Impact of Viral Infectivity on Phototrophic Microbes for Biofuel Applications

by

Kyle Kraft

A Thesis Presented in Partial Fulfillment
of the Requirements for the Degree
Master of Science

Approved November 2014 by the
Graduate Supervisory Committee:

Morteza Abbaszadegan, Chair
Absar Alum
Peter Fox

ARIZONA STATE UNIVERSITY

December 2014

ABSTRACT

Research in microbial biofuels has dramatically increased over the last decade. The bulk of this research has focused on increasing the production yields of cyanobacteria and algal cells and improving extraction processes. However, there has been little to no research on the potential impact of viruses on the yields of these phototrophic microbes for biofuel production. Viruses have the potential to significantly reduce microbial populations and limit their growth rates. It is therefore important to understand how viruses affect phototrophic microbes and the prevalence of these viruses in the environment. For this study, phototrophic microbes were grown in glass bioreactors, under continuous light and aeration. Detection and quantification of viruses of both environmental and laboratory microbial strains were measured through the use of a plaque assay. Plates were incubated at 25º C under continuous direct florescent light. Several environmental samples were taken from Tempe Town Lake (Tempe, AZ) and all the samples tested positive for viruses. Virus free phototrophic microbes were obtained from plaque assay plates by using a sterile loop to scoop up a virus free portion of the microbial lawn and transferred into a new bioreactor. Isolated cells were confirmed virus free through subsequent plaque assays. Viruses were detected from the bench scale bioreactors of *Cyanobacteria Synechocystis* PCC 6803 and the environmental samples. Viruses were consistently present through subsequent passage in fresh cultures; demonstrating viral contamination can be a chronic problem. In addition TEM was performed to examine presence or viral attachment to cyanobacterial cells and to characterize viral particles morphology. Electron micrographs obtained confirmed viral

attachment and that the viruses detected were all of a similar size and shape. Particle sizes were measured to be approximately 50-60 nm. Cell reduction was observed as a decrease in optical density, with a transition from a dark green to a yellow green color for the cultures. Phototrophic microbial viruses were demonstrated to persist in the natural environment and to cause a reduction in algal populations in the bioreactors. Therefore it is likely that viruses could have a significant impact on microbial biofuel production by limiting the yields of production ponds.

TABLE OF CONTENTS

Page

CHAPTER Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

Figure Page

CHAPTER 1 - INTRODUCTION

The Potential of Microbial Biofuels

Petroleum plays such an integral part of our everyday lives. It is used to make fuel for our cars, plastics, jet fuel, and even fertilizers. There has been an increase in global petroleum demand as countries like China and India become more developed. However, it is a nonrenewable resource, and as such will eventually run out. The importance of petroleum combined with global trend of moving towards sustainable energy production has resulted in increased research in biofuels (specifically those derived from oxygenic photosynthetic microalgae and cyanobacteria, which for convenience will be referred to as algae from now on) over the last decade.

There are many motivations for producing a viable alternative to petroleum fuels. Petroleum is a nonrenewable resource, meaning that there is a limited quantity that cannot be readily replenished. This is important because it is used for the production a wide variety of products such as: "transportation fuels, fuel oils for heating and electricity generation, asphalt and road oil, and the feedstocks used to make chemicals, plastics, and synthetic materials found in nearly everything we use today," (U.S. EIA, 2014c). Worldwide petroleum supplies, "about 40% of the world's primary energy and nearly all of the fuel for the world's transportation systems,"(Greene et al., 2006).

For example, in the U.S. gasoline is the number one transportation, with 131 billion gallons being used by the U.S. in 2011 alone (U.S. EIA, 2012). Gasoline accounts for slightly more than 64% of all the energy used for transportation, 46% of all petroleum consumption, and 18% of total U.S. energy consumption (U.S. EIA, 2012). The

consumption of petroleum in the U.S. is not projected to decrease significantly in the near future (see Figure 1), and is rapidly increasing in developing countries like India and China as they become increasingly motorized. According to Greene et al., 2006, "Over the past 30 years, world oil use has increased by 47% despite oil price shocks and economic downturns. Over the next 30 years oil demand is expected to grow by 60%."

