collected from Lake Erie and Georgia coastal water contained fewer viruses after passing through a 0.2 μm filter suggesting the possibility that some phage may bind to particular filter types such as polycarbonate. Since the phage lysate contained no significant difference between the filtered and unfiltered samples, this suggests that the filtering process is filtering out virally infected cells that are causing this decrease.

The production of virus particles in any environment ultimately depends on the successful interaction between the parent virus particle and a permissive host cell. We found

higher virus-host contact rates during the summer compared to the winter due to the significantly higher densities of both bacteria and viruses during the summer sample collection. Cell-specific rates of 77.3-152.8 contacts $cell^{-1}$ d^{-1} were calculated in the winter and 150.1-204.3 contacts $cell^{-1}$ d^{-1} in the summer for total bacteria and total viruses in the system, which is higher than what has been previously reported for Lake Erie (4-11 $cell^{-1}d^{-1}$ in 2000) (Wilhelm and Smith 2000) and marine systems (Wilhelm et al. 1998), but similar to other more eutrophic freshwater systems such as Alte Donau (91-230 $cell^{-1}$ d^{-1}) (Fischer and Velimirov 2002) or Lake Pluβsee (49-180 $cell^{-1}$ d^{-1}) (Weinbauer and Hofle 1998). To our knowledge, this study is the first to estimate contact rates between cyanophages and their potential hosts in a freshwater ecosystem. Unsurprisingly, the total bacteria: total virus contact rates were much higher than the contact rates between putative cyanomyoviruses and their potential hosts. It has previously been shown that at least ~10^4 hosts mL^{-1} is necessary for the minimum contact rate for viral infection and replication (Wiggins and Alexander 1985). Adequate densities for *Synechococcus* are lower than for other heterotrophic bacteria, likely due to the slower growth rate for cyanobacteria (Waterbury and Valois 1993). In the summer, both bacterial and cyanobacterial and viral abundances were high and daily infections of hosts may have lead to the high abundances of cyanomyoviruses found in Lake Erie. What constrains whether an infection will take place is the genetic diversity of the hosts and their susceptibility to viral infection (*i.e.* expression of receptors on host cells). Although cyanobacterial abundances were not determined during the winter, high abundances have been evident in previous winters. Based on the prefiltering experiment from above, it suggested that virally infected bacteria were filtered out or that lysogenic viruses were found during the winter in Lake Erie leading to lower *g20* gene abundances after filtering. Lysogeny in winter months and other unfavorable conditions has been a topic of discussion for many years and conflicting evidence has arisen suggesting a complex relationship of lysogenic bacteria during winter months (McDaniel et al. 2002; Williamson et al. 2002; Lisle and Priscu 2004; Stopar et al. 2004).

Seasonal hypoxia in the central basin of Lake Erie has occurred for over three decades (Charlton and Milne 2004). This "dead zone" may have effects on the extensive fisheries of Lake Erie as well as the grazing rates on the bacterial community (Gobler et al. 2008). Our work showed no significant difference in the total virus abundance in the oxic surface waters from the

hypoxic hypolimnion, but a significant increase in the *g20* gene numbers and a significant decrease in the bacterial abundance in the hypoxic waters. Increased bacterial abundance in oxic waters compared to hypoxic waters has also been evident in several lakes such as Lake Grangent, France (Pradeep Ram et al. 2009) and Mono Lake, California (Jiang et al. 2004). In these same locations, virus abundances were shown to be significantly higher in the anoxic zone compared to overlaying waters which is not what was found in the present study. Others have seen the opposite with a decrease in virus abundance in anoxic waters (Wommack et al. 1992; Taylor et al. 2003). This inconsistency confirms a complex relationship between viruses in oxic vs. anoxic ecosystems. The higher cyanomyovirus population may be due to the high abundance of *Synechococcus* in the hypoxic zone in the central basin of Lake Erie (Wilhelm et al. 2006b). This *Synechococcus* population may account for the increase in putative cyanomyoviruses in these hypoxic waters.

Statistical correlations of *g20* gene copies to other biotic and abiotic parameters in the system showed only a few significant correlations and these came from the summer sampling times. Cyanomyovirus abundance did not significantly correlate to any potential hosts, just chl *a*, which does not elucidate the true hosts of the viruses we were amplifying. Temporal disconnects were likely the cause of low statistical significance in these samples. *g20* gene abundance was, however, significantly correlated to all chl *a* size fractions which suggests a connection to a photosynthetic host. Overall, Lake Erie is a productive system, both during the summer and in the winter. Seasonal dynamics are evident, with decreases in both bacteria and viruses during the winter and a persistent population of putative myoviruses. This quantitative tool will hopefully help elucidate the effects of cyanophage on the host population in other systems, both temporally and spatially.

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Chapter 6 Conclusions

Viruses are abundant in aquatic environments (Bergh et al. 1989) and are important in many ecological processes. They are able to remobilize nutrients and can affect the biogeochemical cycling of nutrients by lytic infections of hosts in marine (Gobler et al. 1997; Poorvin et al. 2004; Mioni et al. 2005) and freshwater ecosystems (Dean et al. 2008) and serve as reservoirs of genetic material through horizontal gene transfer (Hendrix et al. 1999; Fraser et al. 2007). Diversity and abundance of hosts can be controlled by viruses through the "killing the winner" model (Thingstad and Lignell 1997) and can infect the dominant members in a community. Approximately 20-40% of the bacterial stock is infected daily by viruses (Suttle and Chan 1994; Suttle 2005), and multiple methods have been employed to estimate the production rate of viruses in aquatic systems (Weinbauer 2004; Thingstad et al. 2008). Since there are so many methods to estimate virus production rates, it is often difficult to cross examine datasets making a standardized protocol for this method very important in viral ecology.

