RECLAMATION

Managing Water in the West

Viral Treatment of Harmful Algal Blooms

Research and Development Office
Science and Technology Program
ST-2019-0157-01

U.S. Department of the Interior
Bureau of Reclamation
Research and Development Office

9/30/2019

Mission Statements

Protecting America's Great Outdoors and Powering Our Future

The Department of the Interior protects and manages the Nation's natural resources and cultural heritage; provides scientific and other information about those resources; and honors its trust responsibilities or special commitments to American Indians, Alaska Natives, and affiliated island communities.

REPORT DOCUMENTATION PAGE		*Form Approved* *OMB No. 0704-0188*
T1. REPORT DATE: SEPTEMBER 2019	**T2. REPORT TYPE:** RESEARCH	**T3. DATES COVERED** 10/01/2018 – 9/30/2019
T4. TITLE AND SUBTITLE Viral Treatment of Harmful Algal Blooms		**5a. CONTRACT NUMBER** RY.15412019.EN19157
		5b. GRANT NUMBER
		5c. PROGRAM ELEMENT NUMBER 1541 (S&T)
6. AUTHOR(S) Christopher Waechter Alyssa Aligata Yanyan Zhang		**5d. PROJECT NUMBER** ST-2019-0157-01
		5e. TASK NUMBER
		5f. WORK UNIT NUMBER 86-68190
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Christopher Waechter, Civil Engineer U.S. Department of the Interior, Bureau of Reclamation, Technical Services Center, Water Treatment Group PO Box 25007, Denver CO 80225-0007		**8. PERFORMING ORGANIZATION REPORT NUMBER**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) Research and Development Office U.S. Department of the Interior, Bureau of Reclamation, PO Box 25007, Denver CO 80225-0007		**10. SPONSOR/MONITOR'S ACRONYM(S)** **R&D:** Research and Development Office **BOR/USBR:** Bureau of Reclamation DOI: Department of the Interior
		11. SPONSOR/MONITOR'S REPORT NUMBER(S) ST-2019-0157
12. DISTRIBUTION / AVAILABILITY STATEMENT Final report can be downloaded from Reclamation's website: https://www.usbr.gov/research/		
13. SUPPLEMENTARY NOTES		
14. ABSTRACT ***Harmful algal blooms are becoming are more prevalent problem as human presence increases near reservoirs. There are not currently any effective solutions to mitigating harmful algal blooms in large reservoirs. Although there are drawbacks to using biological viral controls to mitigate harmful algal blooms, the potential for viral treatments is worth investigating this approach further.***		
15. SUBJECT TERMS Harmful Algal Blooms, Biocontrol, Cyanobacteria, Cyanophage		

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
			U		Christopher Waechter
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			**19b. TELEPHONE NUMBER**
U	U	U			

S Standard Form 298 (Rev. 8/98)
P Prescribed by ANSI Std. 239-18

BUREAU OF RECLAMATION

Research and Development Office
Science and Technology Program

Water Treatment Group, 86-68190

ST-2019-0157

Viral Treatment of Harmful Algal Blooms

CHRISTOPHER WAECHTER
Digitally signed by CHRISTOPHER WAECHTER
Date: 2019.09.27 12:11:44 -06'00'

Prepared by: Christopher Waechter
Civil Engineer, Technical Services Center, 86-68190

ALYSSA ALIGATA
Digitally signed by ALYSSA ALIGATA
Date: 2019.09.30 08:06:39 -06'00'

Prepared by: Alyssa Aligata
Civil Engineer, Technical Services Center, 86-68190

Yanyan Zhang

Prepared by: Yanyan Zhang, PhD, PEng
Assistant Professor, Dept. of Civil Engineering, New Mexico State University

BOYD.KAYTEE.L.1523615269
Digitally signed by BOYD.KAYTEE.L.1523615269
Date: 2019.09.27 13:15:53 -05'00'

Peer Review: Kaytee Pokrzywinski ERDC-RDE-EL-MS CIV, PhD
Research Biologist, Environmental Laboratory, Engineer Research and Development Center, US Army Corps of Engineers

Acknowledgements

Martin Page
Pei Xu
Jacque Keele
Sherri Pucherelli
Jon Whitler

Acronyms and Abbreviations

HAB – Harmful algal bloom
cyanoHAB – harmful cyanobacterial bloom
MPN – most probable number assay
TEM – transmission electron microscopy
VLPs – viral-like particles
CTAB – cetyltrimethylammonium bromide
PCR – polymerase chain reaction
ORF – Open Reading Frame
MOI – Multiplicity of Infection

Executive Summary

Nutrient pollution issues and HABs are increasing in frequency and magnitude in the last decade, impacting water supplies and costing $1B per year in lost tourism revenues (EPA 2019), with algal blooms specifically having large economic impacts in recent years. The United States had 620 HAB events reported to the Harmful Algal Events Dataset from 1980-2015, which was the second highest number of reported HAB events reported by country (Sanseverino et al., 2016). Typically driven by diffuse sources, nutrient pollution events can quickly turn into massive HAB events with the right environmental conditions for temperature, nutrient levels, sunlight, and other factors. In large water bodies such as Lake Okeechobee in Florida or Lake Erie to the north of Ohio, these blooms can be massive in scale.

There are not currently any practical technical solutions for mitigating such large scale HAB events. However, it may be possible to reduce the scale of these events with early intervention via physical, biological, and/or chemical treatment approaches. If scalable approaches could be made more practical in terms of costs, manpower, and logistics, they could provide an additional tool for communities for HAB control, in addition to longer term programs focused on nutrient limitation at the source.

The use of cyanophages for mitigating large HABs is a biological approach that could potentially be effective, but consideration for mass transfer, logistics limitations, and biological complexity must be given. It is recommended that further research be done in the following areas:

- The behavior of cyanophages and cyanobacteria involved in cyanoHABs
- The interactions between cyanobacteria and their cyanophages, including better understanding the specific strains targeted by cyanophages and the role cyanophages play in the natural cycles of cyanobacterial blooms
- The physical characteristics of a cyanophage infection event and the horizontal spread of a cyanophage infection through a population of cyanobacteria
- Identifying more cyanophages that infect cyanoHAB causing cyanobacteria to potentially develop suites of cyanophages to treat cyanoHABs

Contents

Tables

Figures

Harmful Algal Blooms

Algae are aquatic organisms that can range from microscopic, single-celled organisms (microalgae) to large filament and blade seaweeds (macroalgae). In general, algae are autotrophs, acquiring energy from light and carbon dioxide to carry out metabolic processes, however algae are also capable of mixotrophy and heterotrophy. Algae are ubiquitous in aquatic habitats including lakes, ponds, rivers, oceans, and estuaries. Cyanobacteria are conventionally prokaryotes but are typically lumped in with algae as they have specialized organelles such as phycobilisomes, that have a similar function as true eukaryotic chloroplasts. Like eukaryotic algae, cyanobacteria can also obtain energy through autotrophy, heterotrophy or mixotrophy. Cyanobacteria have a unique set of pigments used in photosynthesis, which can give them a blue-green color; for this reason, they are sometimes referred to as "blue-green algae." An algal bloom forms when there is rapid growth of algae or cyanobacteria in a water body, often resulting in water discoloration. A harmful algal bloom (HAB) is an algal bloom that is producing toxic or harmful effects on the ecosystem. Some types of bloom forming algae or cyanobacteria produce toxins that can kill fish, mammals, or birds. It is also possible for a HAB to produce toxins that can cause illness in humans and even death in extreme cases. Even if a HAB does not release toxins, it could still be harmful to an ecosystem because the algae can consume the oxygen in a water body as it decays, clog the gills of fish, or smother submerged aquatic vegetation (NOAA 2016). HABs can form naturally, however they are becoming more common and showing up in new places due to increasing rates of eutrophication (Anderson et al., 2012; Gilbert et al., 2008; Mehrubeoglu et al. 2014). Toxins from cyanobacteria have been recorded in all but five of the continental states in the USA (Erickson 2013; Loftin et al. 2016). The economic impacts of freshwater HABs are assumed to be significant although they are not well documented today (Erickson 2013).

Blooms can develop quickly if cyanobacterial growth and division rates are fast and/or when water column conditions enable surface accumulation. Accumulation of cyanobacteria can cause adverse effects on the water, such as taste and odor problems and even toxicity. Algal blooms can deplete nutrients in the water, increase turbidity, and use up inorganic carbon (CO_2) and other resources (Paerl et al. 2001). This can lead to a "crash" which means that the bloom starts dying rapidly. The cells decay and a foul-smelling scum that may house a variety of pathogens is left behind. Dissolved oxygen in the water body will also become depleted. In some cases the water can go under hypoxia, which is when the dissolved oxygen concentration falls under 4 mg/l and is known to stress most fauna. The water body can even go anoxic, which is when there is no dissolved oxygen left and most fish die (Paerl et al. 2001). This process is also known as eutrophication. Another adverse effect is that toxic hydrogen sulfide may be released from the dying bloom. Water bodies impacted by algal blooms usually become off limits to recreational use.

