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TECHNOLOGICAL
UNIVERSITY**

**CHARACTERIZATION AND DYNAMICS OF
CYANOPHAGES FROM TROPICAL SURFACE
WATERS**

YEO BEE HUI

SCHOOL OF CIVIL AND ENVIRONMENTAL ENGINEERING

2014

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FROM TROPICAL SURFACE WATERS

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YEO BEE HUI

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School of Civil and Environmental Engineering

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ABSTRACT

Cyanophages are viruses which infect cyanobacteria. Cyanophages are mortality agents of cyanobacteria and play important roles in cyanobacteria evolution, as well as co- evolution between cyanophage and host. Cyanophages are believed to be important players in aquatic ecology, where they regulate biodiversity of the cyanobacteria community and affect the structure of aquatic food webs through lysis of a major producer (cyanobacteria) in aquatic systems. To date, there are only limited reports on freshwater cyanophages, the majority of studies have focused on marine cyanophages. In addition, only a limited numbers of cyanophages from freshwater systems have been isolated.

In this study, isolation was conducted to obtain endemic cyanophages from Kranji Reservoir. Two cyanophages which infect *Anabaena circinalis* and four cyanophages which infect *Anabaena cylindrica* were isolated using the well assay and double-layer plaque assay methods. From the isolation experiments, *Anabaena cylindrica* showed changes in aggregation and algal mat formation when incubated with the cyanophage isolates. Furthermore, cyanophage isolates which infect *Anabaena circinalis* suppressed the growth of their host.

The occurrence and dynamics of cyanophages in Kranji Reservoir in Singapore were studied using the well assay, plaque assay, PCR and qPCR. Cyanophages infectious to *A. cylindrica*, *A. circinalis*, *Microcystis*, and *Synechococcus* were observed in Kranji Reservoir throughout the sampling period (from August 2008 to August 2009). Monsoon seasons can strongly influence the presence of phages which infect *A. cylindrica*. They were found to be absent during the NE Monsoon. The temporal and spatial dynamics of the *Microcystis* cyanophage encoded g91 gene were also observed over the sampling period. The g91 gene concentrations ranged from undetectable to 4.73×10^2 gene copies ml^{-1} in the free- phage fraction, while the g91 gene concentrations in the bacterial cell fraction ranged from below detection limit to 5.81×10^4 gene copies ml^{-1} . The occurrence of cyanophage infectious to *Microcystis* was affected by the threshold of

minimum densities of the host cell, i.e. $3.6 \times 10^3 \text{ ml}^{-1}$ *Microcystis* cells. Pearson's correlation analyses showed that a positive correlation existed between g91 gene abundance (from free- phage fraction and bacterial cell fraction) and *Microcystis* spp.. Environmental parameters, such as chlorophyll- a and turbidity, influenced the g91 gene copy number in the free- phage fraction in a positive way, suggesting that chlorophyll- a/ turbidity may play role as shed to protect cyanophages from UV degradation.

The dynamics of cyanophage abundance from this study demonstrated that cyanophages influenced the replacement of phage sensitive populations by phage-insensitive populations (i.e. changes in intra-species level of host) with no quantitative impact on the entire host population. In this study, the fraction of infected cells was always in a dynamic form, with no obvious changes in the total host cells densities (always between 10^5 to 10^6 gene copies ml^{-1}) was observed. In other words, the percentage of sensitive cyanobacterial populations changes from month to month. However, the total population remains the same.

The cyanomyovirus population dynamics in Kranji Reservoir were examined for a period of seven months. Cyanomyovirus diversity was investigated by the PCR-cloning- sequencing approach with PCR primers targeting the g20 gene which is myoviral-specific. The g20 sequences from Kranji Reservoir, together with g20 sequences from other environments were used to construct a phylogenetic tree by using MEGA 5. Clustering analysis through the phylogenetic tree showed that 73 sequences from Kranji Reservoir were distributed to six major clusters (α to ϵ and PFS), with four unique subclusters identified as KRM-I, KRM-II, KRM-III and KRM-IV.

The cyanophage community in Kranji Reservoir revealed a large degree of diversity because clones obtained from this study showed similarity to clones from many different environments, including oceans, lakes, bays, paddy floodwater as well as the clones from paddy field soil. However, Kranji Reservoir sequences were generally found to be more closely related to g20 sequences of freshwaters and

brackish waters than those from marine environments. The temporal study in Kranji Reservoir revealed shifts in the cyanomyoviral population. The temporal change is likely due to nutrient and biomass fluctuations in the reservoir brought about by seasonal changes (as shown by the PCA score plot) and the role of cyanophages in controlling host community diversity. The rarefaction curves and Chao 1 indices from this study showed that greater diversity of the cyanomyovirus community occurred during the inter-monsoon periods compared with the SW and NE monsoons. Cluster analysis, together with the dynamics of g20 gene concentration, demonstrated that the “kill the winner” phenomenon probably occurred between October and November 2008 at Kranji Reservoir, subsequently creating open niches for other cyanobacterial genotypes to grow. This demonstrates that cyanophages can play a role in controlling the diversity of cyanobacteria.

PUBLICATIONS

Journal paper accepted for publication

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LIST OF SYMBOLS / ABBREVIATIONS

AMGs	auxiliary metabolic genes
AO	acridine orange
APHA	American Public Health Association
ATP	adenosine-5'-triphosphate
BLAST	basic local alignment search tool
bp	base pair
Ca ²⁺	calcium ion
Chl-a	chlorophyll-a
C _t	threshold cycle
CyanoHABs	harmful cyanobacterial blooms
DAPI	4'6-diamidino-2-phenylindole
DCM	deep chlorophyll maximum
df	degrees of freedom
DGGE	denaturing gradient gel electrophoresis
DMS	dimethyl sulfide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DO	dissolved oxygen
DOC	dissolved organic carbon
DOM	dissolved organic matter
Ds	double- stranded
ELM	epifluorescence light microscopy
FC	flow cytometry
FLVP	fluorescently labeled viruses as probe
GPS	global Positioning System
ICTV	International Committee on the Taxonomy of Viruses
IS	insertion sequence
KMO	Kaiser-Meyer-Olkin
KW test	Kruskal-Wallis test
KtW	kill the winner

LB	lysogeny broth
LPS	lipopolysaccharides
Mg ²⁺	magnesium ion
MPN	most probable number
MW	molecular weight
N	nitrogen
NE	Northeast
OD	optical density
OUT	operational taxonomic unit
P	phosphorus
PBS	phycobilisomes
PCA	principal component analysis
PC-IGS	phycocyanin intergenic spacer
PCR	polymerase chain reaction
PE	phycoerythrin
PFGE	pulse- field gel electrophoresis
PFU	plaque forming unit
PKS	polyketide synthases
PS	peptide synthetases
PSI/II	photosystem I/II
POC	particulate organic carbon
POM	particulate organic matter
PUB	Public Utilities Board
qPCR	quantitative real-time PCR
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
rRNA	ribosomal RNA
r_s	Spearman's coefficient
RT-PCR	reverse transcriptase PCR
SEM	Scanning electron microscope
SW	Southwest
TDS	total dissolved solid

TBE	Tris- borate- EDTA
TEM	transmission electron microscope
TFF	tangential flow filtration
T _m	melting temperature
TMD	transmembrane domain
TN	total nitrogen
TOC	total organic carbon
TP	total phosphorus
T-RFLP	terminal- restriction fragment length polymorphism
tRNA	transfer RNA
TSS	suspended solid
USEPA	United States Environmental Protection Agency
UV	ultraviolet
Vis	visible light
VLPs	virus like particles

CHAPTER 1

Introduction

1.1 CYANOPHAGES IN AQUATIC ECOLOGY

Global environmental concerns on the eutrophication issue and global warming have placed a special emphasis on understanding the biogeochemical cycling of carbon and nitrogen. Thus, understanding the physiological and ecological roles of planktonic organisms on global carbon and nitrogen cycles may provide insights into predicting and/or remediating the ecological impacts of large-scale anthropogenic carbon or nitrogen releases. Cyanophages are described as viruses that infect cyanobacteria (Fuhrman 1999; Jassim and Limoges 2013). In the early 1990s, viruses were found to be extremely abundant in the sea and the majority of them were classified as bacteriophages (Bergh et al. 1989; Proctor and Fuhrman 1990; Suttle et al. 1990). A large number of viruses were reported to infect a wide variety of bacteria and primary producers. Host lysis is believed to enhance the flux of bacterial biomass into the dissolved organic matter pool (Colwell and Wommack, 2000), leading to the regeneration of inorganic nutrients and dissolved organic carbon (Miki et al. 2008). High viral concentrations can also cause a short-circuit in the carbon cycle by redirecting carbon back into the loop as dissolved organic carbon (DOC) (Wilhelm and Suttle 1999). As a result, dissolved organic matter is recycled and respired by the bacterial community. This semi-closed loop in the aquatic food web significantly affects material pathways and energy transfer within the pelagic food web. In short, viruses play a critical role in regulating the biogeochemical cycles.

Cyanobacteria are key producers in aquatic ecosystems. Cyanophages play an important role in maintaining an extensive microdiversity of their host through killing of the winner and transferring genes between cyanophages and their hosts (Sullivan et al. 2008). Cyanobacterial dynamics were found to be closely associated

with the abundance of cyanophages in aquatic systems. Cyanophages are capable of entering into a lysogenic relationship with their host and are also capable of infecting a diverse range of hosts so that they become the vector of cyanobacterial evolution. Significant genetic exchange has been observed, from host to phage, phage to host, and within the phage gene pool itself. Encoded photosynthesis genes are commonly found in *Synechococcus* and *Prochlorococcus* cyanophages (Lindell et al. 2007). This co- evolution dynamic generates the genetic diversity of cyanophage communities and influences the evolution of the cyanobacterial photosystem (Sullivan et al. 2006). In addition, cyanophages carry and express the photosynthesis genes, suggesting that cyanophages are not only important in diverting the flow of carbon (short- circuit in the carbon cycle) but may also be directly responsible for carbon fixation (Clokier et al. 2011).

1.2 RESEARCH ON CYANOPHAGES IN AQUATIC ECOSYSTEMS: THE CURRENT STATE

Current studies on cyanophages can be divided into three main parts, (1) examination of cyanophage morphology, genome sequence, physicochemical characteristics, growth pattern and host range, (2) interactions and correlations between cyanophages and cyanobacteria and (3) influence of environmental conditions on the dynamics of the cyanophage community.

The first part usually consists of cyanophage isolation, followed by characterization. Since 1963 when the first cyanophage was isolated by Safferman and Morris, more and more cyanophages have been isolated (Adolph and Haselkorn, 1971; Franche, 1987; Okunishi et al., 2002; Wilson et al., 1993b; Yoshida et al., 2006). Based on examination of the isolates morphology, all cyanophages characterized so far fall under three families in the caudovirales order (tailed phage): *Myoviridae*, *Podoviridae* and *Siphoviridae* (Mann, 2006). To date, 24 marine cyanophages (17 myoviruses, six podoviruses and a siphovirus), five estuarine cyanophages (one podovirus and four siphovirus) as well as four freshwater

cyanophages (two myoviruses and two podoviruses) have been sequenced (Clokier et al., 2011; Dreher et al., 2011; Huang et al., 2012; Yoshida et al., 2008). A very important finding from these genome sequences is the cyanophages' encoded bacterial gene. From physicochemical studies, it has been found that each cyanophage isolate has its own physicochemical properties and responds differently in inactivation tests, even if the isolates are isolated from the same host and have the same morphology. The cyanophage isolates were characterized by determining their phage growth parameters, particularly their adsorption rate, latent period, eclipse period and burst size (Hyman and Abedon, 2009). Cross-infectivity experiments were used to determine the host range of cyanophage isolates, where the cyanophage isolates were screened for their ability to lyse other potential host strains (Deng and Hayes, 2008).

Secondly, current studies examined the interaction and correlation between cyanophages and cyanobacteria. Based on these studies, a few important findings have been concluded on how cyanophages affect cyanobacteria: (1) the lab studies showed that cyanophage infection affects the host cell physiology and tweaks the host metabolism (Suttle 2000b; Chen and Lu 2002; Mann 2006), (2) the *in situ* studies suggested that cyanophages play important roles in regulating cyanobacteria bloom dynamics (Manage et al. 1999; Manage et al. 2001; Breitbart 2012) and influence the replacement of phage-sensitive populations by phage-insensitive populations (Yoshida et al., 2010). In addition, these studies also showed that cyanobacteria affected cyanophage replication (concentration) (Waterbury and Valois 1993; Suttle and Chan 1994; Manage et al. 1999; Okunishi et al. 2003) and diversity (Zhong et al. 2002; Sandaa and Larsen 2006; Chen et al. 2009).

The third part of the study focused on the examination of cyanophage isolates under different environmental variables and examination of *in-situ* dynamics of cyanophage abundance and diversity together with environmental variables. Environmental factors affect viral absorption, replication, burst size and fate of viruses. The fate of cyanophages has been shown to be influenced by environmental conditions: (1) adsorption rate is affected by light (Kao et al. 2005),

nutrient (Mann 2006) and host physiological condition (Brussaard 2004); (2) infectivity is affected by temperature (Franche 1987), divalent cations (Mole et al. 1997) and pH (Franche 1987); (3) latent period and burst size are affected by light (Adolph and Haselkorn 1972; Kim and Choi 1994) and nutrients (Wilson et al. 1996); (4) structural stability and decay rate are affected by temperature (Garza and Suttle 1998; Manage et al. 1999), UV (Liao et al. 2010), divalent cations (Mole et al. 1997), attachment to cells or particles and consumption by protozoa (Suttle 2000a); and (5) life cycle (lytic or lysogen cycle) is affected by light (Clokier and Mann 2006), non-cyclic peptides (Sedmak et al. 2008) and nutrient levels (Danovaro et al. 2011). The spatial and temporal dynamics of *in-situ* studies showed that cyanophage abundance and diversity changed with the depth of column, season and nutrient concentration (Zhong et al. 2002; Frederickson et al. 2003; Dorigo et al. 2004; Clokier et al. 2006; Wang et al. 2010; Matteson et al. 2011)

1.3 RESEARCH AIMS, QUESTIONS AND OBJECTIVES

Kranji Reservoir in Singapore is dominated by cyanobacteria. The occurrence and dynamics of cyanobacteria and their cyanotoxins are investigated by several research groups in Singapore. However, the occurrence, distribution and dynamics of cyanophage populations have not been studied yet. Cyanophages contribute to the bloom and collapse of cyanobacteria and influence the abundance, species composition and diversity of the cyanobacteria community (Manage et al. 1999; Yoshida et al. 2008). In addition, over the last two decades, considerable strides have been made in understanding the importance of cyanophages. However, the majority of these studies focused on marine phages rather than those from freshwater environments. It is important to note that the factors controlling the abundance of viruses differ between lakes and oceans (Clasen et al., 2008). Thus, assessing and monitoring the distribution and dynamics of freshwater cyanophages from Singapore local reservoir are worth exploring further.

This study aims to provide comprehensive data on the occurrence, distribution and dynamics of cyanophages in tropical surface waters, including the relationships between environmental factors and cyanophage abundance, and the interactions between cyanophages and their hosts. To achieve these aims, the following research questions were posed:

1. What are the cyanophage strains present in a Singapore reservoir? Are these cyanophages able to control the cyanobacterial population?
2. How does the cyanophage abundance fluctuate in tropical surface waters?
3. Does the cyanophage community composition change over time? Do the cyanophage populations from a Singapore reservoir have different genetic makeup compared to those in other regions or other aquatic ecosystems, such as marine and estuarine environments?
4. Do cyanophage abundance and diversity correlate well with the environmental variables?

To answer these research questions, the objectives of this study are as follows:

- I. To isolate and characterize cyanophages from Singapore surface waters.
- II. To investigate the potential of these isolated cyanophages as biological control agents for their hosts.
- III. To study the occurrence and dynamics of cyanophages in a Singapore reservoir.
- IV. To study the cyanophage genetic diversity in a Singapore reservoir.
- V. To determine physical, chemical and biological variables (including cyanobacteria concentration and diversity) that are associated with changes in cyanophage abundance and community composition.
- VI. To compare the differences in cyanophage concentration and diversity from a Singapore reservoir with other literature studies.

1.4 THESIS STRUCTURE

This thesis is organized into 6 chapters. Chapter 2 is a detailed literature review of viruses in aquatic ecology, discussing the influence of cyanophages on the cyanobacterial community, isolation and characterization of cyanophages; and influence of environmental conditions on the fate of cyanophages. In addition, the pros and cons of the various approaches used to study cyanophage isolation and cyanophage abundance and diversity dynamics is described. Chapter 3 focuses on method development to isolate indigenous cyanophages from Kranji Reservoir and monitor the changes in cyanobacteria after infection by local isolates. Chapter 4 describes the occurrence and dynamics of cyanophage in Kranji Reservoir. This chapter also investigates the significance of environmental variables on the dynamics of cyanophage abundance. Chapter 5 discusses the genetic diversity of cyanomyovirus population in Kranji Reservoir. Moreover, the influence of environmental conditions on the cyanophage communities is also discussed in this chapter. Finally, Chapter 6 provides the thesis conclusions and some recommendations for future study.

CHAPTER 2

Literature Review

2.1 OVERVIEW

This chapter is organized into five main parts: (1) description of cyanophages; (2) overview of viruses in the aquatic ecology; (3) influence of cyanophages on the cyanobacterial community; (4) isolation and characterization of cyanophages; and (5) influence of environmental conditions on the fate of cyanophages. The pros and cons of the approaches used to study cyanophage isolation and cyanophage abundance and diversity dynamics are also described.

2.2 CYANOPHAGES

Cyanophages are viruses which infect cyanobacteria. All the cyanophages discovered so far have tails, are double-stranded DNA (ds) and fall into three families of viruses. The three families recognized by the International Committee on the Taxonomy of Viruses (ICTV) are: *Myoviridae*, *Siphoviridae* and *Podoviridae* (Mann 2006). Cyanophages from these three families are able to infect both unicellular and filamentous cyanobacteria.

Cyanomyoviruses, i.e. the cyanophages belonging to the family of *Myoviridae*, possess a long contractile tail, long tail fiber and elongated icosahedral symmetrical or symmetrical head. An example of a cyanomyovirus is Ma-LMM01, a cyanophage which infects the toxic cyanobacterium, *Microcystis aeruginosa*. It had an isometric head and a tail complex consisting of a central tube and a contractile sheath with helical symmetry (Figure 2.1A) (Yoshida et al. 2006). The mol% GC content of cyanomyoviruses range from 37 % to 55 %; while the genome molecular weight ranges from 24×10^6 to 57×10^6 daltons with is considerable variation in genome size, e.g. cyanophages N1 has a size of 37 kb while the marine

cyanophage S-PM2 has a size of 196 kb (Suttle 2000a; Suttle 2000b; Mann 2006). Cyanomyoviruses tend to have large genome (Sullivan et al. 2003; Clokie and Mann 2006; Millard and Mann 2006) and vary in size and morphology (Suttle 2000a) compared to cyanosiphoviruses and cyanopodoviruses.

The cyanosiphoviruses have long flexible and non-contractile tails with short tail fibers and isometric heads. This family of cyanophages is the least isolated from both freshwater and marine waters. An example of cyanosiphovirus is N-BS2 (Figure 2.1B), a cyanophage which infects *Nodularia spumigena* (Jenkins and Hayes 2006). Similar to cyanomyoviruses, siphoviruses have variation in mol% GC content ranging from 46 % to 74 %. Their genome sizes range from ca. 40 kb to 100 kb (Suttle 2000a; Suttle 2000b; Mann 2006).

The cyanopodoviruses have short non- contractile tails and icosahedral symmetrical heads. The first cyanophage ever isolated, LPP-1, is a cyanopodovirus which infects and causes lysis of cyanobacteria from the genera *Lyngbya*, *Plectonema*, and *Phormidium*. An example of a cyanopodovirus is S-BBP1 (Figure 2.1C), a cyanophage which infects marine *Synechococcus* spp. (Suttle and Chan 1993). The reported mol% GC content show that cyanopodoviruses have rather less variation, ranging from 53-55 % for LPP-1 to 66-67 % for SM-1; while the genome size is about 42 kb for LPP-1 to 48 kb for P60 (Suttle 2000a; Suttle 2000b; Mann 2006).

Figure 2.1 Examples of cyanophages from different families: (A) Ma-LMM01, *Myoviridae*; (B) N-BS2, *Siphoviridae*; and (C) S-BBP1, *Podoviridae* (Adapted from (Suttle and Chan 1993; Jenkins and Hayes 2006; Yoshida et al. 2006)

2.3 VIRUSES IN AQUATIC ECOLOGY

The discovery of an abundance of viruses in aquatic systems since 1990s has opened up a new research area in viral ecology (Bergh et al. 1989; Proctor and Fuhrman 1990; Suttle et al. 1990). This discovery has changed the traditional conceptual understanding of the functioning and regulation of aquatic ecosystems (Middelboe et al. 2008) with the realization of viruses as important players in aquatic ecosystems. The abundance and dynamics of viruses in water have two major impacts on: (1) biogeochemical cycling and (2) the ecological system.

A fundamental step to understand any ecosystem is gaining knowledge of its trophic levels and food web structure, through which a large proportion of the total flux of energy and matter flow (Fuhrman 1999). Microbes acquire resources for metabolism and growth. Most of the studies of microbial ecology are framed in terms of “bottom up” considerations. Bottom up is where aquatic resources and abiotic conditions drive the distribution and function of microbes. Lytic viruses and grazers as agents of mortality exert strong selection on the population of microbes, contributing to top- down forces (Strom 2008). Thus, top- down and bottom-up force ought to be considered together in the process of understanding microbial ecology. This is especially pertinent as viruses have been identified as integral members of microbial food webs (Wilhelm and Suttle 1999). Moreover, grazing by protists and viral lysis are predicted to bring different biogeochemical effects. Whereas protozoan grazing transfers carbon and nutrients to higher trophic levels, lysis by viruses fuels the microbial loop by releasing dissolved organic matter (DOM) which is subsequently used by heterotrophic bacteria (Breitbart et al. 2008). Viral activity in the biogeochemical cycle will be discussed in detailed below.

The pool of viruses in the ocean is extremely dynamic, with production rates in a day ranging from 10^8 to 10^{11} viruses per liter (Breitbart 2012). Nevertheless, viral abundance is relatively constant on a scale of days to weeks. Thus, new viral progeny must be continuously produced to replace viruses that are destroyed (Wilhelm and Suttle 1999). To obtain the high production rate of viruses in aquatic

systems, a significant lysis of host cells must occur. On the whole, estimates of virus-mediated mortality indicates that approximately 20 % of heterotrophic bacteria; 3 to 5 % of phytoplankton cells (Suttle 1994) and 3 % to 15 % cyanobacteria cells are lost to viral lysis each day (Fuhrman 1999). When the cell bursts upon lysis, progeny viruses plus cellular debris are released. This debris is made up of dissolved molecules (monomer, oligomers and polymers), colloids and cell fragments (Fuhrman 1999). Cellular fragments resulting from viral lysis consist of proteins, carbohydrates, nucleic acid, and other organic nitrogen and phosphorus compounds (Brussaard 2004). The products of viral cell lysis contribute to the dissolved organic matter (DOM) and particulate organic matter (POM) pools (Wommack and Colwell 2000).

Lysed bacterial cells are made bio-available to other bacteria, representing a semi-closed trophic loop. A semi-closed trophic loop will “short-circuit” the flow of energy in food webs. This “short-circuit” phenomenon can be illustrated as viruses diverting the flow of carbon and nutrients from secondary consumers by destroying host cells, with the resulting DOM from the viral lysis effect used as a food source by bacteria (Wilhelm and Suttle 1999). The net effect of this semi-closed loop is called the viral shunt process. The viral shunt is in contrast to consumption of primary producers and heterotrophic prokaryotes by grazers, where it serves as a microbial recycling program, i.e. stimulates nutrients and energy (carbon) cycling (Breitbart 2012). How viruses act as regulators of the carbon cycle and why they are known as catalysts of global nutrient cycles are discussed below.

Carbon can be described as a general tracer of energy flow through biological systems. This is due to all organisms storing energy in the form of chemical bonds within carbon-based complexes (Wilhelm and Suttle 1999). Therefore, it is important to understand the pathways for the supply and recycling of organic carbon in aquatic systems. Viral lysis of primary producers such as algae and cyanobacteria may augment the flux of photosynthetically fixed carbon from phytoplankton biomass into the DOM pool. Viral lysis of bacterioplankton in the Western Gulf of Mexico was recorded to liberate 0.12 to 0.6 $\mu\text{g C/L}$ per day in

offshore waters and 0.72 to 5.2 $\mu\text{g C/L}$ per day in coastal waters (Wilhelm and Suttle 1999). Viral lysis of *Synechococcus* in the Gulf of Mexico was recorded to liberate 0.15 $\mu\text{g C/L}$ per day (Suttle and Chan 1994). Cytoplasmic components from viral lysis will probably enter the dissolve organic carbon (DOC) pool while other structural material from cellular fragments such as lipid bilayers, large proteins and cell walls may be more refractory to biological assimilation and maybe recycled in a manner similar to particulate organic carbon (Wilhelm and Suttle 1999). Virus-mediated cell lysis alters DOC and particulate organic carbon (POC) budget pools in aquatic ecosystems. Much of the DOC is not transferred to higher trophic levels, but, POC could be transferred to higher trophic levels by grazing. The impacts of viral lysis during phytoplankton blooms have been studied by Gobler et al. (1997). In the study, it was shown that viral lysis of a culture of *A. anophagefferens* could increase ambient DOC concentration by 40 μM . This result showed a 160 % increase when compared with uninfected control cultures.

The direct effects of viral lysis on the transfer of carbon through food webs are difficult to quantify, and the alternative way is to model the carbon pathway. Bratbak *et al.* (1992) studied the incorporation of viruses into the budget of microbial carbon transfer. The result suggested that the major difficulty was in balancing bacterial production and bacterial losses due to viral lysis. Fuhrman (1992) designed two contrasting models of carbon flux to investigate the impact of viruses on DOC cycling. The first model was based on the assumption that all bacterial mortality was due to grazing by zooplankton. The second model assumed an equal distribution of mortality between grazers and viruses. These mathematical models indicated that viral lysis resulted in 27 % increase in bacterial production, but a 37 % decrease in export of DOM to nanozooplankton grazers. The model by Wilhelm and Suttle (1999) (Figure 2.2) included viral infection of phytoplankton and bacterioplankton. The results demonstrated that 6-26 % of photosynthetically fixed organic carbon was recycled back to DOM by viral lysis. In addition, the model showed that as much as one- quarter of organic carbon in the sea flows through the viral shunt, which includes carbon in new viruses as well as carbon that is released from host cells during lysis. Miki et al. (2008) developed a new conceptual model.

This model incorporated several aspects such as bacteria- organic matter interactions, lysogenic bacteria, bacterial resistance to viral infection and dependence of protozoan grazing rate on viral infection. The model deduced that the “viral shunt” view is robust, regardless of the specific situations examined in the model analysis. The viral shunt can be defined as viruses which enhance bacterial utilization of organic carbon through regeneration of organic matter but diminishes carbon flow to higher trophic levels (Miki et al. 2008). Thus, viruses are regulators of the carbon cycle in aquatic ecology through the viral shunt process.

Figure 2.2 Model of the influence of viruses on marine carbon cycle. The semi-closed loop and viral shunt activity was demonstrated in dashed arrow (Adapted from Wilhelm and Suttle (1999), Fuhrman (1999) and Breitbart (2002)).

Nutrients, such as inorganic nitrogen and phosphorus (P) are commonly the regulators of primary production. In most freshwater ecosystems, the nutrient regulating the rate of photosynthetic carbon fixation is phosphorus. Virus- driven P is released in freshwater and it has been reported that P- release from virally- lysed bacteria ranges from 122 to 1080 nM per day (Wilhelm and Matteson 2008). Products of viral induced cell lysis are relatively rich in organic nutrients when compared with organic components released extracellularly from phytoplankton (Brussaard 2004). Organisms are composed of more than carbon and lysis of host cells release nucleic and amino acids which are rich in organic nitrogen and phosphorus. Moreover, viral production from host lysis represents an important organic phosphorus pool in seawater (Wilhelm 1995). Therefore, if the virus-bacteria mortality is high, lysis from the host cell is able to increase the pool of dissolved organic matter and limit the flow of nutrients to higher trophic level (Stoddard et al. 2007).

Trace elements, such as iron are known to limit primary production in some aquatic ecosystems. Iron plays a role as an integral component of many enzymes in photosynthesis, electron transport and nutrient acquisition (Wilhelm and Suttle 1999). The study of Gobler et al. (1997) showed that lysis of *A. anophagefferens* increases the concentration of dissolved iron. Moreover, viruses with their small size, make excellent nucleation sites for mineralization of iron (Suttle 2005). Thus, viruses can be known as catalysts of global nutrient cycles, i.e. they accelerate the transformation of nutrient from particulate to dissolved states (Suttle 2005). In short, the consequences of viral lysis in the microbial food web are to increase the bacterial respiration and mineralization of nutrients, reduce the amount of carbon transfer to higher trophic levels and reduce the export of particulate matter to the seafloor (Breitbart et al. 2008).

Other than their role in the microbial food web, viruses also play another profound role in shaping global climate through the release of dimethyl sulfide (DMS) (Hill et al. 1998; Evans et al. 2007). DMS is a volatile compound produced by both prokaryotic and eukaryotic microbes through enzymatic cleavage of

dimethyl sulfoniopropionate (DMSP). DMSP is a chemical signal that is able to reduce grazing, and potentially promote blooms (Strom 2008). DMS is a gas that influences cloud nucleation, increasing cloud cover over the ocean (Strom 2008). Viral lysis causes the release of DMS from host cells. A study on *E. huxleyi* showed that a high level of DMSP inhibited protist grazing and promoted bloom formation, while viruses infecting DMSP- containing blooms enhanced the release of DMS (Fuhrman 1999). Products of cell lysis have other potential geochemical effects, for example, nucleic acids and polymers are released which contribute to 'gel' characteristics that hold aggregates together. One of the direct implications is to facilitate aggregation and sinking of material from the euphotic zone (Proctor and Fuhrman 1991). Empirical evidence for the potential importance of viruses in aggregate formation was given by Peduzzi and Weinbauer (1993). They found that the addition of concentrated natural virus communities were able to stimulate the formation of algal flocs (Fuhrman and Suttle 1993). These potential geochemical effects are due to the chemical and physical properties of the released material and the location where viral lysis occurs in the water column (Fuhrman 1999).

Viruses exert significant control on the bacterial and phytoplankton communities (Fuhrman 1999). Where they act as powerful agents for controlling the bacterial community composition and diversity through horizontal gene transfer and the lysis of specific hosts (Breitbart 2012). Virus and protozoa contribute to bacterial mortality, but their impact on the structure of the bacteria community is different. Omnivorous and heterotrophic protist can graze similar size bacteria. In contrast, phages typically attack specific hosts and influence the bacterial community composition (Breitbart et al. 2008). Virus production is species- specific, significant and mass mortality of one bacterial species potentially leaves room for another species to succeed (Bratbak et al. 1992). As shown above, viral lysis produces nutrients and trace elements and these elements could benefit other non-infected bacteria. The released nutrients from cell lysis acts to fuel new phytoplankton production. Viral lysis of the bloom- forming alga, *Aureococcus anophagefferens* alleviated nutrient limitation in a *diatom* culture (Gobler et al. 1997) and increased bacterial growth rate.

“Kill the winner” (KtW) is the term used to describe the process in which phages specifically lyse the superior competitor among the bacterial (Thingstad and Lignell 1997), thus creating open niches for other bacterial genotypes or stimulating growth of selected members through the organic substrate of lysate (Breitbart et al. 2008). In a lab study demonstrated that initially, heterotrophic bacteria were much lower in abundance than *Synechococcus*. Later, it was shown that *Synechococcus* numbers decreased but the heterotrophic bacteria increased, suggesting that nutrient was released from lysed *Synechococcus* (McDaniel et al. 2006). Through the KtW cycle, the abundance of the actively growing bacterial host was control by phage predation (Breitbart 2012). This may help to control mono-species blooms and increases the microbial diversity. KtW is also a scenario describing the trade-off between resistance (defence specialist) and competition. Bacteria create resistance such as alteration in bacterial membrane proteins or lipopolysaccharide to prevent phage adsorption, but at the same time they may also reduce the cell’s competitiveness, because the receptor molecules are involved in bacterial metabolism. On the other hand, actively growing bacteria are more susceptible to phages (Breitbart et al. 2008). Thus, one cost of phage resistance is reduced physiological capacity. For example, Lennon et al. (2007) showed that there was approximately 20 % reduction in fitness of phage-resistant *Synechococcus* compared with a susceptible strain. This trade-off scenario is describe in a hypothesis: when there is a high level of phage pressure, actively growing bacteria may be rare but are dominated by defence specialist bacteria; when viral pressure is reduce, actively growing bacteria become dominant (Breitbart 2012).

Another main ecological effect of viruses on bacterial population dynamics (bacteria community diversity) is that viruses mediate genetic exchange or transfer. Genetic exchange is via lysogenic conversion and via generalized and specialized transduction (Breitbart et al. 2008). Here, viruses pick up DNA from one host and transfer it to another host (Fuhrman 1999). Phages infecting *Synechococcus* have been found to be capable of enclosing host DNA in their capsid. Thus, cyanophages are potential important horizontal gene transfer agents (Clokier et al. 2003). In

lysogenic infection, viral genetic material of temperate viruses is incorporated into the host genome, and stay in dormant stage (provirus or prophage) for generations until triggered or induced by some external factor to enter into the lysic cycle (i.e. lyses the cell and produce new viruses). During the proviral stage, lysogenic conversion occurs, i.e. the viral gene is incorporated into the host genome that can change the behavior and properties of the host cells, e.g. change the metabolic capabilities and immunological properties (Breitbart et al. 2008). Viruses also play a role in bacterial evolution. This role can be achieved by negative and positive interactions. Negative interaction is when viral infection- mediates mortality and produces selective pressure. Positive interaction is when viral conversion through viral gene codes provides for new host capabilities (Fuhrman and Schwalbach 2003).

In conclusion, viruses play critical roles in the structure and function of aquatic food webs. Moreover, they regulate the biodiversities of the microbial community in aquatic ecosystems. Cyanophages are mortality agents of cyanobacteria and play important roles in cyanobacteria evolution, as well as co-evolution between cyanophage and host (will be discussed at below sections). Thus cyanophages are believed to be an important player in aquatic ecology.

2.4 ROLE OF CYANOPHAGE ON CYANOBACTERIAL COMMUNITY

Cyanophage affects cyanobacterial diversity in various ways: first, cyanophages act as pathogens for cyanobacteria and help to maintain the host community diversity; second, cyanophages affect the host diversity by promoting the emergence of viral resistance and leading to antagonistic co-evolution; and third, cyanophages contribute genetic material to their hosts through horizontal gene transfer.

Cyanophages have been found to affect the most successful host density (superior competitor) in order to maintain the host community diversity.

Cyanophage infection was found to terminate cyanobacteria blooms (Manage et al. 1999; Yoshida et al. 2008), consequently influencing the composition of the phytoplankton species and cyanobacteria population. Yoshida and colleagues (2008) reported that an increase in phage abundance was observed when *M. aeruginosa* numbers declined. Another piece of evidence for the influence of cyanophages on the mass mortality of cyanobacteria was presented by van Hannen et al. (1999). Two laboratory-scaled enclosures with 131 liters of lake water (from eutrophic Lake Loosdrecht) were monitored, and found to have almost-complete lysis of cyanobacterial populations at day 10, together with increases in viral concentration. Tucker and Pollard (2005) found that the density of cyanophages was positively correlated with the rate of *M. aeruginosa* cell lysis, leading to a selective sweep of cyanobacteria species and keeping competitive dominants in check (Tucker and Pollard 2005). These studies clearly demonstrated the ability of cyanophages to kill specific host genotypes and maintain host community diversity.

Cyanophages in surface waters, either as mortality agents to cyanobacteria or as biological control of cyanobacteria community composition, lead to the development and maintenance of host-cell resistance in cyanobacteria population. The mortality rate of *Synechococcus* due to cyanophages varies greatly, with estimates from 0.005 % to 28 % (Stoddard et al. 2007). A few studies have suggested the resistance of cyanobacteria to co- occurring cyanophages at high titer values (Waterbury and Valois 1993; Stoddard et al. 2007). This leads to the complex ecology between cyanophages and cyanobacteria. Resistance to the phage is depending on the host phenotype, e.g. adsorption of the obligate A-1 phage failed when the O- antigen component was altered (Mann 2006). In the research study on cyanophage resistance in marine *Synechococcus* by Stoddard et al. (2007), it was reported that the phenotype changes were stable through several transfers in batch culture, suggesting that the resistance is due to genetic mutation. On the other hand, the phage- resistant *Synechococcus* could be reverted to a phage sensitive state after 2 years of culture in the absence of phages (Waterbury and Valois 1993). This phage- resistance may not be due to genetic mutation as it is reversible. A detailed study on viral resistance in *Synechococcus* revealed a mutation point in the glucose-

1-phosphate thymidyl- transferase gene in *Synechococcus*. This mutation could regulate proteins exposed outside the cell wall and subsequently, could affect the phage attachment to the host (Marston et al. 2012).

Yet, the potential of virus- resistant bacteria becoming prevalent sets up a paradox in the phage- host interaction. If virus- resistant bacteria is prevalent, what prevents resistant bacteria from outcompeting sensitive bacteria and driving viruses extinct, thereby allowing coexistence of both sensitive and resistant bacteria? Therefore, research suggests that virus resistance should be accompanied with an evaluation of fitness/selection cost (Lennon et al. 2007), as discussed at Section 2.3.

Cyanophage infection affects the host cell physiology and tweaks the host metabolism. Ultrastructural changes in unicellular and filamentous cyanobacteria after infection were recorded where the infection of filamentous cyanobacteria resulted in the displacement of the photosynthetic lamella. In contrast, viral synthesis in the unicellular cyanobacteria showed no obvious changes in the thylakoids (Suttle 2000a). One of the key features of the host physiology affected by phage infection is nutrient transport (Mann 2006). As shown by AS-1 infection, the infected host cell's normal control over its response to energy starvation has been lost. Phage replication needs nucleotides and amino acids. Strategies to obtain the required nucleotides are to degrade the host nucleic acids, and shut off the host protein synthesis (Mann 2006). These strategies can be achieved by AS-1 via increasing DNase and RNase activities during infection; on the other hand, P60 modifies the host cell's biosynthesis of nucleotides via encoded enzymes involved in nucleotide biosynthesis, such as ribonucleotide reductase and thymidylate synthase (Chen and Lu 2002). For effects on photosynthesis and respiration, *Nostoc muscorum* (filamentous cyanobacteria) infected by N-1, resulted in carbon dioxide decline and reduced ferredoxin: NADP⁺ oxidoreductase activity and these changes all affect the photosynthetic activity of host. In contrast, respiratory oxygen consumption is not affected (Mann 2006).

Cyanophages are a major force in cyanobacterial evolution (Suttle 2000b) and in shaping the evolution of key metabolic processes of cyanobacteria (Zeidner et al. 2005). They potentially influence the genetic diversity of cyanobacterial communities through selection pressure and virus-mediated genetic exchange such as lysogenic conversion. Detailed studies have been carried out on the host range and it has been found that some cyanophages have a broad range of host (Suttle 2000b; Sullivan et al. 2003; Jenkins and Hayes 2006). For example, Sullivan et al. (2003) found that some cyanophages isolated from *Synechococcus* host strains were able to cross-infect low-light-adapted *Prochlorococcus* strains. A study by Deng and Hayes (2008) reported that some cyanophage isolates are able to infect hosts from more than one genus. Consequently, cyanophage may have the potential to move genes between lineages within cyanobacteria populations and provide a mechanism for the genetic information transfer between evolutionarily distant cyanobacteria lineages (Deng and Hayes 2008) and in promoting changes in the genetic structure of the host population (Jenkins and Hayes 2006).

A study was conducted on the Ma-LMM01 phage (specifically infecting *Microcystis aeruginosa*), where an insertion sequence (IS) element named IS607-cp, encoded by Ma-LMM01 phage showed high nucleotide similarity with a partial sequence in *Cynotheca* sp. and a few strains of the *M. aeruginosa* genome. IS elements are known to lead to genomic rearrangements, thus driving chromosomal diversification in some prokaryotes species and bringing plasticity into the genome. Ma-LMM01-type phages were believed as donors of IS elements, i.e. Ma-LMM01 transfer the IS607-cp into *M. aeruginosa* genome, then contribute to the plasticity of the *M. aeruginosa* genomes (Kuno et al. 2010). More detailed studies on the role of cyanophages in the vast gene pool of cyanobacterial communities and co-evolved with their hosts through horizontal gene transfer (HGT) (Kuno et al. 2010) will be discussed below (Section 2.4.1).

Cyanophage (e.g. Ma-LMM01) genomes code for a homologue of a specific recombinase and may exhibit a prophage state in some hosts (Yoshida et al. 2008).

Temperate¹ phages at the prophage² state can alter the phenotype of the infectious host via the phage conversion phenomena, meaning that there is a temporary phenotypic alteration in the phenotype of the host. Temperate prophage was found could enhances the nitrogenase activity of host cells (Suttle 2000a). Lysogeny seem to be the survival strategy during the times of low host abundance and productivity (Breitbart 2012).

In summary, there is sufficient evidence to show that cyanophages take part in influencing the occurrence and diversity of cyanobacterial community, as well as evolution of cyanobacteria.

2.4.1 Cyanophage encoded bacterial genes

Phage- encoded bacterial genes, or more recently often referred to as auxiliary metabolic genes (AMGs) (Clokier et al. 2011) are the latest finding in cyanophage research. These add another layer of complexity, leading to reciprocal genome evolution through a dynamic co-evolution process. Cyanobacteria are oxygenic photoautotrophs, therefore impacts of phage infection are on the host's photosynthetic metabolism. Conversely, the photosynthesis impacts on phage replication are also of interest in investigating this phage- host relationship. The photosynthesis genes encoded by cyanophages include the *psbA/psbD* gene- D1 and D2 protein; *petE* and *pet F* gene- photosynthetic electron transport; *hli* gene- high light inducible proteins (Clokier and Mann 2006); and *nbla* gene, essential for the degradation of phycobilisome (Yoshida et al. 2008). This observation raises a question: what is the likely fitness^{3,4} benefit for cyanophages that possess these genes?

¹ Temperate phage: Bacteriophages that can infect bacteria and establish a lysogenic relationship rather than immediately lysing their host (Goldman, 2008).

² Prophage: The form of the genome of a lysogenic bacteriophage integrated in the host cell chromosome (Wagner and Hewlett, 2004).

³ Fitness: is a central concept in evolutionary theory. It describes the capability of an individual of certain genotype to reproduce, and usually is equal to the proportion of the individual's genes in all the genes of the next generation ([http://en.wikipedia.org/wiki/Fitness_\(biology\)](http://en.wikipedia.org/wiki/Fitness_(biology))).