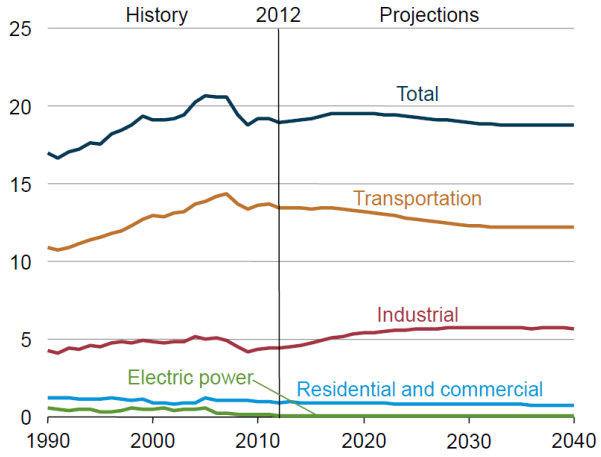


Figure 1. U.S. Consumption of petroleum and other liquid fuels by sector, 1990-2040 (U.S. EIA, 2014a)

Furthermore, they found that

> "Peaking of conventional oil production is almost certain to occur soon enough to deserve immediate and serious attention. If peaking is already underway and oil supplies are as limited as the pessimists believe, the world is facing a drastic transition for which it is unprepared. If peaking is one to three decades away, it is not too soon to begin efforts to understand and prepare for the transition to other energy sources." (Greene et al., 2006)

Therefore we could be rapidly heading toward a future where there is no longer petroleum, or where it has become too cost prohibitive to use. This necessiatates the need for viable alternative such as biofuels.

Economic. There are many benefits to using algae for the production of biofuels. Algae require significantly less land per unit oil produced, than traditional row crops,

such as soy and corn. They can be grown on non-arable, nutrient-poor land that won't support traditional agriculture. This means that the production of algae for biofuels doesn't have to compete over arable land needed for food production. Also, they do not require fresh water for irrigation, or application of petroleum-based fertilizers, limiting their environmental impact. Furthermore, algae produce high yields due to their ability to grow quickly at a large scale. They can potentially generate up to 50 times more oil per acre than row crops, like corn and soybeans, which produce vegetable oil (UC San Diego, 2014). According to Li et al, 2008, "Microalgal biofuel production is potentially sustainable… [and it] is possible to produce adequate microalgal biofuels to satisfy the fast growing energy demand within the restraints of land and water resources."

The U.S. Energy Information Administration (EIA) projections for global production of the petroleum and other liquid fuels over the next 26 years are presented in Figure 2. They project that 33 million barrels a day of additional liquid fuel supply will be needed in 2040 compared to 2010 to satisfy growing demand for liquid fuels (U.S. EIA, 2014b).

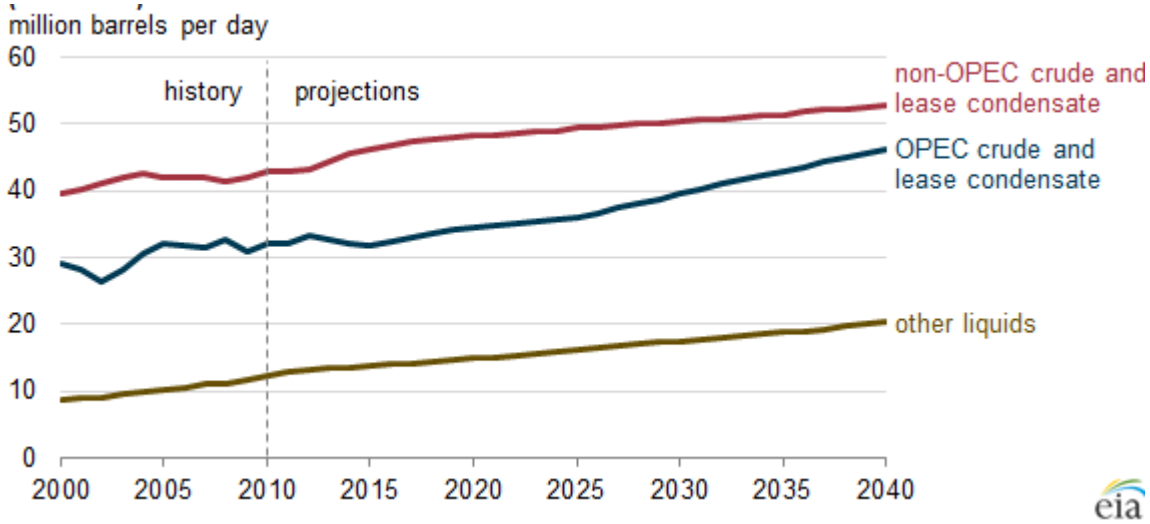


Figure 2. Petroleum and other liquids production by region and type in IEO2014, (U.S. EIA, 2014b)

> "Other liquid resources—including natural gas plant liquids, biofuels, coal-to-liquids, and gas-to-liquids—currently supply a relatively small portion of total world petroleum and other liquid fuels, accounting for about 14% of the total in 2010. However, they are expected to grow in importance, rising to 17% of the world's total liquids supply in 2040." (U.S. EIA, 2014b)

This shows that there is large expected growth for alternative fuels sources, such as biofuels.