Bloom Growth Factors

Factors that contribute to cyanoHAB growth are increased nutrient loadings, food web alterations, water flow modifications, introduced species, and climate change (NOAA 2016). Blooms can form when wind and water currents are favorable. Some cyanoHABs appear after environmental phenomena like high water temperature, extreme weather (i.e., hurricanes, floods,

drought), and/or poor water circulation. A bloom can also form when nutrients, primarily nitrogen and phosphorus, from sources such as lawns and agriculture flow into water bodies and build up at a rate that "overfeeds" the algae that already exist in the environment (NOAA 2016). High rates of nutrient loading in addition to water residence time long enough to support high reproductive rates, combined with low grazing rates of predators to cyanobacteria, represent optimal conditions for bloom development (Paerl et al. 2001).

Recently, cyanoHABs have been forming in waters that were previously bloom free possibly due to anthropogenic nutrient loadings (mainly N and P). In many places around the world, cyanobacterial bloom taxa have been proliferating in areas experiencing increases in agricultural runoff, groundwater, and atmospheric loading of nutrients. Genera that produce toxins and taste/odor problems, such as *Microcystis*, are becoming more widespread in aquaculture operations (Paerl et al. 2001). Eutrophication can occur when nutrients like nitrogen and phosphorous are added to a water body. When nitrogen and phosphorous are in excess, the rate of primary production can increase and surpass the rate at which secondary consumers (i.e., fish and invertebrate) can eat it. The surplus algal matter accumulates and forms a cyanoHAB event. When the cyanobacteria die off and decay, dissolved oxygen is consumed. Taste and odor of the water is also impacted due to the poor water quality. Hypoxia and anoxia could occur in the water body, which may cause shellfish and fish mortality (Paerl et al. 2001).

Phosphorous loadings have been shown to be a driver for eutrophication in freshwater systems. Eutrophication that is driven by phosphorous can support growth of both N_2-fixing and non-N_2-fixing cyanobacteria. This is especially true in situations where waters have a long residence time, surface water temperatures periodically exceeding 20ºC, and vertical temperature stratification (Paerl et al. 2001).

Seasonal variations can change whether N_2-fixing or non-N_2-fixing cyanobacteria are dominating. Usually, late winter and early spring have high precipitation, which leads to raised nutrient loading in the form of nonpoint source surface runoff. These conditions favor non-N_2-fixing bloom species. In dry summer months nonpoint source runoff is very low, so point-sources like wastewater treatment plants are the predominant source of nutrients. Phosphorous enrichment (lower N:P ratios) happens when reduced nonpoint source and higher point source loading occurs. These conditions favor N_2-fixing diazotrophic species.

Cyanobacteria

Many different types of algae can form blooms, but cyanobacteria are the most notorious freshwater HAB formers (Paerl et al. 2001). Cyanobacteria are prokaryotic, meaning that they have no defined nucleus and that their cellular structure resembles that of bacteria. Cyanobacteria in freshwater environments can be found in different morphological groups: unicells, colonies of multiple single cells, undifferentiated non-heterocystous filaments, and filamentous forms containing differentiated cells called heterocysts (nitrogen-fixing cells) (Paerl et al. 2001). Some cyanobacteria are adapted to nutrient deficiencies and can evade nitrogen limited conditions by using atmospheric dinitrogen gas (N_2) as a nitrogen source through biological N_2 fixation. They are also able to uptake phosphorous in excess of growth requirements to store it for future use when phosphorous is limited. A trait that is particularly useful after pulse nutrient inputs typically observed after storm/runoff events. Cyanobacteria are also able to regulate their buoyancy throughout the water column and can often be observed at the surface during the day for photosynthesis and in nutrient rich waters below the thermocline in the evenings for respiration. This buoyancy regulation can also be useful to avoid high irradiances and high

temperatures. Additionally, cyanobacteria have developed specialized mechanisms to adapt to both light quantity (intensity) and quality (spectral color) (Averina et al., 2018). Collectively, these characteristics allow cyanobacteria to thrive in both nutrient deficient and enriched environments (Paerl et al 2001). Blooms caused by cyanobacteria may be toxic and can cause serious problems to water quality, fisheries, aquaculture, and animal and human health (Chorus and Bartram, 1999; Collins 1978). More than 60 different toxins can be released from cyanobacteria including neurotoxins, hepatoxins, cytotoxins, gastrointestinal toxins, and skin irritants (Kuster et al., 2006). In 50 countries and 35 US states, toxins from cyanobacteria have caused human or animal illness (Graham et al., 2015). These blooms can also cause water bodies to undergo hypoxia and anoxia, which can kill off many underwater plants and fish and result in waterway closures that have a negative impact on tourism and local economies. Recreational use and aesthetic values of water bodies may be negatively affected as well.

Cyanobacterial Growth Cycle

Cyanobacterial growth is defined as an increase in the number of cells (or size of a colony) rather than the size of individual cells. In general, algae and cyanobacteria have four growth phases: lag phase, exponential growth, stationary phase, and decay. In batch cultures in the laboratory, upon inoculation into a new medium the cyanobacteria will be in lag phase. This means that the cell sizes are increasing but the population size stays the same. The cells are metabolically active and synthesize enzymes needed for cell division and population growth in the new environment. Next, cyanobacteria will enter the exponential growth phase, also called the log phase. This happens until the nutrients in the medium are depleted. The population growth rate slows or stalls entering scenescence or stationary phase, at this point there is no net difference between cell division and cell death. The decay phase happens when the cell death exceeds the formation of new cells.

Microcystis aeruginosa

Microcystis aeruginosa is a unicellular, colonial freshwater cyanobacterium that often forms blooms in warmer months in eutrophic lakes and reservoirs. This species can produce microcystins, which are a group of cyclic heptapeptide toxins that can kill animals and humans. Microcystin production is influenced the cell growth rate. Factors that affect the growth rate are trace metal supply, nitrogen and phosphorus, light and temperature, and pH (Long et al. 2001). Shortly after inoculation, when *M. aeruginosa* cells are in log phase (exponential growth) is when the maximum amount of microcystins are produced. The minimum amount of microcystin is produced when the cells are in the stationary phase (Long et al. 2001).

According to the US EPA the most commonly occurring HAB forming genera of cyanobacteria in the United States is *Microcystis.* This report is focused specifically on *Microcystis aeruginosa*, one of the most common potentially toxic bloom-formers in eutrophic freshwater (Carmichael 1992). This species is regularly found in high densities at the surface of water bodies in the spring and summer and is one of the most ecologically damaging species due to its potential toxicity to aquatic and terrestrial organisms. This strain can produce cyclic peptide hepatotoxins known as microcystins. *M. aeruginosa* cells, like some other cyanobacteria cells, have gas-filled vesicles that provide buoyancy and keep the cells at a certain level in the water column to obtain optimum sunlight and carbon dioxide (Hense 2006). For this reason, *M. aeruginosa* can thrive in lakes and reservoirs, causing blooms and releasing toxins. This creates many water quality issues as well as harm to animals and humans if toxins are present.

Cyanophages

Cyanophages are a type of bacteriophage. Bacteriophages are one of the most diverse and pervasive entities in the biosphere (McGrath, 2007). Bacteriophages are a type of virus that occurs in many different viral families. Bacteriophages are viruses that infect bacteria and archaea, often infecting a single type of bacteria, and in some cases only one strain within a species. Bacteriophages have a general structure that includes the DNA package, or genome, of the bacteriophage encased in a protein shell, called a capsid. The majority of bacteriophages (96%) are tailed, but they can also be filamentous or pleomorphic (Hendrix, 2002). Bacteriophages are very stable compared to other types of virion, with many bacteriophages inhabiting some of the most extreme environments on Earth. Bacteriophages are a key factor in controlling bacterial populations and are thought to be responsible for 10 – 80% of bacterial mortality in aquatic ecosystems (Weinbauer, 2004).

Morphology

Many cyanophages follow a T4 phage archetype, including the Ma-LMM01 cyanophage which is of the most interest for controlling HCBs. The T4 archetype is based on the *Escherichia* virus T4 species of bacteriophage in the subfamily *Tevenviridae*, a subfamily of the family *Myoviridae* (Leiman et al., 2010). The T4 archetype is a relatively large virus and includes all the available features for a cyanophage. Other cyanobacterial archetypes are missing some of the features of the T4 archetype. As shown in figure 2, the T4 archetype consists of an icosahedral head capsid, a tail tube and sheath, a baseplate, and tail fibers.