Photoinhibition is a phenomenon where photosynthesis is unable to continue due to the D1 protein in the photosystem II (PSII) repair cycle not being in a normal condition, i.e. the D1 inactivation and damage rate due to solar effect is higher than D1 degradation and concomitant replacement rate (Bailey et al. 2004). D1 and D2 are hetero-dimers in the heart of PSII, where D1 and D2 comprise the *psbA* and *psbD* gene. Infection of cyanophages has led some cyanobacteria to rapidly reduce or eliminate the expression of host gene following infection, destroying the structural protein for photosynthesis such as the D1 protein and leading to photoinhibition (Lindell et al. 2005). Yet, surprisingly, photosynthesis was found to still occur even after the decline of bacteria's D1 protein after infection. Photoinhibition has been reported to destroy cyanophages in two ways. First, production of radicals and toxic oxygen species can lead to direct damage. The second way is the starvation of energy for phage replication due to photosynthesis inhibition (Mann 2006). Unicellular cyanobacteria phages have a long latent⁵ period. During the latent period, the energy source and carbon source produced by cyanobacteria through photosynthesis are needed. Therefore, it is very important to sustain photosynthesis even after infection to maximize cyanophage production. With the finding of *Synechococcus* phage, S-PM2, it was found that the genome possesses the *psbA* and *psbD* homolog genes, which encodes for the D1 and D2 polypeptide for photosynthesis (Bailey et al. 2004). Therefore, researchers have hypothesized that cyanophages evolved to solve the photoinhibition problem with encoded proteins that play a pivotal role in host photosynthesis.

Lindell *et al.* (2005) recorded that phage *psbA* and *hli* genes are expressed during infection. Although the host photosynthesis gene declines, subsequently the amount of phage D1 protein increases steadily concomitant with a decrease in host D1 protein. These results suggest that the phage gene is functional in photosynthesis and serves to boost photosynthetic performance of the cyanobacteria during

⁴ Fitness: To describe an organism's vigor, or the degree to which organisms "fit" into their environments (Sober, 2001).

⁵ Latent Period: The initial phase in the one- step- growth experiment in which no phages are released (Goldman, 2008).

infection. This finding coincides with Zeidner et al. (2005), where the evidence of *psbA* is likely to be functional- conservation of phage amino sequences through purifying selection (Zeidner et al. 2005). In addition, Lindell et al. (2007) has done a genome- wide expression study between *Prochlorococcus* and T7- like cyanophage P-SSPS. They reported that the phage genes- *psbA*, *hli*, *talc* (transaldose gene) and *nrd* (nucleotide reductase gene) genes are co- transcribed with phage DNA replication genes. This observation suggests these reactions make up a functional unit involving energy and deoxynucleotide production for phage replication in a resource- limited environment. These findings collectively show a striking example of the interaction of proteins encoded from two distinct genomes but function together in a single metabolic complex.

It has been suggested that increasing phage fitness by supplementing the host production of this rapidly degraded protein D1 can lead to a more efficient and/or prolonged production of viral particles (Sullivan et al. 2006). *PsbA* and *psbD* genes are considered to provide fitness advantage to cyanophage. Thus, it is expected to be an important gene among the cyanophages requiring an energy source from photosynthesis. In the occurrence study by Sullivan et al. (2006), it was showed that *psbA* is a common gene for marine cyanophages (*Synechococcus* and *Prochlorococcus* as host) but is not universal, where 88% of cyanophages from 33 samples bear this gene. In contrast, *psbD* gene showed a smaller percentage (50%) among the isolated samples and the *psbD* gene was only found in isolated cyanomyoviruses containing the *psbA* gene, although not in all. The study subsequently showed that the *psbD* gene widespread among the cyanomyoviruses has broad- host- range, i.e. 17 of 18 broad- host- range bear this gene and just 1 of 21 narrow- host- range phages do so.

Sullivan et al. (2006) suggested that the phage encoded photosynthesis gene is related to the latent period: too short a latent period (1h) is not beneficial for *psbA* expression and thus P60- *Synechococcus* podovirus lacks the *psbA* gene. Another unicellular cyanobacteria phage, AS-1, does not have the phage- encoded photosynthesis gene, yet AS-1 can produce energy from photophosphorylation but

at a reduced level. Ma-LMM01 has a latent period which is as long as other cyanophages having the *psbA* gene (6-8h), but Ma-LMM01 lacks this gene. However, this cyanophage encodes a homologue of the *nblA* genes of cyanobacteria and red algae. This gene is proposed to function to reduce the absorption of excess light energy via degradation of the phycobilisomes (Yoshida et al. 2008). In cyanobacteria *nblA* binds to the key structure of phycobilisomes (PBS) and triggers the PBS degradation during nitrogen starvation. The PBS degradation helps to prevent photodamage caused by the adsorption of excess light energy. Moreover, PBS constitutes up to 50 % of the total soluble protein, thus degradation of PBS provides the substrates for protein synthesis during the acclimation process (Yoshida et al. 2008). Yoshida and colleagues (2008) proposed that the *nblA* encoded by cyanophages was expressed with this *nblA* gene and functions as the host gene, where photodamage can be prevented during phage replication and the degraded PBS becomes important amino acids for phage protein synthesis. In short, cyanophages use diverse genetic strategies to control their host's photosynthesis with the same purpose, i.e. to make the phage replication cycle continue.

Other than photosynthesis genes, cyanophages have encoded other bacterial genes (these genes are thought to respond to harsh environments) which enable the cyanophages to replicate when in stressed environments, including the *MazG*/*SpoT*- pyrophosphohydrolase (Clokier and Mann 2006; Dreher et al. 2011); *talc*-transadolase gene (Sullivan et al. 2005), and *phoH* – phosphate inducible gene (Mann 2006). *MazG* was shown to hydrolyse guanosine 3',5' bispyrophosphate (ppGpp). The ppGpp is a global regulator of gene expression in bacteria and is responsible for adapting to stressed conditions, such as nutrient starvation (Clokier and Mann 2006). To make phage replicate in bacteria, it may be necessary to modify the physiological state of the cell and optimize the physiological condition for progeny phage production. In a nutrient starved environment, the *MazG* gene encoded by phages may reduce the ppGpp pool during infection to make the condition of host cell mimic the state where the cell is replete with nutrients (Clokier and Mann 2006). The *MazG* gene is also suggested to prevent stationary phase in the host cell (Dreher et al. 2011). Thus, it is suggested that while the host activates in

the pathways of macromolecular synthesis, the phages continue to optimize and modify the process (Mann and Clokie 2012). The second full genome sequence of a cyanophage (S-CRM01) which infect freshwater cyanobacteria (*Synechococcus*) was reported to encode the SpoT gene. SpoT pyrophosphatase is able to remove alarmone (an intracellular signal molecule that is produced in harsh environments) guanosine pentaphosphate ((p)ppGpp) during amino acid starvation (Dreher et al. 2011). Thus, *MazG* and *SpoT* genes eliminate the stress triggers on the host cell, subsequently allowing phage replication. Besides the SpoT gene, S-CRM01 was found to encode another *E. coli* gene, i.e. NusG gene (Dreher et al. 2011). This viral NusG may benefit S-CRM01 by influencing the elongation and termination phases of transcription of phage and host genome.

The carbon metabolism gene, *talc* gene, has been revealed in *Synechococcus* cyanophage S-RSM2 and *Prochlorococcus* cyanophages, P-SSM2 and P-SMM4. The *talc* gene codes for key enzymes in the non-oxidative branch of the pentose phosphate pathway in *Escherichia coli* (Sullivan et al. 2005). The non-oxidative branch in cyanobacteria oxidizes glucose to produce ribulose-5-phosphate for nucleotide and amino acid. If this phage-encoded gene is expressed and functions in cyanobacteria, it could facilitate alternative routes of carbon metabolism during infection and metabolize carbon substrate for phage biosynthesis and as a source of phage replication energy (Sullivan et al. 2005). Other than the *talc* gene, glucose-6-phosphate dehydrogenase (*zwf*) and 6-phosphogluconate dehydrogenase (*gnd*) (which are genes encoding enzymes found in the pentose-phosphate pathway) are found widespread in marine cyanomyoviruses (Mann and Clokie 2012). The functions of these genes in cyanophages have not been experimentally determined, but it is suggested that these genes are involved in stress response and mobilization of carbon stores during infection through optimization of NADH and ATP necessary for phage replication (Clokie et al. 2011).

In summary, the role of phage encoded photosynthesis genes that maintain or optimize the photosynthetic efficiency of the cyanobacteria host and other genes used to optimize the physiology of cyanobacteria and efficiency of phage genome

transcription, all have the same purpose, i.e. to enable cyanophages to have enough energy to replicate and survive.

2.5 ISOLATION AND CHARACTERIZATION OF CYANOPHAGES

The isolation of cyanophages which can infect different types of cyanobacteria brings the study of phage-host systems to a whole new dimension. Since 1963 when the first cyanophage was isolated by Safferman and Morris, more and more cyanophages have been isolated (Adolph and Haselkorn 1971; Franche 1987; Wilson et al. 1993; Okunishi et al. 2002; Yoshida et al. 2006). These isolates were used to discover the novel interaction between cyanophages and their hosts, particularly in the presence of light (Clokier and Mann 2006). Moreover, the cyanophage isolates and hosts make excellent models in the study of coevolutionary processes of viruses and hosts. Results from these studies have shown the existence of phage-encoded bacterial genes and this leads to new insights into the coevolution process between bacteria and phages (Sullivan et al. 2005; Zeidner 2005; Sullivan 2006; Wang and Chen 2008).

Genomic characterization of these isolated phages have shed new light on the conserved gene of cyanophages (Fuller et al. 1998; Takashima et al. 2007; Chen et al. 2009). A number of genetic probes have since been developed, for example: genetic probes targeting the major capsid protein (Baker et al. 2006; Jenkins and Hayes 2006); portal protein (Fuller et al. 1998; Zhong et al. 2002); sheath protein (Takashima et al. 2007) and DNA polymerase of cyanopodoviruses (Chen et al. 2009). These probes are used to investigate the abundance and diversity of cyanophages in natural communities, as well as to understand the relationship between phage and host in a natural system. Several studies have shown high genetic diversity in spatial and temporal variations (including freshwater, marine and floodwater systems) of cyanophage assemblages, suggesting that cyanophages respond to changes in the composition of the cyanobacteria community (Zhong et al. 2002; Marston and Sallee 2003; Chen et al. 2009; Wang et al. 2010). Moreover, the

sheath protein probe has been used in an ecological study to reveal that cyanophage dynamics may cause shifts in toxin producing and non-toxin producing cyanobacteria populations (Yoshida et al. 2008). It is only through continuing isolation and purification of cyanophages that these important functions can be elucidated (Millard 2009).

The isolation process comprises of isolation and characterization. There are three approaches used in cyanophage isolation, namely plaque assay, liquid bioassay and liquid enrichment assay (Middelboe et al. 2010) . Two main factors influencing the method selection for isolation are the natural abundance of infectious cyanophage and the ability of host to grow in a solid medium (Suttle 2001; Wilhelm and Poorvin 2001). These isolated cyanophages have been characterized based on their morphology (Smith et al. 1967; Hu et al. 1981; Kuznetsov et al. 2010), genetic analysis (Wilson et al. 1993; Yoshida et al. 2006), growth characteristics (Suttle and Chan 1993; Middelboe et al. 2010) as well as host range or cross infectivity (Jenkins and Hayes 2006) .

2.5.1 Isolation: plaque assay, liquid bioassay and liquid enrichment assay

The plaque assay, which was first described by Felix d'Herelle, has become a tool for the study of bacteriophage since 1917 (Emanuel 2008). Subsequently, this technique was refined from the monolayer plaque assay to double layer plaque assay (Mocé-Llivina et al. 2004). The double layer plaque assay is also known as the double agar overlay method or soft agar overlay method. This method was first described by Gratia in 1936 and then formalized by Mark Adams in the year 1959 (Adams 1959).

In 1963, the first cyanophage was isolated through the plaque assay (Safferman and Morris 1963). Since then, the plaque assay has been successfully used to detect and isolate cyanophages from freshwater (Kim and Choi 1994; Deng

and Hayes 2008) and marine environments (Suttle and Chan 1993; Waterbury and Valois 1993; Sullivan et al. 2003; Jenkins and Hayes 2006). The agar environment was suggested to be a spatially structured environment. This is because such an environment significantly limits the movements of phage and bacteria, resulting in a localized expansion of the plaque (Abedon and Yin 2009).

One of the advantages of plaque assay is that the presence of phages can be identified easily from the pigmented lawns (Middelboe et al. 2010). In addition, target viruses are also easier to purify and clone, as serial dilution is not necessary with the plaque assay since a plaque is a single infection event (Millard 2009; Middelboe et al. 2010). The disadvantages of the plaque assay are that the hosts cell must be culturable and provide a confluent lawn on the solid media (Wilhelm and Poorvin 2001; Middelboe et al. 2010). In addition, plaque formation and plaque size are affected by an array of factors (Karin 2004; Abedon and Yin 2009) which will be discussed below.

The attachment of a single phage particle onto a susceptible host will result in successful infection for viable virus to multiply, resulting in the lysis of the host, and the release of phage progeny (Millard, 2009) and plaque formation. To form a plaque, the first step is the initial adsorption of phage to host. Factors affecting initial adsorption are the initial density of bacteria, phage adsorption rate (Abedon and Yin 2009) and host physiological condition (Brussaard 2004). The growth of the plaque is influenced by the concentration of the top agar layer (affecting the diffusion of phage), thickness of bottom and top agar layers, phage growth characteristics (latent period and burst size), lawn density of bacteria (Karin 2004; Abedon and Yin 2009) and the nutrients in the medium (Mole et al. 1997). The last phase of plaque development is when the viral multiplication ceases. The phage growth ceases when the growth of the host cell stops. This is because it is the host cell's metabolism that produces the energy needed for viral multiplication (Karin 2004; Millard and Mann 2006).

Liquid bioassay is another approach for cyanophage isolation. This approach has been used in several studies to isolate and detect cyanophages (Suttle and Chan 1993; Okunishi et al. 2002; Wilhelm et al. 2006; Yoshida et al. 2006). The assay can be carried out in media with volumes from microliters (using multiwell plates) up to hundreds of milliliters. Liquid bioassay is also known as well assays when multiwell or microtiter plates are used to do the isolation (Millard 2009). Multiwell plates or microtiter plates (consisting of 24 or 96 wells) assay are suitable for the screening of many environmental samples. The assay requires less culture volume and incubation space (Middelboe et al. 2010) and the dilution steps are expandable easily (Wilhelm and Poorvin 2001). However, the disadvantage of the well assay is that there is a decrease in sensitivity (minimum detection limits) as less samples are used for screening (Wilhelm and Poorvin 2001).

In principle of liquid bioassay, environmental water sample is added to the target host and incubated under suitable conditions and monitored over time for signs of infection or lysis (Millard 2009; Middelboe et al. 2010). Signs of lysis can be observed by eye and are detected when there are signs that show a clearing in the treated culture or decrease in pigmentation of the culture compared to the control culture (Middelboe et al. 2010). Lysis can also be observed when there are changes in cyanobacteria biomass (chlorophyll *a*) of the treated culture compared to the control culture (Monegue and Philips 1991). Samples with lysis effect will be centrifuged (to remove lysate) and serially diluted for a further round of liquid bioassay. It is important to produce series dilution to extinction, in order to ensure only clonal or a single phage is isolated (Millard 2009). In addition, it is important for the phage diluents or the bacteria culture medium used for preparing the serial dilutions to be isotonic to prevent phage rupture due to osmotic shock (Karin 2004).

Liquid bioassay is simple and inexpensive; the host need not be axenic and can be used for all aquatic cyanobacteria (Middelboe et al. 2010). In addition, by using liquid bioassay, the resulting progeny phages are not restricted in movement by the agar, enabling complete lysis of the entire culture (Millard, 2009). The disadvantage of this assay is that extinction dilution is necessary to obtain a single

isolate (Millard 2009). Liquid enrichment assay is suitable for regular qualitative monitoring of the presence-absence of particular phages because it is simple, inexpensive and has high sensitivity (Grabow 2001). Liquid enrichment assay generally is a more efficient way of isolating cyanophages from the low titer in natural environments (Middelboe et al. 2010).

Enrichment culture is based on adding or enriching nutrients and target bacterium into a pre-filtered water sample. This mixture is then incubated for a time period (as determined by the growth characteristics of the host), to allow any lytic phages present in the sample to propagate (Grabow 2001). After incubating for a period of time, the lytic phages present in the sample that have propagated to a high titer are ready to be isolated by plaque assay (Grabow 2001) or to continue propagating several times to dilute out non-replicating viruses (Middelboe et al. 2010).

Liquid enrichment assay allows larger volumes of sample (up to 500 ml) to be investigated, thus enabling the isolation of rare phages (Grabow 2001; Middelboe et al. 2010). It also allows the combination of different target hosts (different strains) to be in the same incubation, enabling phage isolates to infect one or both strain(s) of host (Middelboe et al. 2010). There are two main disadvantages of this assay: 1) extreme sensitivity (rich in nutrients) and thus vulnerable to contamination with phages from the environmental water sample (Grabow 2001); 2) similar to the liquid bioassay, end-point dilution (dilute to extinction) steps are required to obtain pure clonal isolates (Middelboe et al. 2010).

2.5.2 Characterization: morphology, physiochemical characteristics, host range, growth characteristics and genetic analysis

In 1965, a virus classification committee was founded (P.C.N.V. 1965) and then developed into the International Committee of Taxonomy of Viruses (ICTV). To date, ICTV uses any of the characteristics of about seventy properties (nucleic

acid, morphology, host range, etc) for classification. For example: nucleic acid (e.g., DNA/ RNA, linear/ circular, genome map) and gross morphology (e.g., shape, presence of envelope, capsid symmetry) to determine the order and family appurtenance of a virus, while host range, physiochemical test (e.g. molecular weight, G+C content), inactivation test, restriction endonuclease digestion pattern and physiology test (e.g. adsorption rate, burst size), are identifiers for species and strains (Ackermann 2009). Below are the properties that are most frequently used to characterize cyanophage isolates.

2.5.2.1 Morphology

The morphology of bacteriophage isolates is determined by an electron microscope, mainly by the transmission electron microscope (TEM). TEM with the best resolution of 0.5 nm is used to study the structure or morphology of viruses, bacteria gas vacuoles, prokaryotic flagella, shape of organelle within microorganism and DNA (Willey et al. 2008). TEM can present internal and external virus structures in two-dimensions (Biel and Gelderblom 2004). In cyanophage isolation studies, TEM is used to determine the isolated cyanophage morphology (Suttle and Chan 1993; Chen and Lu 2002; Jenkins and Hayes 2006; McDaniel et al. 2006) and the thin section images of infected cyanobacteria cells. The thin section images reveal the the changes of cyanobacteria organelles and morphological aspects of the cyanophages during the infective process (Smith et al. 1967; Mackenzie and Haselkorn 1972; Okunishi et al. 2002; Yoshida et al. 2008).

Based on the cyanophage isolates morphology studies by using TEM, all cyanophages characterized so far fall under the caudovirales order (tailed phage) and are under three families: *Myoviridae*, *Podoviridae* and *Siphoviridae* (Mann 2006). Table 2.1 shows the dimensions of cyanophage isolates from different environments.

Scanning electron microscope (SEM) was seldom used to study the morphology of biological specimens before 1990s because it offered a much lower

resolution than TEM. However, it later became a powerful tool for biological specimen research with the improvement of the instrument's resolution and advances in specimen preparation techniques. For example: a new specimen preparation method, called osmium-dimethylsulfoxide (DMSO)-osmium method was introduced and this method is effective in revealing intracellular structures (Tanaka 1989). In addition, an ultra-high resolution SEM (UHS-T1) with a resolution of 0.5 nm on a biological specimen coated with platinum was developed in 1985. SEM had been proved as a simple method to enumerate T4 bacteriophages. At the same time, the phage images obtained from SEM are similar to TEM images by other investigators (Modla et al. 2008). SEM, however, has still not yet been used for cyanophages.

Table 2.1 Morphology of cyanophage isolates

Strain	Family	Host For Isolation	Area of Isolation	Morphology (nm)		References
				Head diameter	Tail length	
N-BM1	<i>Myoviridae</i>	<i>Nodularia spumigena</i>	Swedish coastal waters	80 x 80	133	Jenkins and Hayes (2006)
N-BM2	<i>Myoviridae</i>			79 x 118	126	
N-BS1	<i>Siphoviridae</i>			137 x 120	834	
N-BS2	<i>Siphoviridae</i>			53 x 53	245	
N-BS3	<i>Siphoviridae</i>			54 x 60	188	
N-BS4	<i>Siphoviridae</i>			127 x 122	888	
N-1L	N.A	<i>Nostoc</i>	Domestic sewage, Kuwait	70	130	Sallal et al. (1987)
N-2S		<i>muscorum</i>		50	20	
N(S)1	<i>Podoviridae</i>	<i>Nostoc</i>	Tropical soil, Australia	50	10	Frache (1987)
N-1	N.A	<i>Nostoc muscorum</i>		61.4	100	Adolph and Haselkorn (1973)
AN-10	N.A	<i>Anabaena</i> and <i>Nostoc</i>	Sewage settling pond, Michigan	60	108	Hu et al. (1981)
AN-15		<i>Anabaena</i> and <i>Nostoc</i>		63	100	
S-BM1	<i>Myoviridae</i>	<i>Synechococ cus</i> (WH7803)	Coastal Water, Bermuda	88	57	Wilson et al. (1993b)
S-BS1	<i>Siphoviridae</i>			90	165	
S-PM1	<i>Myoviridae</i>		Coastal Water, Plymouth , UK	88	57	
S-PS1	<i>Siphoviridae</i>			90	165	
S-WHM 1	<i>Myoviridae</i>		Coastal Water, Wood Hole, Mass	88	108	
S-BBS1	<i>Siphoviridae</i>	<i>Synechococ cus</i> (BBC1 G)	Gulf of Mexica	50	230	Suttle and Chan (1993)
S-BBP1	<i>Podoviridae</i>	<i>Synechococ cus</i>		50	-	

Table 2.1 Morphology of cyanophage isolates (continued)

S-PWP1	<i>Podoviridae</i>	(BBC2_G) <i>Synechococcus</i>		65	-	
S-KM1	<i>Myoviridae</i>	(SNC1_G) <i>Synechococcus</i> (KFM001)	Kagoshima Bay, Japan	83	166	Okunishi et al. (2002)
S-CRM01	<i>Myoviridae</i>	<i>Synechococcus</i> (LC16)	Klamath River, Northern California	85-100	140-170	Dreher et al. (2011)
S-CBS1	<i>Siphoviridae</i>	<i>Synechococcus</i> (CB0201)	Chesapeake Bay water	55	80	Huang et al. (2012)
S-CBS2	<i>Siphoviridae</i>	<i>Synechococcus</i> (CB0204)		50 x 90	170	
S-CBS3	<i>Siphoviridae</i>	<i>Synechococcus</i> (CB0202)		55	80	
S-CBS4	<i>Siphoviridae</i>	<i>Synechococcus</i> (CB0101)		72	200	
SM-2	N.A	<i>M. aeruginosa</i> NRC-1	Twin Lakes (a flood control reservoir)	50- 55	130-140	Fox et al. (1976)
Ma-LBP _a	<i>Podoviridae</i>	<i>M. aeruginosa</i> (isolate)	Lake Baroon, Australia	42	-	Tucker and Pollard (2005)
Ma-LBP _b	<i>Podoviridae</i>	<i>M. aeruginosa</i> (isolate)		52	-	
Ma-LMM01	<i>Myoviridae</i>	<i>M. aeruginosa</i> (NIES298)	Lake Mikata in Fukui, Japan	86	90	Yoshida et al. (2006)
Ma-LMM02	<i>Myoviridae</i>	<i>M. aeruginosa</i> (NIES298)	Lake Mikata in Fukui, Japan	96	90 (contracted sheath)	Takashima et al. (2007)

Table 2.1 Morphology of cyanophage isolates (continued)

Ma-LMM03	<i>Myoviridae</i>		97	223 (elongated sheath)
				88 (contracted sheath)
Ma-HPM05	<i>Myoviridae</i>	Hirosawa noike Pond, Japan	97	227 (elongated sheath)
				88 (contracted sheath)
				227 (elongated sheath)

N.A: No assigned

2.5.2.2 Physicochemical characteristics

Physicochemical parameters are divided into two categories. They are, physicochemical properties of cyanophage particles and factors influencing cyanophage stability (inactivation).

In nature, viruses contain either double-stranded DNA (dsDNA), single-stranded DNA (ssDNA), double-stranded RNA (dsRNA) or single-stranded RNA (ssRNA). Vast majority of viruses contain dsDNA, while viruses with dsRNA are very rare (Ackermann 2009). All cyanophages contain dsDNA, (Mann 2006), which are shown by the cyanophage isolates' morphology as all caudovirales contain dsDNA. Physicochemical properties of cyanophage particles such as molecular weight of major protein, guanine plus cytosine (G+C) content, buoyant density in CsCl are important to understand the uniqueness of cyanophage isolates (Padan and Shilo 1973). Major protein molecular weight is observed through protein analysis and is conducted using the SDS-polyacrylamide gel electrophoresis (PAGE) system, which shows the polypeptides profile of each isolate. The major protein molecular weight is obtained from the protein profile which shows the protein mass location (Adolph and Haselkorn 1971; Benson and Martin 1984; Wilson et al. 1993). Wilson et al. (1993a) revealed that isolates belonging to cyanomyoviridae and cyanosiphoviridae have a clear difference in polypeptide profile. Whole virus and DNA buoyant density of cyanophage isolates are determined by linear 10-40 % sucrose gradient and stepwise CsCl gradient respectively. The G+C content in the DNA can be calculated by determining its density in CsCl using the equation of DeLey and Kropinski, or through ultraviolet spectral analysis (Benson and Martin 1984). Another physicochemical property of cyanophages is genome size. The genome size of isolates can be determined by pulsed- field gel electrophoresis (PFGE) (Millard and Mann 2006; Takashima et al. 2007). 87 cyanophages have been isolated from *Synechococcus* through plaque assay, PCR (myoviral- specific primers) and TEM results identified these isolates as cyanomyoviruses. 67 of these 87 genome size of cyanophages were determined by PFGE and the genome sizes ranged from 150 kb to 210 kb (Millard and Mann

2006). These physicochemical properties enable differentiation of isolates with similar morphology.

Physicochemical parameters such as temperature, pH, and cation influence the stability of cyanophage structure and infectivity ability. An inactivation test is conducted on isolates to investigate the influence of these physicochemical parameters, including the effect of temperature and pH (Fox et al. 1976; Franche 1987; Sallal et al. 1987). For the effect of temperature, the samples are incubated at a temperature between 30 °C and 75 °C for different time periods (e.g., 5 min, 30 min and 1hr). To test the effect of pH, the isolates' diluents are adjusted to different pH. The phage titers are enumerated by plaque assay to investigate the stability of cyanophages under different pH conditions. These studies have shown that different isolates possess different thermal inactivation, e.g., N-1L is more sensitive to temperature compared to N-2S and N(S)1, where N-1L is inactive at 55 °C, while N-2S is inactive at 60 °C and N(S)1 at 75 °C. The isolates also have revealed different pH inactivation: N(S)1 is less sensitive compared to N-1L and N-2S and it is stable between pH 5 to pH 11, while both N-1L and N-2S show complete inactivation at pH 10 (Franche 1987; Sallal et al. 1987).

Cation requirement of cyanophages SM-1, SM-2, AS-1 (Benson and Martin 1984) and AN-15 (Mole et al. 1997) are determined by dialyzing against distilled and deionized water, divalent cation (Mg^{2+} or Mn^{2+} or Ca^{2+}) solution and monovalent cation (Na^{+}) solution. Benson and Martin (1984) demonstrated that SM-1, SM-2 and AS-1 require cations for stability. In that study, cyanophage SM-2 also showed loss of all infectivity after incubation in distilled and deionized water, and divalent cations conferred greater viral stability than monovalent cations. Mole et al., (1997) found that cyanophage AN-15 require either 1 mM calcium ion (Ca^{2+}) or 1 mM magnesium ions for the maintenance of viral stability. Moreover, AN-15 has shown specific requirement for Ca^{2+} to exist in order to permit infection of *Anabaena* PCC7120 (host).

In summary, each isolate has its own physicochemical properties and responds differently in the inactivation tests, even if the isolates are isolated from the same host and have the same morphology.

2.5.2.3 Host range

Cross- infectivity experiments are designed to determine the host range of cyanophages isolates, where the cyanophage isolates are screened for their ability to lyse other potential host strains (Deng and Hayes 2008). The host range of cyanophages is relatively complex with both extremely narrow and extremely broad host range (Jenkins and Hayes 2006). One study showed that the narrowest host range of the isolate is one strain of *Synechococcus* infected out of twenty-five isolated *Synechococcus*, while the widest host range was reported to be seventeen different strains of *Synechococcus* infected out of twenty-five isolated *Synechococcus* (McDaniel et al. 2006). The variation of the host range is due to several reasons: 1) morphology or structure of cyanophage, 2) strategy in phage translation, 3) host of isolation and, 4) the depth at which the isolate was obtained from.

Infection begins with the phage attaching to the host cells with its tail, leading to irreversible adsorption (Samimi and Drews 1978). Therefore, the tail fibre affects the initial attachment of phage to the host with adhesion specificities. Previous studies on the tail fibre bacteriophages revealed that phages carrying more than one tail fibre gene enable them to infect a wider host range (Mann 2003).

Most of the cyanomyoviruses are reported to have a broad host range (Sullivan et al. 2003; Clokie and Mann 2006) whereas cyanopodoviruses (Sullivan et al. 2003; Limor-Waisberg et al. 2011) and cyanosiphoviruses (Wilson et al. 1993) are host specific. Recently, a novel study was conducted to investigate the two strategies employed by cyanophages to enhance their translation efficiencies of the viral genome when confronting different hosts (Limor-Waisberg et al. 2011). These

two strategies are named “specialization” and “adaptation”. Cyanophages of the T7-like *Podoviridae* family employ a “specialization” strategy, i.e. they adjust the GC content and the codon usage to the GC content and codon usage to the infected host; whereas cyanophages of the T4-like *Myoviridae* family apply an “adaptation” strategy, i.e. they maintain low GC content but retain their specific myoviral tRNAs (Limor-Waisberg et al. 2011). The employment of different strategies by these two families resulted in broader host range. For example, “the specialization” strategy encoded by podovirus is advantageous due to the readily efficient translation of its genes. However, it may also restrict the host range as this strategy restricts its ability to infect hosts with different GC contents. On the other hand, myoviruses with low GC content are readily efficient in gene translation in hosts with similar low GC content. For high GC content hosts, myoviruses will supplement their own set of tRNAs into the hosts’ tRNA pool. Once expressed, this enhances the viral genome translation efficiency.

Marine cyanomyoviruses have shown wide variation in their host range (Sullivan et al. 2003; Clokie et al. 2010). While some cyanomyovirus isolates infect only their original host, other isolates have the ability to infect multiple hosts not only from the same genus, but also by cross-infecting strains of other cyanobacteria genus (Suttle and Chan 1993; Waterbury and Valois 1993; Clokie et al. 2010). However, there is no infection against any freshwater strains for the isolates from the marine environment (Suttle and Chan 1993). This variation of host range was suggested to be strongly influenced by the host used for isolation (Millard and Mann 2006), and this difference could be due to the fact that the hosts may possess different restriction modification systems (Mann 2006). Lu *et al.* (2001) reported that high host range cross-infectivity was found among the cyanophages infecting phycoerythrin-containing (PE) *Synechococcus* strains compared to cyanophages infecting phycocyanin-containing (PC) *Synechococcus* strains. Moreover, Sullivan et al. (2003) reported that phage isolated from *Synechococcus* host strains have broader host range when compared to phages isolated from *Prochlorococcus*. In a study by McDaniel et al. (2006), it was shown that infectivity was related to depth

of cyanophage isolation, whereby the surface isolates demonstrated a broader host range.

2.5.2.4 Genetic analysis

Isolated viruses are indistinguishable based on morphology and host range but can be confirmed through genetic analysis (Hu et al. 1981). In addition, genetic analysis can be used to determine the diversity of the isolates, where morphological characteristics are inadequate for this type of study (Okunishi et al. 2002).

Large genetic diversity among viruses in the sea has been reported, where 60-80 % of the sequences were dissimilar (E -value >0.001) to a known gene (Suttle 2005). Therefore, to study the genetic diversity of the cyanophage community, more genomes from cyanophage isolates' need to be sequenced, followed by detailed genome comparison (Yoshida et al. 2008). The genomic sequence of phages can be accomplished using the whole genome shotgun approach with preparation of a phage DNA fragment library, where genome fragment sequences are assembled using the Phred/Phrap/Consed software (Summer 2009).

To date, 24 marine cyanophages (17 myoviruses, six podoviruses and a siphovirus), five estuarine cyanophages (one podovirus and four siphovirus) as well as four freshwater cyanophages (two myoviruses and two podoviruses) have been sequenced (Yoshida et al. 2008; Clokie et al. 2011; Dreher et al. 2011; Huang et al. 2012). All marine and estuarine cyanophages were isolated from *Synechococcus* and *Prochlorococcus*. Two of the freshwater cyanomyoviruses Ma-LMM01 and S-CRM01, were isolated from *Microcystis aeruginosa*, and *Synechococcus* respectively; while two podoviruses (Pf-WMP3 and Pf-WMP4) were isolated from the freshwater cyanobacterium, *Phormidium foveolarum*. These genome sequences and genome comparison studies have provided great insights into the roles phages are playing in host biology.

One of the most novel findings to emerge from genomic analysis of cyanophages is that cyanophages contain homologues to photosynthesis genes (*psbA* and *psbD* gene) (Suttle 2005). Interestingly, all marine cyanomyoviruses isolated from *Synechococcus* and *Prochlorococcus* (Chen and Lu 2002; Mann 2005; Sullivan et al. 2005; Pope et al. 2007; Weigele et al. 2007; Sullivan et al. 2010) and a cyanophage isolated from freshwater *Synechococcus* (Dreher et al. 2011) genomes contain *psbA* and *psbD* photosynthetic genes from cyanobacteria, but in Ma-LMM01 (*Microcystis aeruginosa* infectious phage), they are absent. The Ma-LMM01 genome lacks a homologue of these photosynthesis genes but it contains a homologue of the *nblA* gene found in cyanobacteria (Yoshida et al. 2008), *nblA* gene is proposed to function to reduce the absorption of excess light energy and preventing photodamage. Four siphoviruses from an estuary were isolated from *Synechococcus*. These cyanosiphoviruses do not carry photosynthesis genes but carry metabolism and replication genes which are homologues with their host (Huang et al. 2012). The genomic analysis of cyanophage isolates was able to show this surprising lateral gene transfer between phage and host. However, cyanophages with similar morphology (*Myoviridae*) or isolated from the same species (*Synechococcus*) may not acquire the same cyanobacterial genes.

Huang et al. (2012) study is showed no continuous collinearity⁶ across five cyanosiphovirus genomes whereas in contrast, 17 marine cyanomyovirus genomes were collinear and shared around 63 core genes, while 15 core genes were detected among seven cyanopodoviruse genomes (Huang et al. 2012). The conservation of genome among the cyanomyoviruses and cyanopodoviruses allowed the development of group-specific gene markers (as primers) and then use in molecular studies, such as detection of certain groups of cyanophages and their genetic diversity without culture.

PCR is a quick and effective way to identify the presence of specific bacteriophages from environmental samples (Clokier 2009). The study of genetic

⁶Collinear: Sharing or lying on a same line, such as a sets of points (<http://www.thefreedictionary.com/collinearity>)

variation and relationship between different isolates from different environments can be done easily through PCR amplification. An individual cyanophage is first categorized according to the family it belongs to through an electron microscope examination. For example, if the phage is a cyanomyovirus, then the g20 or g43 gene product can be expected from PCR with PCR primers CPS1/CPS2 or CAP1/CAP2 (Fuller et al. 1998; Clokie 2009). On the other hand, if the phage is cyanopodovirus, then it may contain a conserved gene (cyanopodovirus- like DNA polymerase gene sequence) which can be amplified by using CP-DNAP-349F/CP-DNAP-533R primers (Chen et al. 2009; Huang et al. 2010). The PCR product is then sequenced and aligned with other known cyanophage genes. Finally, the percentage of identity between the sequences and the evolutionary distance between isolates are obtained. For example, primers were used to amplify the viral g20 gene for sequence analysis of 40 cyanophage isolates (cyanophages were isolated from *Synechococcus* WH7803, WH8012, WH8018 and WH8113) from Rhode Island's coastal waters (Marston and Sallee 2003). Sequence or phylogenetic analysis showed that these isolates were distributed to three different clusters (I, II and III). Isolates within the same cluster may not have similar host range. For instance, S-RIM10 and S-RIM33 isolates were clustered into cluster I, with S-RIM10 showing the ability to infect *Synechococcus* WH7803, WH8012 and WH8018 whereas S-RIM33 could only infect *Synechococcus* WH7803. On the other hand, S-RIM10 and S-RIM12 have the same host range but clustered to two different clusters. Thus, phylogenetic analysis from targeted gene sequences obtained from PCR is able to differentiate cyanophage isolates with the same host range,

Restriction endonuclease digestion followed by gel electrophoresis is a simple and yet sensitive approach to screen isolate samples for differences in their base sequences, as the DNAs of phages have specific cleavage sites. In brief, purified cyanophage DNA is treated with restriction endonucleases such as *EcoRI*, *HindIII*, *AccI*, *EcoRV* etc., and then the restriction fragments are separated in agarose or gradient polyacrylamide gel with lambda phage DNA fragments as molecular weight standard (Hu et al. 1981; Franche 1987; Wilson et al. 1993; Lu et al. 2001). Three cyanophage isolates (A-1(L), N-1 and AN-10) from heterocyst

forming, filamentous cyanobacteria of the genera *Anabaena* and *Nostoc*, have been examined (Hu et al. 1981). These three cyanophage isolates show similarity in the host range but different patterns of fragments generated from the DNAs of the isolates under treatment with *EcoRI* and *HindIII*. Therefore, it was suggested that these three isolates are distinguishable. In an experiment where DNA from five isolates were cut with a selection of restriction endonucleases, three distinct patterns of restriction fragments were observed: S-BM1 and S-PM1 with the same morphology (*Myoviridae*) had a highly related pattern, as did S-BS1 and S-PS1 with the same morphology (*Siphoviridae*); but another cyanomyovirus S-WHM1 had a unique pattern (Wilson et al. 1993). Thus, restriction endonucleases can be a good alternative tool to differentiate isolates with similar morphology and host range. A limitation of the restriction enzyme digestion approach is that many of the restriction endonucleases are not successful in digesting the cyanophage DNA into a clear definable band pattern. This could be due to the absence of restriction sites or due to the presence of a base modification such as methylation (Wilson et al. 1993; Lu et al. 2001).

2.5.2.5 Growth characteristics

The growth and life cycle of cyanophage isolates may be divided into two main periods: 1) adsorption period and 2) latent period. The isolates can be characterized by determining their phage growth parameters, particularly their adsorption rate, latent period, eclipse period and burst size (Hyman and Abedon 2009).

Adsorption rate is critical in inferring the significance of cyanophage infection on the mortality of cyanobacteria (Mann 2003). The adsorption period begins with virion extracellular search for potential host, then attachment to a susceptible host and ends with irreversible virion attachment to the host (Hyman and Abedon 2009). The latent period is the delay between virion attachment of the host cell and subsequent phage progeny release when lysis occurs. The latent period

spans both eclipse (i.e., during when the infections synthesize phage nucleic acid and proteins) and post-eclipse periods (i.e., during phage progeny maturation or accumulation) (Hyman and Abedon 2009).

There are a number of factors that affect the adsorption rate, which include host size, likelihood of phage attachment given collision (contact rate), adsorption condition and rate of phage diffusion (Hyman and Abedon 2009). The adsorption rates are presented as adsorption constants (k), which is given by the probability of adsorption of a single phage to a single susceptible host within a single unit volume of fluid over a single unit of time (Hyman and Abedon 2009). It is calculated based on the adsorption kinetic which is obtained by an assessment, of free phage loss as a function of time (Mann 2003). The number of free phages (remaining percentage of unadsorbed phage) as a function of time is recorded to develop an adsorption curve. The slope of the adsorption curve is determined using natural-log (i.e., $\ln$) and is used to calculate the adsorption constant. The adsorption constant is equal to the negative slope divided by the host density (N) in homogenous adsorption medium (that is, $k = -\text{slope}/N$) (Hyman and Abedon 2009). Suttle and Chan (1993) showed that the adsorption rate constant of cyanophage S-BBS1 for one hour incubation is 0.035 in a minute, meaning that about 88 % of the viruses are adsorbed after one hour. In an earlier study, Samimi and Drews (1978) showed that the adsorption rate of cyanophage AS-1 varied with three different host cells. The adsorption percentages of, AS-1, after one hour incubation with three different host cells were 51 %, 67 % and 73 %, respectively.

In early cyanophage studies, the electron microscope was used to investigate the ultrastructure of LPP-1 in filamentous cyanobacteria (*Plectonomena boryanum*) (Smith et al. 1967) and SM-1 in unicellular cyanobacteria (*Synechococcus*) (Mackenzie and Haselkorn 1972) during eclipse and post-eclipse periods. The virus LPP-1 attaches to its host and injects its DNA into the nucleoplasm of the host. The viral DNA then moves to the spaces between photosynthetic lamellae where helices begin to form without protein coats. The helices then move into the virogenic stroma, where the protein coat is assembled round the helix and is compressed into

the shape (near spherical) of a mature particle. Virogenic stroma is a space formed by the displacement of the photosynthetic lamella (Smith et al. 1967). On the other hand, SM-1 virion development occurs only at the nucleoplasm, where LPP-1 particles appear to be physically excluded. In addition, no virogenic stroma formation was observed in the *Synechococcus* cell (Mackenzie and Haselkorn 1972) after SM-1 infection. These two cyanophages showed two different features during eclipse to post-eclipse periods and they manipulated the host organelles for replication differently.

Measurement of the latent period may be accomplished either by employing a one-step growth experiment (i.e., detecting the liberation of phage virions) or by turbidometric measures (i.e., detecting the destruction of bacterial infection) (Hyman and Abedon 2009). The one-step growth experiment is used to determine the duration of infectious cycle and the yield of virus (burst size) per infected host cell. This experiment begins with preparing the infective centers by first synchronizing the initiation of phage infections (to prevent subsequent adsorption to uninfected bacteria) and employing an optimized phage multiplicity (to assure a reasonable approximation of singly infected bacteria), then diluting the phage and bacteria into a prewarmed growth media to inhibit subsequent secondary phage adsorption and followed by plaque forming unit (pfu) enumeration (Hyman and Abedon 2009).

The latent period is also known as constant period in the one-step growth curve (Figure 2.3). The duration of latent period is the beginning of the rise minus the time of initiation of infection (Hyman and Abedon 2009). During the “rise” period, all progeny phages are released from the host cell and the number of phage particles released is called the burst size (Rédei 2008). Burst size can be determined by number of pfu well past the initiation of lysis minus the number of pfu at post-rise, when the phage titers stabilize (Hyman and Abedon 2009).

Figure 2.3 One-step growth curve (Adapted from Rede, 2008)

Cyanophage isolates, N-1 (Adolph and Haselkorn 1972) and N(S)1 (Franché 1987), are phages that infect filamentous cyanobacteria. These isolates have different latent period and burst size, where the latent period of N-1 and N(S)1 is 7 hours and 20 hours respectively; and the burst size of N-1 and N(S)1 is 100 pfu/cell and 70 pfu/cell respectively. The growth of AS-1M and S-BBS1 on a unicellular cyanobacterium (*Synechococcus*) has been examined through one-step growth experiments as well. AS-1M demonstrated a latent period of five hrs and a burst of 100 pfu/cell (Kim and Choi 1994), while S-BBS1 demonstrated a latent period of nine hours and a burst size of 250 progeny phages per infected host cell (Suttle and Chan 1993). The burst size of cyanophage infecting *M. aeruginosa* (Ma-LBP) is 28 viral particles per host cell and the Ma-LBP replication time (latent plus rise time) is 11.2 hours (Tucker and Pollard 2005).