The *g20* portal vertex gene has been used since 1998 to analyze the diversity and richness of cyanomyoviruses in marine, estuary, and freshwater ecosystems. High diversity has been shown, but little is known about the abundance of these phage on a temporal or spatial scale. Previously there have only been two reports using qPCR to quantify cyanophages (Sandaa and Larsen 2006; Takashima et al. 2007). Given that cyanobacteria, most notably *Synechococcus* and *Prochlorococcus*, are prevalent in aquatic environments, it is expected that cyanomyoviruses are abundant as well. Overall, this dissertation aimed to 1) Establish methodology in analyzing the production rate of viruses and use this in a natural system ; 2) Quantify cyanomyoviruses in aquatic ecosystems; 3) Analyze the diversity of cyanomyoviruses throughout a phytoplankton bloom. Through this work we have demonstrated:

i. Several different methods have been employed to estimate the production of viruses in aquatic ecosystems. The dilution and reoccurrence technique (Wilhelm et al. 2002) has become the most popular method (Weinbauer et al. 2010) and has been used to estimate production rates in various environments. While detailed written protocols are extremely helpful, it can still take weeks or months to fully develop the method. To alleviate any issues with the methodology, a video demonstrating the dilution method was made in the

laboratory and produced by the Journal of Visualized Experiments. The video shows every step of the process, including data analysis which can often be troublesome to many researchers.

ii. Several spatial surveys of virus production have been completed, but to our knowledge no temporal surveys of pelagic systems have been completed. To put the dilution and reoccurrence method to the test, we analyzed the production rate of viruses during the FeCycleII cruise in the subtropical Pacific Ocean during a seasonal spring phytoplankton bloom. At the onset of our study the system was N-limited and, unlike most of the world's oceans, contained moderate levels of Fe. During our survey a large mixing event introduced N (but not Fe) from deep waters resulting in a different plankton community. High virus production rates and abundances were observed, with production rates from 1.36×10^{10}-2.06×10^{11} L^{-1} d^{-1} and abundances from 2.11×10^{10}-1.59×10^{11} L^{-1}. Production rates correlated to several ecological parameters in an unexpected way which suggests a complex relationship or temporal disconnects with viruses during this bloom. A significant proportion of bioavailable nutrients were produced through the lysis of virally-infected hosts which would be important in this N-limited system.

iii. The abundance of putative cyanomyoviruses from two distinct marine ecosystems were compared and found that they are ubiquitous and abundant in marine environments. Samples were collected in the Sargasso Sea and during a phytoplankton bloom from the FeCycleII cruise and were compared using qPCR for the *g20* gene. Up to 25% of the total virus population consisted of cyanomyoviruses in these environments.

iv. Correlations between *g20* abundance (and therefore putative cyanomyoviruses) with various biotic and abiotic parameters was performed to determine the factors affecting cyanophage abundance as well as potential hosts for these viruses. Based on previous phylogenetic analyses of *g20* amplicons it was suggested that some *g20* sequences came not from cyanomyoviruses, but possibly other bacteriophage (Short and Suttle 2005). To

elucidate potential hosts, Pearson correlation data found that likely cyanobacteria was the host for putative cyanomyoviruses in the Sargasso Sea, but the true host is still unclear in the southern Pacific Ocean based on correlations to only abiotic parameters.

v. The diversity of cyanomyoviruses in the southern Pacific Ocean was determined from samples collected prior to, during and after the formation of a *Synechococcus* bloom. Diversity was as expected and clustered with other marine cyanophage isolates as well as uncultured environmental clones. Unifrac analysis was able to determine phylogenetic differences between the three time points and demonstrated that there was a change in the cyanomyovirus community throughout the bloom.

vi. Samples were collected in Lake Erie in August and February of 2009 to observe potential seasonal changes in the bacterial and virus communities. Significant decreases in total bacteria and virus abundances were evident in the shift from summer to winter samples, but the lake was still quite a productive system during the winter with high chl *a* concentrations. qPCR analysis was performed on samples from both months and found abundances of cyanomyoviruses (up to 4.3×10^6 mL^{-1}) which were much higher than those found in their marine counterparts.

These conclusions confirm that viruses are extremely abundant and can be found in various ecosystems. Despite having no equivalent to the 16S or 18S rDNA gene for evolutionary relationships (Paul et al. 2002), we can use individual conserved genes among different phage families to study both the richness and evenness of phages. The work here also provides several avenues by which future studies can be performed. The work presented here attempted to test the specificity of the *g20* gene primers (for only cyanomyoviruses) employing statistical examinations of data from the Sargasso Sea, the southern Pacific Ocean and Lake Erie. Our results at this point leave us unable to demonstrate that *g20* amplicons resulted from bacteriophage other than *cyanomyoviridae*. This is likely in part due to temporal disconnects between viruses and hosts that were unable to be resolved. It would be beneficial to find several locations for rigorous temporal sampling to determine the true hosts in each environment.

Furthermore, work may be done using qPCR to analyze the production rate of cyanomyoviruses to determine what percentage of the population is infected by cyanomyoviruses and the recycling of nutrients solely to cyanophages.

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Vita

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