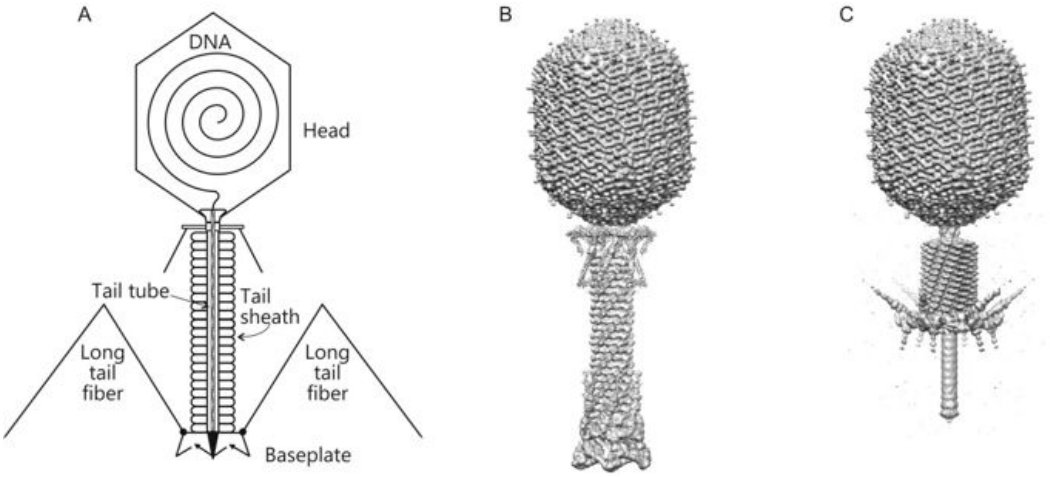

Figure 1: Structure of bacteriophage T4 (Leiman et. al., 2010)

The three main families of cyanophages are separated based on tail morphology. *Podoviridae* includes phages with short, non-contractile tails, *Siphoviridae* includes phages with long non-contractile tails, and *Myoviridae* includes phages with long contractile tails (Leiman et.al., 2010). Figure 1 shows CryoEM-derived models of a contractile sheath (B) prior to host cell attachment, and (C) upon host cell attachment (Leiman et.al., 2010). Figure 2 is a good example of a *Myoviridae* long contractile tail as Ma-LMM01 would have. Ma-LMM01 differs from the T4

archetype according to TEM observations in figure 3; no tail fibers have been observed in Ma-LMM01 (Yoshida et.al., 2006).

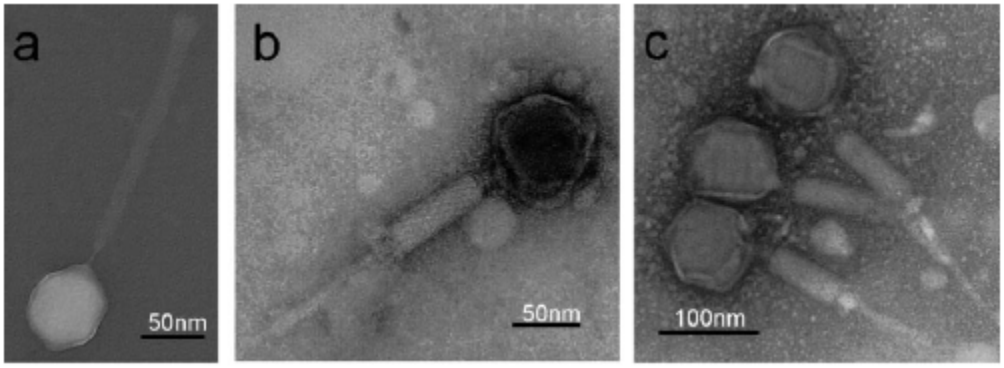

Figure 2: TEM of cyanophage Ma-LMM01 (Yoshida et. al., 2006)

Lifecycle

The lifecycles of cyanophages are similar to the lifecycles of bacteriophages infecting heterotrophic bacteria (Ni and Zheng, 2016). Those bacteriophages are called T-even phages. Cyanophages have two dominant lifecycles, the lytic cycle and the lysogenic lifecycle. A lytic phage can only replicate through the lytic lifecycle, while the lysogenic phage, or temperate phage, can enter either the lytic lifecycle or the lysogenic lifecycle. This is illustrated in the lytic lifecycle produces lysis of the host cell releasing a large number of phages into the aquatic environment. The lysogenic lifecycle integrates the phage's genome into the host cell's genome or through the formation of a circular plasmid within the host cell (Campbell and Reece, 2005). Ma-LMM01, the model cyanophage for this study, has a greater abundance within host cells compared to released phages (Kimura et al., 2012).

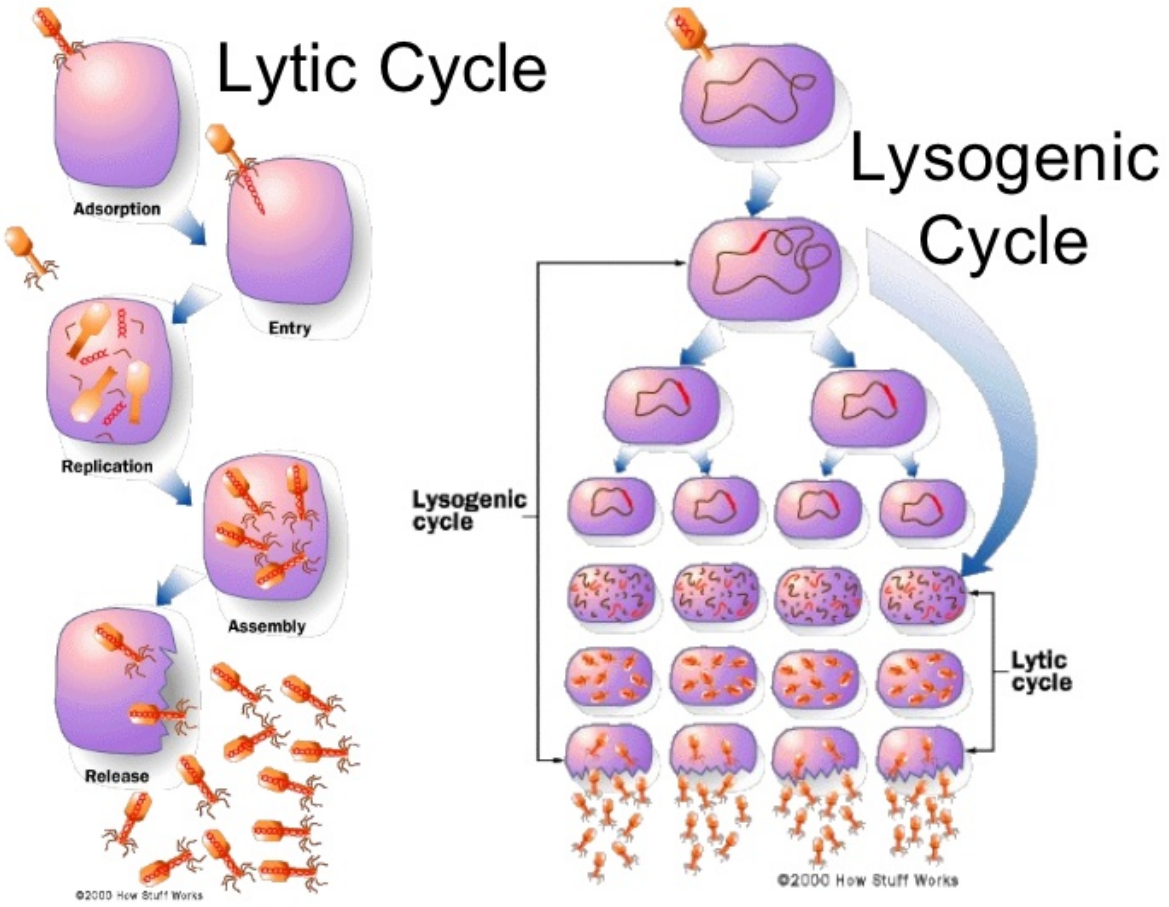

Figure 3: Lytic cycle vs Lysogenic cycle ©2000 How Stuff Works

Yoshida et.al, 2006, has concluded that Ma-LMM01 is a lytic phage similar to P-SSP7, a *podovirus* with an integrase that is responsible for lysogeny (Sullivan et al., 2005). The integrase allows the cyanophage's prophage to be integrated into the host's genome. It is hypothesized that at the end of summer stratification when nutrients are limited, that the cyanophages will integrate their genome into the host genome, but this has not been validated experimentally (Sullivan et al., 2005).

Adsorption is the first step in the lifecycle of the cyanophage. The phage tails recognize specific receptors on the exterior of the host cell, commonly a pili, flagella, membrane protein, or lipopolysaccharide (Labrie et al., 2010). Which structure the phage attaches to can be a way for the phage to recognize the specific hosts it infects. Cyanophages use receptor binding proteins to recognize the surface protein on the cell to which it attaches (Ni, T. and Zeng, Q, 2016). The phages are not motile; they find a host cell by random collision.