The burst size of phage infecting a filamentous host is reportedly difficult to be determined from the one-step growth curve. This is because the effective cell concentration is high, where phages released from a cell in a filament can infect an adjacent cell of the same filament. This phenomenon may lead to multiple cycles of infection (Hu et al. 1981). Hu et al. (1981) performed two one-step growth

experiments of AN-20: (i) AN-20 infecting intact filaments of *Anabaena variabilis* (host) and (ii) AN-20 infecting fragmented filaments (one or two cells) of host cells. The results showed that the burst size of AN-20 with intact filaments was three times higher than AN-20 with fragmented filaments.

Cyanophage isolates can be profiled by their growth characteristics such as adsorption constant rate, burst size, latent period and one-step growth curve. However, growth characteristics alone are insufficient to characterize cyanophage isolates, and other characteristics such as morphology, physicochemical characteristics, host range and genetic profile must also be considered to fully profile a cyanophage isolate.

2.6 INFLUENCE OF ENVIRONMENTAL CONDITIONS ON THE FATE OF CYANOPHAGES

Environmental conditions affect the physiological conditions of cyanobacteria and subsequently, the interaction between host and phage. Besides that, the success rate of frequency of adsorption, viral life strategies (burst size, latent period and enters into lysogen or lytic cycle) and decay rate of cyanophages are also affected by environmental conditions. Below, a few environmental factors and their influence on the fate of cyanophages are discussed.

Temperature is a variable that influences the stability of infectivity and survival of cyanophages. At a temperature above 50 °C, infectivity is rapidly lost; while some thermosensitive cyanophage strains have been reported to be unable to form plaques at 35 °C (Suttle, 2000a). N(S)1 cyanophage was reported stable in infection up to 65 °C, but at 70 °C, a 40 % loss in titer was observed (Frache 1987). Increase of virus decay rates from winter to summer had been shown (Garza and Suttle 1998; Manage et al. 1999). Temperature affects the viral life strategy, e.g. the transition from lysogenic to lytic life stage, where temperature has been shown to induce AS-1 prophage conversion from lysogenic to lytic cycle (Chu et al. 2011).

All phases of the cyanophage life cycle are influenced by light. Light affects the rates of cyanophage adsorption (Mann, 2006) and replication. Studies with AS-1 have demonstrated that adsorption to host cells is two- fold faster in the light than in the dark (Suttle, 2000a & Kao et al., 2005). Cyanobacteria growth and reproduction in the natural environment depend on light as a source of energy, and this indirectly affects the cyanophage burst size. (Clokier and Mann 2006). Adolph and Haselkorn (1972) reported that the effect of withdrawal light on the growth N-1 infected *Nostoc* cells, resulted in reduced burst size; longer latent period and decrease in phage release rate. Kim and Choi (1994) found similar result, where a longer latent period and smaller burst size of AS-1M was reported when one-step growth experiments were performed in the dark. Continuous exposure of cyanophage to high light intensity could also induce prophage from lysogenic to lytic cycle (Clokier and Mann, 2006). Kao et al. (2005) found that an increase in light intensity resulted in an increase in the production of progeny phage.

One of the most important parameters dictating the fate of cyanophages is solar radiation/ sunlight, because the major loss of viruses in aquatic system is due to the destruction by UV (Garza and Suttle 1998; Suttle 2000a). The effect of solar radiation on the inactivation of cyanophages in the environment also depends on other cofactors such as water transparency (secchi depth), solar angle and radiation (Suttle, 2000a). Variation in solar radiation (e.g. the increase of irradiation from winter to summer) was suggested to increase the loss rate of infectivity (Garza and Suttle, 1998). Garza and Suttle (1998) showed that in natural environments, cyanophages during summer were more UV- resistant than isolated samples. When placed in the same conditions, the decay rate of the isolates was two times more than environment samples. Both filamentous and unicellular cyanobacteria have very efficient light-dependent mechanisms and photoreactivation that can repair- UV damaged cyanophages and restore virus infectivity between 38 % up to 78 % (Clokier and Mann, 2006). As a result, this is able to maintain high cyanophage concentrations in surface waters (Graze and Suttle, 1998). An assessment of UV-B damage on cyanophage PP (cyanophage infecting *Plectonema boryanum* and

Phormidium foveolarum) conducted by Liao and colleagues (2010), showed that UV-B induced capsid destruction and decreased viral adsorption ability, thus affecting the survival of cyanophages (Liao et al. 2010).

Divalent cations are cofactors in cyanophage structure stability, infection ability and adsorption to host. Cyanophage AN-15 (cyanophage infecting *Anacystis* and *Nostoc* cyanobacteria) has shown a requirement for divalent cations for the purpose of maintaining capsid structure (Mole et al. 1997). Concentrations of cations also influence cyanophage infectivity. For example, Ca^{2+} ion was required to permit infection by AN-15 to its host (Mole et al. 1997); and in the presence of Mg^{2+} , LPP-1 and SM-2 were much more stable during infection than when resuspended in deionized water (Suttle 2000a). In contrast, other cyanophages have shown no cation requirement, e.g. SM-1 and N(S)-1 remained infectious when resuspended in distilled water (Frache 1987; Suttle 2000). Thus, cations may play a role in the distribution of some freshwater cyanophages, wherein some cyanophages are limited by the availability of cations. Salinity represents the ionic strength in a water system and can influence viral life strategies, e.g. an increase in the concentration of sodium chloride led to an increase in phage latent period and virus adsorption to particles (Danovaro et al. 2011). However, only one study has shown the effects of salinity on cyanophage distribution, i.e. the highest concentration of *Synechococcus* W7803 infective cyanophage was found near the mouth of the river where salinity was highest (Lu et al., 2001). In addition, cyanophages isolated from the coastal water had much more diverse morphotypes than cyanophages isolated from the upstream river, i.e. salinity could affect the morphological diversity of cyanophages (Lu et al., 2001).

Physiological conditions of the host can influence the susceptibility of the phage to the host (Brussaard, 2004), by affecting the surface binding sites of cyanophage. Adsorption of phages to the cell envelope is by phage recognition and adhesions to specific cell- surface receptors (Mann, 2006). Nutrient changes in the aquatic environment lead to changes in the nature of cell surface (Mann, 2006). Theoretically, this affects the binding site and susceptibility of cyanophage with the

host. However, research on the effects of nutrient starvation on cyanophage adsorption is few. From these few studies, the results have been conflicting: one of the study showed no effect on phosphate depletion on cyanophage adsorption, with phages binding to all *Synechococcus* (Wilson et al. 1996); another study reported that only 10 % of phages were bound to *Synechococcus* during phosphate depletion (Mann 2006). Nutrient availability also affects viral life strategies, including entrance into the lysogenic or lytic cycle and latent period of the virus (Danovaro et al. 2011). Wilson et al. (1996) demonstrated that cell lysis of *Synechococcus sp.* was delayed by 18 hours and decreased the S-PM2 burst size by 80 % during phosphate depletion. They also showed that only 9.3 % of phosphate-depleted cells lysed, while 100% of phosphate-replete cells lysed. Therefore, Wilson et al. (1996) suggested that phosphate depletion decreases the cyanophage burst size and extends the latent period. Moreover, cyanophages establish lysogeny in response to growth of phosphate- depleted host cells.

Phage inactivation is also affected by pH, N(S)-1 was reported to be stable in host infection between pH 5 and pH 11. However, N(S)-1 started to lose infectivity at pH 4 and pH 12, and was completely inactivated after 5 min at pH 2 or pH 13 (Franche 1987). AS-1, SM-1 and LPP-1 isolates were reported to be remarkable stable from pH 5 to pH 11 (Padan and Shilo 1973; Suttle 2000). Cyanophage isolates are infective over a broad pH range compared with many bacteriophages which lose their infectivity when the pH exceeds 8. This is in accordance with the optimal growth of cyanobacteria at pH above 8 (Padan and Shilo 1973; Suttle 2000). Non- cyclic peptides, such as planktopeptine and anabaenopentin, are found to act as a trigger agent to induce temperate cyanobacteria into a lytic cycle, resulting in the collapse of a cyanobacteria bloom (Sedmak et al. 2008). Planktopeptine and anabaenopentin are polypeptides produced by cyanobacteria at high concentrations when cyanobacteria bloom. The decay or loss rate of cyanophages also depends on attachment to host cells or particles and consumption by protozoan grazers (Suttle, 2000a).

In summary, the fate of cyanophages is influenced by environment conditions: (1) adsorption rate is affected by light, nutrient and host physiological condition; (2) infectivity is affected by temperature, divalent cations and pH; (3) latent period and burst size are affected by light and nutrients; (4) structural stability and decay rate are affected by temperature, UV, divalent cations, attachment to cells or particles and consumption by protozoa; and (5) life cycle (lytic or lysogen cycle) is affected by light, temperature, non-cyclic peptides and nutrient levels. Sections 2.6.1 and 2.6.2 present a review of recent studies on the spatial and temporal dynamics of cyanophage abundance and diversity and their relationships with environmental conditions.

2.6.1 Spatial and temporal dynamics of cyanophage abundance

2.6.1.1 Approaches to enumerate cyanophage concentration

Enumeration of viruses is a fundamental and important measurement parameter in aquatic ecology, especially in studies concerning the distribution and activity of viruses at the community level. Methods that are currently used to estimate the abundance of viruses in aquatic samples include plaque assay, most probable- number assay (MPNs); direct counting approach (by using transmission electron microscopy (TEM), epifluorescence light microscopy (ELM) flow cytometry (FC)), real-time PCR (qPCR) and real-time reverse transcription PCR (RT-PCR). Plaque assay and MPN assay can be categorized as cell-culture approaches and qPCR and RT-PCR can be categorized as molecular approaches.

Direct counting approach

In this section, the development and principle of direct counting by transmission electron microscope or epifluorescence light microscopy or flow cytometry and their pros and cons is discussed. Transmission electron microscope

(TEM) can present internal and external virus structures in two- dimensions. This is the most common method for direct counting of viruses before the emergence of the fluorescence stains specific to dsDNA and molecular methods (Biel and Gelderblom 2004). In cyanophage studies, TEM is mostly used in the determination of isolated cyanophage morphology (Chen and Lu, 2002), while for site sampling, TEM has been used to determine the distribution of phage- infected cyanobacteria (Proctor and Fuhrman 1990). A severe limitation of the TEM method is that the detection limit for direct counting is high- 10^6 viruses ml^{-1} (Biel and Gelderblom, 2004). This is because of the high magnification of TEM, and hence, TEM is not suitable for direct virus enumeration for samples collected from oligotrophic systems, where the concentration of viruses may be below the detection limit (Wommack and Colwell, 2000). Furthermore, the precision of virus counting is lost because of uneven staining and non homogenous distribution on the grids (Wommack and Colwell 2000). The first limitation can be solved by concentrating environmental samples and the second limitation can be improved by practical experience. However, the most important limitation of this method is the high cost of equipment and tedious sample preparation.

Epifluorescence light microscopy (ELM) methods were developed to overcome the limitation of TEM, such as losses due to preparation and slow and tedious sample preparation. The ELM method requires viruses to be collected on to a small pore-size filter (normally $0.2 \mu\text{m}$) and stained with fluorescent dye. The light from the fluorescent dye results in a glowing particle, such that the size of the glowing particle is larger than the actual size. Thus, viruses can be counted by using lower magnification (e.g. ELM with 100x objective) (Ortmann and Suttle 2008).

DAPI (4'6-diamidino-2-phenylindole) was the first staining agent of ELM for virus quantification, but this method is prone to inaccuracy because DAPI does not produce bright staining of virus particles. Over the years, several different dsDNA binding fluorochromes, such as SYBR Green I, SYBR Gold and Yo- Pro have also been used in this approach (Ortmann and Suttle 2008). These stains have low background staining, higher quantum yield and higher stability than DAPI. The

Yo- Pro stain agent is brighter and more stable compared to SYBR Green, but the downside of Yo- Pro is that this dye needs 2 days of incubation time and is not suitable for use with aldehyde preserved samples (Patel et al. 2007). However, it was reported that the cumbersome 2 days incubation time can be reduced to 4 min by using microwave pretreatment of filtered (0.2µm) samples (Bettarel et al. 2000). This improvement not only reduces the incubation time but also helps in reducing the decay of fixed samples (Wen et al. 2004). On the other hand, SYBR green staining time takes only 15 minutes and can be used on aldehyde- preserved samples. SYBR green I stains are less stable than Yo-Pro, SYBR Green I fades very quickly (Bettarel et. al., 2000) and thus, needs to be chemically stabilized with an anti- fade agent. SYBR Gold stain is more stable and the fluorescent signal is twice as high as SYBR Green I (Chen et al. 2001). However, it emits a yellowish light and may not be a good option when counting samples with humics or sediments (Ortmann and Suttle 2008).

Wen et al. (2004) showed that aldehyde fixation results in a rapid decrease in viral abundance when stored on postfix slides at 4 °C. This raises an important issue in virus enumeration by ELM or flow cytometry method, i.e. for stored fixed samples, the abundance of viruses could be underestimated. The drop in abundance of virus estimation is due to several reasons. First, protease in the fixative may break down viral protein. Second, aldehydes may cross-link the viral protein and interfere with stain penetration. Finally, it could be due to attachment of viruses to other particles or bacteria or with other viruses as well (Wen et. al., 2004). Wen et. al. (2004) reported that there is no significant difference in the estimation of fixed and unfixed samples when the slides were made immediately. Estimation can yield accurate results within 2 weeks with unfixed samples when the stained sample was stored at -20 °C. If storage is needed and immediate slide preparation is impractical, the study recommends that after fixation, samples should be flash frozen in liquid nitrogen and stored at -86 °C. In short, Yo- Pro, SYBR green and SYBR Gold can be used to obtain accurate estimates of viral abundance with careful preparation and appropriate protocols.

ELM estimation was reported to be higher than TEM enumeration. One of the reasons why ELM estimation is higher than TEM counts is the interference of free nucleic acid. Bettarel et al. (2000) suggested that the interference of free nucleic acid to the viral concentration count is about 15.4 %. The ELM approach by fluorochrome staining to DNA may stain non- viral like free DNA and RNA leading to an overestimate of the viral concentration. Bettarel et. al. (2000) estimated the inference of nucleic acid by treating the sample with DNase (enzyme to breakdown free DNA) and RNase (enzyme to breakdown free RNA). Based on the results, 39 to 53 % of the particles considered as viruses were, in fact, free nucleic acid. However, subsequent experiments, which used TEM on the treated samples showed that the interference effect might lead to an overestimation. TEM results also showed losses in viral concentration, i.e. 18.7 % reduction for DNase treated sample and 16.1 % on RNase treated sample. These results demonstrated that DNase and RNase not only eliminated the free nucleic acid but also broke down true viral particles. Therefore, the author proposed that DNase and RNase were not suitable to be included in the routine counting ELM protocol (Bettarel et. al., 2000).

Fluorescently labeled viruses as probe (FLVP) has also been used to detect specific bacteria in mixed marine microbial communities (Hennes et al. 1995). Tucker and Pollar (2005) used labeled cyanophages to observe viral infection and lysis of *Microcystis aeruginosa*. The labeled cyanophages were observed over 12 h infecting susceptible host cells, where sensitive hosts became labeled and uninfected hosts were not labeled (Tucker and Pollard 2005). This technique allows counting of potentially infectious cyanobacteria by specific cyanophages and plays a role in phage typing.

Collectively, ELM is a groundbreaking method to enumerate virus samples, and as a method of phage typing, this tool enables the observation of cyanophage infection to be clearer. The disadvantages of this method are that only total viruses can be enumerated but not the infective viruses (Ortmann and Suttle 2008). Moreover, differentiating small bacteria cells from viral particles is difficult and

confusing with direct counting of environment samples. This may lead to an underestimate of the actual number of virus, due to difficulty in obtaining a uniform focal plane for all viral particles (Chen et. al., 2001) However, ELM may also lead to an overestimate of the actual number of virus due to the interference of free nucleic acid.

Discovery and development of high fluorescence- yield and nucleic- acid- specific stains combined with rapid enumeration equipment such as flow cytometry (FC), enables the identification and enumeration of viruses in aquatic system more rapidly (Brussaard 2004). Moreover, flow cytometry with sensitive nucleic acid stains or fluorochrome has allowed the detection and discrimination of a wide variety of viruses with different morphology and size, e.g. flow cytometric analysis revealed at least four viral subpopulations for Lake Erie (Chen et al. 2001). Furthermore, a combination of flow cytometry and immunofluorescence staining, speed of analysis and sensitivity of detection and enumeration of specific viruses (e.g. by using specific monoclonal antibody probes) in flow cytometry showed rapid and sensitive detection and quantitative analysis of cytoplasmic iridovirus in cell culture (Qin et. al, 2005).

Plaque assay and most probable number (MPN)

Infective viruses can be quantified by cell culture assay, i.e. plaque assay and most- probable-number (MPN). Culture assay requires the observation of interaction between the virus and their host. Cell culture assay can only be used when the host is cultivable and lytic viral activity destroying the host cells is observed (Wilhelm and Poorvin 2001). Cell culture methods have proven to be very useful in cyanophage studies (Wommack and Colwell, 2000). Selection of assay (plaque assay or MPN) depends on the sample and the balance between virus sensitivity, precision and accuracy in the estimation (APHA 1995). Here, we discuss the principles of plaque assay and MPN and their limitations.

Plaque assay is used to estimate the titers and abundance of cyanophages when their host can be grown on solid medium. The plaque assay has become a tool for the study of bacteriophages since 1917. Subsequently, this technique was refined from monolayer plaque assay to double layer plaque assay (Emanuel 2008). The double layer plaque assay is also known as the double agar overlay method or soft agar overlay method. This method was first described by Gratia in 1936 and then formalized by Mark Adams in the year 1959 (Adams 1959). The double layer plaque assay consists of a basal plate (growth medium and agar/agarose) and a molten agar which contains a phage- bacteria mixture without growth medium. The concept behind this double layer plaque assay is that the molten agar in semi- solid is able to fix the phage particle and the host. This mixture (molten agar, bacteria and host) is uniformly poured onto the agar plates and a complete lawn of microorganisms on the agar plate will be produced. Bacteria susceptible to each individual phage will be killed and a zone of clearing is formed, known as the “plaque”. Each plaque represents a single infectious unit. Thus the infectivity titer of the viral sample is recorded as plaque forming unit (PFUs) per milliliter (Wilhelm and Poorvin 2001; Emanuel 2008; Peter 2008).

The MPN assay is used to estimate the abundance of viruses in a sample by diluting the sample in a serial of log-based dilution with the theoretical assumption that a single infectious virus can destroy a sensitive host population (Wilhelm and Poorvin 2001). This assay is mainly used in the enumeration of samples that do not grow well on solid substances (Peter 2008). Several assumptions are considered when the MPN assay is used. For example, the microbes to be estimated are considered to be distributed randomly and evenly within the sample tube; the microorganisms to be estimated are also assumed to be separated individually and not in clusters, the conditions of growth for the microorganism is optimized including optimal growth medium, incubation temperature, and incubation period (Peter 2008). Briefly, a serial dilution is made with a filtered (to eliminate protozoa or protozoan grazers) environmental sample. Then, each of these dilutions are added to the host cells in exponential growth phase and incubated under optimal condition. After several days, the positive (visible turbidity) and negative (clear)

test results are recorded and the viable cell counts are then compared to MPN statistical tables to interpret the results (Wilhelm and Poorvin 2001; Peter 2008).

Plaque assay is considered to be more precise and accurate than MPN because of the visible large numbers of individual infectious units that form plaques which can be counted directly as discrete entities. For sensitivity, MPN is more sensitive than the monolayer plaque assay but is less sensitive than the double layer plaque assay (APHA 1995). Okunishi et al. (2003) reached the same conclusion when using *Synechococcus* strain KFM001 as a host for cyanophage enumeration, they found that the mean value of plaque forming unit (PFU) to be 4.1 fold higher than MPN results. The plaque assay method has another advantage, in that it provides an indication of potential lysogens (temperate phage) in the presence of turbid plaques (Wilhelm and Poorvin 2001). The disadvantages of plaque assay compared to MPN are the requirements that host cells are cultivable and can produce confluent lawns on agar (Wilhelm and Poorvin 2001); and the difficulty in distinguishing certain phage plaques, e.g. plaques which are small in size and incomplete lysis (Kropinski et al. 2009).

The disadvantages of enumerating phages by cell culture assay are that it is selective for viruses infectious for a specific host and it is time- consuming (Brussaard 2004). Moreover, the culture based method tends to underestimate cyanophage abundances because the phage infecting a single strain of host will not represent all the infectious cyanophages in the water system (Okunishi et al. 2003). Suttle and Chan (1994) enumerated cyanophage abundance from coastal seawater, where five different strains of *Synechococcus* were used in the MPN assay. They showed that cyanophage abundance depended strongly on the host that was screened, e.g. concentration of cyanophages infecting DC2 was about 1 order of magnitude higher than cyanophages infecting SNC. In addition, breakthrough of unwanted organisms through the filter to the sample will be enumerated too and together with viral retention during filtration could result in inaccurate enumeration of viruses (Wilhelm and Poorvin 2001).

Molecular tools: Real-time polymerase chain reaction (qPCR) and real-time reverse transcription-PCR (RT-PCR)

Molecular methods, i.e. real-time polymerase chain reaction (qPCR) and real-time reverse transcription-PCR (RT-PCR) have been used to enumerate of cyanophage since 2006 and thus, they are still relatively new methods. To date, only two sets of primers (CPS1/CPS2 and SheathRTF/SheathRTR) have been used in studying the spatial and temporal variation in cyanophages, and these primers are only limited to specific cyanophages.

The g20 gene primers (CPS1 and CPS2) designed by Fuller et al. (1998) specifically amplify a gene encoding the capsid assembly protein (gp20) belonging to cyanophages in the *myoviridae* family with product 165-bp PCR amplicon. These primers have successfully amplified the g20 gene product from cyanomyoviruses infecting *Synechococcus* (Millard and Mann 2006) and *Planktothrix rubescens* (Deng and Hayes 2008) and thus, used to determine the presence and diversity of these specific viruses (Fuller et al. 1998; Millard and Mann 2006; Wang et al. 2011). These primers have recently also been used to determine the spatial and temporal dynamics of cyanomyoviruses which encode the g20 genes, e.g. in Norwegian coastal water (Sandaa and Larsen 2006) and Lake Erie (Matteson et al. 2011).

Previous studies (Short and Suttle 2005; Wilhelm et al. 2006; Sullivan et al. 2008) have shown that only a subset of g20 amplicons from g20 primers (CPS1/CPS8, CPS4/G20-2) amplification fall into clades containing isolated cyanophages, whereas the rest are not clustered to known isolates. Questions arise as to whether these amplicons may be from other bacteriophages or how specific these primers are? However, to date, there is still no direct evidence showing that the g20 PCR primers detect other bacteriophages. In addition, recently Matteson et al. (2013) verified the specificity of CPS1/CPS2 primers (g20 primers used in this qPCR study) by using DE-METAST-BLAST. Their results confirmed that these primers could only amplify cyanomyoviruses amplicons based on sequences in the Global Ocean Survey data set.

Real-time PCR primers, SheathRTF and SheathRTR, were designed to amplify a conserved region of the gene encoding sheath protein in Ma-LMM01-type phages (Takashima et al. 2007). Ma-LMM01 is a cyanophage which specifically infects *Microcystis aeruginosa* NIES298, a toxic strain of the bloom forming cyanobacterium (Yoshida et al. 2006). The g91 gene primers (SheathRTF and SheathRTR) were used to amplify a 132-bp region of the sheath protein coding gene. These primers were used in real-time PCR (qPCR) (Takashima et al. 2007; Yoshida et al. 2008; Kimura et al. 2012) and quantitative real-time reverse transcription-PCR (real-time RT-PCR) assays (Yoshida et al. 2010). The RT-PCR assay was developed by Yoshida et al. (2010) and is used to detect and quantify the mRNA of cyanophages within host (*Microcystis aeruginosa*) cells, to monitor gene expression. It is suggested that this assay can be used to quantify phage gene transcripts within the host cell to investigate the quantitative effects of phage lysis on overall host abundance. In addition, a comparison between the concentration of g91 expression gene (mRNA inside host cell) and the g91 DNA gene (quantified by qPCR- DNA in free viruses), showed that the g91 copy numbers in a pond determined by qPCR underestimated the actual number of phages lysed from the host (Yoshida et al. 2010). Kimura et al. (2012) enumerated the g91 DNA copy number inside the host fraction (which represents the abundance of mature phages produced within host cells) and free-viruses (released phages from the host), and showed that the g91 abundance from the host cell fraction was 1 to 2 orders magnitude greater than the g91 abundance from free-viral particles. Thus, Kimura et al. (2012) agreed with Yoshida et al. (2010) that the actual number of phages lysed from the host (also called as the level of phage production) may be higher than previously reported abundances which considered only the free-phage abundance.

Thus, the molecular tools enable the enumeration of cyanophage abundance to a new level, where quantification of certain groups of cyanophages (e.g. cyanomyoviruses and Ma-LMM01-type phages) can be made. In addition, the development of RT-PCR has helped to elucidate the diurnal patterns of cyanophage

gene expression inside their host. Spatial and temporal dynamics of cyanophages abundane *in situ* studies are reviewed in the following section 2.6.1.2.

2.6.1.2 Review on the spatial and temporal dynamics of cyanophage abundance

The temporal dynamics of cyanophage abundance have been studied ranging from short- term, diel cycles (Clokier et al. 2006; Kimura et al. 2012) to long-term annual or seasonal cycles (Kimura et al., 2012b; Manage et al., 2001; Manage et al., 1999; Marston and Sallee, 2003; Millard and Mann, 2006; Okunishi et al., 2003; Sandaa and Larsen, 2006; Suttle and Chan, 1994; Wang et al., 2011b; Waterbury and Valois, 1993; Yoshida et al., 2008a; Yoshida et al., 2010). In the case of spatial dynamics, cyanophage abundance corresponding to water column depth profile (Frederickson et al. 2003; Okunishi et al. 2003; Sullivan et al. 2003; Clokier et al. 2006; Millard and Mann 2006), transect from coastal to open ocean (Suttle and Chan 1994; Sullivan et al. 2003) and different locations (e.g. Lake Erie, Chesapeake Bay and British Columbia) (Frederickson et al. 2003; Matteson et al. 2011; Wang et al. 2011) have been studied. Table 2.2 shows a summary of studies conveying the spatial and temporal dynamic of cyanophage abundance from 1993 until 2012. The summary table shows that only cyanophage which infect of *Synechococcus*, *Prochlorococcus* and *Microcystis aeruginosa* have been enumerated for the different time and spatial scales. The dynamics of cyanophages infecting *Prochlorococcus* has only been studied by Sullivan *et al.* (2003). The other 15 studies focused on cyanophages which infect *Synechococcus* and *M. aeruginosa*.

The *Synechococcus* WH7803-phage has been detected in various environments, including British Columbia, The Indian Ocean and Chesapeake Bay. *Synechococcus* WH7803-phage abundance in surface waters of British Columbia ranged from $2 \times 10^3 \text{ ml}^{-1}$ to $3.5 \times 10^5 \text{ ml}^{-1}$ (Frederickson et al. 2003), while in the Indian Ocean ranged from $6 \times 10^2 \text{ ml}^{-1}$ to $1 \times 10^3 \text{ ml}^{-1}$ (Clokier et al. 2006) and in

Chesapeake Bay ranged from 1.05×10^2 to 6.2×10^5 ml⁻¹ (Wang et al. 2011). Comparison of the abundance of cyanomyoviruses densities (by enumeration of g20 gene copies) in marine and freshwater system has also been conducted. In the Norwegian coast, the percentage of cyanomyoviral to the total virus community is < 0.1 % but at Lake Erie, the percentage is 1.3 % during winter and 1.8 % during summer (Matteson et al. 2011). *Microcystis aeruginosa* phages number was only enumerated from sites in Japan, i.e. Furuike Pond, Lake Mikata and Hirosawanoike Pond. The number of phages infecting *Microcystis aeruginosa* in Furuike Pond was enumerated by using plaque assay and ranged from undetectable to 4.4×10^4 PFU ml⁻¹ (Manage et al. 1999; Manage et al. 2001). The Ma-LMM01-type phage (phage infectious to *Microcystis aeruginosa*) in Lake Mikata and Hirosawanoike Pond was enumerated with real-time PCR by enumerating g91 gene copies. Measurements in Lake Mikata ranged from undetectable to 1.8×10^5 copies ml⁻¹ (Takashima et al. 2007; Yoshida et al. 2008), while in Hirosawanoike Pond ranged from undetectable to 9.7×10^5 copies ml⁻¹ (Takashima et al. 2007; Yoshida et al. 2010; Kimura et al. 2012). All these studies show the wide variation in abundance of cyanophages in different water environments.

Studies on the seasonal variation in viral and cyanophages abundance show that viral titers are generally higher in the summer and autumn than in the winter (Waterbury and Valois 1993; Suttle and Chan 1994; Millard and Mann 2006; Wang et al. 2011; Breitbart 2012). Multiyear time series of Woods Hole Harbor (for 27 months) and Chesapeake Bay (for 19 months) studies demonstrated that seasonally recurring patterns in cyanophage abundance (Waterbury and Valois 1993; Wang et al. 2011)

Cyanophage infection and production occurs with a diurnal pattern and dependency on host photosynthesis. This has shown in lab experiments (Benson and Martin 1981; Kao et al. 2005) as well as in situ, although there have only been two studies for the latter. The first study was presented by Clokie et al. (2006), who investigated the changes in cyanophages infecting *Synechococcus* WH7803 abundance within 24 hour period. Cyanophage numbers were determined by plaque

assay. The results showed that the titer of cyanophages from surface water varied remarkably over a 24 hour period with the most abundance phage titer at 0100h (Clokie et al. 2006).

The second in situ diel study was by Kimura *et al.* (2012). Two sets (2 different sampling days) of 24 hour sampling period at 3 hr intervals were carried out to investigate the infection pattern of *Microcystis* cyanophage (Ma-LMM01-type). The g91 (sheath protein gene) DNA gene copy number (representing the number of Ma-LMM01- type phage) was enumerated in two fractions, i.e. the free-phage fraction and host- cell fraction (g91 within the host cell) through real-time PCR. The g91 mRNA (g91 gene expression) was enumerated through reverse transcription real-time PCR (RT-PCR). The raw gene expression was then normalized by the *rnpB* gene of *M. aeruginosa*, to give the relative g91 mRNA abundance. These results suggested that phage genes were transcribed at dawn or daylight, thus, increasing in the g91 mRNA. After the peak abundance of g91 mRNA was reached, it was followed by the formation of mature progeny phages and subsequently, releases from host cells after 6 hours, and increases in phage g91 DNA copy numbers. Thus, cyanophage infection and abundance occurs in a diel cycle and the length of the lytic cycle fits into the hours of daylight (Kimura et al. 2012). Both these temporal studies showed diurnal patterns in cyanophage abundance.

Cyanophage abundance was found to vary throughout the water column, along transects in the Gulf of Mexico, from coastal to open ocean and three inlets in British Columbia (Suttle and Chan 1994; Frederickson et al. 2003; Sullivan et al. 2003; Clokie et al. 2006; Millard and Mann 2006; Matteson et al. 2011). In general, cyanophage abundance was found to decrease with depth (Frederickson et al. 2003; Clokie et al. 2006; Matteson et al. 2011). However, this was not always the case. For example, Millard and Mann (2006) found that the depth of highest titers varied over sampling period. While, Sullivan *et al.* (2003) enumerated phage titers using 11 types of *Prochlorococcus* strains and found that the highest phage titers occurred at 70 m or 100 m, near the depth of maximum *Prochlorococcus* abundance.

In term of horizontal spatial distribution , two studies have been conducted, i.e. a transect (total 9 stations) in the Gulf of Mexico (Suttle and Chan 1994) and a transect from coastal (Woods Hole) to open ocean (Sargasso Sea) (Sullivan et al. 2003). Suttle and Chan (1994) concluded that viruses that infected *Synechococcus* DC2 and SYN48 were most abundant at the surface and 11 km offshore. In contrast, minimum values were found at 97 m depth and 83 km offshore. In Sullivan et al.'s (2003) study, *Synechococcus* cyanophage concentration was higher in mesotrophic environments (coastal waters) compared to oligotrophic environments (Sargosso Sea). *Prochlorococcus* cyanophage was not detected in coastal waters coinciding with the absence of *Prochlorococcus* cells. Therefore, cyanophages varied throughout the water column and along the transect due to the gradient in trophic level, together with changes in cyanobacterial population.

A few studies have suggested that cyanophages play important roles in regulating cyanobacteria bloom dynamics (Breitbart 2012). High numbers of cyanophage-like particles were generally correlated with low host abundance (Manage et al. 1999; Manage et al. 2001). The host cell number remains constant but a rapid decrease in Ma-LMM01- type phages was observed, suggesting that this was due to changes at the intra-species level of *M. aeruginosa* (Takashima et al. 2007). A similar observation has been reported by Yoshida *et al.* (2010), where cyanophages show the ability to influence the replacement of phage-sensitive populations by phage-insensitive populations. Moreover, there is no quantitative impact on the entire *M. aeruginosa* population. Thus, cyanophages are an important factor which can shift the composition of the host community during the bloom season (Yoshida et al. 2010).

While cyanophages are able to control the bloom of cyanobacteria, their abundance has also been reported to be affected by their host densities as well. For example, phage titer oscillated with host concentration after a lag of several days (synchronized shift) (Okunishi et al. 2003) and there was seasonal co-variation between cyanophage and total host (Wang et al. 2011; Kimura et al. 2012). In

addition, a requirement of minimum threshold host densities for efficient viral propagation was shown in several studies. Laboratory studies by Wiggins and Alexander (1985) have indicated that a minimum host threshold density, approximately 10^4 cells ml^{-1} , is needed to allow for phage replication. Okunishi *et al.* (2003) found that the *in situ* threshold density for cyanophage propagation is 5×10^4 cells ml^{-1} which is near the suggested value of Wiggins and Alexander (1985). Waterbury and Valois (1993) reported that the minimum host density threshold is not universal; the differences in minimum threshold densities were due to difference the time for phage adsorption and host growth rate. They observed that during winter months, *Synechococcus* concentration were less than 1000 cells ml^{-1} and at least one out of two strains of cyanophage could be detected (Waterbury and Valois 1993). In contrast, Manage *et al.* (1999) demonstrated a requirement of higher minimum threshold host densities for lysis of *M. aeruginosa*, i.e. *M. aeruginosa* concentration at 1×10^5 cells ml^{-1} . Suttle and Chan (1994) were able to detect infectious cyanophages even when *Synechococcus* was less than 100 cells ml^{-1} . However, they suggested that there is a threshold in host abundance beyond which cyanophages concentration increases dramatically, e.g. when *Synechococcus* concentration was above 10^3 ml^{-1} (threshold) the concentration of infectious cyanophages increased sharply, from about 10^2 to 10^5 ml^{-1} .

In summary, cyanophage abundance undergoes temporal and spatial variability which is linked closely with host cell densities and growth environment. In the temporal variation studies, seasonal co-variation between host and phages showed that host and phage were both at their highest concentration during the summer and lowest during the winter (Waterbury and Valois 1993; Wang *et al.* 2011), and the diurnal fluctuations in cyanophages were linked to the host's photosynthesis during the daytime (Kimura *et al.* 2012). In the spatial variation studies, the physical structure (salinity, temperature and light) of the water column changes with depth and location and this, in turn, influence the communities of host cyanobacteria. Since cyanophages and their host interact in a dynamic manner, the concentration and diversity of cyanobacteria affects the dynamics of cyanophage populations and vice versa (Short *et al.*, 2003). Thus, a decrease in the number of

cyanophages with depth is associated with the decrease in the number of host cells (Suttle and Chan 1994); and *Prochlorococcus* cyanophages are detected in oligotrophic environment (ocean), but are undetectable in mesotrophic environment (coastal), coinciding with the absence of *Prochlorococcus* cells in coastal waters.

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance

Specific cyanophage/ gene	Sampling site	Spatial/ Temporal study and frequency of sampling	Enumeration methods and range of cyanophage concentration	Findings (correlation with biotic and abiotic parameters)	Reference
Phage infecting <i>Synechococcus</i> (WH8012 and WH 8018)	Wood Hole Harbor	Temporal: Three times per month (27 months)	<u>Most- probable number (MPN)</u> WH8012-phage: Undetectable to 1.14×10^4 MPN/ml WH8018-phage: >100 to 6×10^3 MPN/ml (estimated through graph)	<ul style="list-style-type: none"> - Cyanophage titer varies seasonally, i.e. during summer, phage titer was between 10^3 and 10^4 MPN/ml, while during winter, phage titer was <100 MPN/ml or undetectable. - Phage titers began to increase about a month after the onset of the host spring bloom. 	Waterbury and Valois (1993)
Phage infecting five <i>Synechococcus</i> strains (838DG=WH8 007, DC2=WH7803, SNC1, SNC2, and SYN48=WH6501)	Along a transect in the western Gulf of Mexico (from coast to 83 km offshore).	Spatial: One sampling (20 June 1992) 9 stations along the transect, station 1 to 9, number consecutively from the most inshore to the most off shore	<u>MPN (by using 96 well plates)</u> A few to 4×10^5 MPN/ml	<ul style="list-style-type: none"> - Spatial variation was observed, where concentration of infective cyanophage decreased with increasing distance offshore and below depths of 20 to 30 m. - Seasonal variation observed, where infective cyanophage was most abundant when the temperature and salinity were 	Suttle and Chan (1994)
	Four stations	Spatial (depth):	1.5×10^2 to 2.52×10^5		

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

	(Stations 3, 5, 7 and 9)	One sampling (20 June 1992) 4 to 6 different depths at each station	MPN/ml ** only enumerated cyanophages which infect strain DC2	highest.	
	Pier of the Marine Science Institute in Port Aransas, Tx	Temporal/ Seasonal 16 samplings over 28 months (from March 1990 to June 1992)	A few to 1.9×10^5 MPN/ml		
Phage infecting <i>Microcystis aeruginosa</i>	Furuike Pond (hypereutrophic)	Temporal: Twice per month (11 months from March 1997 to January 1998)	<u>Plaque assay</u> Undetectable to 4.4×10^4 PFU/ml	- Two occasions, sharp decrease in <i>M. aeruginosa</i> abundance when the cyanophage concentration was high (negative relationship)	Manage et al. (1999)
Phage infecting <i>Microcystis aeruginosa</i>	Furuike Pond (hypereutrophic)	Temporal: Twice per week from September to November 1998 and once a week from December 1998 to August 1999	<u>Plaque assay</u> $< 5 \times 10^2$ to 7.1×10^3 PFU/ml (undetectable in winter and spring)	- Undetectable when the abundance of host was low. - Occasional sharp increase of cyanophage density coincides with the sharp decline in <i>M. aeruginosa</i> . However, it is not so for every peak.	Manage et al. (2001)
Phage infecting <i>Synechococcus</i>	Three inlets in British	Spatial: One sampling for	<u>MPN</u>	- Abundance of infective cyanophage, <i>Synechococcus</i> and	Frederickson

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

(WH7803=DC 2)	Columbia: Salmon Inlet, Malaspina Inlet and Pandrell Sound	each location. One location with 6 different depths (from surface to 25 m)	$< 2 \times 10^3$ to 3.5×10^5 MPN/ml ** Estimated from graph	total viruses decreased with depth.	et al. (2003)
Phage infecting four <i>Synechococcus</i> strains (WH 7803, WH 8012, WH 8018, WH 8113)	Mount Hope Bay, R.I	Temporal : Every other month from August 1999 through March 2001 and then every month from May 2001 to August 2002 (19 samplings)	<u>MPN (by using 24-well plates)</u> < 100 to 30,000 phage/ml	<ul style="list-style-type: none"> - Cyanophage at lowest concentrations during the winter months. - The cyanophage abundance varied with the host strains. 	Marston and Sallee (2003)
Phage infecting <i>Synechococcus</i> (strain KFM001)	Kagoshima Bay, Japan 2 stations St 1 : in a closed area St 2: at a pier near Kagoshima University	Temporal and Spatial (depth): At St 1 with total of 4 time samplings (March, July, September, and November). 8 depths over 100m.	<u>St 1: MPN</u> Undetectable to 920 MPN/ml	<ul style="list-style-type: none"> - At Station 1, cyanophage was undetectable in March and maximum in November. - At Station 1, maximum cyanophage titer detected at upper layer of 20 m (July and December at 20 m; and September at 0 m/ surface water). No cyanophage could be detected at layers deeper than 40 m. - At Station 2, “synchronized shift” was observed; phage titer 	Okunishi et al. (2003)
		Temporal/ Seasonal St 2: Surface water in autumn: September to December with 3 to	<u>St 2: Plaque assay</u> Undetectable to 7.5×10^3 PFU/ml		

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

		6 days interval (total 25sampling times).	*found the plaque assay was more sensitive than MPN	began to increase after several days when the host reached its maximum density. Subsequently, phage titer oscillated with the host concentration after a lag of several days.	
Phage infecting <i>Prochlorococcus</i> host strains (SS120, MIT9309, MIT9313, MIT9215, NATL2A, MIT 9320, MIT 9515, MED4, MIT9211, NATL1A, MIT9312)	Sargosso Sea	Spatial (depth): one sampling, 6 different depths over 120 m	<u>MPN</u> < 350 phage/ml	<ul style="list-style-type: none"> - SS120-phsge, MIT9303-phage and MIT 9313-phage were not detected over the depth. - Maximum MIT9215-phage and NATL2A-phage concentration at 70 m. - Maximum MIT9320-phage, MIT9515, MED4, MIT9211 and NATL1A-phage concentration at 100 m. - Maximum MIT9312-phage concentration at 120 m. - <i>Prochlorococcus</i> increase from surface to maximum concentration at 70 m, and then concentration decreased from 70 m to 120 m. 	Sullivan et al. (2003)

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

Phage infecting <i>Prochlorococcus</i> or <i>Synechococcus</i> host strains	Surface water transect from coastal (mesotrophic) to open ocean (oligotrophic)	Spatial: 4 sampling sites from coastal to open ocean (coast, shelf, slope and Sargasso)	<u>MPN and plaque assay</u> < 3 x 10 ³ phage/ml for any host strains	<ul style="list-style-type: none"> - <i>Synechococcus</i> cyanophage concentration was found to decrease by an order of magnitude or greater in surface waters between coastal and open ocean. - No <i>Prochlorococcus</i>-cyanophage was detected in coastal samples. - Host strain microdiversity increased but cross-infection ability did not increase concurrently and nutrient availability decreased along the transect. Thus, is the potential explanation for decreasing phage titers from coastal to open ocean. 	
Phage infecting <i>Synechococcus</i> (WH7803)	Indian Ocean	Temporal (diel cycle) and spatial (depth): 24 hours with 6 hrs intervals and each time at 6 different depths (10m, 25m, 50m, 80m, 100m and	<u>Plaque assay</u> <ul style="list-style-type: none"> - 10m: 6x10² PFU/ml to 1x10³ PFU/ml (0100h) - 25m and 50m <2x10² PFU/ml 	<ul style="list-style-type: none"> - Phages were most abundant at 10 m depth and pronounced increase in phage number observed at 0100h. - Short-term fluctuation (diurnal pattern) in cyanophage concentration occurred. 	Clokier et al. (2006)

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

		130m)	<ul style="list-style-type: none"> - Not detected in 50m, 80m, 100m and 130m 	<ul style="list-style-type: none"> - At 25m and 50m, no show strong diurnal variation, maybe due to the difference of radiation intensity over a diel cycle and is not as apparent as at 10 m. 	
Phage infecting <i>Synechococcus</i> (WH7803, WH 8013, RS9906, RS9911)	Gulf of Aqaba, Red Sea	Temporal and spatial (depth): 11 samplings over a nine-month period. One station with varied in depth from surface to 150 m (6-7 points)	<u>Plaque assay</u> Undetectable to 6.08×10^3 PFU/ml	<ul style="list-style-type: none"> - Cyanophages are found throughout the water column but not all four different cyanophage strains are present in the water samples. - The highest concentration of cyanophages is in summer months (July to September). - The maximum concentration of cyanophage in surface waters only occurred once during sampling. 	Millard and Mann (2006)
Cyanomyoviruses (g20 gene)	Raunefjorden, Norway (Norwegian coastal waters)	Temporal: 16 samplings over a nine-month period	<u>Real- time PCR (primers CPS1 and CPS2)</u> < 5.0×10 to 7.2×10^3 phage/ml	<ul style="list-style-type: none"> - Cyanophage abundance increased sharply (peaked) after falls (two occasions) in <i>Synechococcus</i> bloom. 	Sandaa and Larsen (2006)

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

Ma-LMM01 (cyanophages which infect <i>Microcystis aeruginosa</i>) - like cyanophages (g91 gene)	Lake Mikata and Hirosawanoike Pond , Japan	Temporal: Lake Mikata: 4 months (April, May, August and September 2005) - once per month Hirosawanoike Pond: 2 months (October and November 2005) - once per month	<u>Real- time PCR (primers ShealthRTF and ShealthRTR)</u> - Hirosawanoike Pond: 2.2×10^3 and 9.7×10^5 particles/ml - Lake Mikata: Undetectable to 1.8×10^5 particles/ml	- Dynamics of Ma-LMM01- type phages was suggested due to changes in the composition of host population that were susceptible to them. - Phages were not detected in Lake Mikata when host cell number was below the detection limit.	Takashima et al. (2007)
Ma-LMM01 (cyanophages which infect <i>Microcystis aeruginosa</i>) - like cyanophages (g91 gene)	Lake Mikata, Japan	Temporal: Monthly sampling from April to December (2006)	<u>Real- time PCR (primers ShealthRTF and ShealthRTR)</u> 1.1×10^2 to 1.1×10^4 gene copies/ ml (below detection limit on April : $<1.0 \times 10^2$ copies/ml)	- Cyanophage abundance was negative correlated with <i>M. aeruginosa</i> cell numbers.	Yoshida et al. (2008)
Ma-LMM01 (cyanophages which infect <i>Microcystis aeruginosa</i>) - like	Hirosawanoike Pond , Japan (surface water)	Temporal: Monthly sampling for 3 years (2006 to 2008) but just involved month May to November.	<u>Real- time PCR (primers ShealthRTF and ShealthRTR)</u> Quantify g91 DNA and g91 mRNA	- No clear relationship between phage abundance (g91 DNA) and the host. - During 2007 and 2008, phage RNA transcripts and phage	Yoshida et al. (2010)

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

cyanophages (g91 gene)			<p>-g91 DNA: 1.5×10^0 to 1.7×10^3 gene copies/ml</p> <p>- Relative abundance of g91 mRNA: 2.3×10^{-5} to 7.6×10^{-2} (<i>rnpB</i> gene transcripts of the host was using to normalize the g91 mRNA data)</p>	abundance showed a similar temporal pattern to host numbers (10^3 to 2×10^6 PC gene copies/ml).	
Cyanomyoviruses (g20 gene)	Lake Erie (Laurentian Great Lake), North America	Temporal and Spatial: One sampling in winter (February) and one sampling in summer (August), 4 sampling sites each sampling sites 2 different depths.	<p><u>Real- time PCR (primers CPS1 and CPS2)</u></p> <p>1.3×10^5 to 4.3×10^6 gene copies per ml</p>	<ul style="list-style-type: none"> - Negative correlation between g20 densities and depth during summer. - Strong correlation between g20 densities and all size classes of chlorophyll-a ($0.2-2 \mu\text{m}$ 2-20 μm and $<20 \mu\text{m}$). - Cyanomyovirus with g20 gene were abundant in both summer and winter. 	Matteson et al. (2011)
Phage infecting <i>Synechococcus</i> WH7803	Chesapeake Bay station 858=	Temporal/ Seasonally: 19 Samplings from	<u>MPN (by using 96 wells plate)</u>	<ul style="list-style-type: none"> - High phage titers in summer and low titers in the winter. 	Wang et al. (2011)

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

	northern bay station 804= middle bay and station 707= southern bay	August 2003 to February 2007	- 105 to 6.2×10^5 MPN/ml	- Seasonal co-variation between cyanophage abundance and total <i>Synechococcus</i> host cell density observed in 2005 and 2006.	
Phage infecting <i>Synechococcus</i> CB0101			- Undetectable to 1.0×10^4 MPN/ml		
Ma-LMM01 (cyanophages which infect <i>Microcystis aeruginosa</i>) - like cyanophages (g91 gene)	Hirosawanoike Pond, Japan (surface water)	Temporal (diel cycle): 24 hours at 3 hours interval (2 different date, 2 samplings)	<u>Real-time PCR and real-time RT-PCR (primers: ShealthRTF and ShealthRTR)</u> quantify g91DNA (host cell fraction and free-phage fraction) and g91 mRNA Diel first sampling g91 DNA in the <u>Free-phage fraction</u> : 1.3×10^2 to 3.9×10^2 (1800 h) copies/ml <u>Host-cell fraction</u> : 5.2×10^3 to 1.3×10^5 (1500 h) copies per ml <u>Relative abundance of g91 mRNA</u> : 0.0007 to 0.0051 (1200 h)	- Phage proliferation was suggested to depend on host photosynthesis performance. Thus, dynamics of cyanophage abundance is affected by diel cycle. - From monthly sampling, it was found that phage abundance fluctuates with host abundance.	Kimura et al. (2012b)

Table 2.2 Summary of spatial and temporal dynamic of cyanophage abundance (continued)

			<p>Diel second sampling g91 DNA in the <u>Free-phage fraction:</u> 1.3×10^2 to 2.2×10^3 (2100 h) copies per ml <u>Host-cell fraction:</u> 2.6×10^4 to 1.1×10^6 (0300 h) copies per ml <u>Relative abundance of g91 mRNA:</u> 0.0007 to 0.017 (1500 h)</p>		
		<p>Temporal / Seasonal: Monthly (April to November 2009)</p>	<p>Seasonal g91 DNA in the <u>Free-phage fraction:</u> below detection limit to 8.2×10^2 copies/ml <u>Host-cell fraction:</u> 2.5×10^1 to 1.6×10^6 copies/ml</p>		

2.6.2 Spatial and temporal dynamics of cyanophage diversity

2.6.2.1 Approaches to study genetic diversity of cyanophage communities

Earlier studies have used PCR to amplify virus marker genes from environmental samples to study the genetic diversity of specific groups of viruses (Short et al. 2010). The viral capsid gene (g20 gene) and deoxyribonucleic acid (DNA) polymerase gene (DNA *pol* gene) are widely used as genetic markers for cyanophage diversity studies. Viral capsid gene primers have been used to study the diversity of cyanomyoviruses, while the DNA *pol* gene primers were used to study the diversity of cyanopodoviruses communities.

The first phylogenetic diversity study of marine cyanophages was conducted by Zhong et al., (2002) with the CPS1 and CPS8 primers targeting the g20 gene. Subsequently, CPS1 and CPS8 primers have been widely used to investigate the cyanomyoviruses community composition of various environments, including lakes, marine water, paddy floodwater, paddy soil and bay water (Marston and Sallee 2003; Dorigo et al. 2004; Wang and Chen 2004; Sandaa and Larsen 2006; Wilhelm et al. 2006; Wang et al. 2010; Wang et al. 2011). The spatial and temporal dynamics of cyanomyovirus diversity has been investigated by another three sets of primers also, i.e. CPS4/CPS5 (Frederickson et al. 2003; Mühling et al. 2005), CPS4/G20-2 (Short and Suttle 2005) and CPS1.1/CPS8.1 (Sullivan et al. 2008; Jameson et al. 2011) with these primers also targeting the g20 gene. The CP-DNAP-349F and CP-DNAP-533R primers were designed by Chen et al. (2009) based on the DNA polymerase gene of seven cyanopodoviruses. CP-DNAP-349F and CP-DNAP-533R primers have been used to investigate the genetic diversity of cyanopodoviruses from Chesapeake Bay, North Atlantic Ocean, Pacific Ocean and South China Sea (Chen et al. 2009; Huang et al. 2010).

Application of primers targeting the cyanomyovirus and cyanopodovirus genes, either in combination with denaturing gradient gel electrophoresis (DGGE)

or terminal- restriction fragment length polymorphism (T-RFLP) or by cloning combined with sequencing, has successfully revealed both temporal and spatial cyanophage community variations in various water systems. The fingerprint from PCR- DGGE approach is useful for determining the relatedness of cyanophage communities from the same environment or different environments (Frederickson et al. 2003). PCR-DGGE was suggested as a more sensitive method to enumerate the diversity of phage communities when compared to restriction digests of a short region of phage genome. However, DGGE bands could only represent a minimum estimate of richness and was especially limited due to use of non- degenerate primers (Frederickson et al. 2003) and the number of fragments in a DGGE gel may not represent the actual number of different target sequences amplified (Short and Suttle 2005).

In general, primers of marker genes are amplified from nucleic acids of viruses to obtain the different target gene sequences. To separate individual gene fragments, cloning or DGGE can be used. After separation of gene fragments, sequencing is conducted, involving several steps, i.e. sequence editing, sequence alignment, phylogenetic inference, drawing phylograms and lastly calculating diversity indices (Short et al. 2010). From PCR- cloning/ DGGE- sequencing- sequence/ phylogeny analysis, the evolution distance and similarity of cyanophage communities between various environments can be obtained. These are reviewed in the following section 2.6.2.2.

2.6.2.2 Review on the spatial and temporal dynamics of cyanophage diversity

The temporal variation in cyanophage communities from various environments (e.g. Lake Bourget, Chesapeake Bay, the Red Sea, Norwegian coastal water, paddy flood water and paddy field soils) has been demonstrated by using g20 clones (cyanomyoviruses) (Dorigo et al. 2004; Wang and Chen 2004; Mühling et al. 2005; Sandaa and Larsen 2006; Wang et al. 2010; Wang et al. 2011) and DNA *pol*

clones (cyanopodoviruses) (Chen et al. 2009). Temporal variation was demonstrated via formation of a unique cluster, shifting of the dominant cluster and dissimilarity of DGGE or T-RFLP profiles between the sampling periods. Mühling et al. (2005) demonstrated that cyanomyovirus population diversity (DGGE analysis) was greater during the spring and winter compared to the summer and autumn seasons, co-varying with *Synechococcus* diversity. In addition, when *Synechococcus* and phage titer were at their maxima (July 1999), they found that the lowest numbers of genotypes of *Synechococcus* and cyanomyoviruses communities obtainable. Thus, Mühling et al. (2005) suggested that myoviral genetic richness is in parallel with their host.

The spatial variation in cyanophage communities studies have been demonstrated that population structure in estuary and open ocean waters differed from each other (Zhong et al. 2002; Huang et al. 2010) and the clonal diversity changed from surface water to the deep chlorophyll maximum (DCM) layer (Zhong et al. 2002). The highest diversity of the g20 sequences has been described at the deep chlorophyll maximum (DCM) layer (Zhong et al. 2002) in the Atlantic Ocean at the 1 % light depth (Jameson et al. 2011). Zhong et al. (2002) suggested that the diversity variation was due to different host population in response to different water conditions such as light intensity, nutrient, and also dynamic interaction between cyanophage and cyanobacteria population. Jamerson et al. (2011) suggested that at 1 % light depth, solar radiation is insufficient to affect significant viral decay, thus increasing the survival of various cyanophages. Other than light, nutrient was shown to affect cyanophage communities. Paddy floodwater samples collected from four plots in the subjected to different fertilizer treatment gave different DGGE profiles (refer to Table 2.3) (Wang et al. 2010).

Wang and Chen (2004) showed similar T-RFLP profiles at four stations in Chesapeake Bay when g20 clone diversity was investigated. No spatial variation in DNA *pol* clones distribution was shown in the Chesapeake Bay when phylogenetic analysis was conducted on samples of three stations in the bay (Chen et al. 2009). Chen et al. (2009) found that no spatial variation in cyanophage communities was

consistent with weak spatial variation of bacterial communities in the Chesapeake Bay. Zhong et al. (2002) suggested that cyanophage diversity increased from surface water to the DCM layer, due to a more diverse host population at depth in stratified water columns (Zhong et al. 2002). Sandaa and Larsen (2006) showed that cyanophage diversity was increased after *Synechococcus* bloomed. Thus, host abundance and diversity were influencing the diversity of the cyanophage communities.

From phylogenetic studies, researcher showed that different environments or biomes consisted of unique clusters or clades. Wilhelm et al. (2006) showed that Clade 1 consisted of g20 clones from marine environments, and Clade C3 consisted of only freshwater clade. Four unique subclusters (PFS- I and PFS IV) containing g20 clones from paddy field soil (Wang et al. 2011) have been identified. Sullivan et al. (2008) suggested that the g20 clones were distributed according to geographic segregation. However, Short and Suttle (2005) reported that in both marine and freshwater environments, environments which differed greatly in temperature and salinity were able to show clone sequences more than 99 % similarity. They suggested that this nearly identical g20 gene indicates closely related hosts and the viruses infecting them are distributed widely due to horizontal exchange occurs among phage population from different environments. Thus, similar g20 clones can be obtained from various environments but g20 diversity distribution pattern (such as phylogenetic tree) from different environments are different and unique clades/cluster can be obtained from each individual location.

In summary, spatial and temporal variation of cyanophage population diversity in *in-situ* studies were examined through the g20 (cyanomyoviruses) and DNA *pol* (cyanopodoviruses) clones distribution. Cyanopodovirus distribution has only been studied in marine environments. These *in-situ* studies showed that temporal variation was greater than spatial variation if limited to a confined location such as Chesapeake Bay. However, when comparing different biomes such as estuarine water, oceanic water, lakes and paddy floodwater or soil, different distribution patterns and unique subclusters were easily obtained from each biome.

Light, nutrient, host abundance and diversity have been shown to correlate with cyanophage population diversity. Other than light (UV degradation), nutrient, host abundance and diversity, Jamerson et al. (2011) suggested that host resistance/susceptibility, host starvation, mixing and diurnal cycle are the external influences in the ocean which affect cyanophage diversity. Chen and Lu (2002) believed that the genetic diversity of lysogenic phages is much higher than lytic phages due to higher frequencies of lateral genetic exchange between the lysogenic phage and host. In other words, the replication of lysogenic phages would be more host dependent than that of lytic phages. Table 2.3 summarizes the previous studies on the temporal and spatial variation of genetic diversity within the cyanophage community.

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community

Specific gene / primers	Spatial/ temporal study and frequency of sampling	Methods to study the cyanophage community and results of the cyanophage community distribution	Findings: correlation between cyanophage diversity and water conditions	Reference
g20 gene/ CPS1 and CPS8	<p>Spatial: Only one sampling from each station</p> <p><u>Sampling sites</u> Six sampling sites from the river estuary to the open ocean (salinity ranged from 33 % to 36.7 %)</p> <ul style="list-style-type: none"> - Skidaway Esturine surface sample (SE1)- salinity: 33 % - Gulf Stream surface sample (GS26) – salinity : 36.2 % - Gulf stream deep chlorophyll maximum (DCM) (83 m) (GS27) sample – salinity: 36.4 % - Sargasso Sea surface sample (SS48) – salinity 36.7 % 	<p><u>Method: PCR-Cloning-Sequencing – Phylogeny analysis</u></p> <ul style="list-style-type: none"> - Designed primers CPS1 and CPS8. - CPS1 and CPS8 primers were tested on 11 cyanophage isolates, these isolates were isolated from oceanic <i>Synechococcus</i> strains. These isolates were then assigned to three clusters, i.e. I, II and III from the phylogenetic analysis. - Six natural virus concentrates were amplified with CPS1 and CPS8, 114 different g20 homologs were assigned to nine clusters, i.e. I to III, A to F and I. - Clusters A to F and cluster I contained only clones from natural environments (virus concentrates). None of the 	<ul style="list-style-type: none"> - This study showed that genetic structures or cyanophage population structures vary greatly in different marine environments, such as estuarine water (SE1) versus open ocean (GS26 and SS48) and in oceanic surface water (GS26 and SS48) versus oceanic DCM layer (GS27, SS40 and SS47). - SE1 (cluster A, E, F, I, III) showed more diversity than GS26 (cluster B, E, III) and SS48 (cluster B, C, E). SE1 dominant cluster is cluster 1 while GS26 and SS48 are dominated by cluster E. Unique phylogenetic clusters were found for each environment, i.e. for SE1 	Zhong et al. (2002)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<ul style="list-style-type: none"> - two DCM samples (SS40- 134 m and SS47- between 100 and 148 m) 	<p>clones belong to any cyanophage isolates.</p> <ul style="list-style-type: none"> - Cluster I consisted of 5 isolates and 15 clones from SE1 only. Cluster A and F included only clones from SE1. - Cluster II consisted 1 isolate and 8 clones from DCM (GS27, SS40 and SS48). Cluster D only included oceanic DCM communities (GS27, SS40). 	<p>sample is cluster I, A and F and for opean ocean samples are cluster B, C, D, and II.</p> <ul style="list-style-type: none"> - Samples from DCM layer showed higher diversity (distributed to 4 to 6 clusters) than oceanic surface water samples (assigned to 3 clusters). Moreover, the dominant cluster shifted from cluster E to cluster B. - GS26 and SS48 were similar on g20 gene diversity. These two stations have fewer phyletic groups, thus indicating that oceanic surface waters are less diversified compare to estuarine water and DCM layer. 	
g20 gene/ GC-CPS4 and CPS 5	<p>Spatial: One sampling for one location. One location with 6 different depth (from surface to 25 m)</p>	<p><u>Method: PCR – ¹DGGE</u></p> <p>15 to 20 DGGE bands (PCR product: ca. 200 bp)</p>	<ul style="list-style-type: none"> - DGGE band pattern of Salmon and Malaspina inlets at surface water (temperature was higher, and salinity was lower) showed significance difference compare with 	Frederickson et al. (2003)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<p><u>Sampling sites</u> Three inlets in British Columbia:</p> <ul style="list-style-type: none"> - Salmon Inlet, - Malaspina Inlet - Pandrell Sound. <p>These three inlets connected to the same body of water, the Straits of Georgia</p>		<p>others depth (from surface to 25 m total 6 different depths were investigated).</p> <ul style="list-style-type: none"> - However, other depths within Salmon and Malaspina Inlets showed a high degree of similarity in the DGGE band pattern. - Pandrell Sound with broader differences in temperature and salinity throughout depth (shift of 12.5 °C and change of 14 ppt) showed differences in community composition at different depths (based on DGGE band patterns). - Three DGGE band patterns showed some cyanophages to be widespread (observed at all depths); while others were observed at specific depth. - In Malaspina Inlets and Pandrell sound, unique bands were observed which coincided with high 	
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

			chlorophyll fluorescence.	
g20 gene/ CPS1 and CPS4 or CPS1 and CPS8 or CPS1 and CPS2	<p>Temporal: Alternate month sampling (August 1999 to March 2001) Monthly sampling (May 2001 to August 2002)</p> <p><u>Sampling site:</u> Mount Hope Bay, Rhode Island</p> <p>Spatial <u>Sampling sites:</u> Mount Hope Bay (June to August 2002) Narragansett Bay (June and August 2002)</p>	<p><u>Method: Plaque purification (isolation)- PCR - RFLP- cloning-sequencing-phylogenetic analysis</u></p> <ul style="list-style-type: none"> - 36 different cyanophage g20 genotypes were identified over a 3-year period of 190 cyanophage isolates. - Phylogenetic analyses: 3 clusters (Cluster I, II and III). - Nucleotide sequence similarities among the isolates ranged from 47.3 to 98.6 %. - Amino acid sequence similarities ranged from 70.4 to 100 % 	<ul style="list-style-type: none"> - Temporal changes in both the overall composition of the cyanophage community and the relative abundance of cyanophage g20 types were observed. For example S-RIM14, SRIM12, SIRM29 and S-RIM28 were present in June samples (from both bay) and represented more than 47 % of each sample, but none of these specific cyanophage genotypes were detected in August samples. - Seasonal patterns in the appearance and disappearance of particular genotypes were not detected. - No correlation was observed between season and the presence of genotypes in different phylogenetic groupings. 	Marston and Sallee (2003)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

g20 gene/ CPS1 and CPS8	<p>Temporal/ seasonal: Monthly sampling (September 2002 to January 2003)</p> <p><u>Sampling site:</u> Lake Bourget, France Approximately 5 m depth (Freshwater)</p>	<p><u>Method: PCR – cloning - sequencing – phylogeny analysis and PCR- DGGE</u></p> <p>A total of 47 sequences were produced.</p> <ul style="list-style-type: none"> - Nucleotide analyses: 35 distinct genotypes from these sequences. - Plylogenetic analyses: 6 clusters (Cluster 1 to Cluster 6). One sequence stood apart from these clusters, thus 7 OTUs were identified. - Nucleotide sequence similarity in each cluster was > 96 %, while the similarity between different clusters ranged from 54 to 60 %. <p>DGGE was performed on the same samples as those used for cloning- sequencing</p>	<ul style="list-style-type: none"> - A clear seasonal pattern variation in the composition of the cyanophage community was observed through sequencing and DGGE analysis. For example: Cluster 1 was only obtained from samples in September and October. In addition, these two months have similar DGGE band patterns. Dominance of sequences for November samples belonged to Cluster 2. 	Dorigo et al. (2004)
g20 gene/ CPS1 and CPS8	<p>Spatial One sampling at these 4 stations (November 2000)</p> <p><u>Sampling sites</u></p>	<p><u>Method: PCR – cloning – ²RFLP (enzyme: RsaI) – sequencing – phylogeny analysis</u></p> <ul style="list-style-type: none"> - g20 gene fragments with 569 to 	<ul style="list-style-type: none"> - 11, 8, 8 and 7 different RFLP patterns were found at Pier 5, CB908, CB818 and CB 707 respectively. 	Wang and Chen (2004)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<p>4 stations in Chesapeake Bay</p> <ul style="list-style-type: none"> - Pier 5 in the Baltimore Inner Harbor: surface water (salinity: 20%) - CB908: 3 m depth (salinity: 18.15%) - CB818: 3 m depth (salinity: 19.45 %) - CB707: 3 m depth (salinity: 29.03 %) <p>(Estuarine water)</p> <p>Temporal Monthly sampling from Inner Harbor, total (March 2001 to May 2002)</p>	<p>599 bp were amplified from the samples.</p> <ul style="list-style-type: none"> - 180 clones randomly selected to run RFLP. 15 distinguishable RFLP patterns were identified and assigned as ⁵OTU1 through OTU15. - Representative clones from each station distinguished by RFLP were sequenced. - OTU1 to OTU 15 with previous g20 clones from Zhong et al. (200) were used to construct the phylogentic tree. Cluster N1 to N4 contained only g20 clonal sequences from the bay and only OTU 12 was closely related to known cyanomyovirus isolates (in cluster I). - Cluster N2, consisting of 42 % of 180 clones, was suggested to be related to clone in Skidaway Estuary. - Cluster N3 (OTU 3 and OTU 9) was suggested to be closely related to a Sargasso Sea DCM clone (SS4705) 	<ul style="list-style-type: none"> - Similar T-RFLP profiles were observed from these four stations. But, the relative abundances of TFs varied between the stations. This fingerprint results suggested that no significant variation in genetic diversity of the cyanomyoviruses in the bay in November 2000. - 9 months (which excluded winter samples due to no amplification of g20 gene (February, April and May 2002) or poorly resolved of T-RFLP profile (March 2001)) of T- RFLP profile from Inner Harbor demonstrated huge variation in genetic diversity. There is seasonal variation, and this variation was suggested to be correlated with changes in the host population. - Maximum levels of both <i>Synechococcus</i> abundance and phage titers (phage 	
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

		<p><u>Method: PCR (CPS1 and 5' hex-labeled CPS8 primers) -³T- RFLP analysis</u></p> <ul style="list-style-type: none"> - A total of 20 unique T-RFs were identified from 46 representative sequences (15 OTUs, 10 cyanomyovirus isolates and 21 environmental clones) chosen from 200 g20 gene sequences in the GenBank. - These 20 unique T-RFs (predicted T-RFs) were used to identify the T-RFLP peaks from the environmental samples. Thus, T-RFLP profiles demonstrated the distribution of cyanomyoviruses community in the bay. - Diverse g20 genotype in the bay was suggested due to > 25 T-RFs obtained from the temporal profile. 	<p>infectious to <i>Synechococcus</i> CB0101) together with 12 major T-RFs (consider more diverse) were observed between July and September 2002 (summer). Temperature was suggested to affect cyanophage community indirectly due to influence of host- cell density.</p>	
g20 gene/ GC-CPS4 and CPS 5	<p>Temporal</p> <p>15 samplings between March 1999 and January</p>	<p><u>Method: PCR – cloning – DGGE</u></p> <ul style="list-style-type: none"> - Only eight samples were chosen for genotypic analysis. 	<ul style="list-style-type: none"> - <i>Synechococcus</i> showed greater diversity in the spring and winter (11 and 17 RFLP types), similar to the 	Mühling et al. (2005)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<p>2000</p> <p><u>Sampling site:</u> Gulf of Aqaba, Red Sea (depth – 10 m)</p>	<ul style="list-style-type: none"> - 36 clones of each clone libraries were screened by DGGE, only different DGGE clones were sequenced. 	<p>cyanophage population, i.e. 20 DGGE types in the spring and 18 DGGE types in the winter. Both of the diversities showed decline in richness towards the summer and autumn.</p> <ul style="list-style-type: none"> - The seasonal changes in myoviral genetic ⁶richness paralleled with the host. In addition, strong correlation ($r^2 = 0.74$, $P < 0.01$) had been found from the ⁶evenness measure. This indicates when the periods of dominance by particular viral genotypes tend to co- occur with specific <i>Synechococcus</i> genotypes. - Myoviral diversity and abundance was correlated with co- occurring <i>Synechococcus</i> diversity and abundance. The correlation was even higher when 1 month lag- time was introduced. 	
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

g20 gene/ CPS4 and G20-2	<p>Spatial</p> <p>* Marine and Freshwater</p> <p><u>Sampling site - depth, salinity:</u></p> <p>Marine</p> <ul style="list-style-type: none"> - Gulf of Mexico (GOM01)- 110 m, 36.6 ppt - Beaufort Sea, Arctic Ocean (BES02A) – 35 m, 34.0 ppt - Beaufort Sea, Arctic Ocean (BES02B) – 25 m, 30.0 ppt - Chuckchi Sea, Arctic Ocean (CHS02) – 3,246 m, 35.0 ppt - Antarctic peninsula (ANT98) – 0.5 m, 34.1 ppt - Coast of Colombia, SE Pacific (COL00) – 1-3 m, 33.5 ppt - Coast of Chile, SE Pacific (CHI00) – 1-3 	<p><u>Method: PCR- DGGE</u></p> <ul style="list-style-type: none"> - Designed G20-2 primer, and pair with CPS4 to amplify g20 fragment myoviruses infecting <i>Synechococcus</i> spp. - DGGE profiles patterns resulted in the separation of 3 to 18 bands. - 99 DGGE bands were excised to continue with cloning and sequencing. <p><u>Method: Excise DGGE band – cloning- sequencing- phylogeny analysis</u></p> <ul style="list-style-type: none"> - Cluster I, II and III (Zhong et al, 2002) were group as cultured <i>Synechococcus</i> phage (CSP) group in this study, because all g20 sequences for cyanomyoviruses isolates infecting <i>Synechococcus</i> fell into this group. The g20 sequences outside the cultured <i>Synechococcus</i> phage (CSP) group may not be from 	<ul style="list-style-type: none"> - Nearly identical bacteriophage structural gene sequences (> 99 % identical nucleotides) were recovered from environments which differed greatly in location, salinity and temperature. For example, sequence from Gulf of Mexico (GOMO1-16) shared 99 % identity with g20 clone from Lake Constances (LAC95A-1). - 2 of the 4 new clades, i.e. G and J contained only freshwater g20 sequences. 	<p>Short and Suttle (2005)</p>
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<p>m, 34.4 ppt</p> <ul style="list-style-type: none"> - Salmon Inlet, NE Pacific (SAI99) – 0.5 m, 17.9 ppt - Pendrell Sound, NE Pacific (PES99) – 6.2 m, 22.3 ppt - Malaspina Inlet, NE Pacific (MAI00) – 1 m, 25.6 ppt - Teakerne Arm, NE Pacific (TEA00) – 7-10 m, 24.0 ppt <p>Freshwater – salinity : Not applicable (NA)</p> <ul style="list-style-type: none"> - Cultus Lake, BC, Canada (CUL02M) – 10 m - Cultus Lake, BC, Canada (CUL02H) – 18 m - Chilliwack Lake, BC Canada (CHL02E) – 3 m - Catfish Pond, Crowley La (CAT02) – NA - Shore Pond 	<p>cyanophages.</p> <ul style="list-style-type: none"> - Four new clades have been identified, they are G, H, J and K. - Only 2 of 15 sequences from these three lakes were assigned into CSP group. 		
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<p>cyanobacterial Mat, Arctic Ocean (SPM02) – NA</p> <p>- Lake Constance, Germany (LAC95A/ B) – 3 m</p>			
<p>g20 gene/ CPS1 and CPS2 or CPS1 and CPS8</p>	<p>Temporal:</p> <p>16 samplings over a nine-month period, from March to November 2004</p> <p><u>Sampling site</u> Marine Raunefjorden, Norway (Norwegian coastal waters) Depth of 2 m.</p>	<p><u>Method: ⁴PFGE- PCR (CPS1 and CPS 2)</u></p> <p>- DNA extracted from 25 PFGE bands and tested with CPS1 and CPS2 primers. 15 PFGE bands give positive signal. These positive bands ranged from 26 to 380 kb.</p> <p><u>Method: PCR (CPS1 and GC-CPS8) – DGGE</u></p> <p>- Temporal DGGE profiles were undergoing cluster analysis, and two main groups obtained. March to June DGGE profiles had been designated in group II, and group I comprises samples from August to November 2004.</p>	<p>- Cyanophage diversity increased after the <i>Synechococcus</i> bloom.</p> <p>- Seasonal shift in cyanophage community has been revealed.</p>	<p>Sandaa and Larsen (2006)</p>

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

		<p><u>Method: PCR products from PFGE/ DGGE bands – sequencing - phylogeny analysis</u></p> <ul style="list-style-type: none"> - All PFGE/DGGE products were grouped into Clade I, II and III (as Zhong et al.) and another clade which displayed highest similarity with the cyanophage S- BnM1. Thus g20 genes all clustered within cultured <i>Synechococcus</i> phage group (CPS). - All sequences from PFGE bands were clustered in clade II. 		
g20 gene/ CPS1 and CPS8	<p>Spatial</p> <p><u>Sampling site: Lake Erie</u></p> <ul style="list-style-type: none"> - Sandusky Bay (UTK 63 and UTK 147: virus concentrates; MC9-MC 11: particulate samples; MC33: whole water) - Lake Erie, station 357 	<p><u>Method: PCR – cloning - sequencing – phylogeny analysis</u></p> <ul style="list-style-type: none"> - C1, C2 and C3 clades have been assigned from phylogenetic analysis of g20 amino acid sequences. - All g20 sequence of cyanophages isolated from marine <i>Synechococcus</i> strain WH7803 and g20 sequences of 	<ul style="list-style-type: none"> - The phylogeny of g20 sequence in this study showed that the amplicons from natural environments as list under sampling site are related to marine cyanophage, i.e. cluster with marine clones in C2 clade however, in some cases they form a unique clade, i.e. C3. 	Wilhelm et al. (2006)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<p>(UTK 64 and UTK 145: virus concentrate)</p> <ul style="list-style-type: none"> - Grand River (UTK147/MC31: virus concentrate) - Burns Harbor (MC38, MC42: ballast samples) - Thunder Bay (MC40: ballast sample) 	<p>previously known isolated cyanoviruses are assigned to cluster C1.</p> <ul style="list-style-type: none"> - Clade C1 only consisted of cyanophages isolated from marine <i>Synechococcus</i> spp., clones from marine environment, and there is no direct amplicon from this study. Thus C1 is suggested to be exclusively a marine clade. - No marine clones from previous studies were identified in C3 clade, and C3 was suggested to be a freshwater clade. 		
g20 gene/ CPS1 and CPS8	<p>Spatial and temporal:</p> <p>*paddy floodwater (PFW)</p> <p><u>Sampling site:</u> Paddy field located in Aichi- ken Anjo, Japan.</p> <p>Four plots in the paddy field were chosen. These plots had been subjected to</p>	<p><u>Method: PCR (GC-CPS1 and CPS8)- DGGE</u></p> <ul style="list-style-type: none"> - Many DGGE bands were observed in every sample. - Many DGGE bands indicated that the g20 sequence structure of PFW was diverse. <p><u>Method: Excise DGGE band- PCR – cloning- sequencing- phylogeny</u></p>	<ul style="list-style-type: none"> - DGGE profiles of different plots were different on 19 June and 10 July but similar on 30 July. - Dissimilarity in DGGE band patterns between the plots and sampling dates was observed. - Unique branches of PFW g20 clones were found from the 	Wang et al. (2010)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<p>different fertilizers</p> <ul style="list-style-type: none"> - Plot without fertilizers (NoF plot) - Plot with P and K but no N (CF(-N)) - Plot with N, P and K chemical fertilizers (CF plot) - Plot with amended N, P and K chemical fertilizer and rice straw compost (CM plot) <p>Three samplings (19 June, 10 July and 30 July 2008) were conducted, where the paddy floodwaters were collected from the middle part of each plot.</p>	<p><u>analysis</u></p> <ul style="list-style-type: none"> - DGGE gave different g20 clones; in total 77 bands were excised for sequencing. - Length of PCR products: 546 bp (86%), 549 bp (10%) and 555 bp (4%). - Clones from paddy floodwater showed the highest identities of g20 sequence from 67% to 98% with the cyanophage isolates and clones obtained from previous studies. - Five major clusters have been identified from the phylogenetic analysis, i.e. α, β, γ, δ and ε. Clone PFW-CM14 stood apart from these five clusters. - Eight subclusters which contained only clones from this study were designated, PFW-I to PFW-VI and CSP- PFW1 and CSP- PFW2. - Cluster δ in this study coincided with the CSP cluster (cluster cultured <i>Synechococcus</i> phage) designated by Short and 	<p>phylogentic tree. These clones were more closely related to freshwaters clones than to clones from marine waters.</p>	
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

		<p>Suttle (2005).</p> <ul style="list-style-type: none"> - Cluster α was suggested to be clustered specific to g20 for freshwater environment (exclusively included PSW and freshwater clones except two clones from the marine environments). 		
g20 gene/ g20_CPS1.1 and g20_CPS8.1	<p>Spatial</p> <p><u>Sampling site:</u> Along a North- South Atlantic Ocean transect: 10 sampling sites were collected from 8 locations. The 8 sites spanned from the northern temperate ocean to the southern boundary of the south Atlantic gyre. Samples were carried out during the Atlantic Meridional Transect cruise (AMT-15) from Southampton (UK) to Cape Town (South Africa)</p> <ul style="list-style-type: none"> - 6 (1 % at depth 50 m) - 12 (1 % at depth 75 m) 	<p><u>Method: PCR-Cloning-Sequencing – Phylogeny analysis</u></p> <ul style="list-style-type: none"> - 628 of g20 clones were obtained from 10 samples. - The g20 PCR products ranged from 541 bp to 550 bp: variations between sequences were by up to 9 bases or 3 amino acids. - Phylogenetic analyses were conducted on 1416 g20 sequences, including 628 g20 clones and 2 sequences from AMT- 15 <i>Prochlorococcus</i> lysates from this study and 786 g20 sequences from previous studies (Zhong et al. 2002; Dorigo et al., 2004; Sullivan et al., 2008) and g20 sequences 	<ul style="list-style-type: none"> - The highest <i>Prochlorococcus</i> cell numbers were found at 1% light depth. - The Atlantic Ocean had higher g20 clones diversity than previously reported and with significant variation in diversity between sampling sites. - Myoviral (g20 sequences) richness appeared to be lowest in the samples collected from the north and south transects. At these stations, lower <i>Prochlorococcus</i> diversity was also reported. 	Jameson et al. (2011)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<ul style="list-style-type: none"> - 21 (14 % at depth 55 m and 1 % at depth 117 m) - 44 (1 % at depth 71 m) - 47(1 % at depth 50 m) - 56 (1 % at depth 75 m) - 68 (14 % at depth 55 m and 1 % at depth 126 m) - 91 (1 % at depth 112 m) <p>14 % and 1 % represent the percentage of surface irradiance.</p> <p>Site selection was primarily based on the abundance of <i>Prochlorococcus</i> and distribution of LL <i>Prochlorococcus</i> along the transect. Sites selected were representative of different oceanic province. <i>Prochlorococcus</i> was the dominant cyanobacterium at all sampling sites except site 6 (1%).</p>	<p>from the cyanomyoviruses isolates.</p> <ul style="list-style-type: none"> - The g20 sequences obtained from this study were distributed to 10 clusters, i.e. Cluster II to Cluster XI. Cluster I was used to root the tree, i.e. enteric coliphage T4. - Cluster II to Cluster XI contained sequences from previously described clades (Zhong et al. 2002 and Sullivan et al. 2008), except for cluster IX and sub cluster Xa, Xb and Xc. These were new clades defined in this study. - Cluster VI, VII, Xb and XI are the clusters contain phages isolated from <i>Prochlorococcus</i> and <i>Synechococcus</i>. 	<ul style="list-style-type: none"> - Higher correlation (Spearman rank correlation) was found between myoviral diversity and <i>Prochlorococcus</i> diversity, compare to correlation between myoviral diversity and <i>Synechococcus</i> diversity. However, they were not significant ($r = 0.64$, $P = 0.47$). - No significant correlation between myoviral diversity and environmental parameters was found through multivariate statistical analysis. 	
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

<p>g20 gene/ CPS1 and CPS8</p>	<p>Spatial and temporal:</p> <p>*Soil samples</p> <p><u>Sampling site:</u> Three paddy fields in Japan</p> <ul style="list-style-type: none"> - Kuroishi (KuCf-Apr 13 and KuCf- Jun26) - Omagari (OmCf- Apr 14 and OmCf- Jul27) - Anjo (AnCf- April11 and AnCf- Sep 04) <p>Two samplings were conducted at each station, one was conducted before land preparation, and another one was conducted during the flooding period of rice growth.</p>	<p><u>Method: PCR- cloning- DGGE- sequencing- phylogeny analysis</u></p> <ul style="list-style-type: none"> - DGGE gave 70 clones with different g20 sequences. - The average g20 recovery efficiency of the samples was 93%, i.e. 166 positive clones were sequenced, and 154 clones were identified as g20 fragments. - The length of PCR products: 546 bp (74%), 549 bp (6%) and 552 bp (20%). - The g20 sequences in paddy field soils were diverse and distributed to seven clusters: Cluster α, ϵ, β, as designated by Wang et al. (2010) and another four newly designated clusters, i.e. Cluster-1, -2, -3 and -4. - These newly formed clusters were obtained with only clones from this study, lakes and paddy floodwater. - Four unique subclusters were established within Cluster β and ϵ, i.e. PFS-I to PFS IV. These 	<ul style="list-style-type: none"> - The PCA analysis of the g20 clones showed that cyanophage communities were different among the paddy fields and sampling times. - Cyanophage communities from paddy field soils were different from freshwater, marine water and paddy floodwater, from the evidence of distribution of g20 sequences in phylogenetic tree. Four new clusters were formed and four unique subclusters were identified in the previous designated clusters. 	<p>Wang et al. (2011)</p>
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Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

		subclusters formed by g20 clones were exclusively identified from paddy field soils.		
DNA <i>pol</i> from cyanopodiviruses/ CP-DNAP-349F and CP-DNAP-533R	<p>Spatial and Temporal</p> <p>*Chesapeake Bay (Estuarine environment)</p> <p><u>Sampling site (sampling month)</u></p> <ul style="list-style-type: none"> - Station 858, upper bay (February and July 2005) - Station 804, middle bay (February and July 2004; February and July 2005) - Station 707, lower bay (February and July 2005) <p>*February represents winter month; and July represents summer.</p>	<p><u>Method: PCR- cloning- sequencing- phylogeny analysis</u></p> <ul style="list-style-type: none"> - Designed PCR primers based on DNA polymerase gene sequence of 7 marine (coastal and oceanic) cyanopodoviruses, i.e. CP-DNAP-349F, CP-DNAP-533R. Expected amplicon size ca. 550 -600 bp. - A total of 221 DNA <i>pol</i> clones obtained from eight samples. - These clones together with 8 known cyanopodoviruses sequences were divided into two large clusters (MPP-A and MPP-B) and 10 subclusters (Subcluster I to X). - These 10 subclusters were designated as the Marine Picocyanobacteria Podovirus Cluster (MPP). - 72% of the DNA <i>pol</i> clones from Chesapeake Bay fell into 	<ul style="list-style-type: none"> - Three cyanopodoviruses (S-CBP1, S-CBP3 and S-CBP4) infecting the same host (<i>Synechococcus</i> CB0101) were divided into three different DNA <i>pol</i> subclusters (I, III and IV). - Based on a comparison of pairwise similarity between samples (S-LIBSHUFF analysis), four samples from February 2004 and 2005 formed the winter group and four samples from July 2004 and 2005 formed the summer group. - The composition of cyanopodoviruses in Chesapeake Bay showed seasonal variation, i.e. distinct winter and summer DNA <i>pol</i> clone distribution patterns 	Chen et al., (2009)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

		<p>two subclusters (I and VIII).</p> <ul style="list-style-type: none"> - Majority of clones for Subcluster I were from summer clones (66), 23 winter clones fell in Subcluster I. In contrast, subcluster VIII contained more winter clones (59) and less summer clones (12). 	<p>and changes in the dominant cluster between seasons. These changes are indicative of seasonal variation of cyanophage populations.</p> <ul style="list-style-type: none"> - No spatial variation in DNA <i>pol</i> clones distribution was shown in the bay even through an environmental gradient existed across the bay. However, this was consistent with weak spatial variation of bacterial communities in the bay. 	
DNA <i>pol</i> from cyanopodiviruses/ CP-DNAP-349F and CP-DNAP-533R	<p>Spatial:</p> <p>*Ocean</p> <p><u>Sampling site</u></p> <p>North Atlantic Ocean</p> <ul style="list-style-type: none"> - UTK202 - UTK211 - UTK220 - UTK229 - UTK240 <p>Pacific Ocean</p>	<p><u>Method: PCR- cloning- sequencing- phylogeny analysis</u></p> <ul style="list-style-type: none"> - A total of 303 DNA <i>pol</i> clones were recovered from 10 ocean samples. - These 303 DNA <i>pol</i> gene sequences were distributed to five subclusters (II, III, VIII, IX and XI- assigned by Chen et al., 2009). Around 50 % of the clones fell into subcluster VIII. 	<ul style="list-style-type: none"> - Cyanopodoviruses in the open ocean showed lower genetic diversity compared to an estuary. In addition, the composition of DNA <i>pol</i> genotypes also differed between these stations. - From the multidimensional (MDS) analysis no distinct geographic patterns were observed for the UTK samples, i.e. nine samples 	Huang et al. (2010)

Table 2.3 Temporal and spatial variation of genetic diversity of the cyanophage community (continued)

	<ul style="list-style-type: none"> - UTK250 - UTK255 - UTK262 - UTK295 <p>South China Sea</p> <ul style="list-style-type: none"> - SCS1 	<ul style="list-style-type: none"> - 81 of 303 (26.7 %) DNA <i>pol</i> gene sequences shared > 97 % amino- acid identity with P60, i.e. a lytic phage that infects <i>Synechococcus</i> WH7805 and with a short latent period. These 81 clones fell into subcluster XI. 	from Atlantic and Pacific Oceans were clustered into three groups. However, the South China sample (SCS1) was discriminated from these three groups.	
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¹DGGE: denaturing gradient gel electrophoresis

²RFLP: Restriction fragment length polymorphism

³T-RFLP: terminal- restriction fragment length polymorphism

⁴PFGE: pulse- field gel electrophoresis

⁵OTU: operational taxonomic units

⁶Two components of diversity – richness and evenness were used in diversity measures. Richness- the number of entities present and the evenness- the distribution of individual among entities (Mühling et al. 2005)

2.7 SUMMARY OF LITERATURE REVIEW

Earlier studies showed that viruses play critical roles in the structure and function of aquatic food webs. Viruses also regulate the biodiversities of the microbial community in aquatic ecosystems. Cyanophages are mortality agents of cyanobacteria and play important roles in cyanobacteria evolution, as well as co-evolution between cyanophage and host. Cyanobacteria are important primary producers in aquatic ecosystems, and harmful cyanobacterial blooms in particular can cause human or ecosystem health problems. Thus, investigation on the dynamic and ecological roles of cyanophages in the aquatic environment is important due to their influence on the dynamics of cyanobacterial populations and biogeochemical cycling of nutrients.