Injection of the cyanophage genome is the second step. Once the cyanophage has attached to the host cell, in the case of Ma-LMM01 and the cyanobacterial *myoviruses* (*cyanomyoviruses*), the phage then contracts it tail and injects the genome of the phage into the host cell as illustrated in figure 1, part C. Once the cyanophage genome is injected into the host cell, the capsid and tail are left attached to the cell as a remnant. When there are a large number of phages present, multiple phages can attach to the same host cell and deliver their payload.

In the lysogenic cycle, once the prophage is injected into the cell, it then incorporates itself into the host DNA. When the host cell replicates, it copies the prophage into the daughter cells along with the host cell DNA. The daughter cells which contain the prophage are called lysogens. The lysogens can remain in the lysogenic phase for multiple generations (Watson et al., 2008). The lysogens can switch over to the lytic lifecycle at any time by induction (Watson et al., 2008). In the induction process, the prophage is cut out of the host genome and is transcribed and translated to start manufacturing cyanophages and regulate the lytic growth (Watson et al., 2008). While lysogenic activity has not been observed in a laboratory setting, the Ma-LMM01 genome encodes all the mechanisms for lysogeny and induction (Stough et al., 2017). It has been observed that in metatranscriptomic data from cyanoHABs in Lake Tai in Taihu, China, genes associated with lysogeny have been observed. The expression of genes associated with lytic activity were negatively correlated (Stough et al., 2017).

The next step in the lytic cycle once the genome has been injected is to reduce protein synthesis by the host cell. Ma-LMM01, through the expression of the nb1A gene, rapidly degrades the PBSs of the host cell (Yoshida-Takashima et al., 2013). This has a two-fold effect. It reduces the absorption of extra light energy under stress conditions and provides amino acids for phage replication. Once the PBSs have been degraded and there are available amino acids, transcription begins to start the assembly of new cyanophages.

In Ma-LMM01, the early transcription units are translated and transcribed first, creating the mechanisms inside the host cell to provide nucleotides as building blocks to assemble new copies of the cyanophage. The early transcription activity reaches its maximum around 3 hours post infection (pi) and continued to occur at the same rate until lysis (Yoshida-Takashima et al., 2013). The late transcription units are translated and transcribed, creating the proteins that are the building blocks needed to assemble the complete phages. Infection proceeds with assembly of new phages. First the cyanophage genome is replicated and a protein coat is assembled around the genome into a procapsid. Once the procapsid is formed, the tail is then assembled to create a new cyanophage. This process occurs throughout the inside of the host cell simultaneously, until the cell lyses. The assembly occurs during the “LG” late transcriptions.

Ma-LMM01 does not have a homolog of the T4 genes for reprogramming the host RNA polymerase needed for late transcription (Yoshida et al., 2008). Ma-LMM01 may use a different, unidentified mechanism to regulate transcription of its genome during infection (Yoshida-Takashima et al., 2013). There are many ORFs in the Ma-LMM01 genome that have no known homologs, and there is much work left to do to decode the genome of Ma-LMM01 to better understand the molecular mechanisms used during infection.

Lysis occurs as a two-part process, involving two proteins. First, a membrane protein, called holin, first creates a lesion in the cytoplasmic membrane. Once the lesion becomes a hole in the cytoplasmic membrane, a murein hydrolase, or lysin, passes through the lesion to the murein layer (Young, 1992). The lysin compromises the outer murein layer of the cell resulting in the rupture of the cell, or lysis.

Upon lysis, the host cell is destroyed and the new generation of phages is released. This completes the lifecycle of the cyanophage, and the new cyanophages disperse and find new hosts.

The expression of lysogenic genes by Ma-LMM01 and the presence of down-regulated lytic genes needs to be further studied to better understand the infection mechanisms of *M. aeruginosa* cyanophages. In order to consider the mitigation of HABs using cyanophages, the specific

infection mechanisms of cyanophages need to be better understood to most effectively exploit the natural predation of cyanobacteria by cyanophages.

Genomics

Currently identified cyanophages, as a group, consist of small clusters of phages identified as having host strains within several different cyanobacterial families. All the positively identified cyanophages that have been isolated mainly occur within three families, *Myoviridae*, *Siphoviridae*, and *Podoviridae*; all of the order *Caudovirales* (Yoshida et. al., 2006). The order *Caudovirales* collectively includes phages that possess a tail. The three families are divided based on tail morphology (Leiman et. al., 2010).

There are currently seven identified cyanophages which are known to infect the genus *Microcystis* (Jaskulska and Mankiewicz-Boczek, 2019). Table 1 identifies the cyanophages that have been identified to date in primary literature.

Table 1: Microcystis Cyanophages

Cyanophage	Microcystis aeruginosa Host Strain	Family	Morphology			Sample Location	Source
			Archetype	Capsid diameter (nm)	Tail Type & Length (nm)		
Ma-LBP	unnamed	Podoviridae	T7	10 - 52	bulky & short	Baroon Lake, Australia	Tucker and Pollard (2005)
Ma-LMM01	NIES-298	Myoviridae	T4	86	contractile, 90-209	Mikata Lake, Fukui Prefecture, Japan	Yoshida et al. (2006)
MaMV-DC	FACHB-524	Myoviridae	T4	70	contractile, 160	Dianchi Lake, Kumning City, China	Ou et al. (2013)
VLP(unnamed)	PCC 7820	Podoviridae	T7	~52	non-contractile	Rostherene Mere Lake, Chesire, U.K.	Hargreaves et al. (2013)
VLP(unnamed)	BC84/1	Siphoviridae	B1	~84	~158	Rostherene Mere Lake, Chesire, U.K.	Hargreaves et al. (2013)
MaCV-L	HAB1801	Corticovirus-like particles	-	47 - 53	-	Donghu Lake, Wuhan City, Hubei, China	Li et al. (2013)
ϕMHI42	BC84/1	Podoviridae	T7	100 - 120	non-contractile, short	unnamed lake, Hayling Island, Hampshire, U.K.	Watkins et al. (2014)

The cyanophage known as ***M***. ***a****eruginosa* **- L**ake **M**ikata **M**yoviridae **01**, or Ma-LMM01, has been confirmed as infectious to *M. aeruginosa* strain NIES-2198. NIES-298 is known as a toxin forming strain of *M. aeruginosa*. This makes Ma-LMM01 an ideal target for further study to potentially control toxin releasing *M. aeruginosa* blooms. Another cyanophage, ΦMHI42, has been observed to infect the *M. aeruginosa* strains BC84/1 and 1450/8 (Watkins et.al., 2014).; it is a broad-range cyanophage that also infects *Planktothrix* spp. and other *Microcystis* spp. (Watkins et.al., 2014). Continued exploration of these cyanophages is needed to determine their potential for controlling a variety of strains for successful transition to the field.

Ma-LMM01 consists of a terminally redundant, linear dsDNA genome 162,109 bp in length in a circularly permuted package (Yoshida et.al., 2008). Most of the T4 core genes for co-opting the host cell's photosynthesis are missing in Ma-LMM01 (Yoshida et al., 2008). The most current hypothesis suggests that Ma-LMM01 infection causes degradation of the host cells' phycobilisomes (PBSs), the light harvesting apparatus of *M. aeruginosa*, to decrease the absorption of light energy to prevent photodamage during phage replication (Yoshida et.al., 2008). The degradation is caused by the gene nb1A, which encodes a protein that helps degrade the PBSs (Ou et al., 2013). The nb1A gene is also expressed by MaMV-DC (Ou et al., 2015). The nb1A gene is a potential target for identifying the presence of cyanophages infecting *M. aeruginosa* because nb1A may have been introduced into the *M. aeruginosa* cyanophage genomes by horizontal transfer from a common *Microcystis* strain (Ou et al., 2015). Another gene, g91, encodes a sheath protein and has previously been chosen as a molecular marker to study *Microcystis* specific cyanophages from Central Europe and Eastern Asia (Jaskulska and Mankiewicz-Boczek, 2019).

Proteins are created from open reading frames (ORFs), discrete sections of DNA that can be translated into a protein. Analysis of Ma-LMM01 indicated that there are two phases of protein transcription, early transcription units and late transcription units. The early transcription units correspond to the "UG" region, which compromises the upper portion of the circular plasmid that contains the Ma-LMM01 genome as shown in Figure 1. The "UG" region early transcription units mostly contain ORFs involved in DNA processing and nucleotide metabolism (Yoshida, 2008). The "LG" region late transcription units mostly contain ORFs that encode phage structural proteins (Yoshida, 2008). The "LG" region corresponds to the lower side of the plasmid in figure 1. Red and blue arrows indicate putative ORFs. Pale blue and pink lines inside the circle show G+C and A+T contents, respectively (Yoshida et al., 2008).