Earlier lab studies showed that the fate of cyanophages was influenced by environmental conditions such as light / UV, phosphate concentration, temperature, divalent cations, non-cyclic peptides and pH. Previous *in-situ* studies demonstrated temporal and spatial variation of cyanophage communities in various environments, showing that seasons, diurnal cycles (day time), salinity, temperature, light and host cell densities influence cyanophage abundance. The cyanophage diversity was influenced by the light, nutrient, and host cell diversity.

Most of the existing studies on the dynamics of cyanophages in a particular site have only investigated particular species, e.g. in the Indian Ocean, only *Synechococcus*' phages have been investigated, while at Furuike Pond at Japan, only *Microcystis*' phages were investigated. However, environmental conditions can affect different types of cyanophages differently. The diversity of cyanophage communities was also studied in various environments, but mostly are from oligotrophic and mesotrophic environments. Only two studies covered eutrophic environments, i.e. paddy floodwater and paddy field soils. Furthermore, earlier studies showed that the diversity distribution pattern and unique clades could be obtained from each location. Hence, discovering the distribution of cyanophage

communities in eutrophic environments in Singapore local tropical water system would bring new knowledge to this field.

Isolation of phages serves as a crucial initial step towards further experimental procedures to achieve the aim of understanding the intricate relationships between cyanobacteria and their respective cyanophages. Isolation and characterization of cyanophages started in 1963, but cyanophage isolates are still very limited, especially from freshwaters. In addition, earlier studies showed that different species have different impact on their host. The interaction studies between phage and cyanobacteria also can hardly be found from earlier studies. Hence, in order to investigate the potential of local cyanophages to control cyanobacterial blooms (especially harmful cyanobacterial blooms), it is necessary to isolate and understand the characteristics of indigenous cyanophages found in Singapore local aquatic environments. Moreover, the established cyanophage isolation methods only target unicellular cyanobacteria infecting cyanophages such as *Synechococcus*-infectious cyanophages. The challenge of isolating cyanophages which infect filamentous cyanobacteria is the multicellular physiological condition of the host, which can significantly affect the infectivity process. Thus, it is essential to develop isolation protocols which are able to target the filamentous cyanobacteria.

CHAPTER 3

Isolation of Cyanophages which Infect *Anabaena circinalis* and *Anabaena cylindrica* in a Tropical Reservoir

3.1 INTRODUCTION

Singapore has very little natural freshwater reserves. However, annual high rainfall averages 2,358 mm, making it a viable source of freshwater (Hu et al. 2003). As such, one of the important national water sources is water from local catchment areas, i.e. harvesting urban stormwater on a large scale. A major challenge faced by Singapore is utilizing resources to optimize rainwater collection despite spatial limitations, and preserving the quality of these resources. Rainwater is collected through a comprehensive network of drains, canals and stormwater collection ponds before it is channeled to reservoirs for storage (Public Utilities Board 2012). One of these reservoirs is the Kranji Reservoir. The trophic status of Kranji Reservoir has been categorized between eutrophic and hypereutrophic (Te and Gin 2011). In a previous study, the *Anabaena* 16S rRNA gene was detected over a period of 18 months in this Reservoir (Te and Gin 2011). Thus, *Anabaena* species is an ordinary species of the cyanobacteria population in Kranji Reservoir and is believed to contribute to the blooms in this reservoir. In this study, *Anabaena circinalis* and *Anabaena cylindrica* which have different filament morphology (Pan et al. 2008) were selected to represent toxin producing and non-toxin producing *Anabaena* species.

Anabaena circinalis and *Anabaena cylindrica* belong to the *Anabaena* species which are found to be filamentous, heterocystous cyanobacteria. They possess unique capabilities to evolve oxygen by carbon dioxide fixation and nitrogen fixation simultaneously. This capability may have led to cyanobacterial blooms in surface waters (Dugdale and Dugdale 1962) which are prone to cultural eutrophication (Paerl 1979). *A. circinalis* blooms are a major problem worldwide,

because of the algae's ability to produce a range of toxins including paralytic shellfish poisons (PSPs) and neurotoxins (anatoxin-a) (Fergusson and Saint 2000). The filaments are generally solitary, straight or coiled (Pan et al. 2008). In contrast, *Anabaena cylindrica* trichomes are complexly intertwined with each other and easily form algal mats (Hori et al. 2002). Hori et al. (2002) also observed that *A. cylindrica* ATCC 9414 excreted a kind of exopolysaccharide that can strengthen the filamentous network of the algal mat.

Filamentous cyanobacteria have differentiated cell types that exhibit different cell shapes. Most of the cell shapes are controlled by the cell wall. Other than vegetative cells, filamentous cyanobacteria have two other prevalent development cell types: heterocysts and akinetes. These are morphologically, structurally (Lang 1965; Miller and Lang 1968), physiologically and biochemically (Talpasay and Bahal 1967) distinct from vegetative cells. The differentiation of heterocysts has been observed during nitrogen limitation (Kumar et al. 2010). Heterocysts are larger, rounded and have thicker cell envelopes compared to vegetative cells (Kumar et al. 2010; Singh and Montgomery 2011). From the physiological and biochemical perspective, heterocysts express genes that synthesize constituents for different metabolic pathways functioning in the assimilation of molecular nitrogen (Cohen and M. Gurevitz 2006) giving the cell the ability to fix nitrogen. At the same time, heterocysts lose their carbon dioxide fixing and photosynthesis capacity (Aziz 2006; Cohen and M. Gurevitz 2006) and become nitrogen suppliers for the ecosystem. An akinete is a thick-walled resting cell; it shows resistance to adverse environments and does not have a functional photosystem (Fay 1969). Depletion of iron and carbon can induce the formation of akinetes (Hori et al. 2002; Kaplan-Levy et al. 2010; Singh and Montgomery 2011). The ratio of C:N is also observed to regulate akinete formation (Tyagi 1978). Akinetes also act as a seed for the next generation of bloom formation (Hori et al. 2002).

Cyanophage infection has been implicated as an important factor in cyanobacterial community development (Monegue and Philips 1991; Suttle and

Chan 1994; Tucker and Pollard 2005; Yoshida et al. 2008). Cyanophages are recognized as important mortality agents which can terminate cyanobacteria blooms (Safferman and Morris 1963; Manage et al. 1999) and are a major force in cyanobacterial evolution (Suttle 2000). Filamentous cyanobacteria have been found lysed *en masse* due to viruses, leading to changes in the bacterial and eukaryotic community structure in Lake Loosdrecht, The Netherlands (van Hannen et al. 1999). Therefore, cyanophages not only affect the cyanobacterial community, they also influence microbial diversity and the biogeochemical nutrient cycle in aquatic environments. Isolation of phages serves as a crucial initial step towards further experimental procedures to achieve the aim of understanding the intricate relationships between cyanobacteria and their respective cyanophages, and understanding their impacts and roles in local ecosystems.

The first cyanophage isolated was by Safferman and Morris in 1963. This cyanophage infected filamentous cyanobacteria but was not heterocystous. The first report of isolated cyanophages infecting filamentous, heterocystous cyanobacteria (C-1 and AR-1) was in 1967 (Singh and Singh 1967). Adsorption of phage to host is the key step in phage infectivity. Adsorption of phage to the cell envelope is by phage adhesion, recognition and binding to specific cell- surface receptors (Mann 2006). The nature of the cell surface and physiological condition of the host is likely to be influenced by nutrient stress as discussed above. Physiological conditions of the host can influence the susceptibility of the phage to the host (Brussaard 2004; Chibani-Chennoufi et al. 2004). This is due to changes in surface binding sites of cyanophage to cyanobacteria. Nevertheless, divalent cations were also reported to directly affect cyanophage infectivity and structural stability. Divalent cations such as magnesium ion (Mg^{2+}) or calcium ion (Ca^{2+}) are required by some cyanophages for structural stability, for example, AN-15, SM-2 and LPP-1 require the presence of cations to maintain their capsid structure (Goldstein et al. 1967; Padan and Shilo 1973; Martin and Benson 1988; Mole et al. 1997). In the same study by Mole *et al.* (1997), it was shown that the requirement of Ca^{2+} ion permitted infection by AN-15 to the host, implying that cations influence cyanophage infectivity.

The objectives of the present study is to isolate the cyanophages infecting *A. circinalis* and *A. cylindrica* from a tropical reservoir and to investigate the potential of cyanophages as a biological control to these filamentous, heterocyst forming cyanobacteria. The challenge of isolating cyanophages which infect filamentous cyanobacteria is the multicellular physiological condition of the host, which can significantly affect the infectivity process. Thus, this study shows the importance of understanding the growth pattern of host in addition to optimizing the isolation methods.

3.2 MATERIALS AND METHODS

3.2.1 Sampling location

Kranji Reservoir is one of the 17 reservoirs in Singapore. The water is mainly supplied by four tributaries: Tengah River, Kangkar River, Peng Siang River and Pang Sua River. Three sampling stations were chosen for this study, i.e. KR1 (1°24'49.640"N; 103°43'49.247"E), KR3 (1°25'48.508"N; 103°44'41.399"E) and Peng Siang (1°23'35.495"N; 103°44'05.364"E).

3.2.2 Sample collection

Around 12 liters of water sample were collected monthly from Kranji Reservoir between August 2008 and August 2009 for the isolation of *A. circinalis* and *A. cylindrica* cyanophages. Samples were concentrated from reservoir water using ultrafiltration. All water samples were initially filtered through 50 µm pore size using low binding protein membrane filters (Pall Corporation) to remove particulate matter, phytoplankton and zooplankton. Subsequently, the filtrate was filtered through 0.2 µm pore size membrane (Pall Corporation) to remove most of the bacteria. After undergoing pre-filtering, samples were stored at 4 °C in a cool

room. The viruses in the filtrate of about 10 L of sample were concentrated to a final volume ranging from 250 to 500 ml, using tangential flow filtration (TFF) with a 30 kDA membrane cassette (Pall Corporation). Concentrated samples were believed to contain virus like particles (VLPs).

3.2.3 Host strains and media

Axenic strains of *Anabaena circinalis* and *Anabaena cylindrica* (CSIRO CS-172) were used in this study to ensure that the isolated cyanophages were specific to the target cyanobacteria strains. *A. circinalis* and *A. cylindrica* were maintained in MLA medium in conical flasks under a light dark (LD) cycle. Growth curves were constructed for these two hosts to understand their growth patterns by using optical density (OD). OD readings were obtained by subjecting the samples to wavelengths of 750 nm. This wavelength has been used to monitor cyanobacterial growth in previous experimental studies (Fay et al. 1984; Pomati et al. 2003). Thereafter, the constructed growth curves were used to determine the stage of growth and to determine whether the cultivated hosts were ready for infection. It is important to know which growth phase was attained by the cyanobacteria host to determine if it was ready for cyanophage inoculations.

3.2.4 Well assay

Three cycles of *Anabaena circinalis* infection were performed through well assay to isolate a clonal infectious agent. Infections were preferably done on the host *A. circinalis* in early log phase stage of growth. The first cycle was carried out to ascertain which of the environmental water samples had *A. circinalis* cyanophages present. Samples with positive lysis effect were then analyzed with the extinction dilution method (Millard 2009) in the well assay for another 2 cycles to obtain a clonal cyanophage. The well-plating method of infection with 12-well plates was used, as the host *A. circinalis* was found to grow in well plates.

Disposable sterile Pasteur pipettes were used to transfer 12 ml of early log phase *A. circinalis* into culture flasks, and subsequently added with 1.2 ml of the environmental sample. For the control, 1.2 ml of MLA medium was used in place of the environmental sample.

The culture flasks were incubated in an incubator shaker at 25 °C, 100 rpm and exposed to light for at least 1.5 hrs. This was to ensure homogenous mixing and optimum contact between host and cyanophage. Samples were then transferred to the 12 well-plates, whereby 1 ml of sample was placed in each well plate. Plated samples were then transferred to the growth racks in reactor room and observed for lysis. The well plates were subjected to light dark diel cycles at 12 hr intervals, at about 22 °C (± 1 °C). Throughout the infection period, the multi-well plates were frequently incubated in the incubator shaker. This step was to ensure maximum contact rate between host and the phage. The placement of the plates was randomly changed to optimize light exposure to the well-plates, and to increase the consistency of light exposure to each well-plate.

Every 5 or 9 days, optical density (OD) readings were obtained from the well plates by randomly removing samples from 2 wells from each well-plate. The plates were incubated and monitored for 30 days. The plates were monitored for the lysis of *A. circinalis* and compared to the negative control. The lysis effect was observed using OD tests combined with visual observation using an inverted light microscope. The filament number of *A. circinalis* was counted under a microscope using a hemocytometer.

Epifluorescence light microscope (ELM) images were taken from randomly chosen samples while the cleared well (after lysis) was observed. The sample and controls were diluted with 0.2 μm filtered sterilized distilled water and stained with acridine orange (AO). Acridine orange is a nucleic acid stain and is normally used for bacteria counts using epifluorescence light microscope. AO bound to nucleic acids in bacteria cells show as green or yellow or orange or red fluorescence. The

fluorescence colour depends on how the AO molecule interacts with the nucleic acid.

The cleared lysate from the well plate was centrifuged for 10 min at 6,000 x g to remove the cell debris and remnant cyanobacteria prior to storage and continued to the next cycle. After centrifugation, supernatant was serially diluted (10- fold dilutions) and used for further rounds of well assay. Quadruplicate were prepared of each dilution. The cleared lysate from the most diluted well plate was serially diluted for the third cycle of well assay. The cleared lysate from the most diluted well plate in the third cycle was a clonal cyanophage and used for phage propagation.

3.2.5 Double- layer plaque assay

The double layer plaque assay was used to determine and isolate the cyanophage infecting *Anabaena cylindrica* (Abedon and Yin 2009; Millard 2009). *Anabaena cylindrica* was cultured to exponential phase (OD >0.4, at 750 nm) and subsequently concentrated to OD >1 (at 750 nm), by which time the culture was ready for infection. Briefly, 160 ml of *A. cylindrica* at exponential phase was distributed to four 50 ml centrifuge tubes. Then the culture was centrifuged at 6000 x g for 15 min at 25 °C to pellet the *A. cylindrica* cells. Supernatant was poured out leaving approximately 5 to 10 ml of cell pellets left in each centrifuge tube. Cell pellets in the first 50 ml centrifuged tube were resuspended with 10ml fresh MLA medium, and then this suspension (i.e. cell pellets together with the fresh medium) was used to resuspend the remaining cell pellets in the second 50 ml centrifuge tubes. This step was repeated sequentially for the third and fourth tubes. The combined suspension solution (containing concentrated host and 10 ml of fresh medium) was transferred to a new centrifuge tube. Another 5 ml of fresh MLA medium was used to wash any remaining cells from the sides of the centrifuge tubes and transferred to the same new centrifuge tube. The optical density reading of the

concentrated host was recorded to ensure that the host concentration was high enough for infection.

One or 0.5 ml of concentrated environmental samples containing the VLPs was added to three ml of the concentrated host strain. This mixture was subsequently incubated and shaken for one hour at 25 °C to aid the phage-host adsorption. To prevent temperature shock from happening, the mixture was placed in a water bath at 40 °C for 10 min prior to addition of 1.5 ml of molten 0.6 % (w/v) agarose at 40 °C. The mixture was gently mixed with a swirling action. The mixture was poured onto the top of a solid 0.8 % MLA agar plate. The molten soft agar was allowed to set for 1 to 2 hours before it was inverted and placed in the growth chamber under the conditions described above. The plates were monitored daily for the appearance of plaques. Visible plaque formation varied from 3 to 10 days. A Pasteur pipette was used to pick plaques and suspend them in 1 ml of MLA medium. Two to three plaques were picked from each plaque formation observed on the plate, to avoid the unintended selection of phage mutants or contaminants (Karin 2004). The suspension was left in the dark at 4 °C for at least one hour, to ensure enough time for phage resuspension (Millard 2009). The phage would diffuse into the fresh medium to form a lysate. The lysate was serially diluted and then used in the second cycle of plaque assay. This process was repeated twice to obtain the clonal cyanophage.

3.2.6 Cyanophage propagation

High titer cyanophage stocks were prepared for electron microscope imaging and molecular study. Propagation was done in liquid media (*A. circinalis*) and on plates (*A. cylindrica*). For propagation in liquid media, the cleared lysate from the well assay of the third cycle was centrifuged at 13,000 x g for 5 min to pellet cell debris. The supernatant was then used for propagation. The well assay as described above was used for propagation of *A. circinalis* infecting cyanophage; the well assay was repeated until 50 to 80 ml of lysate was obtained. On the other hand,

the propagation of *A. cylindrica* cyanophage in Petri dishes utilizes the double layer agar method, followed by resuspension of phage in phosphate buffer solution (PBS) (Mesquita et al. 2010). The plate infection was continued until confluent lawn lysis was observed. For phage collection, 10 ml of PBS was poured on to the top of each plate. The plate was flooded with PBS at room temperature for three to five hours with intermittent gentle shaking to allow phages to diffuse in to the solution. Supernatant was decanted carefully to avoid cellular debris and agar from being decanted with the phages.

Culture from the liquid media and plate propagation (50 to 80 ml) were transferred to individual glass bottles (100 ml). A few drops of chloroform were added to 50 ml of culture, mixed gently and incubated at room temperature for two hours. Lysate was transferred to a centrifuge tube with care to ensure that chloroform was not included. The lysate was centrifuged at 8000 x g for 20 min at 4 °C to pellet the cellular debris and agar. Phage suspensions were filtered through 0.45 µm and 0.2 µm and filtrates were kept in the dark at 4 °C. Further concentration was done on the isolated cyanophage by ultrafiltration (30 kDa Amicon Ultra centrifugal filter device, Milipore). Concentrated cyanophage isolates were fixed with glutaraldehyde (1 % final concentration) and stored at 4 °C before visualization and characterization of phages by transmission electron microscope (TEM).

3.2.7 Transmission electron microscope

The fixed isolates were harvested onto 200 mesh formvar covered nickel support grids. Then each grid was negatively stained with 1% phosphotungstic acid (PTAS) at room temperature for 4 minutes and dried on a filter paper. Grids were examined with TEM (JEOL JEM-1010) at magnification between 60 000 and 100 000 x. The photographic negatives were scanned with Adobe Photoshop and cyanophage dimensions were determined using IMAGEJ software.

In naming cyanophage isolates from this study, the following nomenclature was used: the first and second letter indicate the host species, where Ac = *Anabaena circinalis*, Ay = *Anabaena cylindrica*; the third letter indicates the sampling location, where K = Kranji Reservoir; the forth letter indicates the phage morphotype, where P = podovirus, S = siphovirus and M = myovirus; and the number is used to distinguish between the same type of isolates.

3.3 RESULTS

3.3.1 Cyanophage Isolation

A total of six lytic cyanophages from Kranji Reservoir have been isolated. Two lytic cyanophages were isolated from *A. circinalis* and four from *A. cylindrica*. Well plate assays were used to detect the presence of *A. circinalis* phages. Figure 3.1 shows the OD readings obtained from the well plates every 5 to 9 days until the wells became clear compare to the control. Clearing of the well and declining of OD values are assumed to be due to the dying off of host *A. circinalis* cells by lysis. The plate's mixtures of *A. circinalis* and VLPs became clear on day 9 (infection started during the early log phase) and day 19 (infection started during the lag phase), while the controls showed a greenish color.

The numbers of trichomes were enumerated when lysis was observed on the ninth day when infection started during the early log phase. The trichome number per ml of *A. circinalis* in the control was 1.03×10^4 , whereas the sample with VLPs was 800, i.e. 92.2 % less in the sample with VLPs compares to the control. The trichome number per ml was continually monitored until day 15; the trichome number of *A. circinalis* in the control was 7.44×10^4 , whereas sample with VLPs was 700. From day 9 to day 15, the control showed a significant increase in trichome number, but the sample with VLPs showed a decrease in the trichome number.

The color of cell fluorescence (under ELM) was reported to change from green to yellow to red to orange or vice versa, this change could be accounted for by bacterial metabolic activity and bacteria growth. However, the relationship between the colors of cell fluorescence and the physiological state of a bacterium is not exactly clear. In most cases show red-orange fluorescence occurs for rapidly growing cells because of the predominance of RNA. In contrast, inactive cells or slow growing bacteria typically show green fluorescence (Yeh et al. 1987). The lysed culture of *A. circinalis* was colorless and contained a suspension of cellular debris (Figure 3.2A) and the cellular debris with green fluorescence (inactive growing phase) when it was observed under the epifluorescence light microscope (ELM) (Figure 3.3E to 3.3H). In contrast, the control was greenish in color and filaments were long (more than 5 cells per filament) when viewed under an inverted light microscope (Figure 3.2B). Under the ELM, Figure 3.3 (A-D) show images of the control samples with red- orange fluorescence suggesting that the culture was in an active growing phase.

On day 9 of incubation, no positive lysis effect (clearing of well) was observed when the well plate assay was conducted on the *Anabaena cylindrica* that had been exposed to the environmental sample. Indeed, wells of the plates appeared to show a decrease in the volume of solution until the surface of the well dried completely when the incubation was continued to day 14 (Figure 3.4B). Figure 3.5 shows that *A. cylindrica* in wells with samples containing potential *A. cylindrica* infecting cyanophages (confirmed with the plaque assay) showed differences in their aggregation compared to the controls. In Figure 3.5B and Figure 3.5C, KR1 and KR3 plates showed a clearing of lawn (lysis occurred), but not for the PS plates; the wells (row 3 and 4) with samples from KR1 (column 2) and KR3 (column 3) had shown no or less aggregation when compared with the wells without VLPs (controls, column 1) and wells with PS samples (negative result in plaque assay, column 4).

From observation of the plaque assay, a small plaque was observed during early lysis, which was barely visible to the eye. The plaque size increased very rapidly and the entire confluent lawn was lysed within two to three days from the time the small plaque first appeared. The regrowth of *A. cylindrica* was observed at day 21 when the plate was incubated further (Figure 3.6). Regrowth of *A. circinalis* was not observed from any well plates that had been left in normal growth conditions after lysis (Figure 3.7).

Figure 3.1 Changes in optical density (OD) in well assay with (open triangles) and without (filled rectangles) cyanophages isolates. Well assays were conducted when *A. circinalis* is in lag phase (dotted lines) and early exponential phase (solid lines). The insert shows the *A. circinalis* growth curve.

Figure 3.2 Lysis of *Anabaena circinalis* cells viewed under inverted light microscope at 400x magnification. (A) VLPs were added to *A. circinalis* and the clearing of the well plate was observed. The lysed culture contained a suspension of cellular debris. (B) Well contained *A. circinalis* only (control) where the trichomes were long and abundance.

Figure 3.3 Epifluorescence light microscope images of *Anabaena circinalis*: (A- D) Control; (E- H) incubated with VLPs. These images were taken when cell lysis was observed. Images A- D show the shape of *A. circinalis* as long trichomes and a majority of them were fluorescing red-orange. Conversely, images E- H show only cellular debris with mostly green fluorescence.

Figure 3.4 Effectiveness of using well plate assay. Lysis of *A. circinalis* was observed in (A), whereas no lysis of *A. cylindrica* was observed in (B), where the well plates became dried after incubation for fourteen days.

Figure 3.5 Plaque and well assays showing the infection of *Anabaena cylindrica* by cyanophages: plaque assays (top 2 plates) and well assays (bottom 2 plates) for (A) controls and *A. cylindrica* with environmental samples from (B) KR1, (C) KR3 and (D) PS.

Figure 3.6 *Anabaena cylindrica* controls (A and C) compared with *A. cylindrica* inoculated with cyanophage isolate (B and D). After nine days of incubation, the clearing of confluent lawn occurred in the plate containing a mixture of *A. cylindrica* and cyanophage isolate (as shown in B) and regrowth of *A. cylindrica* was observed on the same plate (as shown in D) on day 21.

Figure 3.7 Comparison of duplicate controls (solid filled bars) with *A. circinalis* inoculated with cyanophage isolate (gradient filled bars). No regrowth of *A. circinalis* was observed.

3.3.2 Transmission Electron Microscope

Four isolated cyanophages were examined under transmission electron microscope (TEM). These isolated cyanophages fell into two different double-stranded DNA tailed phage families: the *Myoviridae* and *Podoviridae* as described by Ackermann (2009) (Ackermann 2009). Ac- KP1 (Fig. 3.8A) and Ac-KP2 (Fig. 3.8B), the cyanophages infecting *A. circinalis*, are short tailed phages and deemed to be *Podoviridae*. Ay- KP1 (Fig. 3.8C) and Ay- KM1 (Fig. 3.8D) are cyanophages which infect *A. cylindrica*. Ay-KP1 is a short tailed phage, and therefore, placed in the *Podoviridae* family. Ay- KM1 has a contractile tail sheath and is deemed to be myovirus.

Ay- KM1 had a head diameter of 96 x 123 nm; while the head diameter of Ay-KP1, Ac-KP1 and Ac-KP2 were 130 nm, 143 nm and 170 nm respectively.

Figure 3.8 Transmission electron micrographs of four isolated cyanophages from Kranji Reservoir: (A) Ac-KP1, (B) Ac-KP2, (C) Ay-KP1 and (D) Ay- KM1. The scale bars are 100 nm.

3.4 DISCUSSION

The well plate assay with extinction dilution was used as the isolation method to isolate *Anabaena circinalis* cyanophages in this study. This is because no growth of *A. circinalis* was observed when cultured on MLA medium agar, even though the agar concentration was reduced to 0.6 %. In addition, the well plate assay provided rapid and significant lytic effect. In this study, *A. circinalis* was exposed to environmental samples containing VLPs during lag phase, early- exponential phase and mid-exponential phase. It was found that the effect of *A. circinalis* phages infecting host cells was more effective when inoculation to the host was carried out in lag phase and early exponential phase than during the middle of exponential phase (data not shown). In fact, the lysis effect occurred more rapidly in the sample that was exposed during early exponential phase than the sample exposed during the lag phase, as shown in Figure 3.1. These results suggest that early exponential phase is the optimum physiological condition to carry out phage infection, resulting in a rapid and significant lytic effect.

Both of the well plates showed clearing of wells when phages were inoculated against *A. circinalis* in lag phase and early exponential phase. However, the clearing of host cells was not observed when inoculation started at mid-exponential phase. Monegue and Philips (1991) showed that the cyanophage AC1 lowered the chlorophyll a concentration in *A. circinalis* by 95% (final concentration: 0.25 µg/ml), 28 days after inoculating into a lag phase culture; whereas inoculation of AC1 into a mid-log phase culture resulted in a 70 % (final concentration: 1.5 µg/ml) drop in concentration compared to the control (Monegue and Philips 1991). This suggests that the clearing of host cells is less likely to occur when the plate assay is carried out during the host's growth in the mid-exponential phase. The reason that the clearing of host cells was not observed when inoculation started at the mid-exponential phase. This may be due to nutrient limitation occurring just after a few days of incubation. The cell number of *A. circinalis* increased dramatically during mid-exponential phase and nutrient uptake is generally faster

than in the early log phase and lag phase. Nutrient limitation regulates the cellular differentiation of filamentous cyanobacteria (Singh and Montgomery 2011) with the cell wall becoming more rigid (Marcus et al. 1982) and induction of akinet and heterocysts formation (Kaplan-Levy et al. 2010; Singh and Montgomery 2011). The physiology, biochemistry, morphology and molecular metabolism of cyanobacteria changes to maximize energy production and increase survivability (Singh and Montgomery 2011). These changes in the physiology and cell wall of *A. circinalis* influences the cell surface receptor for cyanophage adsorption (Brussaard 2004; Mann 2006). Inactivation of cyanophage C-1 and AR-1 to infectivity in heterocysts and spores has been reported (Singh and Singh 1967). The thick cell walls of heterocysts have been suggested to be one of the reasons behind failure of cyanophage attachment and infectivity of cyanobacteria. Another reason for the lack of cyanophage infectivity to heterocysts is the host cells' decrease in photosynthetic capacity. Photosynthesis can produce adenosine triphosphate (ATP), and ATP is one of the requirements for viral replication. If the heterocysts lose the ability to provide ATP for viral replication, the phages may 'sense' which cells to infect and which to avoid. In addition, it has been reported that the lack of cations in the media over long incubation periods may lead to cyanophage structural instability and failure to infect (Goldstein et al. 1967; Padan and Shilo 1973; Martin and Benson 1988; Mole et al. 1997). The optical density of the *A. circinalis* culture exposed to the phages during the early exponential phase decreased faster than that at lag phase. This could be due to the host growing more actively in the early exponential phase: thus providing greater energy for phage replication than during the lag phase.

This study has shown that for *A. cylindrica* infection, the clearing of the well plate fails. However, from the well plate result we were still able to observe the difference between the control and the samples with and without lysis through the aggregation of *A. cylindrica*. The aggregation of the culture was not observed or was less so in the sample with positive lysis effect, compared to the control and sample without lysis (Figure 3.5). This difference may be due to the ability of

cyanophages to affect the production of exopolysaccharide in order to strengthen the filamentous network of the algal mat (aggregation).

In this study, the lysis effect for *A. cylindrica* exposed during the exponential phase and at high concentrations to VLPs in the plaque assay was rapid and effective. Plaque sizes increased very rapidly and the entire culture lawn was lysed within 2- 3 days only from the time small plaque first appeared. This plaque characteristic was also observed when the C-1 phage infected *Cylindrospermum*: the plaque of C-1 increased very fast and the entire algal lawn on the plate is lysed within a few days (Singh and Singh, 1967). The dramatic increase of the plaque size in this study was attributed to the filamentous network of the algal mat *A. cylindrica*. Due to the high cell concentration of host cells, phages that are released from one cell in a filament could easily infect the juxtaposed cells or cells of filaments intertwined together. The contact rate and efficiency of infection is thus much higher than for unicellular cyanobacteria or filamentous cyanobacteria which are unable to intertwine filaments tightly together like the *A. cylindrica*.

Monegue and Philips (1991) suggested that addition of cyanophage to *A. circinalis* could dramatically decline standing crops in non-expanding populations. This opinion is consistent with our finding: the concentration of *A. circinalis* in the early log-phase was reduced by 92.2 % when inoculated with cyanophages. Moreover, this study has shown that cyanophages have the ability to suppress the development of *A. circinalis* population at the early stage of exponential growth. This ability is important as it helps to prevent akinete germination at the later growth stages of *A. circinalis*. A single akinete has the ability to generate new cycles of cyanobacteria blooms when the environmental conditions become appropriate (Hori et al. 2002; Zapomělová 2006).

The regrowth of *Anabaena* PCC 7120 in lysed culture after 10 days was observed in a study by Mole et al., (1997). They suggested that this was due to the phage-immune derivatives of *Anabaena* PCC7120 to cyanophage AN-15. Figure 3.7 shows there was no regrowth of *A. circinalis* in our lysed culture, implying that

A. circinalis in our study was not able to mutate to resist the infection by cyanophage isolates. Conversely, *A. cylindrica* cyanophages have shown their ability to collapse the host population rapidly and effectively, but the regrowth of *A. cylindrica* has also been observed (Figure 3.6D), implying that *A. cylindrica* in our study was able to mutate to resist infection by isolated cyanophages. Therefore, cyanophage isolates from this study have the ability to prevent or inhibit *A. circinalis* bloom formation in our aquatic system but may not be able to prevent the formation of *A. cylindrica* blooms.

The head size of Ay- KP1 is similar to cyanopodovirus infecting *Arthrospira plantensis* (*Spirulina*), which has a capsid of 120 nm (Jacquet et al. 2013). However, the capsid of Ac-KP1 and Ac-KP2, which infect *A. circinalis*, and cyanomyovirus Ay-KM1 which infects *A. cylindrica* are larger than previously recorded cyanophages infecting the filamentous, heterocystous cyanobacteria (Franche 1987; Sallal et al. 1987; Jenkins and Hayes 2006). The head diameter difference between the two *A. circinalis* phages; Ac- KP1 and Ac- KP2, is 27 nm. These two phages may be considered as two different clonal cyanophages. Cyanophage isolates infects *A. circinalis* effectively in the early growth phase and *A. circinalis* was not able to resist Ac-KP1 and Ac- KP2 infectivity even at the later stage. On the other hand, Ay-KM1 and Ay-KP1 can effectively infect *A. cylindrica* during the exponential growth phase. However, *A. cylindrica* is able to mutate successfully and resist infection from Ay-KM1 and Ay-KP1, eventually beginning a new cycle of *A. cylindrica* population expansion.

3.5 CONCLUSION

To obtain the cyanophages that infect *A. circinalis* and *A. cylindrica*, two isolation methods have been established based on the understanding of the characteristics of the host cell. The clearing of well plates (from greenish to no color) is important to determine the presence of specific cyanophages in the well plate assay. When *A. circinalis* growing in the mid- log phase was exposed to

VLPs, the lysis effect was rapid at first but subsequently, no obvious changes in lysis effect were observed when nutrient limitation occurred after a few days of incubation. This was evident by the lack of clearing of well plates. Therefore, we suggest that the early log phase of *A. circinalis* should be used to determine the presence of the phages in the well plate assay. On the other hand, exponentially growing *A. cylindrica* (OD >0.4, 750 nm) at high concentrations is ideal for producing a confluent lawn on the agar plate. Under these conditions, the infectivity of phages was observed to be rapid and effective.

Through these two isolation methods, a total of six lytic cyanophages have been isolated from Kranji Reservoir. Two lytic cyanophages were isolated from *A. circinalis*, while four lytic cyanophages were obtained from *A. cylindrica*. This study showed that the cyanophage isolates have the ability to affect the host physiology. For example, *A. cylindrica* experienced changes in aggregation and algal mat formation when inoculated with the cyanophage isolates. This research indicates that cyanophage isolates from the reservoir could reduce the *A. circinalis* concentration by 92.2 % and suppress the growth of *A. circinalis* effectively. In addition, no regrowth of *A. circinalis* culture was observed after the lysis. These results suggest that two of the cyanophage isolates from this study are potential biocontrols to regulate the development of *A. circinalis* populations in aquatic environments.

CHAPTER 4

Occurrence and dynamics of cyanophages in Kranji Reservoir

4.1 INTRODUCTION

Cyanobacteria are classified as oxygenic photosynthetic bacteria with chlorophyll-a and phycobiliprotein being the primary photosynthesis pigments of cyanobacteria (Waterbury 2006). The prevalent genera of cyanobacteria that have been identified in Kranji Reservoir, Singapore, during the study period were *Microcystis*, *Synechococcus* and *Anabaena*, whereas *Microcystis* was the dominant genus (Te 2012). *Microcystis* and *Synechococcus* are unicellular cyanobacteria, while *Anabaena* is multicellular. *Microcystis* and *Anabaena* are among the potential genera which cause harmful cyanobacterial blooms. Harmful cyanobacterial blooms (CyanoHABs) occur when the dominant species in a water system produce secondary metabolites which may be toxic to other bacteria and eukaryotes. They have been reported to produce foul odors and tastes and cause hypoxia, fish kills and altered food web dynamics, leading to serious problems in water quality, fisheries resources, animal and human health. *Microcystis* and *Anabaena* are among the potential cyanobacteria genera which produce toxins that lead to harmful cyanobacterial blooms (Paerl and Fulton 2006).

Cyanophage infection is an important mechanism to terminate cyanobacteria blooms (Manage et al. 1999; Yoshida et al. 2008), consequently influencing the population composition of cyanobacteria. The infectivity of cyanophage available depends on host density and ability of the cyanophages to kill specific host genotypes. As a result, this leads to a selective sweep of cyanobacterial species, keeping competitive dominants in check (Tucker and Pollard 2005). Cyanophages are reported to have the potential to move genes between lineages within cyanobacterial populations, providing a mechanism for the genetic information transfer between evolutionarily distant cyanobacterial lineages (Deng and Hayes

2008) and promoting changes in the genetic structure of the host population (Jenkins and Hayes 2006). Thus, cyanophages are believed to take part in influencing the occurrence and diversity of the cyanobacterial community, as well as the evolution of cyanobacteria.

Environmental conditions are known to affect the physiological conditions of cyanobacteria (Mann 2006) and subsequently affect the binding sites of cyanophage to cyanobacteria (Brussaard 2004). In addition, the adsorption rate of cyanophage to cyanobacteria (Kao et al. 2005), viral life strategies (including entrance into lysogenic or lytic cycle and latent period of virus) (Danovaro et al. 2011), and decay rates (Frache 1987; Liao et al. 2010) of cyanophages are also affected by environmental conditions. *In-situ* studies have been carried out to investigate the temporal and spatial dynamics of cyanophage abundance. For temporal studies, a seasonal variations in cyanophage abundance have been observed, i.e. higher in the summer and lower in the winter, with phages occasionally undetectable during the winter (Waterbury and Valois 1993; Suttle and Chan 1994; Millard and Mann 2006; Wang et al. 2011). On shorter time scales, cyanophage abundance showed a diurnal pattern and the length of the lytic cycle fits into the hours of daylight (Clokier et al. 2006; Kimura et al. 2012). Spatial variability has also been observed, i.e. cyanophage abundance was found to vary throughout the depth of the water column and it is generally greater in coastal environments and decreases toward open ocean environments (Suttle and Chan 1994; Frederickson et al. 2003; Sullivan et al. 2003; Clokier et al. 2006; Millard and Mann 2006; Matteson et al. 2011). To date, studies on the occurrence and dynamics of cyanophages in freshwater are scarce and often restricted to cyanophages infecting *Synechococcus* (Matteson et al. 2011) or cyanophages infecting *Microcystis aeruginosa* (Manage et al. 1999; Manage et al. 2001; Takashima et al. 2007; Yoshida et al. 2008; Yoshida et al. 2010; Kimura et al. 2012).

There are two goals in this study: (1) to investigate the occurrence of cyanophages which infects unicellular and multicellular cyanobacteria in Kranji Reservoir; and (2) to better understand the significance of environmental variables

on the dynamics of cyanophage abundance. To achieve the first goal, monthly sampling was conducted in Kranji Reservoir (a total of 39 samples for 13 months from 3 locations) and the presence of cyanophages infecting *Anabaena*, *Microcystis* and *Synechococcus* were examined. To meet the second goal, the author quantified the (i) cyanomyoviruses (cyanophages under the myoviridae family) by employing the g20 gene as a molecular proxy, (ii) phage infectious to *Microcystis aeruginosa* (Ma-LMM01-type phage) by employing the g91 gene as a molecular proxy and (iii) measured several physical and chemical parameters, such as total nitrogen, total phosphorus total organic carbon, and chlorophyll- a concentration. Subsequently, statistical analyses were carried out to identify how the occurrence of cyanophage correlates to cyanobacteria (*Microcystis* and *Anabaena*), cyanotoxin and environmental variables. Cyanobacteria and cyanotoxin results were taken from Te (2012). To my knowledge, this is the first study that examines the presence of cyanophages infecting different genera of cyanobacteria in an aquatic ecosystem. Additionally, this is also the first study which demonstrates the dynamics of g20 and g91 genes together in an aquatic ecosystem.

4.2 MATERIALS AND METHODS

4.2.1 Sampling site and sample collection

Rainfall in Singapore begins to increase in the month of October (monthly mean: 158.8 mm) and peaks in the month of December (monthly mean: 329.5 mm) during the Northeast Monsoon (NEA 2009). In Singapore, rainwater is collected through a comprehensive network of drains, canals and stormwater collection ponds before it is channeled to reservoirs for storage (Public Utilities Board 2012). One of these reservoirs is the Kranji Reservoir. Kranji Reservoir is mainly supplied by three tributaries in the northern part of the reservoir: water flows from Tengah River, Kangkar River and Peng Siang River. Water flow from Pang Sua River was diverted into Kranji Reservoir at the end 2005 as the fourth tributary to Kranji Reservoir at the southern end of the reservoir (Chua et al. 2009).

A total of 39 water samples were collected from Kranji Reservoir over a year between 8 August 2008 and 27 August 2009. Field sampling was conducted monthly at three sampling stations, i.e. KR1 (1°24'49.640"N; 103°43'49.247"E), KR3 (1°25'48.508"N; 103°44'41.399"E) and Peng Siang (1°23'35.495"N; 103°44'05.364"E) (Figure 4.1). Sampling was conducted from 10 am to 12 pm except for March 2009 which was conducted at 3 pm. Twelve liters of surface water samples were collected with autoclaved, sample-rinsed carboys and transported to the laboratory immediately. The samples were then stored at 4 °C before processing. All the samples were filtered within 24 hrs. For each sampling, another 1L of surface samples were collected for the purpose of chemical and chlorophyll-a measurement. *In-situ* measurements included: water temperature, dissolved oxygen (DO), salinity, conductivity, secchi depth and total dissolved solid (TDS). A YSI meter (EC300) was used to measure the water temperature, salinity and TDS, and a YSI model 52 (YSI probe 5739) was used to measure the concentration of dissolved oxygen. Secchi depth or water transparency was determined with a Secchi disc.

Figure 4. 1 Map of Kranji Reservoir and sampling stations

4.2.2 Environmental variables

At each station, one liter of sample was collected and stored in an ice box during transportation from site to the laboratory. Water quality parameters of each sample were analyzed in duplicate or triplicate according to standard methods, i.e. American Public Health Association (APHA 1998) and United States Environmental Protection Agency (USEPA 1993). Water quality parameters examined in the lab included total nitrogen (TN), total phosphorus (TP), total carbon (TC), inorganic carbon (TIC), calcium ion, magnesium ion, turbidity, total suspended solid (TSS), pH and chlorophyll-a. The pH of samples was determined in the laboratory with a Thermo pH meter (Orion 555A) and the turbidity was measured by using a turbidity meter (Euteoh Instruments, TN-100). Pre-treatment and the standard methods for TN, TP, TC, TOC, ion calcium and ion magnesium have been summarized in Table 4.1. TSS was analyzed according to APHA 25400 (APHA 1995). The chlorophyll-a was measured according to APHA 10200H (APHA 1995) with modification. In brief, 50-250 mL of water sample with MgCO_3

(0.001 % w/v) added was filtered through a cellulose nitrate membrane. Subsequently, 10 mL of 90 % acetone was added to the membrane, then sonicated for 3 min and incubated in the dark for 24 hours. After 24 hours, the sample was then centrifuged at 2800 rpm for 15 min. A clear supernatant (chlorophyll-a extracted by acetone) was obtained and measured with a spectrophotometer (Backman Coulterm DU 640B). Total toxin, intracellular and extracellular toxin were tested by Te Shu Harn (refer to Te (2012)). In addition, in-situ measurements were also conducted for temperature, dissolved oxygen (DO), salinity, conductivity, secchi depth and total dissolved solid (TDS).

TN, TP and carbon parameters are important factors influencing the growth of cyanobacteria. Hence, changes in these parameters will see an alteration in the composition of the cyanobacterial assemblage, which will thus affect the cyanophage community. The TN to TP ratio represents the trophic status of the sampling site. Additionally, chlorophyll-a indicates the abundance of phytoplankton and biomass. As discussed in Chapter 2, calcium and magnesium ions play a vital role in maintaining the structure of cyanophages and therefore, the infectivity of cyanophage on cyanobacteria.

Table 4.1 Standard methods for each analyst

Analyst/s	Sample Pre-treatment	Preservations and storage conditions	Time-frame for analysis	Standard Method
Total Nitrogen	Filtration not required.	35 ml of unfiltered samples was stored in plastic sample vial, and kept in -20 °C freezer until analysis.	Within 28 days	USEPA 353.2 (after manual persulfate digestion)
Total Phosphate				APHA 4500P.E (after manual persulfate digestion)
Total Carbon		80 ml of unfiltered sample was stored in 2 combusted amber glass vial, acidified with 2 drops of concentrated HNO ₃ and kept in 4 °C refrigerator until analysis.	Within 28 days	APHA 5310. B
Inorganic Carbon				APHA 5310. B
Calcium ion*	Filtration through 0.45µm syringe membrane	10 ml of filtrate was stored in 15 ml centrifuge tube, acidified with 2 drops of concentrated HNO ₃ (pH < 2) and kept in 4 °C refrigerator until analysis.	Within 28 days	APHA 3500- Ca C.
Magnesium ion*				APHA 3500- Mg. C

*Analyzed by Mrs Pang (lab technician) at Environmental Lab, Department Civil and Environmental Engineering, Nanyang Technological University.
Total organic carbon = TC- IC

4.2.3 Concentration of viral communities

Natural virus communities were obtained from concentrating 12 liters of reservoir water using an ultrafiltration system. Briefly, 12 liters of samples were pre-filtered using a depth filter system (Pall Corporation) with 50 or 20 μm pore size low binding protein membrane (polypropylene) to remove particulate matter, phytoplankton and zooplankton. Subsequently, the filtrate was filtered through a 0.2 μm membrane cassette by using tangential flow filtration (TFF) (Pall Corporation) to remove most of the bacteria. After undergoing pre-filtering, samples were stored in a 4 °C cool room. The viruses in the filtrate of about ten liters of sample were concentrated to a final volume ranging from 250 to 500 ml, using TFF with a 30 kDA membrane cassette (Pall Corporation). Concentrated samples were believed to contain virus like particles (VLPs). These concentrated samples were stored in the dark at 4 °C, ready for well/ double- layer plaque assay or secondary concentration. A secondary concentration was carried out by using an Amicon Ultra centrifugal filter device (Milipore). Briefly, the TFF- concentrated sample was loaded to the filter device (10 kDA) and centrifuged at 5,000 x g for approximately 10 minutes according to manufacturer's instruction. The final volume of each concentrated water sample was approximately 4 ml from 200 ml for the DNA extraction purpose.

4.2.4 Well assay

The well-plating method of infection with 12-well plates was used for detection of phage infectious to *A. circinalis*. Disposable sterile Pasteur pipettes were used to transfer 12 ml of early log phase *A. circinalis* into culture flasks, and subsequently added with 1.2 ml of environmental sample. Prior to addition, the concentrated environmental samples were vortexed to ensure resuspension of all cyanophages in the liquid. For the controls, 1.2 ml of MLA medium was used in place of the environmental sample. The culture flasks were incubated in an incubator shaker at 25°C at low shaking speeds of about 100 rpm and exposed to light for at least 1.5 hrs. This was to ensure homogenous mixing and optimum

contact between host and cyanophage. Samples were then transferred to the 12 well-plates, where 1 ml of sample was placed in each well. Plated samples were then transferred to growth racks and observed for lysis. The well plates were subjected to light- dark cycles at 12 hours intervals, at about 22 °C (± 1 °C). Throughout the infection period, the multi-well plates were frequently incubated in the incubator shaker at 25 °C, at 100 rpm and exposed to light. This step was to ensure maximum contact rate between host and the phage. The placement of the plates was randomly changed to optimize light exposure to the well-plates, and to increase the consistency of light exposure to each well-plate.

Every 5 or 9 days, optical density (OD) readings were obtained from the well plates by randomly removing samples from 2 wells from each well-plate. The plates were incubated and monitored for 30 days. The plates were monitored for the lysis of *A. circinalis* and compared to the negative control for 30 days. The lysis effect was observed using OD tests combined with visual observation using an inverted light microscope to ensure that infection had taken place. The inoculated cultures were incubated until significant mortality of the *A. circinalis* host was visually apparent (i.e. clearing of the well). Clearing of the well was assumed to be due to the dying of host *A. circinalis* cells by lysis and indicated the presence of lytic agent, such as cyanophage.

4.2.5 Double layer plaque assay

Anabaena cylindrica was cultured to exponential phase (OD >0.4, at 750 nm) and subsequently concentrated to OD >1 (at 750 nm), by which time the culture was ready for infection. Three ml of the concentrated host strain was then added to 1 ml/ 0.5 ml of concentrated environmental sample containing the VLPs. This mixture was subsequently incubated and shaken for one hour at 25 °C to aid phage-host adsorption. To prevent temperature shock, the mixture was placed in a water bath at 40 °C for 10 min prior to addition of 1.5 ml of molten 0.6 % (w/v) agarose at 40 °C. The mixture was gently mixed with a swirling action and poured onto the top of a

solid 0.8 % MLA agar plate. The molten soft agar was allowed to set for 1 to 2 hours before it was inverted and placed in a growth chamber under the conditions described above (well assay). A confluent lawn of overlay agar was prepared. The plates were monitored daily for the appearance of plaques within the lawn for 30 days. Plaques observed or clearing of lawns indicated the presence of a lytic agent, such as cyanophage.

4.2.6 DNA extraction

To determine the potential contribution of viruses within the bacterial cells, the DNA in the bacterial cells were extracted. DNA was extracted from host cells (cyanobacterial) using a DNA purification kit (Wizard Genomic DNA Purification Kit, Promega). Before extraction, 50 to 500 ml of water sample (depending on the concentration of phytoplankton) was filtered through a glass microfiber filter (Whatman 934-AH). The cyanobacterial cells were harvested on the glass microfiber filter and frozen at -20 °C. DNA extraction was carried out according to manufacturer's instruction with some modifications in the steps of cell disruption: 600 µL of nuclei lysis solution was added to the glass microfiber filter (provided in the DNA purification kit) and ground twice (at a speed of 42 for 8 s) mechanically (Biospec Product Inc.) with 2 µm glass beads (Sigma-Aldrich). This was followed by incubation at 80 °C for 5 mins, and centrifugation (14,000 rpm for 1 min), after which the supernatant was collected. The steps of cell disruption were repeated to get a total supernatant volume of 1.2 mL. DNA was then extracted from the supernatant by using the DNA purification kit. Extracted DNA was kept in storage at -20 °C until the molecular work was performed.

Viral DNA (free viral fraction) was extracted from 200 µl of concentrated sample by using the DNA extraction kit (Qiagen, QIAamp DNA Mini Kit). DNA extraction was carried out according to manufacturer's instruction. Viral DNA was eluted in 60 µl Buffer AE. Extracted DNA was kept in storage at -20 °C until the molecular work was performed. For each sample, two sets of DNA analysis were performed.

4.2.7 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a general molecular approach in identifying and detecting microorganisms. The primer sequences used to target the g91 gene and g20 gene are shown in Table 4.2. A pair of g20 gene primers (CPS1 and CPS2) designed by Fuller et al. (1998) specifically amplifies a gene encoding a capsid assembly protein (g20) belonging to cyanophages in the myoviridae family, with a product of 165-bp PCR amplicon. These primers have successfully amplified the g20 gene product from cyanomyoviruses infecting *Synechococcus* (Millard and Mann 2006) and *Planktothrix rubescens* (Deng and Hayes 2008) only. Other pair of primers (SheathRTF and SheathRTR) were designed to amplify a conserved region of the gene encoding sheath protein of Ma-LMM01-type phages (Takashima et al. 2007). Ma-LMM01 is a cyanophage which specifically infects *Microcystis aeruginosa* NIES298, a toxic strain of the bloom forming cyanobacteria. (Yoshida et al. 2006).

To develop the PCR assay, optimization of the amplification programme and master mix for the PCR reaction was performed by testing a series of conditions and the optimum conditions were fixed. From this process, it was observed that environmental samples contained inhibitors that interfered with the PCR reaction. To counter this problem, the concentration of taq polymerase was increased in the PCR master mix. To increase the sensitivity, the annealing temperature was lowered and the cycle of amplification was increased. After several trials in optimizing the PCR programme and master mix condition, we found that PCR targeting the sheath protein gene (g91 gene) is feasible in detecting cyanophages which infect *Microcystis aeruginosa* with a concentration of more than 1 genome copies per ml. The following programme was used: pre-incubation at 95 °C for 3 min; amplification for 36 cycles consisting of denaturing at 95 °C for 45 s, annealing at 60 °C for 30 s, extension at 72 °C for 30 s; and final extension at 72 °C for 7 min. The master mix of the PCR reaction consisted of 3.0 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM for both primers, 2.5 unit (U) taq polymerase, 1x of PCR buffer, 5 µl DNA

template. The optimized programme of g20 gene amplification: pre- incubation at 95 °C for 2 min; amplification for 36 cycles consist of denaturing at 95 °C for 45 s, annealing at 45 °C for 45 s, extension at 72 °C for 30 s; and final extension at 72 °C for 5 min. The master mix of the PCR reaction consisted of 4.0 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM for both primers, 2.5 unit (U) taq polymerase, 1x of PCR buffer, and 5 µl DNA template. Positive (targeting gene plasmid) and negative controls (DNA-free water) were included in every reaction performed with PCR to ensure the performance of assays.

Table 4.2 PCR and qPCR primer sets used in this study

Targeted primers	group/	Sequence (5' – 3')	Reference
<i>Microcystis aeruginosa</i> infectious phage			
SheathRTF		ACA TCA GCG TTC GTT TCG G	(Takashima et al. 2007)
SheathRTR		CAA TCT GCT TAG GTA GGT CG	
<i>Cyanomyoviruses</i>			
CPS1		GTA GWA TTT TCT ACA TTG AYG TTG G	(Fuller et al. 1998)
CPS2		GGT ARC CAG AAA TCY TCM AGC AT	

PCR products were run on a 3 % agarose gel in 1x Tris- borate- EDTA (TBE buffer) stained with ethidium bromide at 100 volts for one hour. The gels were photographed under UV transillumination using a camera (Kodak DC 290). A positive control was successfully obtained from the PCR product. The PCR reaction was used to amplify the concentrated virus DNA, and a positive result was obtained with the correct location of base pairs. To further verify the PCR product, sequencing was done on a few random selected PCR products. Multi- sequences were observed from direct sequencing of the g20 gene PCR product. This was found to be due to the degeneracy primer design of CPS1 and CPS2. Thus, cloning of the g20 amplification PCR product, followed by the plasmid DNA produced from cloning was required. Sequencing results were compared with those available in BLAST. The g20 PCR product showed highest similarity (85 %) with

Synechococcus phage S-RIM at the amino acid level. The plasmid DNA from cloning of the g20 was used as the positive control. For the g91 PCR product, only a single sequence was obtained from direct sequencing and BLAST results showed a 100 % match with *Microcystis aeruginosa* cyanophage (Ma-LMM01). To obtain positive controls, the g91 amplification PCR product was cloned. The plasmid DNA from cloning of the g91 was used as the positive control. Appendix 4.A shows the sequencing results of g91 with the g20 gene PCR product used as a positive control in PCR experiments.

4.2.8 Quantitative Real-time PCR (qPCR)

The qPCR method was developed using the SYBR Green I dye. SYBR Green I is a DNA- binding dye, which intercalates into double- stranded DNA (dsDNA), as well as PCR product. After binding with dsDNA, the fluorescence increase and can be detected by LightCycler 2.0 (Roche Applied Science). This instrument is able to monitor six different fluorescence detection channels (530, 560, 610, 640, 670 and 705nm) simultaneously, in real- time so that the amplification cycle and fluorescence changes can be observed.

The standard used to determine the gene copy number of *M. aeruginosa* phage was based on the cloned plasmids using the specific sequence for g91, and the standard curve was obtained from the calculated plasmid concentration at various dilutions. The g20 gene standard curve was also built in a similar manner with known plasmid concentrations. The plasmid DNA was extracted and the concentration was calculated in terms of genome copy number and the equation used for this calculation is as follows:

$$\text{Number of genome copier per } \mu\text{l} = \frac{(6.022 \times 10^{23} \times \text{DNA concentration})}{(\text{Genome molecular weight})}$$

where 6.022×10^{23} is the number of genome copies per mole of genome, DNA concentration was measured by spectrophotometer at wavelength of 260 nm and the

genome molecular weight equals the genome size multiplied by a molecular weight of 1 bp (660 g/mol). For example, for a DNA concentration equal to 22.25 ng μ l⁻¹; the genome size is approximately 3147 bp, and therefore the genome concentration is 6.2045 x10⁹ genome copies μ l⁻¹. The extracted plasmid DNA was diluted to provide ten standard concentrations for constructing a standard curve. These known concentrations were 6.2 x 10⁵, 6.2 x 10⁴, 6.2 x 10³, 6.2 x 10², 6.2 x 10, 6.2, 1.2 genome copies/ μ l, where the standard range was based on measurements obtained from environmental samples. The standards were run with the known concentration g91/ g20 gene to obtain cycle threshold values (C_t value) and used to establish the standard curve. C_t value was reported as the fluorescence intensity of the SYBR Green I dye. The amplification efficiency for the standard curve was calculated with software (LightCycler 4.05). The efficiency calculation should have a maximum value of 2 because DNA copies were doubled at each amplification. The amplification efficiency was calculated as the average of all replicates by the software.

In this study, two sets of primers were used, i.e. sheathRTF/sheathRTR and CPS1/CPS2. SheathRTF and sheathRTR are non-degenerate primers, while CPS1 and CPS2 are degenerate primers. Primers are known as degenerate primers when the primer sequences differ in at least one base, therefore causing a difference in the melting temperature. One of the main functions of melting curve analysis in the qPCR programme is to differentiate between the PCR target product and primer dimers, and to differentiate target products with non- specific amplification. Thus, melting curve analysis was performed to assess the specificity of the amplified product. The specific β - globin product melts at a higher temperature than primer dimers.

Quantification of the Ma-LMM01 type phages (g91 gene) and cyanomyoviruses which encoded the g20 gene were performed with 5 μ l of DNA template (free viral DNA and host cell fraction DNA), 0.5 μ M of both primers, 10 μ l of reaction master mix and sterilized distilled water to a final volume of 20 μ l. The thermal cycle and measurement of SYBR Green fluorescence binding were

performed in a Light- Cyclor qPCR using the following programme: pre- incubation at 95 °C for 15 min; amplification for 45 cycles consisting of denaturing at 95 °C for 15 s, annealing at 58 °C for 20 s, extension at 72 °C for 30 s; melting curve analysis (1 cycle amplification) heated to 95 °C, annealing at 65 °C for 60 s, extension at 95 °C. Only 11 samples from each station were enumerated for g20 gene concentration, i.e. samples from August 2008 to July 2009 with the exception of December 2008 samples (this latter was due to a change in the qPCR machine leading to inconsistent results).

Other than using the melting curve the targeted product was also confirmed with gel electrophoresis. The qPCR products were run on a 3 % agarose gel in 1x Tris- borate- EDTA (TBE buffer) stained with ethidium bromide at 100 volts for one hour. The gels were photographed under UV transillumination using a camera (Kodak DC 290). The correct g91 qPCR amplicon was placed at ca. 132-bp and the g20 qPCR amplicon was placed at ca.165-bp.

4.2.9 Statistical Analysis

Data from all locations (i.e. KR1, KR3 and PS) were processed together to identify non-local specific trends. Before the compilation of data, outliers were identified through box- plot analysis (Devore and Farnum 2005) for each variable at each station. Pearson's correlation analysis (PASW Statistic 18, SPSS Inc) was conducted to identify the correlation between environmental variables such as abundance of host (*Microcystis* and *Anabaena* concentrations were given by Te Shu Harn), total nitrogen, total phosphate, chlorophyll-a, toxin (toxin concentrations were given by Te Shu Harn) and cyanophages concentration.

RESULTS

4.3.1 Water Conditions

Temperature of the Kranji Reservoir surface water was stable over the sampling period, i.e. between 28 °C and 31 °C. Site observation and descriptions of weather, visual color of the surface water, scum appearance, etc. at the site were recorded as shown in Appendix 4.B. The corresponding data of physical and chemical conditions are attached in Appendix 4.C.

The physical and chemical conditions which experienced relatively significant dynamic changes are shown in Figure 4.2. Significantly higher concentration of total nitrogen (TN) and total phosphorus (TP) were noted at site PS compared with sites KR1 and KR3, especially between October 2008 and May 2009 (Figure 4.2A and B). Dramatic increased of chlorophyll- *a* concentrations were noted at site PS from October 2008 to November 2008 (Figure 4.4D). Site KR3 had the lowest concentration of Chl *a*, turbidity and TSS in most of the sampling periods. Total organic carbon (TOC) dynamic of these three site was significantly different (Figure 4.2F), e.g. in April 2009, site KR1 had the lowest TOC concentration, but KR3 had the highest TOC concentration.

Figure 4.2 Dynamics concentrations of environmental variables in Kranji Reservoir from August 2008 to August 2009. Error bars represent the standard deviation of duplicate or triplicate data from chemical analysis.

4.3.2 QPCR methods

The detection limit of the qPCR reaction was examined using known concentrations of the Ma-LMM01-type phage tail sheath- g91 gene, and the cyanomyoviruses vertex portal protein, g20 gene. The linear amplification result for the concentration of gene plasmid, g91 gene, is between 2 and 6×10^6 gene copies per μl . For the g20 gene, the linear amplification was obtained between plasmid concentrations of 5×10^0 and 1×10^7 gene copies per μl . One gene copy per μl or five gene copies per reaction were tested for the g91 and g20 qPCR assay but both showed no amplification. Therefore, the lowest detection limit of the qPCR assay for this study is two gene copies per μl for g91 the qPCR assay and five gene copies per μl for the g20 qPCR assay.

The qPCR reaction standard curves were developed at the beginning of the qPCR assay. Linear regression was obtained between the C_t (threshold cycle) value and known gene copy concentrations from the standard curve. Using the linear regression formula, the concentration of the target gene within environmental samples can be enumerated. The average R^2 for the linear regression between C_t value and gene copy number and the average efficiency for the replicate efficiency for Ma-LMM01-type phage tail sheath- g91 gene, and cyanomyoviruses vertex portal protein- g20 gene were: $R^2 = 0.999$, efficiency = 92.6 % and $R^2 = 0.99$ and efficiency = 85 % respectively (Figure 4.3). A new standard curve was built when any reaction buffers were changed or the positive control with known concentration had a difference of >5% when back calculated with the standard curve.

Figure 4.3 QPCR standard curve for (A) the Ma-LMM01-type phage tail sheath- g91 gene and (B) the cyanomyoviruses vertex portal protein- g20 gene

Melting temperatures of the g91 gene and g20 gene qPCR amplification were determined from the standard melting peaks (Figure 4.4). Table 4.3 shows the summary of melting temperatures for the plasmid and environmental samples. The melting temperature and the correct base pair (determined using gel electrophoresis) used to determine the targeting gene was amplified. In contrast, if the melting temperature obtained from qPCR amplification was not at this specific temperature or not located at the correct base pair, it was considered as non-specific binding and not the targeting gene.

Table 4.3 Comparison of melting temperatures (T_m) values for qPCR products using reference strains and environmental samples.

Target Genes	<u>qPCR melting temperature °C</u>	
	Ma-LMM01- type phage tail sheath, g91 gene	Cyanomyoviruses vertex portal protein, g20 gene
Plasmid	84.27±0.06	82.34±0.08
Environmental Sample (Free viral fraction)	83.86±0.18	82.72±0.39
Environmental sample (host cell fraction)	84.27±0.13	-

Figure 4.4 An example of qPCR results (Ma-LMM01-type phage tail sheath g91 gene). The melting peaks of the positive control and environmental samples are at around 84 °C and no melting curve or melting peaks were observed for negative controls. Melting curves (A) and melting peaks (B) are to verify the environmental samples at correct melting temperature (T_m). The corresponding PCR products were examined with gel electrophoresis to double confirm the amplification products are the targeted gene. Gel electrophoresis (C) is to confirm the environmental samples at correct base pair, ca. 132 bp for g91 gene amplicon. No band was observed for the negative control (lane 2). Environmental samples are from lane 3 to 14, positive control is lane 15. Lanes 1 16 was the 100 bp molecular marker. From the melting curve/ peak and the gel electrophoresis, Ma-LMM01- type phage tail sheath- g91 gene was detected from the environmental samples.

4.2.3 Cyanophages which infect unicellular and filamentous cyanobacteria

We monitored the presence of various types of cyanophages in Kranji Reservoir from August 2008 to August 2009 (Table 4.4). Cyanophages which infect *Anabaena circinalis* were only detected from PS station, and only in samples from October 2008 to December 2008. During these three months, a dramatic change in chlorophyll-a (chl-a) concentration was observed, i.e. dramatically increase of chl-a concentration from October to November 2008 (from 110.83 µg/L to 1584.74 µg/L), followed by significantly decrease from November to December 2008 (131.71 µg/L).

Cyanophages which infect *Anabaena cylindrica* showed a continuous presence at site KR3 from August 2008 to November 2008. Site KR3 showed the highest percentage (38 %) of the presence of this phage compared to sites KR1 and PS (23 %). Cyanophages which infect *A. cylindrica* were detected in November 2008 and April 2009 from all stations, i.e. before the Northeast Monsoon and just after the Northeast Monsoon. In contrast, these were absent in the samples from December 2008 and March 2009, i.e. the Northeast Monsoon. These three stations demonstrated an increasing trend in *Anabaena* spp. (refer to Te (2012)) for the same period.

Ma-LMM01-type phages (i.e. cyanophages which infect *Microcystis aeruginosa*) were detected at all samples (except for the PS sample in August 2008) using the PCR assay and the qPCR assay. Cyanomyoviruses encoding the g20 gene (i.e. representing *Synechococcus* or *Plankthothrix* infect phages) were detected in all samples (except for the PS sample in November 2008) using PCR assay. The g20 gene was detected in the PS November 2008 sample when using qPCR assay, but below the limit of quantification and thus suggesting that the g20 gene in this sample is in very low concentration.

Although KR1, KR3 and PS are three sites in the same reservoir, they show are spatial variability in terms of their community of cyanophages. Site KR1 is relatively stable in the abundance of cyanophages which infect *Microcystis* (g91 gene) compared with the other two stations, i.e. site KR1 showed a one log difference in g91 concentration over 13 sampling periods, while sites KR3 and PS showed a three log difference. Site KR3 showed a relatively higher percentage of the presence of cyanophages which infect *A. cylindrica*. In addition, site KR3 appears to be the most seasonally variable site, with the disappearance of phages infectious to *A. cylindrica* and phages infectious to *Microcystis* under the detection limit during the Northeast Monsoon. This temporal variability is suggested to be due to the physical characteristics of site KR3. Site KR3 is located at the end of the reservoir has the deepest point in Kranji Reservoir (14 to 18 m), and is close to the dam which separate the reservoir from the Straits of Johor. During the rainy season, the gate at Kranji Reservoir is opened upon reaching a certain water level and therefore, it is possible that the flushing effect at site KR3 cause the highest temporal variability in phages compared to KR1 and PS. Cyanophages which infect *Anabaena circinalis* were only detected at site PS from October 2008 to December 2008.

Table 4.4 The occurrence of specific cyanophages at sampling stations KR1, KR3 and PS, was examined with well plate assay for cyanophage which infect *A. circinalis*; plaque assay for cyanophage which infect *A. cylindrica*; and PCR and qPCR assay for Ma-LMM01-type phages and cyanomyoviruses. 13 samples were analyzed at each station for the entire study period. The total number of these three stations is 39.

Sampling Date	Ma-LMM01-type phage tail sheath, g91 gene			Cyanomyoviruses vertex portal protein, g20 gene			Phages which infect <i>Anabaena circinalis</i>			Phages which infect <i>Anabaena cylindrica</i>		
	KR1	KR3	PS	KR1	KR3	PS	KR1	KR3	PS	KR1	KR3	PS
8-Aug-08	+	+	-	+	+	+	-	-	-	-	+	-
23-Sep-08	+	+	+	+	+	+	-	-	-	-	+	+
21-Oct-08	+	+	+	+	+	+	-	-	+	-	+	-
20-Nov-08	+	+	+	+	+	+	-	-	+	+	+	+
19-Dec-08	+	+	+	+	+	+	-	-	+	-	-	-
16-Jan-09	+	+	+	+	+	+	-	-	-	-	-	-
19-Feb-09	+	+	+	+	+	+	-	-	-	-	-	-
26-Mar-09	+	+	+	+	+	+	-	-	-	-	-	-
17-Apr-09	+	+	+	+	+	+	-	-	-	+	+	+
29-May-09	+	+	+	+	+	+	-	-	-	-	-	-
30-Jun-09	+	+	+	+	+	+	-	-	-	+	+	-
21-Jul-09	+	+	+	+	+	+	-	-	-	-	-	-
29-Aug-09	+	+	+	+	+	+	-	-	-	-	-	-
Total	13	13	12	13	13	13	0	0	3	3	6	3

*sample was showed positive amplification using qPCR assay but negative when using PCR assay.

4.2.4 Dynamics of cyanophage abundance

The three stations showed different g91 gene and g20 gene fluctuation patterns. The g91 DNA copy number was enumerated in two types of fractions, i.e. the host cell fraction and free-phage fraction. For the free-phage fraction, the g91 gene abundance varied between stations and months across the range of undetectable to 4.7×10^2 gene copies ml^{-1} of environmental sample (Figure 4.8). Only one sample was absent of the g91 gene, i.e. the August 2008 sample from site PS. The concentrations of the samples from site KR3 between November 2008 to February 2009 and the sample from site PS in September 2008 were under the qPCR detection limit (i.e. showed amplification but the Ct value was higher than the standard curve) but showed the presence of the g91 gene. In general, our g91 concentrations (undetectable to 4.73×10^2 copies ml^{-1}) were comparable to the g91 gene concentrations found in Hirosawanoike Pond, Japan (below detection limit to 8.2×10^2 copies ml^{-1}) (Kimura et al. 2012) but lower than the g91 gene concentrations in Lake Mikata, Japan (below detection limit to 1.1×10^4 gene copies ml^{-1}) (Yoshida et al. 2008).

For the host cell fraction, copy numbers of the Ma-LMM01-type phage- g91 gene ranged from below detection limit to 5.81×10^4 gene copies ml^{-1} . The g91 gene in site KR3 samples from December 2008 and January 2009 and the site PS samples from August and September 2008 were all under the qPCR detection limit. The only other study which has reported the g91 gene in the host-cell fraction was from the site of Hirosawanoike Pond, Japan, where the g91 gene ranged from 2.5×10^1 to 1.6×10^6 gene copies ml^{-1} (Kimura et al. 2012). Comparing the copy numbers of the g91 gene from the host cell fraction and free- phage fraction, it was demonstrated that the host- cell fraction was always 2 to 3 orders of magnitude higher than the free-cell fraction (Figure 4.6). Site PS was the only site which showed an increase in g91 gene concentration (in free- viral fraction) from December 2008 to February 2009.

The estimation of putative cyanomyovirus abundance based on the g20 gene showed that the range varied between stations and months from below the detection limit to 3.1×10^3 gene copies ml^{-1} . Only the sample in November 2008 from the PS site was below detection limit but showed the presence of the g20 gene. Only two sampling sites from previous studies enumerated the g20 gene by using qPCR, i.e. a Norwegian coast (Sandaa and Larsen 2006) and Lake Erie (Laurentian Great Lake, North America) (Matteson et al. 2011). Concentrations of the g20 gene in Norwegian coastal water was reported to range from $< 5.0 \times 10$ to 7.2×10^3 gene copies ml^{-1} (Sandaa and Larsen 2006). In another study, g20 gene densities in Lake Erie was found to range from 1.3×10^5 to 4.3×10^6 gene copies ml^{-1} (Matteson et al. 2011). Our study showed that the g20 gene densities in Kranji Reservoir is closer to Norwegian coastal water and lower than Lake Erie. Figure 4.7 shows the comparison between the g91 gene and g20 gene in Kranji Reservoir, where the concentration of the g20 gene is always higher than that of the g91 gene except three occasions, i.e. May 2009 at site KR1; November 2008 and February 2009 at site PS.

Figure 4.5 Temporal dynamics of g91 gene copies at Kranji Reservoir from August 2008 to August 2009. For KR3 station, the g91 gene was detected in samples from November 2008 to February 2009 the concentration was under the qPCR detection limit. For PS station, the g91 concentration was under qPCR detection limit for September 2008 and the g91 gene was not detected for sample August 2008. Error bars represent the standard deviation of triplicate data from qPCR analysis.

Figure 4.6 Monthly changes in the abundance of cyanophages in both the free-viral fraction (bars in black) and host cell fraction (bars in shading) at (A) KR1, (B) KR3 and (C) PS. Samples that were below detection limit and undetectable are not reflected. Error bars represent the standard deviation of triplicate data from qPCR analysis.

Figure 4.7 Comparison between Ma-LMM01-type phage abundance (g91 gene- bars in black) and cyanomyovirus abundance (g20 gene- bars in shading) at (A) KR1, (B) KR3 and (C) PS. Samples that were below detection limit and undetectable are not reflected. Error bars represent the standard deviation of triplicate data from qPCR analysis.

4.2.5 Correlations between cyanophage abundance and environmental conditions

Correlation analysis was conducted to identify environmental variables which were significantly correlated with cyanophage abundance. The Pearson's correlation was selected to correlate cyanophage abundance with environmental variables. Two types of *Microcystis aeruginosa* infectious phages were enumerated, i.e. the g91 gene copies from both the free-viral and bacterial DNA fractions. Table 4.5 shows the Pearson's correlation analyses of cyanophage abundance (g91 gene concentration) versus the environmental variables.

Concentrations of g91 gene from the free- viral fraction were positively correlated with *Microcystis* 16S rRNA gene copy number, *Microcystis mcyE* gene copy number, chlorophyll-a, TN, DO, Ca^{2+} , pH, conductivity, turbidity, TSS and TDS but negatively correlated with Secchi depth. Concentrations of the g91 gene from the host- cell fraction were positively correlated with the same variables as above with a few exceptions, i.e. no correlation with DO and pH. However, unlike the free- viral fraction, the g91 gene copy number for the host cell fraction showed a positive correlation with extracellular toxin, TP and Mg^{2+} . In general, the abundance of g91 genes from the bacterial- cell fraction showed relatively strong relationships with *Microcystis* spp. and the *Microcystis mcyE* gene. In contrast, the abundance of g91 genes from the free-viral fraction showed relatively strong relationships with chlorophyll-a, turbidity, Secchi depth and Ca^{2+} .

Table 4.5 Pearson's correlation analyses of cyanophage abundance (g91 gene concentration) versus the environmental variables. The values represent the Pearson's coefficient (r) for variables correlated with *Microcystis aeruginosa* infectious phage- gene (g91) from the free- viral and bacterial cell fractions. Only significant correlations at 0.05 or 0.01 level are shown.

Variables	<i>Microcystis aeruginosa</i> infectious phage- g91 gene, free- viral fraction (gene copies/100ml)	<i>Microcystis aeruginosa</i> infectious phage- g91 gene, bacterial cell fraction (gene copies/ml)
<i>Microcystis</i> 16S rRNA (gene copies/ml)	0.49**	0.77**
<i>Microcystis mcyE</i> (gene copies/ml)	0.48**	0.68**
<i>Anabaena</i> 16S rRNA (gene copies/ml)		
Chlorophyll-a (µg/L)	0.84**	0.70**
Intracellular toxin (µg/L)		
Extracellular toxin (µg/L)		0.375*
Microcystin concentration (µg/L)		
Total nitrogen, TN (mg N/L)	0.59**	0.63**
Total Phosphorus, TP (mg P/L)		0.53**
Total Carbon, TC (mg/L)		
Total Organic Carbon, TOC (mg/L)		
Total Inorganic Carbon, TIC (mg/L)		
Dissolved oxygen, DO (mg/L)	0.59**	
Ca ²⁺ (mg/L)	0.57**	0.50**
Mg ²⁺ (mg/L)		0.37*
pH	0.47**	
Conductivity (us/cm)	0.40*	0.40*
Turbidity (NTU)	0.82**	0.58**
Secchi depth (cm)	-0.51**	-0.39*

Total suspended solid, TSS (mg/L)	0.75**	0.56**
Total dissolved solid, TDS (g/L)	0.35*	0.35*

** indicates the correlation is significant at the 0.01 level (2-tailed).

* indicates the correlation is significant at the 0.05 level (2-tailed).

4.2.6 Correlation between *Microcystis* infectious cyanophage (Ma-LMM01- type phage) and *Microcystis*

Microcystis aeruginosa- infectious cyanophage (such as Ma-LMM01 isolate) encodes the g91 gene. Here, the g91 gene concentration is used to represent the concentration of cyanophage which infects *Microcystis aeruginosa*. Figure 4.8 shows the dynamics of *Microcystis*, toxigenic *Microcystis*, g91 gene in the free viral fraction and g91 gene in the host-cell fraction. Monthly co-variation between the abundance of g91 gene in the host- cell fraction and *Microcystis* host cell density was observed in most of the sampling months. Occasionally, a dramatic increase in g91 gene density (free-viral fraction) with *Microcystis* abundance was observed.

The cyanophage-to- *Microcystis* ratio is presented in Figure 4.9. This ratio was calculated with g91 gene densities in the host-cell fraction and *Microcystis* densities (16S rRNA). This ratio represents the fraction of infected cells in a cyanobacterial population. The peaks of g91 gene in the free viral fraction (Figure 4.8) were always located in the month (or succeeding months) of the peaks of cyanophage- to- *Microcystis* ratio, for example the first peak of g91 gene in the free viral fraction of KR1 in November 2008, KR3 in October 2008 and PS in February 2009 coincided with the peaks of cyanophage-to-*Microcystis* ratio. In general, an increase (peak) in ratio in a particular month followed by a decrease of the ratio in the subsequent month was observed (Figure 4.9).

Figure 4.8 Abundance of *Microcystis* spp (16S rRNA gene- filled triangles), toxigenic *Microcystis* (*mcyE* gene- opened triangles) and cyanophage (g91 gene) in the both free-phage fraction (opened squares) and host cell fraction (filled squares) at (A) KR1, (B) KR3 and (C) PS. *Microcystis* 16S rRNA and *mcyE* genes concentrations are from Te (2012).

Figure 4.9 Cyanophage- to- *Microcystis* ratio. Here, the cyanophage concentration is measured in- g91 gene copies ml⁻¹ in the host-cell fraction and *Microcystis* is measured in- cell number (two copies of *Microcystis* 16S rRNA are equivalent to one *Microcystis* cell (Te (2012)).

4.3 DISCUSSION

4.3.1 Occurrence and dynamics of cyanophages

The temporal and spatial variability in the cyanophage population was observed in Kranji Reservoir from August 2008 to August 2009. Based on the culture based methods, i.e. plaque assay and well assay, the occurrence of cyanophages infectious to *Anabaena cylindrica* and *Anabaena circinalis* was examined. Cyanophages infectious to *Anabaena cylindrica* appeared before (i.e. November 2008) and after (i.e. April 2008) the NE monsoon (December 2008 to early March 2009), but were absent during the NE Monsoon. During NE Monsoon, the host cells, *Anabaena* spp., showed an increase from December 2008 to February 2009 (Te, 2012). Moreover, it is interesting to note that the fraction of infected *Microcystis* cells (Figure 4.9) showed the lowest value at all three stations during the wet season in the NE Monsoon, i.e. December 2008 and Jan 2009. In a study by Sawstrom and Pollard (2012), a negative relationship between percentage of heterotrophic prokaryotes and picocyanobacteria containing intracellular viruses and rainfall was reported. Results from this study are also in agreement with Sawstrom and Pollard (2012), in that heavy rainfall in during the wet season (i.e. NE Monsoon) coincided with low fraction of infected *Microcystis* cells. This could be due to changes in the virus- host interaction such as the contact rate between phage and host which in turn, reduce infectivity. Thus, the concentrations of host, *Anabaena* spp., increase during the disappearance of the phage infectious to *A. cylindrica*. Another possibility is the change in *Anabaena* population during the NE Monsoon, from a phage sensitive community to a phage resistant community, thus leading to a disappearance of cyanophages infectious to *A. cylindrica* and increase in *Anabaena* spp..

Ninety percent of the g20 concentrations were higher than those of g91 gene for the period under study. This could be due to the better specificity of the primers. Degenerate primers CPS1 and CPS2 were designed by Fuller et al. (1998) to detect

the g20 gene, and were able to positively amplify 23 strains of isolated cyanophages belonging to the *Myoviridae* family (Fuller et al. 1998). These 23 cyanophages were isolated from seven different *Synechococcus* strains. In addition, CPS1 and CPS2 have also been shown to detect the *Planktothrix* isolate, P-Z1 (Deng and Hayes 2008). In contrast, the primers, sheathRTF and sheathRTR (non-degenerate primers), were designed based on four strains of cyanophage isolates, which were isolated from the same *M. aeruginosa* strain NIES 298 (Takashima et al. 2007). Degenerate primers CPS1 and CPS2 potentially target a broad set of cyanophages compared to the non-degenerate primers, sheathRTF and sheathRTR. If sheathRTF and sheathRTR are only able to detect cyanophages infecting *M. aeruginosa* strain NIES 298 or similar phages, while CPS1 and CPS2 are able to detect a family (*Myoviridae*) of cyanophages infecting *Synechococcus* or *Planktothrix*, then it is not surprising that the g20 gene concentrations were always higher than the g91 gene in Kranji Reservoir. There were three occasions when the g91 gene was higher than the g20 gene. This may be due to an increase of the sensitive *Microcystis* spp. population. The most noticeable was at site PS on November 2008, when a cyanobacterial bloom (with obvious scum at site) and *Microcystis* concentration (1.08×10^8 gene copies/ml) were observed. *Microcystis* concentration was about 2 log higher than other months (Te 2012) and this corresponded with the lowest concentrations of g20 gene, i.e. below the detection limit.

The concentrations of cyanophages infectious to *Microcystis* in this study were comparable to those obtained in the study of Hirosawanoike Pond, Japan (Kimura et al. 2012), but relatively lower than Lake Mikata, Japan (Yoshida et al. 2008). Hirosawanoike Pond experiences eutrophication and cyanobacterial blooms from early summer to autumn every year (Kimura et al. 2012). The highest nutrient concentration at Lake Mikata is $0.0507 \text{ NO}_3\text{-N mg L}^{-1}$ (Yoshida et al. 2008), and it may be considered less eutrophic compared with Hirosawanoike Pond and Kranji Reservoir. Total *Microcystis* cell counts in Hirosawanoike Pond ranged from 3×10^7 to 3×10^8 copies 16S rRNA ml⁻¹ (Ha et al. 2008), comparable to *Microcystis* concentrations in Kranji Reservoir, which ranged from 1.43×10^3 to 1.08×10^8 16S rRNA ml⁻¹ (Te, 2012). Total *Microcystis aeruginosa* in Lake Mikata ranged from

2.7×10^1 to 2.0×10^5 copies ml^{-1} (based on the phycocyanin intergenic spacer (PC-IGS)) (Yoshida et al. 2008) while Hirosawanoike Pond had concentrations from 6.1×10^4 to 3.9×10^7 copies ml^{-1} (Kimura et al., 2012). Cell numbers of *M. aeruginosa* were not enumerated in Kranji Reservoir, but only total *Microcystis* was obtained.

It is interesting to note that Lake Mikata had higher g91 gene (cyanophages) concentrations but lower host cell numbers of *M. aeruginosa* compared to Hirosawanoike Pond. At the same time, a negative relationship between cyanophages and their host was observed in Lake Mikata (i.e. a sharp increase in cyanophage abundance when *M. aeruginosa* numbers decreased); while Kranji Reservoir and Hirosawanoike Pond showed positive correlation between g91 gene and *Microcystis* numbers. Kranji Reservoir has similar eutrophic conditions and comparable *Microcystis* cell numbers with Hirosawanoike Pond. Author suggests that nutrient conditions together with host population numbers play important roles in the dynamics of cyanophages.

Several past studies showed that the abundance of free cyanophages (filtered through $1 \mu\text{m}$ / $0.2 \mu\text{m}$) in water systems were generally underestimated. Matterson et al. (2011) found that cyanomyovirus in whole water was significantly higher in density than in filtered (through $1 \mu\text{m}$) water. Kimura et al. (2012) demonstrated that the copy numbers of phage g91 gene in the host cell fraction were 2 to 3 orders of magnitude higher than the free-phage fraction (filtered through $0.2 \mu\text{m}$). This study also showed a similar difference, i.e. the copy numbers of phage g91 gene in the free- phage fraction were 2 to 4 orders of magnitude lower than the g91 gene in host cell fractions. This difference between the replication number of phage (in the host cell fraction) and the availability of mature cyanophages (in the free- phage fraction) has been attributed to several reasons: (a) progeny phages could have been trapped by host lysates or attached to the next hosts for infection; (b) progeny phages could have attached to nonspecific particles and (c) the progeny phages may could have been degraded by UV radiation (Kimura et al. 2012). Thus, phages produced in the water column by the host are suggested to be higher than what we enumerated from the free-viral fraction. Another reason for the difference between

the concentration of replicating phages and mature phages could be due to infected cells with replicating phages trapped on the filter during processing of samples. Another possibility for underestimating the cyanophage concentration is due to the diurnal pattern of infection and production of cyanophages. Cyanophages were shown to have a diurnal pattern (24 hrs) in cyanophage abundance, the most abundance of phage titers were found at 0100 h (Clokier et al. 2006), 1800 h and 2100 h (Kimura et al. 2012). Thus, it is likely that sampling time plays an important role in determining phage abundance in an aquatic system. Hence, this temporal variation should be taken into consideration for any routine sampling program.

4.3.2 Impact of environmental conditions on cyanophage abundance

Analysis of the combined data set (n =39) showed that the g91 gene abundance either in the free- phage fraction or host- cell fraction were positively correlated with concentrations of *mcyE* gene and total *Microcystis* (Table 4.5). However, g91 genes in the host- cell fraction had relatively stronger correlation with the potential host cells. This is probably due to losses in the free- phage titer, e.g. UV radiation. The losses in the free-phage due to UV radiation is supported by the positive correlation observed between g91 gene copy number and chlorophyll- a/ turbidity. In where, chlorophyll- a/ turbidity may play role as shield to protect cyanophages from UV degradation.