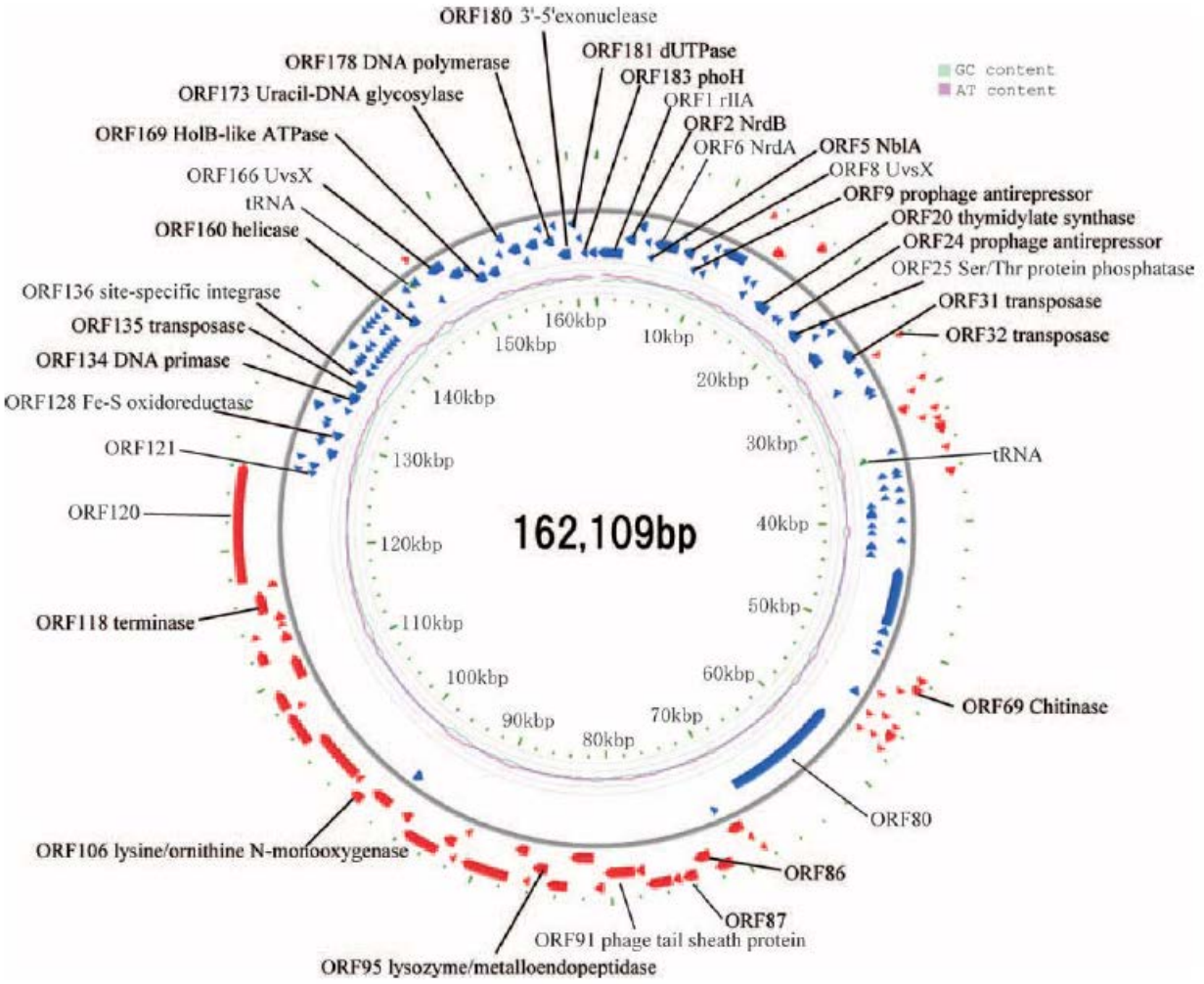

Figure 4: Ma-LMM01 genome organization. (Yoshida, T., 2008)

Population Kinetics

Cyanophages in controlling cyanobacteria

Several studies have reported that cyanophage could be a major natural control mechanism of cyanobacteria population in aquatic ecosystems. Cyanophages with high abundance and genetic diversity have been isolated from lakes and ponds, which can infect cultures of known bloom-forming freshwater cyanobacteria. For instance, eleven lytic cyanophages were isolated on *Microcystis* while 12 species of lytic cyanophages each were found to infect *Anabaena* and *Planktothrix* (Deng and Hayes 2008).

Tucker and Pollard (2005) found that isolated natural lake viral cocktail could decrease the abundance of *M. aeruginosa* culture by 95% within 6 days in a laboratory study. The density of the cyanophage was positively correlated with the rate of *M. aeruginosa* cell lysis. The cyanophage replication time was 11.2 h, with an average burst size of 28 viral particles per host cell (Tucker and Pollard 2005). Additionally, outdoor mesocosm study has been also applied to show the effectiveness of natural phage cocktails on killing *M. aeruginosa* in a hyper-eutrophic

pond. The cell density of *M. aeruginosa* decreased 10-fold under the impact of phages with different genome sizes (Honjo et al. 2006). Another study also demonstrated that infection by cyanophages may have a substantial effect on cyanobacterial succession in a hypereutrophic pond (Manage, Kawabata, and Nakano 1999). A clear increase in the phage abundance was observed when the host *M. aeruginosa* numbers declined in Lake Mikata (Yoshida et al. 2008), showing that the abundance of *M. aeruginosa* was negatively correlated with the cyanophage abundance.

Infection impacts

In addition to reducing cyanobacterial biomass, cyanophages also have a wider community-level impact due to the release of nutrients by cyanophage-induced cell lysis. Released intracellular nutrients could influence planktonic food web structure, community dynamics, and even biogeochemical cycles. One study reported that cyanophage dynamics may also affect shifts in microcystin-producing and non-microcystin-producing populations (Yoshida et al. 2008). The cyanophages with genotypes (Ma-LMM01, Ma-LMM02, Ma-LMM03, and Ma-HPM05) that specifically infect only a microcystin-producing *M. aeruginosa* strain may have been responsible for the fact that the microcystin-producing subpopulation was outcompeted by non-microcystin-producing populations during the summer. Given *M. aeruginosa* populations have a high degree of genetic diversity at the intraspecies level, the different genotypes of cyanophages could influence the seasonal shift in the composition of the different *M. aeruginosa* populations (Yoshida et al. 2008).

In addition to their hosts, the cyanophages could also affect the seasonal dynamics of other species. The role of a cyanophage in the ecological dynamics of its host *Nodularia* and competitor *Synechococcus* was investigated in a set of microcosm experiments. Initially phage-induced cell lysis decreased the number of *Nodularia* cells in the cultures. Nitrogen was released from the *Nodularia* cells as a consequence of phage lysis, resulting in a 7-fold increase in *Synechococcus* cell density. To sum up, the presence of the cyanophage altered the ecological dynamics in the cyanobacterial community, causing the evolution from a population dominated by susceptible cells to a population dominated by resistant ones (Coloma et al. 2017). Additionally, some heterotrophic bacteria can utilize the debris of cyanobacteria and convert it to dissolved organic matter (DOM), resulting in additional energy becoming available to higher trophic levels. Once the heterotrophic bacteria become abundant, they turn out to be superior competitors for phosphorus, which will negatively impact the population of cyanobacteria (Drakare 2002).

Environmental factors affecting infection

To explain changes in the abundance of cyanophages and determine their impact (or dependence) on the presence of *Microcystis*-dominated blooms, it was necessary to investigate the influence of environmental conditions including both biotic and abiotic factors. A five-year study showed the impact of environmental parameters on *Microcystis*-specific cyanophages. It was found that the increase of the water retention time, rise of the water temperature, optimum nutrient concentrations, and the predomination of *Microcystis* blooms could result in high cyanophage abundance ($>10^4$ gene copy number per microliter) (Mankiewicz-Boczek et al. 2016). Cyanophages and *Microcystis* hosts, including toxic genotypes, were positively correlated

in 4 of the 5 years analyzed. An increase of cyanophage abundance was observed when a *Microcystis* bloom developed in late July when the water retention time increased from 16 days to 32 days and total phosphorus (TP) concentration rose to 0.37 mg/L from 0.25 mg/L in the reservoir. Additionally, the occurrence of the highest gene copy number of cyanophages was preceded by a sudden increase in temperature (from 19.79 to 23.68 °C).

It was also found that the relative phage gene expression within host cells showed a peak during the daylight hours and was lowest around midnight in a Japanese pond, implying a diurnal pattern of cyanophage infection depending on photosynthesis (Kimura et al. 2012). The phage g91 DNA copy numbers in host cell fractions also increased in the afternoon, followed by an increase in the free-phage fractions (lytic phages). It was inferred that *Microcystis* cyanophage infection occurs in a diel cycle, which may depend on the light cycle (Kimura et al. 2012).