Anabaena sp. and TN were reported to have a positive correlation with total *Microcystis* and toxigenic *Microcystis* abundance (Te and Gin, 2011). This study showed cyanophage abundance (g91 gene copy number) has no correlation with *Anabaena* sp. but did have a positive correlation with TN. The absence of correlation between g91 gene copy number and *Anabaena* is not surprising, since the g91 gene is encoded by cyanophages which infect *Microcystis aeruginosa*. Nutrients are important in cyanobacterial population dynamics, and high nutrient supply especially TN, favours *Microcystis* sp., because *Microcystis* sp. are not nitrogen fixing cyanobacteria. Thus, it is suggested that TN influences the

production of *Microcystis* sp in Kranji Reservoir and this in turn, affects the cyanophage titers. Positive correlation between TP and g91 gene copy number in the host- cell fraction was also found. Literature reports show that *Synechococcus* cyanophages extend the latent period and decrease the burst size when phosphate is depleted (Wilson et al., 1996). Longer latent period and smaller burst size directly affect the phage replication number (i.e. cyanophages in the host cell fraction). Thus, it is possible that the replication number of *Microcystis aeruginosa*- phages in Kranji Reservoir is also influenced by TP. Earlier studies have shown that AN-15 (cyanophages which infect *Anacystis* and *Nostoc*) required Ca^{2+} to maintain structural stability and permit infectivity (Mole et al., 1997). In this study, a positive correlation between Ca^{2+} and g91 gene abundance was observed in both fractions, suggesting the requirement of Ca^{2+} in maintaining infection ability and structural stability of the *Microcystis aeruginosa*- phages.

4.3.3 Temporal trends in cyanophages infectious to *Microcystis* (Ma-LMM01- type phage) and *Microcystis* sp.

Cyanophages are reported to be able to control cyanobacterial blooms and diversity. However, cyanophages are also known to be affected by their host density as well. For example, minimum threshold host densities are required for efficient viral propagation. Manage et al. (1999) demonstrated the need for threshold host densities, e.g. there was no lysis of *M. aeruginosa* by cyanophages when *M. aeruginosa* cells were less than $1 \times 10^5 \text{ ml}^{-1}$ in Furuike Pond, Japan. In our study, when the *Microcystis* concentration was at 1.4×10^3 16S rRNA ml^{-1} , Cyanophages (g91 gene copy number) were not detected in the free-viral fraction. However, cyanophages infectious to *Microcystis* were detected when the *Microcystis* concentration at 7.2×10^3 16S rRNA ml^{-1} . Therefore, a minimum threshold density of *Microcystis* suggested for lysis to occur in our local reservoir, at about 7.2×10^3 16S rRNA ml^{-1} (or 3.6×10^3 *Microcystis* cells, where two copies of *Microcystis* 16S rRNA genes are equivalent to one *Microcystis* cell). The minimum host threshold density in this study is lower compare to Furuike Pond, Japan. Waterbury and

Valois (1993) reported that the minimum host threshold density is not universal and that differences in minimum threshold density could be due to difference in time for phage adsorption and host growth rates (Waterbury and Valois 1993). The differences also could be due to sensitivity of detection methods. In this study real-time PCR was used, which has been shown to be more sensitive compared to plaque assay. Thus, the presence of cyanophages at low concentrations can be detected with qPCR but not with plaque assay (used by Manage et. al., 1999).

In this study, occasionally, the cyanophage abundance increased sharply when *Microcystis* spp. were observed. This result is in contrast with Manage et al. (1999) and Yoshida et al. (2008) who observed that occasional sharp increases in cyanophage density coincided with sharp decreases in *M. aeruginosa* abundance. However, our findings are similar to Okunishi et al. (2003), Wang et al. (2011) and Kimura et al. (2012) where phage titers oscillated with host concentrations after a lag of several days (Okunishi et al. 2003) and there were a seasonal co-variation between cyanophage and host (Kuznetsov et al. ; Wang et al. 2011; Kimura et al. 2012). From the results of both the dynamics of cyanophage abundance using either the g91 gene copy number in the free- viral fraction or host- cell fraction, and also the fluctuation pattern of fraction of infected cells, two main hypotheses were concur: (1) this study agree with the suggestion by Manage et al. (2001) that cyanophages can substantially reduce the bloom. In Te (2012) study, *Microsystis* sp. in Kranji Reservoir generally fluctuated between 10^5 and 10^6 gene copy numbers ml^{-1} . However, on three occasions where *Microsystis* numbers exceeded 10^7 gene copy numbers ml^{-1} , in the subsequent month, the *Microcsystis* concentration decreased to 10^5 or 10^6 gene copy numbers ml^{-1} ; (2) this study agree with Takashima et al. (2007) and Yoshida et al. (2010), that cyanophages influence the replacement of phage sensitive populations by phage- insensitive populations (i.e. changes in intra-species level of host) with no quantitative impact on the entire host population. In our study, we observed that the fraction of infected cells was always in a dynamic form, with no obvious changes in the host cells densities, suggesting that the percentage of sensitive populations changes from month to month, however,

the total population remain the same unless bloom happen ($> 10^7$ gene copy numbers ml^{-1}).

4.4 CONCLUSION

Cyanophages infectious to *A. cylindrica*, *A. circinalis*, *Microcystis*, and *Synechococcus* were observed in Kranji Reservoir throughout the sampling period (from August 2008 to August 2009). In general, at least one type of cyanophage could be detected in each sample. Phages which infect *Anabaena cylindrica* showed a strong influence by the monsoon season, phages were absent during the NE Monsoon.

The temporal and spatial dynamics of cyanophages infectious to *Microcystis* (g91 gene) was also observed over the sampling period. The g91 gene concentrations ranging from undetectable to 4.73×10^2 gene copy ml^{-1} in free-phage fraction; while the g91 gene concentrations in bacterial- cell fraction ranged from below detection limit to 5.81×10^4 gene copy ml^{-1} . The copy numbers of phage g91 gene in the free- phage fraction were 2 to 4 orders of magnitude lower than g91 gene in host cell fractions. The occurrence of cyanophage infectious to *Microcystis* was affected by the threshold of minimum densities of the host cell, i.e. $3.6 \times 10^3 \text{ ml}^{-1}$ *Microcystis* cells. In addition, cyanophage abundance was affected by host cell numbers, i.e. a positive correlation existed between g91 gene abundance and *Microcystis* spp.. Environmental parameters, such as chlorophyll- a and turbidity, influenced the g91 gene copy number in the free- phage fraction in a positive way, suggesting that free- phages were protected by turbidity and phytoplankton from UV degradation. This study is the first *in situ* study to show the relationship between Ca^{2+} and cyanophage abundance. The results demonstrated a positive correlation between Ca^{2+} and cyanophage abundance, which is consistent with the laboratory studies done elsewhere. This study also suggests that cyanophage infectious to *Microcystis* play an important role in changing the intra-species community of *Microcystis* sp. in Kranji Reservoir.

CHAPTER 5

Population dynamics of Cyanomyovirus in Kranji Reservoir

5.1 INTRODUCTION

The discovery of an abundance of viruses in aquatic systems since the 1990s re-initiated investigation into the ecological roles of viruses (Fuhrman 1999; Wilhelm and Suttle 1999). These re-initiated investigations have changed the traditional conceptual understanding of the functioning and regulation of aquatic ecosystems (Middelboe et al. 2008) with the realization of viruses as important players in aquatic ecosystems. To better understand the diversity and distribution of viruses in the aquatic environment, culture-independent approaches and molecular techniques have been chosen. Culture approaches requires the maintenance and growth of the host organism (Short et al. 2010) for viruses to infect. However, to date, 95% of bacteria reported cannot be cultivated (Clokie et al. 2011). In contrast, molecular techniques are allowing researchers to characterize complex viral consortia directly (Short et al. 2010).

The gene encoding the vertex portal protein (g20) in the myoviridae family has been exploited to examine the diversity of natural cyanophage communities (McDaniel et al. 2006). A pair of family- specific gene primers (CPS1 and CPS8) have been developed by Zhong et al. (2002). These primers were used to study the diversity of cyanophages under the *Myoviridae* family (the term, cyanomyoviruses will be used for the sake of convenience). No cyanophages from the *Podoviridae* or *Siphoviridae* families and other bacteriophages can be amplified by these primers. Another efficiency and specificity test of the CPS1/CPS8 primers was carried out by Sullivan et al. (2008). The study revealed that the CPS1/CPS8 primers could only amplify g20 genes from cyanomyoviruses infectious to *Synechococcus* and *Prochlorococcus*, but were unable to amplify g20 genes from 12 non-cyanophages and cyanophages from the *Podoviridae* family. However, not all cyanomyoviruses

infectious to *Synechococcus* and *Prochlorococcus* were able to be detected by CPS1 and CPS8 primers (Sullivan et al. 2008).

Genetically similar g20 clones were detected at distant sites with different nutrient status, temperature and salinity (Short and Suttle 2005; Jameson et al. 2011). For example, clones from the Gulf of Mexico (marine environment, 110 m depth) share 99 % identity with g20 clones from Lake Constance (freshwater environment, 3 m depth) (Short and Suttle 2005). Thus, this suggests that some g20 clones are widely distributed with no apparent geographical segregation (Jamerson et al., 2011) or that the horizontal gene exchanges occur among cyanophage communities (Short and Suttle, 2005). However, Sullivan et al. (2008) suggested that the g20 clones were distributed according to geographic segregation. For example, population structure in an estuary and open ocean were different from each other and clonal diversity changed from surface to the deep chlorophyll maximum (DCM) layer, as shown by Zhong et al. (2002). Thus, similar g20 clones can be obtained from various environments but g20 diversity distribution patterns (such as phylogenetic trees) from different environments are different and unique clades can be obtained from each individual location.

The temporal variation of cyanomyoviruses had been demonstrated from various environments e.g. Lake Bourget, Chesapeake Bay, the Red Sea, Norwegian coastal water, paddy flood water and paddy field soils (Dorigo et al. 2004; Wang and Chen 2004; Mühling et al. 2005; Sandaa and Larsen 2006; Wang et al. 2010; Wang et al. 2011). For example, Mühling et al. (2005) demonstrated that cyanomyovirus population diversity was greater during the spring and winter than summer and autumn, and co-varied with *Synechococcus* diversity. In addition, when *Synechococcus* and phage titer were at their maxima (July 1999), they found that the lowest numbers of genotypes of *Synechococcus* and cyanomyoviruses communities were obtained. Thus, previous studies did showed both spatial and temporal variation in g20 clone distribution.

Singapore is a country with a tropical rainforest climate, divided into four periods according to the average prevailing wind direction: Northeast Monsoon (December to early March), Inter Monsoon Period (late March to May), Southwest Monsoon (June to September) and second Inter Monsoon Period (October to November). Kranji Reservoir is one of the drinking-water reservoirs in Singapore and is situated in the northwestern part of Singapore. Kranji Reservoir is part of the Kranji catchment, which consists of various different land-uses including residential areas, agriculture, forests and reserve area (Granger 2010). Chua et al. (2009) demonstrated that surface runoff loadings from Kranji catchment were positively correlated with peak flow. In addition, Gin et al. (2000) suggested that heavier precipitation during the North-East monsoon affected the environmental parameters by dilution. Therefore, reservoir waters in Singapore face temporal changes due to the monsoon seasons. Kranji Reservoir has been categorized as a lake between eutrophic and hypereutrophic status (Te and Gin 2011). Diverse cyanobacteria populations have been detected in the past (Aug 2008 to Feb 2009), and species included *Microcystis*, *Anabaena* and *Synechococcus* (Te 2012).

The objective of this study is to understand the diversity and dynamics of cyanomyoviruses in Kranji Reservoir. Cyanomyovirus diversity was investigated by PCR-cloning-sequencing approach with PCR primers targeting the g20 gene which is myoviral-specific. This study showed the first distribution of g20 clones in tropical reservoir water. Additionally, this study investigated the effect of monsoon seasons on the g20 clones distribution and determined the differences in genetic makeup between cyanomyoviruses populations in a Singapore reservoir and other aquatic environments.

5.2 MATERIALS AND METHODS

5.2.1 Sampling Site and Field Sampling

Kranji Reservoir formed by damming mouth of the Kranji estuary and has a water surface area about 2.8 km². It is mainly supplied by three tributaries in the northern part of the reservoir: water flows from Tengah River, Kangkar River and Peng Siang River. Water flow from Pang Sua River was diverted into Kranji Reservoir at the end of 2005 as the forth tributary to Kranji Reservoir at the southern end of the reservoir (Chua et al. 2009). Three sampling stations were chosen, i.e. KR1 (1°24'49.640"N; 103°43'49.247"E), KR3 (1°25'48.508"N; 103°44'41.399"E) and Peng Siang (1°23'35.495"N; 103°44'05.364"E). These three sampling locations represent different hydrological conditions. Peng Siang station is located in the middle of the Peng Siang tributary; KR1 is located at the confluence of the three northern tributaries; and KR3 is located at the end of the reservoir, close to the Straits of Johor and Kranji Reservoir Park. In addition, these three stations have different depths, with the maximum depth of Peng Siang station at only 1m depth while the deepest point is located at KR3 (Gin and Gopalakrishnan 2010).

Global Positioning System (GPS) was used to locate the selected site. Around 12 liters of water were collected monthly from Kraji Reservoir between August 2008 and February 2009 to investigate the genetic diversity and population dynamics of cyanophage communities. Another 1L of surface water from KR1, KR3 and PS were collected for the purpose of water quality parameter measurements, kept in an ice box (~ 4 °C) and transported to the laboratory. Sampling time, site observation (e.g, weather, visual color of the surface water and scum appearance) and in-situ measurements were also conducted and recorded on site. In- situ measurements included: water temperature, dissolved oxygen (DO), salinity, conductivity, secchi depth and total dissolved solid (TDS). A YSI meter (EC300) was used to measure water temperature, salinity and TDS and a YSI model

52 (YSI probe 5739) was used to measure the dissolved oxygen concentration. Secchi depth or water transparency was determined with a Secchi disc.

5.2.2 Concentration of Viral Communities and DNA Extraction.

Twelve liters of surface water were collected with autoclaved, sample-rinsed carboys and transported to the laboratory immediately. The samples were then stored at 4 °C before processing. All samples were filtered through 0.2 µm within 24 hrs and subsequently concentrated with an ultrafiltration system. Briefly, 12 liters of samples were pre-filtered using depth filter system (Pall Corporation) with 50 or 20 µm pore size low binding protein membrane (polypropylene) to remove particulate matter, phytoplankton and zooplankton. Subsequently, the filtrate was filtered through a 0.2 µm membrane cassette by using tangential flow filtration (TFF) (Pall Corporation) to remove most of the bacteria. After undergoing pre-filtering, samples were stored in a 4 °C cool room. The viruses in the filtrate of about ten liters of sample were concentrated to a final volume ranging from 250 to 500 ml using tangential flow filtration (TFF) with a 30 kDA membrane cassette (Pall Corporation). Secondary concentration was performed using an Amicon Ultra centrifugal filter device (Milipore). Briefly, the TFF- concentrated sample was loaded to the filter device (10 kDA) and centrifuged at 5,000 x g for approximately 10 minutes according to manufacturer's instruction. The final volume of each concentrated water sample was approximately 4 ml from 200 ml for DNA extraction purposes. Concentrated samples after the second concentration were stored in the dark at 4 °C until used. These samples were believed to contain virus like particles (VLPs) ready for DNA extraction.

Viral DNA was extracted from 200 µl of concentrated sample by using the DNA extraction kit (Qiagen, QIAamp DNA Mini Kit) according to manufacturer's instructions. Viral DNA was eluted in 60 µl of DNase-, RNase- free buffer and stored at -20 °C until the molecular work was performed.

5.2.3 Polymerase Chain Reaction (PCR)

PCR amplification was performed with CPS1/CPS8 primers (Zhong et al. 2002) to detect the g20 gene. Amplification reactions were carried out in 50µl volumes containing 1x Taq polymerase buffer (Promega), 4 mM MgCl₂, 0.2 mM of deoxynucleosidetriphosphate (dNTP) mix, 0.5 µM of each primer, 2.5 units Taq polymerase with hot start (Promega) and 5 µl template DNA. PCR amplification was carried out with a Mastercycler®pro thermal cycler from Eppendorf and performed with the following cycle profile: initial denaturation at 94 °C for 3 minutes, 36 cycles of denaturation at 94 °C for 45 s, annealing at 36 °C for 45 s, extension at 72 °C for 1 min, and a final extension at 72 °C for 5 min.

Final PCR products were run on a 1.5 % agarose gel in 1x Tris-Borate-EDTA stained with ethidium bromide for 1 hour at 90 volts. The gel was visualized under UV trans-illumination and photographed to confirm the target amplicon. The correct base pair for the CPS1/CPS8 amplicon is ca. 592 bp.

5.2.4 Cloning

The PCR amplicons from CPS1/CPS8 amplification were purified using Wizard® SV Gel and PCR clean up system (Promega, Corporation, Madison, WI). Purified PCR products were subsequently cloned into the pGEM-T Vector System II (Promega, Corporation, Madison, WI) and the construct was transformed into JM109 competent cells according to manufacturer's instructions. At least eight clones were randomly picked from the JM109 cell's plate and the correct target fragments were confirmed by using PCR. Successful transformants were magnified overnight in LB broth with ampicillin for plasmid extraction. Plasmid DNA was extracted from the competent cells by using Wizard® SV Minipreps DNA Purification System (Promega, Corporation, Madison, WI). The extracted plasmid DNA was then sent for sequencing. All sequencing services were provided by AITbiotec Company.

5.2.5 Phylogenetic analysis

All nucleotide sequences were edited and aligned with the BioEdit sequence alignment editor (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). After editing, sequences were compared to other reference sequences available in the GenBank database by using BLAST software. Subsequently, all sequences from this study together with some previously obtained cyanomyovirus isolates' g20 gene sequences and uncultured g20 clone sequences from other environments were aligned with ClustalW Multiple Alignment and saved in FAS format. A phylogenetic tree was constructed with MEGA 5 (Tamura et al. 2011) by using the aligned sequences. The phylogenetic tree was drawn using the neighbor-joining method with p-distance and 1000 bootstrap replication. The g20 sequences from this study were deposited in the GenBank database. The GenBank accession numbers for the sequences are KC485882 - KC485966.

The Chao-1 index and a rarefaction curve were obtained for monsoons using PAST software (<http://folk.uio.no/ohammer/past>).

5.2.6 Statistical analysis

A Principal Component Analysis (PCA) was carried out using PASW 18 software package (SPSS Inc.). PCA analysis was used to compare samples collected from different sampling locations and different sampling period. Five environmental variables (i.e. TN, TP, Chl, Turbidity and TSS) were chosen in this PCA analysis. These variables are significant affected cyanobacteria concentrations in Kranji Reservoir (Te 2012).

5.3 RESULTS

5.3.1 Analysis of Nucleotide and Amino Acid g20 Sequences

In total, 88 g20 clones were sequenced in this study and 86 clones were sequenced as g20 fragments (Appendix 5A) with 33 clones from KR1, 27 clones from KR3 and 26 clones from PS. The remaining two clones' sequences showed 80 % similarity to environmental clones, but both of these sequences encoded one stop codon, which were absent in the other sequences. Therefore, these two clones were taken out from the phylogenetic analysis and were not considered as the g20 fragment. As a result, the g20 recovery efficiency was 97.7 % (86/88).

Nucleotide sequence analysis revealed seven pairs of identical sequences and one set with three sequences identical at nucleotide level (100%). All pairs of identical nucleotide sequences originated from the same sample, such as KRA1008M1 and KRA1008M2 which were 100% identical in their sequences and were from KR1 on 21 October 2008. The 3 clones with identical nucleotide sequences were obtained from the same sampling date (16 January 2009) but from three different locations. Amino acid sequence analysis revealed five pairs of identical amino acid sequence. Only one of the five pairs was from the same sample, where the rest were from different sampling dates, locations or both.

Sequence analyses of a total of 86 different g20 segments from three sampling stations with 21 viral DNA samples across the Kranji Reservoir revealed that within the examined gene fragment, the most variable region consisted of an insertion/deletion site, which varied between sequences by up to four amino acids. The lengths of the g20 fragment varied among clones (between the primers): 546 bp for 64 clones (74.5 %), 549 bp for 7 clones (8 %), 552 bp for 14 clones (16.3 %) and 554 bp for 1 clone. The identity within the clones in this study at the amino acid level ranged from 50 % to 99 %. 546 bp is the normal g20 fragment lengths. Note that g20 fragments with 549 bp were identified from g20 clones from paddy

floodwater and paddy field soils (Wang et al. 2010; Wang et al. 2011), while 552 bp were reported only from paddy soil samples.

The BLAST search within NCBI showed that all our sequences at amino acid level were under a putative conserved domain called Phage_T4_Gp20_Superfamily. Table 5.1 shows a summary of the closest relatives at the amino acid level, revealing that this study had the highest identities varying from 60 % to 96 % compared with the g20 clones obtained from other environments (as listed in the table 5.1). A total of 11 clone sequences showed the lowest identities lower than 80 %, where three were from KR1, five from KR3 and three from PS. The remaining 76 clones obtained had the highest identities greater than 80 %. The clone names were labeled based on their location, sampling date, family (myoviridae) and sample number. For example, KRA0808M1 means clone sample 1 of g20 fragment (M- *myoviridae*) from location KR1 collected on August 2008. KRB represents KR3 and KRC represents PS respectively.

Table 5.1 shows the clones from our study mostly had the highest similarity with the clones from freshwater environments (e.g., Dianchi Lake, Cultus Lake, Lake Bourget, paddy field floodwater and paddy field soil) and brackish environments (e.g., Sandusky Bay, Chesapeake Bay, Skidaway Estuarine and ballast water). Only four clones (<5 %) from this study had the highest similarity with the marine environments (i.e. Atlantic Meridional Transect, DCM of Sargasso Sea and Beaufort Sea, Arctic Ocean). The highest identity clones compared favourable with the study by Wilhelm et al., (2006), especially clones from KR3: 65.4 % of the KR3 clones had the highest similarity with the ballast water and Sandusky Bay clones obtained by Wilhelm et al., (2006). There were only two clones from KR3 which showed the highest similarity with paddy field clones. However, KR1 had 13 clones (48 %) and PS had 10 clones (38 %) with the highest similarity with paddy field clones.

Table 5.1 The closest relative of sequenced g20 (cyanomyoviruses) clones of Kranji Reservoir at the amino acid level

Clone Name	Length (amino acid)	Cluster	Closest Relative	Accession Number	Identity % (coverage) ^a	Source (Cluster) ^b	Reference
KRA080 8M1	181	δ	clone MC3	ABC4980 0	95	Laurentian Great Lake	(Wilhelm et al. 2006)
KRA080 8M2	181	δ	g20_12_56_1%_NEQ	ACR5739 1	84	Atlantic Meridional Transect	(Jameson et al. 2011)
KRA080 8M3	181	δ	clone PFW-CF6	BAG8508 6	94	Floodwater of a Japanese paddy field at Aichi, Anjo (CSP/δ-PFW2)	(Wang et al. 2010)
KRA080 8M4	183	β	clone MC38	ABC4982 3	85	Ballast sample, Burns harbor (C3)	(Wilhelm et al. 2006)
KRA080 8M5	181	β	clone d04	AAR1033 4	87	Lake Bourget, France	(Dorigo et al. 2004)
KRA090 8M1/M5	181	β	clone KuCf-Apr13-7	BAJ0747 0	87	Paddy field soil Japan: Aomori, Kuroishi (β- PFS II)	(Wang et al. 2011)
KRA090 8M2	181	β	clone: AnCf-Apr11-5	BAJ0751 3	81	Paddy field soil Japan: Aichi, Anjo(β- PFS I)	(Wang et al. 2011)
KRA090 8M3	183	β	clone OTU7	AAO1320 5	81 (98%)	Chesapeake Bay (N4)	(Wang and Chen 2004)
KRA090 8M4	181	β	clone: KuCf-Apr13-7	BAJ0747 0	86	Paddy field soil Japan: Aomori, Kuroishi (β- PFS II)	(Wang et al. 2011)
KRA100 8M1/M2	183	β	clone MC38	ABC4982 3	81	Ballast sample, Burns harbor (C3)	(Wilhelm et al. 2006)
KRA100 8M3	181	β	clone: KuCf-Apr13-7	BAJ0747 0	84	Paddy field soil Japan: Aomori, Kuroishi (β- PFS II)	(Wang et al. 2011)
KRA100 8M4	181	β	clone: KuCf-Apr13-7	BAJ0747 0	87	Paddy field soil Japan: Aomori, Kuroishi (β- PFS II)	(Wang et al. 2011)

Table 5.1 The closest relative of sequenced g20 (cyanomyoviruses) clones of Kranji Reservoir at the amino acid level (continued)

II)							
KRA100 8M5/110 8M1	181	β	clone: KuCf- Apr13-7	BAJ0747 0	88	Paddy field soil Japan: Aomori, Kuroishi (β - PFS II)	(Wang et al. 2011)
KRA110 8M2	181	PFS	clone CUL02 M-11	AAW487 6	82 (99%)	Cultus Lake, BC, Canada (J)	(Short and Suttle 2005)
KRA110 8M3	181	γ	clone: PFW- CM11	BAG8510 3	91	Floodwater of a Japanese paddy field at Aichi, Anjo (γ - PFW- VI)	(Wang et al. 2011)
KRA110 8M4	181	α	clone: PFW- NoF13	BAG8506 1	96	Floodwater of a Japanese paddy field at Aichi, Anjo (α - PFW-III)	(Wang et al. 2010)
KRA110 8M5	181	PFS	clone CUL02 M-11	AAW487 6	82 (98%)	Cultus Lake, BC, Canada (J)	(Short and Suttle 2005)
KRA120 8M1	181	PFS	clone CUL02 M-11	AAW487 66	83 (98%)	Cultus Lake, BC, Canada (J)	(Short and Suttle 2005)
KRA120 8M2	183	β	clone MC38	ABC4982 3	81	Ballast sample, Burns harbor (C3)	(Wilhelm et al. 2006)
KRA120 8M3	181	ε	clone VC63_A 8	ABC4982 8	85 (98%)	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRA120 8M4	181	β	clone: PFW- CM33	BAG8512 5	86	Floodwater of a Japanese paddy field at Aichi, Anjo (β - PFW-V)	(Wang et al. 2010)
KRA010 9M1	181	ε	clone VC63_A 8	ABC4982 8	92	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRA010 9M3	181	ε	clone VC63_A 8	ABC4982 8	92	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRA010 9M4	181	β	clone DC05 g20	ACX5411 1	93	Dianci lake, Yunnan China	(Xia et al.)
KRA010 9M5	181	ε	clone VC63_A 8	ABC4982 8	92	Sandusky Bay (C2)	(Wilhelm et al. 2006)

Table 5.1 The closest relative of sequenced g20 (cyanomyoviruses) clones of Kranji Reservoir at the amino acid level (continued)

8							
KRA020 9M1	181	α	clone: PFW-NoF13	BAG8506 1	96	Floodwater of a Japanese paddy field at Aichi, Anjo (α - PFW-III)	(Wang et al. 2010)
KRA020 9M2/M3	181	ε	clone o02	AAR1029 3	76	Lake Bourget, France	(Dorigo et al. 2004)
KRA020 9M4	181	PFS	clone CUL02 M-14	AAW487 70	72 (99%)	Cultus Lake, BC, Canada (J)	(Short and Suttle 2005)
KRA020 9M5	182	β	clone MC27	ABC4981 7	95	Sandusky Bay (C3)	(Wilhelm et al. 2006)
KRB080 8M1	182	δ	clone SE5	AAK3168 1	96	Skidaway Estuarine Surface Water (I)	(Zhong et al. 2002)
KRB080 8M2/M4	181	δ	clone MC4	ABC4980 1	94	Laurentian Great Lake	(Wilhelm et al. 2006)
KRB090 8M1	181	α	clone PFW-NoF13	BAG8506 1	96	Floodwater of a Japanese paddy field at Aichi, Anjo (α - PFW-III)	(Wang et al. 2010)
KRB090 8M2	181	ε	clone VC63_A 8	ABC4982 8	84 (98%)	Sandusky Bay (C2)	(Wilhelm, Carberry et al. 2006)
KRB090 8M3	181	β	clone BES02A -4	AAW487 65	83 (99%)	Beaufort Sean, Arctic Ocean (Cluster 6)	(Short and Suttle 2005)
KRB100 8M1	183	β	clone MC38b	ABC4982 4	81	Ballast sample, Burns harbor (C3)	(Wilhelm, Carberry et al. 2006)
KRB100 8M2	183	β	clone MC38	ABC4982 3	81	Ballast sample, Burns harbor (C3)	(Wilhelm et al. 2006)
KRB100 8M3	183	β	clone OTU7	AAO1320 5	96 (98%)	Chesapeake Bay (N4)	(Wang and Chen 2004)
KRB100 8M4	183	β	clone MC38	ABC4982 3	81	Ballast sample, Burns harbor (C3)	(Wilhelm, Carberry et al. 2006)
KRB100 8M5	181	PFS	clone VC63_F 8	ABC4983 6	70	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRB110 8M1	183	PFS	clone PFW-	BAG8510 6.1	59 (99%)	paddy floodwater	(Wang et al. 2010)

Table 5.1 The closest relative of sequenced g20 (cyanomyoviruses) clones of Kranji Reservoir at the amino acid level (continued)

CM14							
KRB110 8M2	181	ε	clone VC63_A 8	ABC4982 8	93	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRB110 8M3	181	PFS	clone VC63_F 8	ABC4983 6	72	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRB110 8M4	181	-	clone SS4716	AAK3174 7.1	60 (99%)	DCM of Sargasso Sea	(Zhong et al. 2002)
KRB120 8M1	181	β	clone KuCf- Apr13-7	BAJ0747 0	88	Paddy field soil Japan: Aomori, Kuroishi (β- PFS- II)	(Wang et al. 2011)
KRB120 8M2	183	β	clone MC38	ABC4982 3	81	Ballast sample, Burns harbor (C3)	(Wilhelm et al. 2006)
KRB120 8M3	181	ε	clone VC63_A 8	ABC4982 8	93	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRB120 8M4	181	β	clone DC05	ACX5411 1	94	Dianchi Lake, Yunnan China	(Xia et al.)
KRB010 9M1	181	ε	clone VC63_A 8	ABC4982 8	85	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRB010 9M2	181	ε	clone VC63_A 8	ABC4982 8	93	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRB010 9M3	181	β	clone DC05	ACX5411 1	93	Dianchi Lake, Yunnan China	(Xia et al.)
KRB020 9M1/M5	182	β	clone MC27	ABC4981 7	95	Sandusky Bay (C3)	(Wilhelm et al. 2006)
KRB020 9M2	181	ε	clone o02	AAR1029 3	76	Lake Bourget, France	(Dorigo et al. 2004)
KRB020 9M3	181	ε	clone VC63_A 8	ABC4982 8	93	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRB020 9M4	181	β	clone VC63_B 8	ABC4982 9	92	Sandusky Bay (C2)	(Wilhelm, Carberry et al., 2006)
KRC080 8M1	182	α	clone MC10	ABC4980 7	96	Sandusky Bay Particulate (C3)	(Wilhelm et al. 2006)
KRC080 8M2	181	δ	clone MC4	ABC4980 1	94	Laurentian Great Lake	(Wilhelm et al. 2006)

Table 5.1 The closest relative of sequenced g20 (cyanomyoviruses) clones of Kranji Reservoir at the amino acid level (continued)

KRC080 8M3	181	δ	g20_44_ 68_1%	ACR5733 0	85	Atlantic Meridional Transect	(Jameson et al. 2011)
KRC080 8M4	183	β	clone OTU7	AAO1320 5	81 (98%)	Chesapeake Bay (N4)	(Wang and Chen 2004)
KRC080 8M5	182	δ	clone PFW- NoF20	BAG8506 8	98	Floodwater of a Japanese paddy field at Aichi, Anjo (CSP/δ-PFW2)	(Wang, Murase et al. 2010)
KRC090 8M1	181	ε	clone VC63_A 8	ABC4982 8	83 (98%)	Sandusky Bay (C2)	(Wilhelm et al., 2006)
KRC090 8M2	181	β	clone MC27	ABC4981 7.	82	Sandusky Bay (C3)	(Wilhelm, Carberry et al.2006)
KRC090 8M3	183	β	clone: PFW- NoF21	BAG8506 9	80	Floodwater of a Japanese paddy field at Aichi, Anjo (β)	(Wang, Murase et al. 2010)
KRC090 8M4	181	β	clone MC27	ABC4981 7	82	Sandusky Bay (C3)	(Wilhelm et al. 2006)
KRC090 8M5	181	β	clone KuCf- Apr13-7	BAJ0747 0.1	85 (99%)	Paddy field soil Japan: Aomori, Kuroishi (β- PFS- II)	(Wang, Asakawa et al., 2011)
KRC100 8M1	181	PFS	clone VC63_F 8	ABC4983 6	65 (98%)	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRC100 8M2/M4	181	PFS	clone VC63_F 8	ABC4983 6	68 (98%)	Sandusky Bay (C2)	(Wilhelm et al. 2006)
KRC100 8M3	181	α	clone PFW- CF1	BAG8508 1	86	Floodwater of a Japanese paddy field at Aichi, Anjo (α-PFW-II)	(Wang, Murase et al. 2010)
KRC100 8M5	181	β	clone KuCf- Apr13-7	BAJ0747 0	83 (99%)	Paddy field soil Japan: Aomori, Kuroishi (β- PFS- II)	(Wang, Asakawa et al., 2011)
KRC120 8M1	181	β	clone KuCf- Apr13-7	BAJ0747 0	84 (99%)	Paddy field soil Japan: Aomori, Kuroishi (β- PFS- II)	(Wang, Asakawa et al., 2011)

Table 5.1 The closest relative of sequenced g20 (cyanomyoviruses) clones of Kranji Reservoir at the amino acid level (continued)

KRC120 8M2	181	α	clone PFW- NoF13	BAG8506 1	96	Floodwater of a Japanese paddy field at Aichi, Anjo (α - PFW-III)	(Wang, Murase et al. 2010)
KRC120 8M3	184	β	clone: PFW- NoF21	BAG8506 9	83 (95%)	Floodwater of a Japanese paddy field at Aichi, Anjo (β)	(Wang, Murase et al. 2010)
KRC010 9M1	181	ε	clone VC63_A 8	ABC4982 8	93	Sandusky Bay (C2)	(Wilhelm, Carberry et al. 2006)
KRC010 9M2	181	β	clone DC05	ACX5411 1	93	Dianchi Lake, Yunnan China	(Xia et al.)
KRC010 9M3/M4	183	β	clone OTU7	AAO1320 5	80	Chesapeake Bay (N4)	(Wang and Chen 2004)
KRC020 9M1	181	α	clone PFW- NoF13	BAG8506 1	97	Floodwater of a Japanese paddy field at Aichi, Anjo (α - PFW-III)	(Wang, Murase et al. 2010)
KRC020 9M2	181	PFS	clone CUL02 M-11	AAW487 66	83	Cultus Lake, BC, Canada (J)	(Short and Suttle 2005)
KRC020 9M3	181	β	clone KuCf- Apr13-7	BAJ0747 0	84	Paddy field soil Japan: Aomori, Kuroishi (β - PFS- II)	(Wang, Asakawa et al. 2011)
KRC020 9M4	181	PFS	clone CUL02 M-11	AAW487 66	83	Cultus Lake, BC, Canada (J)	(Short and Suttle 2005)

* Cluster assigned in this study

^a Percentage of coverage between sequence of this study and previous study

^b Cluster assigned by the originated study

5.3.2 Phylogenetic Analyses

After merging clones with identical nucleotide or amino acid sequence, 73 different g20 segments were obtained and used for phylogenetic analyses in this study. Phylogenetic analyses were carried out based on alignment consisting of these 73 Kranji Reservoir g20 segments and 80 sequences from previously published studies. The addition of the g20 segments from other environmental studies was to ensure that the representative sequences included most of the g20 phylogenetic lineage identified to date. The phylogenetic tree (Figure 5.1) revealed that the g20 clones obtained from Kranji Reservoir were distributed to six major clusters (α to ϵ and PFS). This assignment corresponded to previously reported g20 clones from marine water, freshwater, ballast water, paddy floodwater and paddy field soil environments. In this study, the phylogenetic tree clustering of the g20 clones followed the previous reports, i.e Cluster α to ϵ were clustered according to Wang et al. (2010) while the PFS (paddy field soil) cluster was designated by Wang et al. (2011). One sequence, KRB1108M4, stood apart from all of these six clusters. As a result, a total of seven operational taxonomic units (OTUs) were proposed in this study.

With reference to the phylogenetic tree (Figure 5.1), the following observation were made. Cluster α , with a bootstrap of 69 %, contained seven clones (8 %) from this study, two clones from lakes (Varelis et al. ; Xia et al.), four clones from paddy floodwater (Wang et al. 2010) and two clones from Sandusky Bay (Wilhelm et al. 2006). Cluster α exclusively included clones from Kranji Reservoir and clones from only freshwater environments only. This was supported by Wang (2010), who also found that cluster α was specific to g20 gene from freshwater environments including Sandusky Bay particulates and floodwater from paddy fields.

Cluster β was a largest cluster and strongly supported (96 %): it contained 41 clones (48 %) from this study, 26 clones from freshwater environments (Xia et al. ; Dorigo et al. 2004; Wilhelm et al. 2006; Wang et al. 2010; Wang et al. 2011),

two clones from brackish waters (Wang and Chen 2004) and only one clone (BES02-24) from the marine environment (Short and Suttle 2005). Cluster β was observed for all sampling dates and hence, could be the dominant species in Kranji Reservoir. Two unique subclusters have been identified within Cluster β in this study: they are KRM-I and KRM-II. Kranji Reservoir clones (14 clones, 16.5 %) formed a weakly supporting (47 %) independent sister clade in subcluster KRM-I. This clade with another two sister clades as assigned by Wang et al., (2011), PFS-I and PFS-II, establishing a strong bootstrap (100 %) subcluster KRM-I in Cluster β . KRM-II was a strongly supported (100 %) subcluster that contained seven clones only from this study.

Cluster δ , also known as cluster CSP, was first designated by Short and Suttle (2005). Cluster CSP contained g20 sequences from cyanophage isolates which infect marine *Synechococcus* and *Prochlorococcus* (Short and Suttle 2005; Wang et al. 2010). Cluster δ in this phylogenetic tree consisted of clones from marine waters (Zhong et al. 2002; Sullivan et al. 2008; Sullivan et al. 2010; Jameson et al. 2011), freshwaters (Wilhelm et al. 2006) and paddy floodwaters (Wang et al. 2010). This cluster was strongly supported (99 %) and contained nine clones (11 %) obtained from this study (all clones from Aug 2008 samples).

Cluster γ was the smallest cluster but with strong bootstrap support (100 %). Cluster γ only contained one clone from this study and two clones (PFW- CM11 and PFW- CM12) from paddy floodwaters (Wang et al. 2010), PFW- CM11 and PFW- CM12 formed a unique subcluster, PFW- IV, in Wang et al. (2010) study.

Cluster ϵ was a weakly bootstrap-supported (40 %) cluster which accounted for 25 % of 85 clones. Cluster ϵ consisted of clones from the deep chlorophyll maximum (DCM) of the Sargasso Sea, Gulf Stream (Zhong et al. 2002), Atlantic Ocean (Jameson et al. 2011), Sandusky Bay (Wilhelm et al. 2006) and lakes (Dorigo et al. 2004). Another subcluster, consisting only of Kranji Reservoir clones, has been identified and named as KRM-III within Cluster ϵ . KRM-III is a strongly supported (99%) subcluster which consists of eight clones (9.4%) from this study.

Cluster PFS was a newly identified cluster in this study. Cluster PFS consists of two clusters, i.e. Cluster-2 and Cluster-3, which were first designated by Wang et al. (2011). Cluster PFS with very weak bootstrap support (15 %), contained 12 clones (14 %) from this study, one clone from the DCM of the Sargasso Sea (Zhong et al. 2002), an Arctic cyanobacterial mat (Short and Suttle 2005) and paddy floodwater (Wang et al. 2010), and two clones from bay water (Wilhelm et al. 2006) and lake water (Short and Suttle 2005). A subcluster within Cluster PFS consisting only of Kranji Reservoir clones has been identified, named as KRM-IV. KRM-IV was a strongly supported (96 %) subcluster.

Clone KRB1108M4 was unique and not grouped to any cluster. It was most closely related to the DCM clone from the Sargosa Sea clone (SS4716).

Figure 5.1 Neighbour-joining phylogenetic tree of g20 gene amino acid sequence showing the relationships between g20 amino acid sequences from Kranji Reservoir and other environmental sequences. Black circles indicate clones obtained in this study. The numbers in the parenthesis are the accession numbers of amino acid sequences in the NCBI web site. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Tamura et al. 2011). Bootstrap numbers less than 50 are not shown in the phylogenetic tree.

5.3.3 Spatial changes of the cyanomyoviruses community

In this study, cluster distribution of cyanomyovirus communities were showed across the three locations (KR1, KR3 and PS) in Kranji Reservoir based on the clones produced. Spatial cluster distribution in this study did not reveal differences for overall patterns of the composition of cyanomyoviral assemblages for these three stations in Kranji Reservoir (Figure 5.2).

However, there was a certain degree of different in the propotional of clones for each cluster: (1) cyanomyoviruses which belonged to the cluster ϵ were shown to be in higher proportion at KR3 (7 out of 15 clones), (2) PS had the highest propotion of Cluster α clones (4 out of 7) compared to KR1 and KR3, and (3) PS had the lowest propotion of Cluster ϵ clones (2 out of 15).

Figure 5.2 Cyanomyovirus spatial clusters distribution from the three sampling locations (KR1, KR3 and PS)

5.3.4 Temporal changes of the cyanomyoviruses community

The temporal cluster distribution of the myoviral cyanophage population is shown in Figures 5.3. The temporal cluster distribution profile of seven months exhibited dramatic changes in genetic diversity in term of the g20 genotypes distribution.

There were between three to four g20 genotypes detected in samples collected from August 2008 to February 2009, except for November 2008 and January 2009. 13 clones were sequenced from the August 2008 samples, these 13 clones which were distributed to three clusters (δ , β and α) with 9 clones falling in to Cluster δ . In other words, Cluster δ was the major cluster for August 2008. A total of 13 clones were sequenced from September 2008. These were distributed to three clusters (β , ϵ and α) with 10 clones falling in to Cluster β . 15 clones from October 2008 were distributed to three clusters (α , β and PFS). Cluster β had the

highest weight in September and October 2008 and was the major cluster for these two months.

The November 2008 sample from PS was the only sample that showed no amplification when CPS1/ CPS8 primers were used in PCR. The sample concentration was under the detection limit when CPS1/ CPS2 primers were used in qPCR (please refer to section 4.2.4). The November 2008 samples (from KR1 and KR3) experienced the most genetic diversity compared with the other 6 months. Only 9 clones were obtained from the November 2008 samples, but these clones were distributed to 5 different clusters and six genotypes, where KRB1108M4 was not found in any cluster. In contrast, only two cyanophage genotypes (clusters β and ϵ) were detected in January 2009, when 11 clones had been sequenced. The 11 clones from three stations in December 2008 were distributed to four clusters (α , β , ϵ and PFS) with seven of the clones falling in Cluster β . 14 clones from the February 2009 samples were distributed to four clusters which was similar to December 2008, but the significant fraction of Cluster β did not occur in the February 2009 samples.

The sequences belonging to Cluster δ only occurred in August 2008 and only one clone was obtained in November 2008 belonging to cluster γ . Cluster α was not present in January 2009 while cluster ϵ was absent in August 2008. Cluster α genotype was present for six months but only consisted of seven clones, except for February 2009 where two clones were detected. Cluster β was the only genotype present for all sampling dates, but spatially Cluster β were absent at KR3 in August and November 2008. Cluster β was the dominant fraction in September, October and December 2008, and February 2009 clones

The PCA was performed by using 5 variables based on 21 complete data sets. Kaiser-Meyer-Olkin Measure of Sampling Adequacy (KMO) together with Bartlett's test of Sphericity were used to check the suitability of analysis. The value of KMO was 0.728 (> 0.6) and Bartlett's Test of Sphericity was significant ($P < 0.0005$) showing that the analysis was appropriate (Norušis and SPSS 2008). Figure

5.4 shows the PCA ordination plot, where the first component (PC1=69.2%) and second component (PC2 = 17.2%) explained 86.4 % of the data variation. PC1 is mainly defined by phytoplankton biomass-related variables including Chlorophyll-a, TSS, turbidity and Secchi depth. PC2 is mainly influenced by nutrient (TN and TP).