Seasonal changes in densities of cyanophage infectious to *M. aeruginosa* were also studied (Manage, Kawabata, and Nakano 1999). The copy number of phage molecular marker g91 was hardly detected at the beginning of May, and then its abundance consistently increased from June and reached the maximum in August, followed by rapid decline in September (8.24×10^2 copies/mL), and slight increase again in October (1.12×10^3 copies/mL). After then, the cyanophage abundance decreased until the following April. High activities of cyanophages in summer led to the sharp decrease in *M. aeruginosa* densities in June and September with increased cyanophage density. Densities of cyanophages became undetectable when those of *M. aeruginosa* were at low levels during winter (Manage, Kawabata, and Nakano 1999). Seasonal changes of both lytic and lysogenic *Microcystis* phage were also investigated during large bloom events in Lake Tai in China (Stough et al. 2017). A predominance of lytic virus activity occurred from late July through October, whereas genes associated with lysogeny were strongly expressed in the early months (June-July) and toward the end of bloom season (October). Those results suggest lysogeny may be prevalent in *Microcystis* blooms and environmental conditions drive switching between temperate and lytic life cycles during bloom proliferation. Another study showed that expression of lytic-cycle associated genes was positively correlated to total dissolved nitrogen levels, ammonium concentrations, and salinity while lysogeny-associated gene expression has a clear correlation with pH and total dissolved phosphorous levels (Stough et al. 2017).

Cyanophage resistance

Although cyanophages may be of great importance in aquatic food webs during large cyanobacterial blooms, the host population becomes resistant or insensitive to phage infection, and/or the phage transforms and is rendered inactive against the target host. Although the success of phages in managing cyanobacteria has been demonstrated in the laboratory, there is limited information on the ability of naturally occurring cyanophages to control cyanoHAB events. Although confounding variables, limited phage productivity in natural environments and regulatory guidelines/approvals for using viruses in a field setting result in the limited development of Cyanophage-based control on harmful algal blooms, it is worth noting that cyanophage resistance was developed by cyanobacteria against the infection. Previous reports documented that that lytic cyanophages have a negligible effect in regulating the densities of marine cyanobacteria *Synechococcus* populations despite their diversity and abundance (Waterbury and Valois 1993). It was discovered that those bacterial communities are dominated by cells resistant to their co-occurring phages (Waterbury and Valois 1993). Similarly, a

temporal decline in the *M. aeruginosa* abundance was observed in Lake Mikata in the beginning of summer due to the activity of cyanophage, however, the number of *M. aeruginosa* increased again in July (Yoshida et al. 2008) due to the dominance of phage-resistant *M. aeruginosa*. Likewise, there were larger numbers of Ma- LMM01-type phage infecting *M. aeruginosa* in East Lake of China in August, causing a sharp decrease in the total *M. aeruginosa* abundance in September. In October, however, the population of *M. aeruginosa* may have been replaced by another type of *M. aeruginosa* that is not susceptible to the Ma-LMM01-type cyanophage, resulting in an increase of the total *M. aeruginosa* abundance in October (Xia et al. 2013). Another lab study also showed the cultured host community recovered possibly because the host developed resistance to the cyanophage although isolated cyanophages can reduce the abundance of *M. aeruginosa* in 3 weeks (Tucker and Pollard 2005).

Cyanophages could only infect phage-sensitive cyanobacteria, which is small percentage of the population. It was reported that the average percentage of infected *Microcystis* cells varied between 0.1 and 32 % in Lowland Dam Reservoir (Mankiewicz-Boczek et al. 2016). It was also found that only some strains of *M. aeruginosa* are susceptible to the specific Ma-LMM01-type phage in the East Lake of China, indicating that the cyanophage assemblage might only have the ability to infect a small percentage of the cyanobacteria population present. The ability of phages to infect only a portion of the cyanobacteria population might be related to the simultaneous occurrence of phage-sensitive and insensitive populations. The presence of cyanophages could result in the replacement of susceptible populations by more resistant ones and/or cyanobacteria succession events through the resultant release of DOM, rather than having a quantitative impact on *Microcystis* abundance (Mankiewicz-Boczek et al. 2016).

The abundance of phage-resistant hosts could also impact population dynamics at the community level. One study revealed the frequency of the phage-resistant marine cyanobacteria *Nodularia* genotype determined the dynamics of an experimental plankton community. Cyanobacterial populations with a high frequency (50%) of the phage-resistant genotype dominated the cultures in the plankton community despite the presence of phages. Due to the occurrence of abundant phage-resistant cyanobacteria, most of the intracellular nitrogen were retained in the cyanobacterial cells. In contrast, populations with low frequencies (0% and 5%) of the phage-resistant genotype were lysed and reduced to extinction by the phage, transferring the intracellular nitrogen held by *Nodularia* to *Chlorella* and rotifers, and allowing *Chlorella* to dominate the communities and rotifers to survive. This study shows that cyanophages can have key effects on community composition and eco-evolutionary feedbacks in plankton communities (Coloma et al. 2019).

The evolution of phage resistance in bloom forming cyanobacterial populations especially fresh water cyanobacteria are still poorly studied. Generally, host bacteria can interfere with the phage adsorption by mutation. They may change the structure or exposure of the receptors, reduce the density of receptors, even lose the receptors completely (Donlan 2009). A comparative genomic study shows that *M. aeruginosa* has membrane-like protein involved in phage adsorption associated with phage-sensitivity (Yoshida et al. 2014). An investigation on cyanophage resistance in marine *Synechococcus* strains indicate that resistance is likely due to changes in host receptor sites that limit viral attachment (Stoddard, Martiny, and Marston 2007). Moreover, it was found that selection for resistance to one phage frequently resulted in cross-resistance to other phages. On average, phage-resistant *Synechococcus* strains became resistant to eight other cyanophages even if there was no significant correlation between the genetic similarity of the phages and cross-resistance (Stoddard, Martiny, and Marston 2007).

Laboratory Techniques

To conduct research on cyanophages, several laboratory techniques are required. The first step would be to collect environmental samples to obtain the cyanophage. Environmental water samples can be collected from the field. Baker et al. (2006) gathered 20 L water samples from freshwater lakes and brought them to a laboratory within an hour. The samples were prefiltered through to remove any debris and filtered to isolate particles small enough to be cyanophages.

In microbiology, enumeration is the determination of the number of individual viable microbes in a sample. Four methods are used for enumerating viruses in aquatic environments: plaque assays, most-probable-number assays (MPNs), transmission electron microscopy (TEM), and epifluorescence microscopy (Suttle 1993). Plaque assays and MPNs quantify the abundance of infectious units that lyse a particular host. TEM is used to determine the number of viral-like particles (VLPs) in whole water or in culture medium. Epifluorescence microscopy is used to quantify viral-sized particles containing double-stranded DNA (Suttle 1993).

Once the number of viable microbes is determined, the next step is identification. To identify a cyanophage, one must extract DNA from an environmental sample. Baker et al. (2006) extracted DNA from concentrated water samples using the cetyltrimethylammonium bromide (CTAB) method. Polymerase chain reaction (PCR) is another option that is commonly used to identify and examine pathogenic DNA water samples. PCR uses the natural function of polymerase enzymes and makes it possible to make unlimited copies of DNA from a single copy of initial DNA (G-Biosciences 2012). Baker et al. (2006) used oligonucleotide primers to amplify the major capsid protein gene of freshwater cyanophages.

After the phage is identified to be the cyanophage of interest, isolation is completed to separate the viral strain from a natural, mixed population of living microbes. Successful isolation of a virus depends on combining the host and pathogen to amplify the virus and infect the host. The probability of finding a virus increases with the volume of water screened. Therefore, the chances of isolating a virus by plaque or MPN assays are low. The host density is the largest factor directing the rate of phage propagation. It is beneficial to either grow the host to high density in the water to be assayed or to concentrate the virus using ultrafiltration and adding some of the concentrate to an exponentially growing culture of the host (Suttle 1993). The two main isolation procedures are the amplification procedure and the concentration procedure.

Once the cyanophage is isolated, one can enrich the sample to replicate it. The easiest way to replicate a virus is through plaque purification, as long as the host can grow on solid agar. Serially diluted culture lysate from a virus amplification is plaque assayed. A single well separated plaque is removed from the lawn and eluted in medium overnight. The eluent can be used for another dilution series and plaque assay. This procedure should be repeated multiple times to ensure that the virus is propagating. For hosts that will not grow on solid agar, enrichment must be done by amplifying a single infectious unit in a liquid culture (Suttle 1993). Yoshida (2006) inoculated an exponentially growing culture of *M. aeruginosa* with a sample of cyanophage and incubated the mixture for 4 days. The resulting lysate was filtered with 0.8 µm and then 0.2 µm filters to remove cellular debris. The titer of fresh lysate went through the extinction dilution method. The lysate was stored at -80ºC in the dark to preserve the cyanophage.