The score plot of samples is shown in Figure 5.5, where samples were grouped into four groups. Groups 1 and 2 consisted of samples with high numbers of g20 sequences falling in cluster β and PFS (more closely related to g20 clones from paddy floodwater and paddy field soil). Group 3 consisted of samples with the g20 sequences more closely related to g20 clones from lakes and bays. Group 3 samples were mainly from the NE monsoon. Group 4 samples had the most diverse clones, with clones closely related to different environments, including bays, paddy field soil, ballast water, paddy floodwater and marine water.

Figure 5.6 shows the fraction and distribution of clusters for the three different monsoons, i.e. Southwest (SW) Monsoon, Inter Monsoon and Northeast (NE) Monsoon. The SW Monsoon in this study was represented by six samples with a total of 26 clones. The Inter Monsoon consisted of six samples over two months, with 23 clones produced from 5 samples (note that the PS November 2008 sample did not give any amplification). A total of 36 clones obtained from 9 samples over three months represented the NE Monsoon.

Cluster β was the major cluster for the three different monsoon seasons. Cluster PFS only appeared from October 2008 onwards, and was absent in the SW Monsoon Season (August and September 2008), where a total of 26 clones was investigated. Compared with the NE Monsoon, Cluster PFS was more dominant during the Inter Monsoon period.

The fraction of Cluster ϵ increased significantly in the NE Monsoon. 12 clones from the NE Monsoon fell in this cluster, while only two clones and one clone came from the SW Monsoon and Inter Monsoon respectively. Note that subcluster KRM-III in Cluster ϵ only consisted of clones from the NE Monsoon.

Figure 5.7 shows the rarefaction curves for three monsoon seasons. The SW and NE monsoon rarefaction curves reach an asymptote. However, the Inter-monsoon rarefaction curve did not reach an asymptote, thus suggesting that greater diversity of g20 sequences was present for the Inter-monsoon. The rarefaction curves result was supported by the Chao 1 index. Chao 1 index is a non-parametric richness estimator, which can be used to determine the total richness (Santos and Soares 2011). Chao 1 indices (mean) for SW, NE and Inter- monsoons were 4, 4 and 5.5 respectively. Clones for SW and NE monsoon were distributed to four clusters (taxa) as shown at Figure 5.6. For the Inter- monsoon, clones obtained from this study were distributed to five clusters, and less than the 5.5 Chao 1 index, thus suggesting that greater diversity is present during the Inter- monsoon.

Figure 5.3 Temporal clusters distribution of cyanomyoviruses

Figure 5.4 PCA ordination plot of variables for chlorophyll-a (Chl), total suspended solid (TSS), turbidity, Secchi depth, total nitrogen (TN) and total phosphorus (TP). The first component (PC1) explained 69.2% of the data variance and is mainly defined by Chl-a, TSS, turbidity and Secchi depth. The second component (PC2) is mainly defined by TN and TP and explained 17.2% of the data variance

Figure 5.5 Samples' factor analysis scores on the PCA plot. PS November 2008 (with score PC1:4.21467; PC2: 1.27372) was not showed in the plot, this is due to this extreme case will make the rest of plots huddled together, thus difficult to group the plots. Samples KR1 (opened diamond), KR3 (opened square) and PS (opened circle) are grouped in four groups. Group 1 and Group 2 consist of samples more closely related to g20 clones from paddy floodwater and paddy field soil. Group 3 consist of samples with the g20 sequences more closely related to g20 clones from lakes and bays. The g20 sequences in Group 4's samples with the most diverse clones, these clones closely related to various environments, including clone from bays, paddy field soil, ballast water, paddy floodwater and marine water.

Figure 5.6 Cluster distribution of cyanomyoviruses with different monsoon seasons A) Southwest Monsoon (August 2008 and September 08); B) Inter Monsoon (October 2008 and November 2008) and C) Northeast Monsoon (December 2008 to February 2009)

Figure 5.7 Rarefaction curves of cyanomyoviruses for different monsoon seasons.

5.4 DISCUSSION

Despite the important ecological roles played by cyanophages, data regarding the occurrence of cyanophage in environmental water systems in Southeast Asia countries such as Singapore is lacking. In the present study, the prevalence of cyanophages in an eutrophic local reservoir was studied. Local environmental isolates were obtained and compared with isolates from other environments using phylogenetic analysis. The temporal and spatial variations in cluster distribution were obtained and analyzed according to changes in environmental parameters.

5.4.1 Phylogenetic diversity of g20 gene sequences in Kranji Reservoir

The cyanophage community in Kranji Reservoir revealed a large degree of diversity: Kranji Reservoir clones identity at the amino acid level ranged from 50 % to 90 %. Clones from this study showed the highest identities of g20 sequences varying from 60 % to 96 % compared with the clones from different environments, including oceans, lakes, bays, paddy floodwater as well as paddy field soil (Table 5.1). *Synechococcus* communities in Chesapeake Bay have been found to be more diverse than those from coastal and open- ocean waters (Wang and Chen 2004). A previous study on phytoplankton structure in Singapore showed different *Synechococcus* communities found in the Singapore Strait and Johor Strait (Gin et al. 2006) where the Johor Strait was more eutrophic and had high nutrient levels than the Singapore Strait. Different *Synechococcus* strains were present in the Kranji Reservoir and not just the marine type *Synechococcus*. Theses clones (a total of 9 *Synechococcus* clones) included *Synechococcus* belonging to clones collected from hydroelectric power plant reservoir, lakes, reservoir and Chesapeake Bay (Appendix 5.B). Rich genotype of cyanobacteria in Kranji Reservoir was suggested contribute the large degree of diversity of cyanophage community occurred.

5.4.2 Spatial and temporal changes in the cyanomyoviruses community

CPS1/CPS8 primers have been successful in detecting *Synechococcus* and *Prochlorococcus* phages by detecting the presence of the g20 gene (Zhong et al. 2002; Sullivan et al. 2008). However, to date, no study has shown that these primers are able to detect any *Microcystis* and *Anabaena* phages. Deng and Hayes (2008) successfully amplified the g20 gene of phage P-Z1 (a cyanophage infecting *Planktothrix rubescens*) from among 24 phages of filamentous cyanobacteria. However, CPS1/CPS2 was used in the amplification and not CPS1/CPS8 (Deng and Hayes 2008). Thus, CPS1/CPS8 amplicons are believed to originate from the g20 gene of cyanophages which only infect *Prochlorococcus* or *Synechococcus*.

Prochlorococcus is generally restricted to marine environments (Wang et al. 2010) whereas *Synechococcus* are found in both freshwater and marine water. Based on the size structure of phytoplankton in the coastal waters of Singapore, it was reported that most of the cells in the relevant size range were *Synechococcus*, with no evidence of the smaller *Prochlorococcus* (Gin et al. 2000). However, in the case of freshwater, we did find a clone of *Prochlorococcus* (from the sequencing results given by Te Shu Harn, see Appendix 5.B) in the February 2009 sample from Kranji Reservoir. Cyanobacterial genotypes from the Kranji Reservoir were studied by using ribosomal ITS DNA sequences (Te, 2012). The samples were collected in parallel with this study and hence the cyanobacterial genotypes can be linked with the current cyanomyoviruses study. However, the *Synechococcus* spp. and *Prochlorococcus* spp. diversity study was limited as the diversity of ITS sequences was only examined for samples from August 2008, November 2008 and February 2009 (between August 2008 and February 2009). In addition, the study targeted the ribosomal ITS gene of whole cyanobacteria communities and only clones with different base pairs (from the result of gel electrophoresis) were sequenced for the phylogenetic analysis. From the cyanobacterial genotype study, only one *Prochlorococcus* clone was detected from KR3 on the February 2009 sample and no *Prochlorococcus* clone was detected on either August or November 2008 samples

(Appendix 5.B). Therefore, *Synechococcus* seems to be the likely host for the cyanomyovirus clones from August to November 2008.

The g20 gene was successfully amplified using CPS1/CPS8 primers from all samples except for the November 2008 sample from PS. At this particular sampling site and date, the cyanobacterial bloom (with obvious scum), had the highest chlorophyll- a concentration (1584 µg/L) and was an extreme case in the PCA. In addition, *Microcystis* concentration (1.08×10^8 gene copies ml⁻¹) was about 2 log higher than other months (Te 2012). This suggests that a cyanobacteria bloom had occurred and was dominated by *Microcystis* species. This could have led to a low density of *Synechococcus* and *Prochlorococcus* at this station. Minimum host densities for cyanophage replication have been studied (Waterbury and Valois 1993; Suttle and Chan 1994; Okunishi et al. 2003). These studies showed that cyanophages replication was affected by thresholds of minimum host densities. On November 2008, the density of *Synechococcus* at PS station was likely to be lower than the minimum threshold bacterial density, thus resulting in low numbers of lytic virus which were not easy to detect.

Note that samples from August 2008 in cluster δ/CSP were closely related to known marine cyanomyovirus isolates. In this study, only 9 out of 86 g20 clones belonged to cluster CSP, indicating that marine type *Synechococcus* and *Prochlorococcus* were generally not predominant in Kranji Reservoir except for August 2008 where they were dominant. In Figure 5.3 it was shown that the Cluster β fraction increased sharply from September 2008, with the development of a unique subcluster, KRM-I. This subcluster was found to be most closely related to the clones in PFS-I. Wang et al. (2011) had suggested that Cluster β is the main cluster of the g20 gene from paddy field soils with PFS-I as a unique group. These changes in cluster distribution suggest that the *Synechococcus* community from Kranji Reservoir was more closely related to the *Synechococcus* community from paddy field soil. The PCA score plot (Figure 5.5) also showed that the August 2008 and September 2008 samples were clustered into different groups. This temporal

difference may shed some clues on the role of cyanophages as indicators of shifts in the host community.

5.4.3 Phages and host interaction

In previous studies, it was shown that when *Synechococcus* and phage titers were at their maxima (July 1999), the lowest numbers of genotypes of *Synechococcus* and cyanomyoviruses communities were obtained (Mühling et al., 2005). Mühling et al. (2005) suggested that the dominance of only one genotype of *Synechococcus* made the whole *Synechococcus* population particularly vulnerable to phage infection. This study agree with Mühling et al.'s (2005) suggestion, between August 2008 and February 2009 the g20 gene concentration for KR1 and KR3 showed a maximum in October 2008 (Figure 4.7) and the proportion of the dominant cluster was 100 % and 80 % respectively. PS showed a maximum concentration in September 2008 and the highest proportion of the dominant cluster (80 %) in the same month as well. Thus, phages present in Kranji Reservoir that were able to infect the vulnerable dominant host could replicate effectively at a high contact rate, resulting in the high concentrations but low numbers of genotypes pf cyanomyoviruses.

The concept of “killing the winner” was proposed by Thingstad and Lignell (1997) through a theoretical model (Thingstad and Lignell 1997). “Killing the winner” means the process by which lytic viruses lyse the most successful population (fast growing population) and allow for the co-existence of less competitive populations and thus, sustaining bacterial diversity (Weinbauer and Rassoulzadegan 2004; Matteson et al. 2013). In November 2008, KR1 and KR3 showed a significant fall in the abundance of g20 genes and the fraction of Cluster β . Simultaneously, nine clones from KR1 and KR3 were classified to five different clusters, revealing a more diverse cyanomyovirus community. It is interesting to note that the PCA score plot shows little difference in water conditions from these two months. It is likely that the “killing the winner” phenomenon probably occurred

between October and November at these locations. Thus, the dominant species or fast growing species was lysed and a new *Synechococcus* community was formed. Mühling et al. (2005) showed that cyanophage infection plays an important role in the succession of *Synechococcus* genotypes. The cyanomyovirus diversity results obtained from this study are consistent with Mühling et al. (2005) observations, demonstrating that cyanophage infection can potentially control the cyanobacterial community in Kranji Reservoir.

Do cyanophages respond to changes in *Synechococcus* assemblages or does phage infection control the *Synechococcus* assemblage? This study shows that fluctuations in water quality affect the growth of the *Synechococcus* community (during August 2008 and September 2008) and subsequently, the genotype of cyanophages. With the emergence of dominant genotypes, cyanophages could be an important factor which controls the diversity of *Synechococcus* populations through “killing the winner” (during October 2008 and November 2008). As the host community diversifies, this in turn, leads to an increase in diversity of the cyanophages. Thus, cyanophage diversity could potentially be used as an indicator of the shift in strains of specific species of cyanobacteria.

In order to deepen the understanding of how cyanophages respond to changes in host population dynamics and vice versa, a comprehensive sampling to capture the development and collapse of relevant genotypes will be required. We suggest that instead of regular monthly sampling, sampling should be conducted intensively during three main periods of the bloom cycle, i.e. initial peaking of the bloom, during the bloom event and the die-off period. This will enable a better understanding of the short- term dynamics of phage and host diversity.

5.5 CONCLUSION

In this study, the viral capsid assembly protein genes (g20) from a tropical reservoir were amplified with PCR using CPS1/CPS8 primers, followed by cloning-sequencing to investigate the genetic diversity of cyanophage communities. In total, 73 different g20 segments were obtained with different amino acid sequences. Through phylogenetic analysis, it was found that these 73 segments were distributed to six major clusters (α to ϵ and PFS), with four unique subclusters identified as KRM-I, KRM-II, KRM-III and KRM-IV. The cyanophage community in Kranji Reservoir revealed a large degree of diversity because clones obtained from this study showed similarity to clones from many different environments, including oceans, lakes, bays, paddy floodwater as well as the clones from paddy field soil. However, Kranji Reservoir sequences were generally found to be more closely related to g20 sequences of freshwaters and brackish waters than those from marine environments.

The major cluster for most of the months (5/7) was Cluster β . The sequences belonging to Cluster δ only occurred in August 2008 and was the major cluster at this time, while the major cluster in November 2008 was Cluster PFS. The rarefaction curves and Chao 1 indices from this study showed that greater diversity of the cyanomyovirus community occurred during the Inter- monsoon as compared to the SW and NE monsoons. A few taxa changes were observed during the seasonal change. Cluster PFS was absent during the SW Monsoon, i.e. August 2008 and September 2008. During the NE Monsoon from December 2008 to February 2009, most of the samples fell in Group 3 in the PCA score plot and the fraction of Cluster ϵ increased significantly. Note that subcluster KRM-III in Cluster ϵ only consisted of clones from the NE Monsoon.

This study showed the temporal changes of cyanomyovirus population in Kranji Reservoir. This temporal change is likely due to nutrient and biomass fluctuations in the reservoir brought about by seasonal changes (as shown by the PCA score plot) and the role of cyanophages in controlling host community

diversity. A significant fall in the abundance of g20 gene and the fraction of Cluster β , and increase in cluster distribution diversity was observed from the November 2008 samples. This study suggests that the “killing the winner” phenomenon probably occurred between October and November 2008 at Kranji Reservoir. The dominant species was lysed which left room for other cyanobacteria to grow, and subsequently, changed the phage community.

CHAPTER 6

Conclusions and Recommendations

6.1 CONCLUSIONS

This study is the first study of cyanophages in Singapore. Local cyanophages were isolated from Kranji Reservoir using two isolation methods based on an understanding of the characteristics of the host cells. During the isolation process, author observed that the cyanophage isolates have the ability to affect host physiology. For example, *A. cylindrica* experienced changes in aggregation and algal mat formation when inoculated with the cyanophage isolates. This study indicates that cyanophage isolates from the reservoir could suppress the growth of *A. circinalis* effectively. In addition, no regrowth of *A. circinalis* culture was observed after lysis. These results suggest that two of the cyanophage isolates from this study could be potential biocontrols to regulate the development of *A. circinalis* populations in aquatic environments.

The occurrence and dynamics of cyanophages were also investigated. The presence of different types of cyanophages in Kranji Reservoir were monitored, including cyanophage infectious to *A. circinalis* and *A. cylindrica*, Ma-LMM01-type- phage (cyanophages which specifically infect *Microcystis aeruginosa* NIES298) and cyanomyoviruses encoding the g20 gene (potential hosts are *Synechococcus* and *Planktothrix rubescens*). In addition, Ma-LMM01-type- phages (by employing the g91 gene as a molecular proxy) and cyanomyoviruses (by employing the g20 gene as a molecular proxy) were quantified, and their spatial and temporal changes were monitored. The g91 DNA copy number was enumerated in two types of fractions, i.e. the host cell fraction and free-phage fraction.

The spatial and temporal changes of the cyanophage communities in Kranji Reservoir were analyzed from August 2008 to August 2009 in term of their occurrence and genotype. Site KR1 was relatively stable in the abundance of

Microcystis- infectious cyanophage (g91 gene) compared with sites KR3, and PS. Site KR3 showed a relatively higher percentage of cyanophages which infect *A. cylindrica*. In addition, site KR3 appeared to be the most seasonally (i.e. monsoon dependent) variable site, with the disappearance of cyanophages which infect *A. cylindrica* and decrease in concentration of cyanophages which infect *Microcystis* to under the detection limit during the Northeast Monsoon. Site PS was the only station which showed the presence of cyanophages infectious to *A. circinalis*.

The g91 gene and g20 gene concentrations from this study were compared with previous studies. The g91 concentrations in the free- phage fraction from this study (undetectable to 1.97×10^2 copies ml^{-1}) were comparable to those in Hirosawanoike Pond, Japan (below detection limit to 8.2×10^2 copies ml^{-1}) (Kimura et al., 2012) but lower than those in Lake Mikata, Japan (below detection limit to 1.1×10^4 gene copies ml^{-1}) (Yoshida et al., 2008). For the host cell fraction, copy numbers of the g91 gene ranged from below detection limit to 5.81×10^4 gene copies ml^{-1} which was lower than Hirosawanoike Pond, Japan (2.5×10^1 to 1.6×10^6 gene copies ml^{-1}) (Kimura et al., 2012). However, the copy numbers of g91- phage in the host cell fraction were 2 to 4 orders of magnitude higher than the free-phage fraction observed in this study as well as Kimura et al.'s (2012) study. The g20 gene concentrations in Kranji Reservoir (below detection limit to 3.1×10^3 gene copies ml^{-1}) were close to Norwegian coastal water ($< 5.0 \times 10$ to 7.2×10^3 gene copies ml^{-1}) (Sandaa and Larsen, 2006) but lower than Lake Erie (3×10^5 to 4.3×10^6 gene copies ml^{-1}) (Matteson et al., 2011).

Pearson's correlation was used to correlate cyanophage abundance (g91 DNA copy number in host cell fraction and free-phage fraction) with environmental variables. Analysis of the combined data set showed that the g91 gene, either in the free- phage fraction or host- cell fraction, positively correlated with concentrations of *mcvE* gene and total *Microcystis*. However, g91 gene in the host- cell fraction had relatively stronger correlation with the host cells. This was probably due to losses in the free- phage titer or mature tite by UV radiation. This possibility was supported by the strong positive correlation between chlorophyll- a/ turbidity and

the g91 gene in the free- phage fraction. In where, chlorophyll- a/ turbidity may play role as shed to protect cyanophages from UV degradation. Monthly co-variation between the abundance of g91 gene in the host- cell fraction and *Microcystis* host cell density was also observed. Occasionally, a dramatic increase in g91 gene in the free-phage fraction with *Microcystis* abundance was observed. The fraction of infected *Microcystis* cells was also calculated, and the peaks concentrations of g91 genes in the free viral fraction were always located in the same month or the subsequent months of the highest fraction of infected *Microcystis* cells. Fractions of infected *Microcystis* cells were observed to fluctuate on a monthly basis. This could be due to cyanophages influencing the replacement of phage sensitive populations with phage- insensitive populations.

The cyanomyovirus population dynamics in Kranji Reservoir were examined with PCR (CPS1 and CPS8 primers) - cloning - sequencing approaches followed by phylogentic analysis. A total of 86 g20 sequences were obtained from 21 viral DNA samples. These 21 viral DNA samples were collected from three stations from August 2008 to February 2009. The BLAST search within NCBI showed that all our sequences at amino acid level were under a putative conserved domain called Phage_T4_Gp20_Superfamily, i.e. our targeted conserved area. 73 different g20 fragments were obtained from the 86 clones. Clones from our study mostly had the highest similarity with clones from freshwater environments and brackish environments, with only four clones (<5%) had the highest similarity with marine environments. These 73 Kranji Reservoir g20 sequences and 80 sequences from previously published studies were used to carry out the phylogenetic analyses. A phylogenetic tree revealed that the g20 clones from Kranji Reservoir were distributed to six major clusters (α to ϵ and PFS), with four unique subclusters (KRM-I, KRM-II, KRM- III and KRM- IV) identified.

The spatial cluster distribution in this study did not reveal differences in overall patterns of the composition of cyanomyoviral assemblages for the three stations in Kranji Reservoir. However, there was a certain degree of difference in the propotional of clones for each cluster: (1) cyanomyoviruses which belonged to

the cluster ϵ were shown to be in higher proportion at KR3 (7 out of 15 clones), (2) PS had the highest proportion of Cluster α clones (4 out of 7) compared to KR1 and KR3, and (3) PS had the lowest proportion of Cluster ϵ clones (2 out of 15).

In contrast to the spatial changes the temporal cluster distribution did reveal a dramatic changing in genetic diversity over the seven months. The sequences belonging to Cluster δ only occurred in August 2008, and it was the major cluster at that time. November 2008 was the month that revealed the most diversity in terms of g20 genotypes distribution, with 9 clones obtained that were distributed to 5 different clusters. The major cluster in November 2008 was Cluster PFS. In contrast, only two clusters (cluster β and ϵ) were detected in January 2009, where 11 clones had been sequenced. Cluster β was the dominant cluster in most of the months, except August 2008, November 2008 and January 2009. Cluster PFS was absent in the Southwest (SW) Monsoon Season (August and September 2008), where a total of 26 clones were investigated. The fraction of Cluster ϵ increased significantly in the Northeast (NE) Monsoon. 12 clones (34 %) from the NE Monsoon fell in this cluster, while only two clones (8 %) and one clone (4 %) fell in Cluster ϵ from the SW Monsoon and Inter Monsoon respectively. Note that subcluster KRM-III in Cluster ϵ only consisted of clones from the NE Monsoon. It is intriguing to note that in November 2008, KR1 and KR3 showed a significant fall in the abundance of g20 genes and the fraction of Cluster β . This coincided with the occurrence of the most diverse cluster distribution. We suggest that the “killing the winner” phenomenon occurred sometime between October and November. Thus, the dominant species was lysed and left room for a new host community to develop, and subsequently changes to the phage community.

From this study, author hypothesizes that cyanophages are controlling the cyanobacteria community in Kranji Reservoir. For example, isolated cyanophages which infect *A. circinalis* showed the ability to suppress the growth of host cells, subsequently preventing the expansion of host populations. Results of *in situ* studies from Chapter 4 showed that *Microcystis* spp. infectious cyanophages potentially play role in changing of intra- species of *Microcystis* spp. in Kranji Reservoir.

Chapter 5 showed the changes in cyanomyovirus genetic diversity over the sampling period and possible links to shifts in the cyanobacterial population. In addition, results from the diversity study strongly support the hypothesis that cyanophages play an important role in controlling the cyanobacteria diversity in Kranji Reservoir.

6.2 RECOMMENDATIONS

From previous studies and this study undertaken, researchers are beginning to understand the importance of cyanophage interactions with their host and their role in ecosystems. However, there are still many unknowns to be unraveled before researchers can fully understand how these cyanophages act as natural control agents of algal blooms and contribute to the overall microbial ecology. In the following section, some recommendations are proposed to enable a better understanding of the role of cyanophages in nature and how to manipulate this natural agent to control cyanobacterial blooms.

Chapter 3 results showed that the isolated cyanophages (phages of *Anabaena circinalis* and *Anabaena cylindrica*) from Singapore reservoir could effectively control the growth of their host cyanobacteria such as *Anabaena circinalis*. However, this chapter also showed the regrowth of the host, *A. cylindrica*, just after a short period. This suggests that the role of cyanophages within different hosts is different. In the future, cyanophages which infect cyanobacteria that produce toxins or off- flavors (geosmin and MIB) (such as *Synechococcus*, *Microcystis* spp) could be isolated and characterized. Isolated cyanophages could be characterized through morphology, physiochemical characteristics, host range, growth characteristics and genome analysis. Subsequently, the characteristics of isolated cyanophages from Singapore could be compared to previously isolated cyanophages from various environments (summarized in section 2.5). Genome analysis is very important at the current stage of cyanophage research as there are only four freshwater cyanophages genomes that have been sequenced and none of

them are from cyanophages which infect *Anabaena* spp. The addition of genomes from isolated cyanophages could certainly help in the verification of previous unknown clones from environmental studies (Jameson et al. 2011). Moreover, bioinformatic tools can be developed based on the homologues structural protein and functional genes of the known cyanophage isolates. Thus, isolation and characterization of cyanophages from diverse cyanobacterial hosts from various environments are important toward a comprehensive understanding of cyanophage diversity in aquatic environments.

One of author interests is to investigate the interactions between cyanophage and host so that a better understanding of the mechanisms of control can be elucidated. From the results of the infection study, the following can be carried out using local isolates: (1) to find the optimum ratio between phage and host for a successful infection and lysis activity, (2) to understand how cyanophages affect the toxin and off- flavor production during infection through the gene expression of cyanobacteria, and (3) to evaluate the impact of different strains of cyanophages on their hosts.

Through the monthly sampling at Kranji Reservoir, the dynamics of abundance and diversity of cyanophages was observed. However, monthly samplings may miss shorter term variability and may decrease accuracy of correlation studies between phages and environmental parameters. Author suggests that instead of regular monthly sampling, intensive and detailed samplings during three main periods of the bloom cycle in a tropical reservoir are conducted to detect the relationship between phage abundance and environmental conditions. These three main periods would include the initial peaking of the bloom, during the bloom event and the die-off period. Before the detailed sampling, it is advisable to conduct diurnal sampling, to monitor the diel cycle of the cyanophages of interest. Previous studies have shown that the concentration of cyanophages experiences diel changes (Kao et al. 2005; Clokie and Mann 2006; Kimura et al. 2012). In addition, the author suggests that the replication of samples is necessary during future sampling

procedures. This will enable the temporal and spatial variation to be studied statistically.

Besides sampling time and sampling pattern, it is also important to monitor differences in the chemical composition of water, especially for secondary metabolites produced by cyanobacteria. Planktopeptine and anabaenopentin are polypeptides produced by cyanobacteria at high concentrations during a cyanobacteria bloom. This polypeptide has been found to act as a trigger agent to induce temperate cyanobacteria into a lytic cycle, resulting in the collapse of a cyanobacteria bloom (Sedmak et al. 2008). The difference in the chemical composition during the three main periods of the bloom cycle could potentially provide an insight into the elements that singularly or collectively work to initiate a bloom, and factors that lead to the collapse of the bloom due to the lytic activity of phages. Understanding the factors that launch cyanophages to attack their host may make it possible to manipulate these factors to control increasing bloom occurrences worldwide.

The correlation between the dynamics of phages and host genetic diversity is important to broaden the characterization of the viroplankton ecological role within aquatic (Chen et al. 2009). Previous studies and this study were not able to give conclusive evidence on the correlation between phage and host genetic diversity. A very important reason is that most of the studies did not conduct the examination of phage and host diversity in parallel. The only study where this was done was by Mühling et al. (2005), but this was on *Synechococcus* (host) and cyanomyoviruses. It is important to note that cyanopodoviruses are more host specific and produce more rapidly compared to cyanomyoviruses (Huang et al. 2010). In addition, strategies employed by myovirus (adaptation) and podovirus (specialization) to enhance their translation efficiencies of the viral genome when confronting different hosts are different (Limor-Waisberg et al., 2011). Thus, it is interesting to investigate which environmental conditions could affect the diversity of cyanomyoviruses and cyanopodoviruses. In the future, more comprehensive studies on the diversity of different cyanophages such as *myoviridae* vs *Podoviridae*,

together with potential hosts, cyanobacteria (16 sRNA) or individual species such as *Microcystis*, *Anabaena* and *Synechococcus* need to be investigated in parallel. Through this, we may gain greater insight into the dynamics and interactions between cyanophages and their hosts, and from there, harness the potential for controlling harmful and nuisance algal blooms.

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Appendix 4.A

Table: Real- time PCR positive controls sequences

Amino acid sequences	
g20 gene sequence	NLPKGKAEQYLRDVMIKYKNKVVDASTGEIRDD RKHMS
g91 gene sequence	TVVGAALNLVEGNSWSIRVNKYVVSVPIFS

Appendix 4.B

Table: Description of sampling points at Kranji Reservoir

Date	Weather			Site observation		
	KR1	KR3	PS	KR1	KR3	PS
8/8/2008	Sunny	NR	NR	NR	NR	Brown color
9/23/2008	Sunny	Cloudy	Cloudy	Greenish	Green water with insects (larvae etc)	Light brown
10/21/2008	Sunny	Cloudy	Sunny	Greenish	Greenish	Greenish, odor
11/20/2008	Sunny	Sunny	Cloudy	Greenish	Greenish	scum, algae bloom, greenish
12/19/2008	Cloudy	Cloudy	Cloudy	Greenish (lighter compare with PS)	Greenish	Greenish but no scum
1/16/2009	Sunny	Cloudy	Sunny	Clearer water compare to last month	Clear	No scum on top
2/19/2009	Sunny	Sunny	Sunny	Greenish	Greenish	Greenish
3/26/2009	Sunny	Sunny	Sunny	Greenish	Greenish	Greenish, blooms, dirty
4/17/2009	Sunny	Sunny	Sunny	Greenish	Not Clear	Scum, dark green
5/29/2009	Cloudy	Cloudy	Cloudy	Dark green, bloom	Bloom	Bloom
6/30/2009	Sunny	Sunny	Sunny	Scum	Brown greenish, algal break	Greenish
7/21/2009	Sunny	Cloudy	Cloudy	NR	NR	Turbid water
8/29/2009	Cloudy	Cloudy	Cloudy	No Scum	No Scum	No Scum

NR: no recording

Appendix 4.C

Table 1 Physical and chemical parameters in KR1

Parameter/ Date	8-Aug-08	23-Sep-08	21-Oct-08	20-Nov-08	19-Dec-08	16-Jan-09	19-Feb-09	26-Mar-09	17-Apr-09	29-May-09	30-Jun-09	21-Jul-09	29-Aug-09
Chlorophyll-a (µg/L)	77.67	98.87	41.94	69.21	66.48	52.95	100.11	71.13	101.91	272.42	109.16	97.04	90.13
TN (mg N/L)	0.64	2.51	0.87	0.93	1.16	1.12	1.18	1.40	1.04	2.07	2.07	1.68	1.37
TP (mg P/L)	0.06	0.07	0.07	0.09	0.07	0.09	0.10	0.04	0.03	0.06	0.06	0.06	0.03
TC (mg/L)	NA	22.38	NA	21.10	16.42	17.25	10.32	11.20	12.48	24.21	21.90	25.58	19.39
TOC (mg/L)	-	-	-	10.29	8.99	6.67	7.76	4.72	3.12	15.40	20.26	21.75	19.01
IC (mg/L)	NA	NA	34.01	10.81	7.43	10.57	2.56	6.48	9.36	8.81	1.64	3.83	0.38
Ca ²⁺ (mg/L)	18.00	19.67	22.77	17.08	16.88	18.47	18.44	18.87	22.48	22.31	20.73	19.46	21.20
Mg ²⁺ (mg/L)	2.41	2.20	2.69	1.88	2.09	2.49	2.77	2.46	2.31	2.91	2.99	2.99	3.32
TN/TP	10.34	35.81	13.12	10.56	17.29	12.54	11.68	36.31	32.18	37.39	34.82	26.80	44.63
Dissolved Oxygen (mg/ L)	5.47	6.44	4.48	5.28	6.87	7.65	6.31	8.74	8.33	6.58	6.34	6.74	6.53
Salinity (ppt)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Conductivity (µs/cm)	213.00	210.20	185.35	185.50	189.80	185.40	241.95	233.90	222.45	238.40	262.50	270.35	289.65
Turbidity (NTU)	25.05	51.60	28.40	27.85	37.65	37.00	41.35	40.65	46.70	55.35	76.40	46.35	43.70
pH	7.93	7.96	7.59	7.83	8.60	8.20	8.80	9.50	8.45	8.85	9.10	8.95	8.90
Total Dissolved Solid, TDS (g/L)	0.13	0.11	0.10	0.10	0.11	0.11	0.13	0.13	0.12	0.13	0.14	0.15	0.16
Total Suspended Solid, TSS (mg/L)	18.08	18.80	17.00	20.10	21.40	25.20	18.00	20.00	23.00	47.00	30.80	25.80	23.40
Secchi Depth (cm)	43	50	67.5	52	57	50	46.5	50	55	40	40	40	47.5

Appendix 4.C

Table 2 Physical and chemical parameters in KR3

Parameter/ Date	8-Aug-08	23-Sep-08	21-Oct-08	20-Nov-08	19-Dec-08	16-Jan-09	19-Feb-09	26-Mar-09	17-Apr-09	29-May-09	30-Jun-09	21-Jul-09	29-Aug-09
Chlorophyll-a (µg/L)	96.75	109.56	77.03	46.18	51.93	51.08	70.77	75.75	50.58	149.49	108.71	99.44	76.87
TN (mg N/L)	0.66	3.67	0.93	0.61	0.88	0.74	1.10	1.31	0.93	3.07	1.47	1.65	1.45
TP (mg P/L)	0.06	0.11	0.07	0.07	0.06	0.03	0.11	0.04	0.03	0.13	0.05	0.07	0.04
TC (mg/L)	NA	27.02	8.05	18.60	15.39	16.41	10.98	12.30	21.74	22.08	14.85	17.41	22.20
TOC (mg/L)	-	-	6.08	6.91	7.84	5.70	6.29	7.89	19.86	13.35	13.20	14.43	15.45
IC (mg/L)	NA	NA	1.97	11.69	7.54	10.71	4.69	4.41	1.88	8.73	1.65	2.98	6.76
Ca ²⁺ (mg/L)	18.97	19.54	21.94	18.41	17.90	20.93	18.36	18.75	20.68	22.77	19.91	18.43	21.41
Mg ²⁺ (mg/L)	2.49	2.15	2.73	1.98	2.16	2.62	2.76	2.41	2.26	2.85	2.84	2.89	3.30
TN/TP	11.85	33.90	14.13	8.40	15.62	27.41	9.93	33.22	27.12	24.46	28.06	23.10	36.19
Dissolved Oxygen (mg/ L)	4.82	7.12	5.64	4.10	6.23	6.29	6.62	9.29	7.53	5.68	4.30	7.54	5.30
Salinity (ppt)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Conductivity (µs/cm)	213.30	213.40	191.70	185.05	187.90	183.60	239.65	230.10	225.00	241.10	261.20	268.90	284.95
Turbidity (NTU)	29.80	86.50	21.40	13.25	24.40	25.75	27.50	36.65	20.79	119.00	44.25	53.90	39.10
pH	8.88	9.01	7.69	7.34	8.30	8.00	7.85	9.50	7.70	8.30	8.15	9.05	8.50
Total Dissolved Solid, TDS (g/L)	0.13	0.12	0.11	0.10	0.11	0.11	0.13	0.12	0.12	0.13	0.14	0.15	0.16
Total Suspended Solid, TSS (mg/L)	26.00	28.00	17.20	16.20	10.80	27.80	18.80	20.40	16.00	31.40	26.80	35.60	22.00
Secchi Depth (cm)	39.50	46.50	64.50	68.50	69.00	60.00	52.50	56.50	75.00	40.00	42.50	40.00	60.00

Appendix 4.C

Table 3 Physical and chemical parameters in PS

Parameter/ Date	8-Aug-08	23-Sep-08	21-Oct-08	20-Nov-08	19-Dec-08	16-Jan-09	19-Feb-09	26-Mar-09	17-Apr-09	29-May-09	30-Jun-09	21-Jul-09	29-Aug-09
Chlorophyll-a (µg/L)	12.84	10.14	110.83	1584.76	131.71	75.90	183.56	298.30	342.38	358.82	150.83	71.62	115.89
TN (mg N/L)	1.01	2.85	1.79	2.36	2.91	1.33	2.43	4.36	2.48	4.05	2.35	1.56	1.89
TP (mg P/L)	0.06	0.08	0.17	0.17	0.11	0.08	0.21	0.15	0.08	0.16	0.07	0.07	0.04
TC (mg/L)	NA	17.92	9.21	18.42	14.56	17.55	13.59	16.98	23.53	23.23	18.44	12.94	18.20
TOC (mg/L)	-	-	7.21	8.51	8.61	6.43	10.18	11.50	11.85	10.47	12.82	12.72	12.71
IC (mg/L)	NA	NA	2.00	9.91	5.94	11.12	3.42	5.47	11.68	12.77	5.62	0.22	5.48
Ca ²⁺ (mg/L)	24.41	23.08	23.86	15.56	19.19	22.54	19.76	UDL	27.68	28.99	21.87	19.83	21.01
Mg ²⁺ (mg/L)	3.65	2.25	2.61	1.04	2.35	3.11	2.93	UDL	2.43	3.62	3.21	3.12	2.67
TN/TP	16.26	37.50	10.29	13.50	25.45	16.00	11.51	29.49	32.21	25.99	33.98	23.88	43.09
Dissolved Oxygen (mg/ L)	2.97	4.27	8.68	4.10	6.95	6.37	7.60	14.40	12.01	6.50	9.49	7.88	8.03
Salinity (ppt)	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Conductivity (µs/cm)	302.85	217.15	181.15	106.50	196.55	195.95	263.50	259.95	243.80	293.10	292.45	279.85	299.70
Turbidity (NTU)	21.00	22.75	64.00	224.50	66.95	51.20	78.05	159.00	105.75	103.00	83.00	46.85	35.85
pH	7.98	7.66	7.97	7.50	9.15	8.40	9.70	10.35	8.35	8.30	9.75	9.45	9.20
Total Dissolved Solid, TDS (g/L)	0.18	0.12	0.10	0.06	0.11	0.11	0.15	0.14	0.14	0.16	0.16	0.15	0.17
Total Suspended Solid, TSS (mg/L)	19.25	10.40	26.00	210.00	30.20	30.80	34.67	80.67	48.00	56.53	34.40	25.80	20.20
Secchi Depth (cm)	70.00	60.00	45.00	19.00	39.50	40.00	30.00	28.00	30.00	40.00	20.00	40.00	50.00

NA: No analyst; UDL: under detection limit

Appendix 5.A

Myovirus g20 amino acid sequences

>KRA0808M1

NLPKIKAEQYLREVMsRYRNKL VYGANTGEIKDDRKFMSMLEDFWLPRREGGRG
TEISTLPGGQNLGELEDVKYFFQKKLYKALNVPSSRLETETTFNIGRSTEITRDELKF
QKFINRLRKRFSDLFQDILKTQLLLKGVMTLEDWDQIKNHIQYDFIADNYFNELKN
MEMMNERMNLVATM

>KRA0808M2

NLPKVKAQYLREVMARYRNKLTYDAGTGEIRDDKKYMSMLEDFWLPRREGGR
GTEITTLPGGQNLGELTDVEYFQKKLYRALGVPESRLNSSDGFNLGRSSDILRDELK
FSKFVGRMRKRFSNLFHDILRSQILKNIVTPEEWDLMSDHIQYNYLYDNHFAELK
NVEIMQERMGVLA AV

>KRA0808M3

NLPKVKAQYLREVMARYRNKL VYDASTGEIRDDKKHMSMLEDFWLPRREGGR
GTEITTLPGGQNLGELKDVEYFRKKLYNSLNLPPSRLTDDNKGFNLGKTTEVLRDE
LKFAKFIGRLRKRFSSELFHDILKTQLILKGVIAPEDWEDMEEHIQYDFLFDNHFNEL
KNVELMKERIALVTQM

>KRA0808M4

NLPKGKAEQYL RDVMVKYRNKMVYDATTGELRDDRKHLSMLEDFWLPRREGGK
GTEITTL PAGQNLGELEDVKYFRKKLLQSLNVPMSRLEPQEGGMIGVGR TTEVTRD
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MEMRDAELLKERLGLLTIV

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TEISTLEGARNLGELEDVKYFQSKLYKSLGVPVSRLEPQQGFSLGRTTEITRDELKF
TKFIQRLRNKFSILFDELLRVQLVLKKVCTEDEWKEFKEDIWYDYKKDNNFEELKE
AELLNVRLDVLTKM

>KRC0209M4

NLPKMKAEQYLRDMMIRYKNRVVYDAATGEIRDDRKFMTMLEDYWLPRREGGR
GTEIDTLPGGQNLGEMADVEYFQKKLYEALGVPPSRLNQEGGFNIGRDSEITRDEV
KFSKFINRLRLKFSQIFLKALEKQVVLKGIMTLDEWNDIVQDIKVIYQDDNFYAELK
DIGIMKERINALTMV

Appendix 5.B

Table: ITS sequences obtained from Kranji Reservoir (provided by Dr Te Shu Harn)

Clone Name	Closet Relatives	Accession Number	Identity (coverage) %	Source	Journal
I/C0808/1	<i>Synechococcus</i> sp. CB0202	AY704678.1	90 (99)	Chesapeake Bay	Appl. Environ. Microbiol. 72 (3), 2239-2243 (2006)
I/C0808/2	Uncultured <i>Synechococcus</i> sp. clone TRITSAD02	FJ849923.1	87 (93)	Tucurui Hydroelectric Power Plant reservoir, Brazil	Unpublished
I/C0808/3	Uncultured <i>Synechococcus</i> sp. clone Kanui-2	EF638720.1	84(99)	Lake Kanui, New Zealand	Limnol Oceanogr Methods 5, 474-483 (2007)
I/B1108/3	<i>Synechococcus</i> sp. 19-3	EF638719.1	98 (89)	Helensville Dam, New Zealand	Limnol Oceanogr Methods 5, 474-483 (2007)
I/B1108/4	Uncultured <i>Synechococcus</i> sp. clone Kanui-2	EF638720.1	99(82)	Lake Kanui, New Zealand	Limnol Oceanogr Methods 5, 474-483 (2007)
I/B1108/5	<i>Synechococcus</i> sp. 19-3	EF638719.1	98 (89)	Helensville Dam, New Zealand	Limnol Oceanogr Methods 5, 474-483 (2007)
I/A0209/4	Uncultured cyanobacterium clone TH320-12-6	EF513348.1	77 (88)	Baltic Sea, 12m depth	Environ. Microbiol. 10 (1), 174-188 (2008)
I/B0209/3	<i>Prochlorococcus marinus</i> str. MIT 9303, complete genome	CP000554.1	94 (84)	NR	PLoS Genet. 3 (12), E231 (2007)
I/B0209/4	Uncultured <i>Synechococcus</i> sp. clone Kanui-2	EF638720.1	91(81)	Lake Kanui, New Zealand	Limnol Oceanogr Methods 5, 474-483 (2007)
I/B0209/7	Uncultured Cyanobium sp. clone 2D-3	EU015873.1	98 (64)	Lake Rotoiti, New Zealand	Unpublished

NR: No record in BLAST search

Sequences clone name were named according to the format X/YZ; where X is the sequence type ('I' for ITS sequence), Y indicate sampling location and date (letters A: KR1 station; B: KR3 station and C: PS station then the figure for month and year) and Z is the different genotype detected in the same sample (Te, 2012)