Case Study

An algal bloom can span hundreds of square miles of lake area at cell densities on the order of 10^5 cells per ml of water. Lake Okeechobee, for example, was 80% covered in an algae bloom with high algal densities reported (Kramer et al., 2018; Rosen et al., 2017). Assuming the algae is predominantly in the top 1 m of the water column, this scenario represents a biomass load of:

$$1500\ km^2 \times 80\% \times 0.0001\ km \times 10^9\ m^3/km^3 \times 10^6\ ml/m^3 \times 10^5\ cells/ml = 1.2 \times 10^{20}\ cells$$

Assuming a multiplicity of infection (MOI) requirement of 10 phages per 1 cell, and that the toxin-producing algae only comprise 10% of the bloom, and a phage stock solution density of 10^{12} phage per L, then approximately 1.2×10^9 liters of phage stock would be needed. This would equate to approximately 60,000 5000-gal tanker trucks.

Clearly, this volume is not feasible. A more realistic approach may be to apply a treatment early in the bloom cycle, when the bloom is perhaps only covering 5% the lake at a density of 10^3 cells/ml. Even under this lower algal density and area scenario, about 39 5000-gal tanker trucks of concentrated phage stock would be needed.

One possible solution to this scale problem may lie in the natural spread of infection through the population. There may be ways to reduce the total required volume of initial phage application by exploiting the mechanisms by which a natural phage infection spreads through a cyanobacterial population during a bloom. Further study will be needed to look at the hydraulics of mixing in a reservoir and how that affects the horizontal movement of cyanobacteria and its associated cyanophages. There also may be solutions in deploying mechanical mixers to more effectively spread phages horizontally across a reservoir.

Conclusion

CyanoHABs are becoming increasingly more pronounced and more frequent as human activities that result in nutrient loading in reservoirs increase. It is critical that the scientific community explore all the possible solutions to this problem in order to develop not just one magic bullet treatment. It is becoming apparent that the interactions in the microbial communities of aquatic environments are very complicated and nuanced. It is important to develop a suite of technologies to address the many different types of cyanoHABs and the different scales at which this problem will need to be addressed.

Using cyanophages to treat cyanoHABs is a promising technology, with the potential to mitigate blooms without chemicals or energy intensive methods like physically filtering out the algae using boats and other treatment processes. This approach is not without its drawbacks. As shown in the case study calculations, applying this process as currently envisioned would require a massive volume of concentrated phage solution for a large reservoir, which is clearly not feasible. The complex interactions at play in a cyanoHAB between the cyanobacteria that is causing the bloom and its associated phages are also a barrier to development of this technology. For example, the Ma-LMM01 cyanophage appears to only infect phage sensitive strains of its host bacterium *M. aeruginosa*, while not affecting phage resistant strains. There are also barriers to developing this technology in the mortality of large numbers of cyanobacteria in the water column, which could cause nutrient release, potentially trading a cyanoHAB for another type of HAB.

This technology is promising, and the potential to use natural, targeted treatments to eliminate HABs is an attractive alternative to the other potential solutions such as using coagulants and/or flocculants to cause the algae to settle to the bottom, or physically filtering the algae from the water. These alternatives use large amounts of chemicals that have a monetary and environmental cost and/or large amounts of labor and energy. From the current state of research in this field, the potential to use cyanophages to treat freshwater cyanoHABs is worth further inquiry and experimentation.

References

1. Anderson, Donald M., Cembella, Allan D., Hallegraeff, Gustaaf M. (2012). *Progress in understanding harmful algal blooms: paradigm shifts and new technologies for research, monitoring, and management*. Annual review of marine science 4: 143-176.

2. Averina, S., Velichko, N., Senatskaya, E. et al. (2018). *Far-red light photoadaptations in aquatic cyanobacteria.* Hydrobiologia 813: 1. https://doi.org/10.1007/s10750-018-3519-x

3. Baker, A. C., Goddard, V. J., Davy, J., Schroeder, D. C., Adams, D. G., Wilson, W. H. (2006). *Identification of a diagnostic marker to detect freshwater cyanophages of filamentous cyanobacteria*. Applied and Environmental Microbiology, 72(9), 5713–5719. https://doi.org/10.1128/AEM.00270-06

4. Bartram, Jamie, and Ingrid Chorus. (1999). *Toxic cyanobacteria in water: a guide to their public health consequences, monitoring and management*. CRC Press, 1999.

5. Bhagavan N.V. (2002). *Nucleic Acid Structure and Properties of DNA*, Medical Biochemistry (Fourth Edition), (Ch. 23) Academic Press, Pages 521-543, ISBN 9780120954407

6. Bickle, T.A., Kruger, D.H., *Biology of DNA restriction* (1993). Microbiological Reviews, 57 (2), pp. 434-450.

7. Campbell and Reece (2005). Biology. San Francisco: Pearson. pp. 338–339.

8. Carmichael, Wayne. (1994). *The toxins of cyanobacteria*. Scientific American, vol. 270, 1994.

9. Coloma, S., Dienstbier, A., Bamford, D., Sivonen, K., Roine, E. and Hiltunen, T. (2017). *Newly isolated Nodularia phage influences cyanobacterial community dynamics*. Environmental microbiology 19(1), 273-286.

10. Coloma, S., Gaedke, U., Sivonen, K. and Hiltunen, T. (2019*). Frequency of virus-resistant hosts determines experimental community dynamics*. Ecology 100(1), e02554.

11. Deng, L. and Hayes, P.K. (2008). *Evidence for cyanophages active against bloom-forming freshwater cyanobacteria.* Freshwater Biology 53(6), 1240-1252.

12. Dittmann, E., Neilan, B. A., Erhard, M., Von Döhren, H., & Börner, T. (1997). *Insertional mutagenesis of a peptide synthetase gene that is responsible for hepatotoxin production in the cyanobacterium Microcystis aeruginosa PCC 7806*. Molecular microbiology, 26(4), 779-787.

13. Donlan, R.M. (2009). *Preventing biofilms of clinically relevant organisms using bacteriophage*. Trends in Microbiology 17(2), 66-72.

14. Francy, D. S., Graham, J. L., Stelzer, E. A., Ecker, C. D., Brady, A. M., Struffolino, P., & Loftin, K. A. (2015). *Water Quality, Cyanobacteria, and Environmental Factors and Their Relations to Microcystin Concentrations for Use in Predictive Models at Ohio Lake Erie and Inland Lake Recreational Sites, 2013-14* (No. 2015-5120). US Geological Survey.

15. G-Biosciences. (2012). *Using Polymerase Chain Reaction to Identify Different Strains of an Organism*, info.gbiosciences.com/blog/bid/155569/using-polymerase-chain-reaction-to-identify-different-strains-of-an-organism.

16. Gilbert, J. A., Field, D., Huang, Y., Edwards, R., Li, W., Gilna, P., & Joint, I. (2008). *Detection of large numbers of novel sequences in the metatranscriptomes of complex marine microbial communities*. PloS One, 3(8), e3042. https://doi.org/10.1371/journal.pone.0003042

17. Grimsley, Nigel H., Thomas, Rozenn, Kegel, Jessica U., Jacquet, Stéphan, Moreau, Hervé, Desdevises, Yves. (2009). *Chapter Nine - Genomics of Algal Host–Virus Interactions,* Advances in Botanical Research, Academic Press, Volume 64, 2012, Pages 343-381, ISSN 0065-2296, ISBN 9780123914996, https://doi.org/10.1016/B978-0-12-391499-6.00009-8.

18. Hargreaves, K.R., Anderson, N.J., Clokie, M.R.J. (2013). *Recovery of viable cyanophages from the sediments of a eutrophic lake at decadal timescales*. FEMS Microbial. Ecol. 83, 450–456.

19. Hendrix RW. (2002). *Bacteriophages: evolution of the majority*. Theor Popul Biol. 2002;61:471–480. doi: 10.1006/tpbi.2002.1590.

20. Hense, I., Beckmann, A. (2010). *The representation of cyanobacteria life cycle processes in aquatic ecosystem models*. Ecological Modelling, 221(19), 2330–2338. https://doi.org/10.1016/j.ecolmodel.2010.06.014

21. Honjo, M., Matsui, K., Ueki, M., Nakamura, R., Fuhrman, J.A. and Kawabata, Z.i. (2006). *Diversity of virus-like agents killing Microcystis aeruginosa in a hyper-eutrophic pond*. Journal of plankton research 28(4), 407-412.

22. Kimura, S., Yoshida, T., Hosoda, N., Honda, T., Kuno, S., Kamiji, R., Hashimoto, R. and Sako, Y. (2012). *Diurnal infection patterns and impact of Microcystis cyanophages in a Japanese pond*. Appl. Environ. Microbiol. 78(16), 5805-5811.

23. Kramer, B.J., Davis, T.W., Meyer, K.A., Rosen, B.H., Goleski, J.A., Dick, G.J., Oh, G. and Gobler, C.J. (2018). *Nitrogen limitation, toxin synthesis potential, and toxicity of cyanobacterial populations in Lake Okeechobee and the St. Lucie River Estuary, Florida, during the 2016 state of emergency event*. PloS one, 13(5), p.e0196278.

24. Labrie, S.J., Samson ,J.E. ,and Moineau, S. (2010). *Bacteriophage resistance mechanisms.* Nat.Rev.Microbiol. 8, 317–327.doi:10.1038/nrmicro2315

25. Li, S., Ou, T., Zhang, Q. (2013). *Two virus-like particles that cause lytic infections in freshwater cyanobacteria*. Virol. Sin. 28 (5), 303–305.

26. Liu, T., Mazmouz, R., Pearson, L. A., & Neilan, B. A. (2019). *Mutagenesis of the Microcystin Tailoring and Transport Proteins in a Heterologous Cyanotoxin Expression System*. ACS Synth. Biol. 2019 8, 5, 1187-1194.

27. Long, B. M., Jones, G. J., & Orr, P. T. (2001). *Cellular Microcystin Content in N-Limited*. Microbiology, 67(1), 278–283. https://doi.org/10.1128/AEM.67.1.278

28. Manage, P.M., Kawabata, Z.i. and Nakano, S.-i. (1999) *Seasonal changes in densities of cyanophage infectious to Microcystis aeruginosa in a hypereutrophic pond.* Hydrobiologia 411, 211-216.

29. Mankiewicz-Boczek, J., Jaskulska, A., Pawełczyk, J., Gągała, I., Serwecińska, L. and Dziadek, J. (2016) *Cyanophages infection of Microcystis bloom in lowland dam reservoir of Sulejów, Poland*. Microbial ecology 71(2), 315-325.

30. McGrath S and van Sinderen D (editors). (2007). *Bacteriophage: Genetics and Molecular Biology* (1st ed.). Caister Academic Press. ISBN 978-1-904455-14-1.

31. Mehrubeoglu, M., Teng, M. Y., & Zimba, P. V. (2013*). Resolving mixed algal species in hyperspectral images*. Sensors (Switzerland), *14*(1), 1–21. https://doi.org/10.3390/s140100001

32. Ni T., Zeng Q. (2016) *Diel Infection of Cyanobacteria by Cyanophages*. Frontiers in Marine Science, 2 (JAN), art. no. 123

33. NOAA. (2016) “What Is a Harmful Algal Bloom?: National Oceanic and Atmospheric Administration.” NOAA, www.noaa.gov/what-is-harmful-algal-bloom.

34. Ou, T., Gao, X.C., Li, S.H., Zhang, Q.Y. (2015). *Genome analysis and gene nblA identification of Microcystis aeruginosa myovirus (MaMV-DC) reveal the evidence for horizontal gene transfer events between cyanomyovirus and host*. J. Gen. Virol. 96 (12), 3681–3697, http://dx.doi.org/ 10.1099/jgv.0.000290.

35. Ou, T., Li, S., Liao, X., Zhang, Q. (2013*). Cultivation and characterization of the MaMV-DC cyanophage that infects bloom-forming cyanobacterium Microcystis aeruginosa*. Virol. Sin. 28 (5), 266–271.

36. Ouellette, A. J., Handy, S. M., & Wilhelm, S. W. (2006). *Toxic Microcystis is widespread in Lake Erie: PCR detection of toxin genes and molecular characterization of associated cyanobacterial communities*. Microbial ecology, 51(2), 154-165.

37. Paerl, H. W., Fulton, R. S., Moisander, P. H., Dyble, J. (2001). *Harmful freshwater algal blooms, with an emphasis on cyanobacteria.* The Scientific World Journal, 1, 76–113. https://doi.org/10.1100/tsw.2001.16

38. Leiman, Petr G., Arisaka, Fumio, van Raaij, Mark J., Kostyuchenko, Victor A., Aksyuk, Anastasia A., Kanamaru, Shuji, Rossmann, Michael G. (2010) *Morphogenesis of the T4 tail and tail fibers*. Virology Journal, volume 7, Article number: 355

39. Rosen, B., Davis, T., Gobler, C., Kramer, B., & Loftin, K. (2017). *Cyanobacteria of the 2016 Lake Okeechobee Waterway harmful algal bloom.* US Geological Survey Open-File Report, 1054(2017), 34.

40. Sanseverino, I., Conduto, D., Srdan,D. and Lettieri, T. (2016*). Algal blooms and their economic impact.* European Commission JRC Technical Reports. EUR 27905 EN.

41. Stoddard, L.I., Martiny, J.B. and Marston, M.F. (2007) *Selection and characterization of cyanophage resistance in marine Synechococcus strains*. Appl. Environ. Microbiol. 73(17), 5516-5522.

42. Stough, J.M., Tang, X., Krausfeldt, L.E., Steffen, M.M., Gao, G., Boyer, G.L. and Wilhelm, S.W. (2017) *Molecular prediction of lytic vs lysogenic states for Microcystis phage: Metatranscriptomic evidence of lysogeny during large bloom events*. PloS one 12(9), e0184146.

43. Sullivan, M.B., M.L. Coleman, P. Wiegele, F. Rohwer, and S.W. Chisholm. (2005). *Three Prochlorococcus cyanophage genomes; signature features and ecological interpretations*. PLoS Biol. 3:e144.

44. Suttle, C. A. (1993). *Enumeration and isolation of viruses*, p. 121–137. In P. F. Kemp, B. Sherr, E. Sherr, and J. J. Cole (ed.), *Handbook of methods in aquatic microbial ecology*. Lewis Publishers, Boca Raton, Fla.

45. Tucker, S. and Pollard, P. (2005*) Identification of cyanophage Ma-LBP and infection of the cyanobacterium Microcystis aeruginosa from an Australian subtropical lake by the virus.* Appl. Environ. Microbiol. 71(2), 629-635.

46. USEPA (2019). The Effects of Nutrient Pollution. https://www.epa.gov/nutrientpollution/issue

47. Waterbury, J.B. and Valois, F.W. (1993) *Resistance to co-occurring phages enables marine Synechococcus communities to coexist with cyanophages abundant in seawater.* Appl. Environ. Microbiol. 59(10), 3393-3399.

48. Watkins SC, Smith JR, Hayes PK, Watts JEM (2014) *Characterisation of Host Growth after Infection with a Broad-Range Freshwater Cyanopodophage*. PLoS ONE 9(1): e87339. doi:10.1371/journal.pone.0087339

49. Watson; et al. (2008). *Molecular Biology of the Gene*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp. 784–786.

50. Weinbauer M. (2004) *Ecology of prokaryotic viruses*. FEMS Microbiol Rev. 28:127–181. doi: 10.1016/j.femsre.2003.08.001.

51. Wommack, K. Eric, Colwell, Rita R. (2000). *Virioplankton: Viruses in Aquatic Ecosystems*. Microbiology and Molecular Biology Reviews Mar 2000, 64 (1) 69-114; DOI: 10.1128/MMBR.64.1.69-114.2000

52. Xia, H., Wang, M., Ge, X., Wu, Y., Yang, X., Zhang, Y., Li, T. and Shi, Z. (2013) *Study of the dynamics of Microcystis aeruginosa and its cyanophage in East Lake using quantitative PCR*. Virol Sin 28, 309-311.

53. Yoshida, T., Shirai, Y., Takao, Y., Hiroishi, S., Nagasaki, K. (2006). *Isolation and Characterization of a Cyanophage Infecting the Toxic Cyanobacterium*. Microbiology, 72(2), 1239–1247. https://doi.org/10.1128/AEM.72.2.1239

54. Yoshida, M., Yoshida, T., Kashima, A., Takashima, Y., Hosoda, N., Nagasaki, K. and Hiroishi, S. (2008*) Ecological dynamics of the toxic bloom-forming cyanobacterium Microcystis aeruginosa and its cyanophages in freshwater*. Appl. Environ. Microbiol. 74(10), 3269-3273.

55. Yoshida, T., Nagasaki, K., Takashima, Y., Shirai, Y., Tomaru, Y., Takao, Y., Sakamoto, S., Hiroishi, S., Ogata, H., *(2008) Ma-LMM01 infecting toxic Microcystis aeruginosa illuminates diverse cyanophage genome strategies,* Journal of Bacteriology, 190 (5), pp. 1762-1772.

56. Yoshida, T., Kamiji, R., Nakamura, G., Kaneko, T. and Sako, Y. (2014) *Membrane-like protein involved in phage adsorption associated with phage-sensitivity in the bloom-forming cyanobacterium Microcystis aeruginosa*. Harmful Algae 34, 69-75

57. Yoshida-Takashima, Y., Yoshida, M., Ogata, H., Nagasaki, K., Hiroishi, S., Yoshida, T., (2012) *Cyanophage Infection in the Bloom-Forming Cyanobacteria Microcystis aeruginosa in Surface Freshwater*, Microbes Environ. Vol 27, No. 4, 350-355

58. Young, R. (1992). *Bacteriophage Lysis: Mechanism and Regulation.* MICROBIOLOGICAL REVIEWS, Sept. 1992, Vol. 56, No. 3. p. 430-481