As with most things, the economics will ultimately be the determining factor for the adoption of biofuels as an alternative to petroleum. One way to determine the economic viability of algal biofuels is with the use of a Life Cycle Assessment (LCA). An LCA is a mechanism to identify and quantify the material and energy flows involved in the lifecycle of a particular product, process, or material(Allenby, 2012). It provides data enabling systematic and rational improvement of performance by identifying areas where improvements can be made (Allenby, 2012).

In fact, a recent LCA paper proclaimed, "that although microalgal biofuel systems remain in an early stage of development, they are now approaching profitability if the co-production systems in the base case, and/or the increased productivities in the projected case can be attained," (Stephens et al., 2010). The coproduction in the base case refers to, "the co-production and extraction of a high value product (HVP; e.g., β-carotene at 0.1% of biomass, $600/kg); and (iv) the sale of the remaining biomass as feedstock (e.g., soymeal or fishmeal substitute),"(Stephens et al., 2010). It minimizes waste, by making use of the byproducts of biofuel production. In contrast, the projected case is intended to represent the microalgal biofuel industry at maturity and no longer incorporates the co-production of HVPs (Stephens et al., 2010). Furthermore, they stipulate that, "improved microalgal productivity approaching the targets identified in the projected case will reduce the reliance on co-production…as the industry matures" (Stephens et al., 2010).

Furthermore they found, "estimates that current technology could produce oil for $84/barrel (with no value attributed to the non-oil fraction), with reasonable advancements in technology reducing this cost to $50/barrel or less. This supports our conclusion that co-production is required in the short term and that at increased oil prices (that is, $100 in this model) an IRR of 15% could be obtained," (Stephens et al., 2010). This means that producing solely biofuels will not be economically viable until there is a further advancement of the current technologies. Its current economic viability is contingent on the co-production of high value products along with the biofuel.

It is important to note that most of the gasoline now sold contains some ethanol. In 2011, the 133.93 billion gallons of gasoline consumed by the United States contained

approximately 12.87 billion gallons of ethanol, accounting for 9% of the volume of gasoline consumed (U.S. EIA, 2012). In general the ethanol percentage will not exceed 10% by volume. Gasoline that contains 10% ethanol by volume is called E10 and gasoline with 15% ethanol is called E15 (U.S. EIA, 2012). E85 is a gasoline that contains 85% ethanol and 15% gasoline. All gasoline vehicles can use E10 gasoline, but currently you need a light-duty vehicle with a model year of 2001 or greater to use E15, and a "flex fuel: vehicle to use gasoline with an ethanol content greater than E15 (U.S. EIA, 2012). Unfortunately, the energy content of ethanol is about 33% less than pure gasoline (U.S. EIA, 2012). So vehicles using E10 may experience a decrease in vehicle mileage by up to 3.3%. This highlights the need for algal biofuels to have energy content similar that of petroleum gasoline, in order to be a good replacement for petroleum.

In the same way that ethanol is currently being blended into gasoline, so to could algal biofuels. This would allow for algal biofuel production plants to have a meaningful impact on gasoline without requiring them to completely supplant petroleum based fuels. Allowing the biofuel industry to grow in concert with the advancements in technology and slowly shift the primary source of gasoline for the U.S. This gradual shift to biofuels gives adequate time for the proper restructuring of the United States' current gasoline infrastructure to accommodate the needs of algal biofuels.

Need for an Understanding on how Viruses can Impact Biofuel Production

It is already known that, "predators such as rotifers are omni-abundant in the environment and predate on the algal communities, dramatically decreasing yields" (Kazamia et al., 2012). However, there has been little to no research on the how viruses

can impact the yield of algal biofuel production. A virally contaminated stock may simply have reduced yields, or it could potentially experience a complete population crash. It is important to establish the impact of viral infectivity of algal cells on production and growth rate. This requires further research to determine if algal phages will indeed have a meaningful impact on production.

Study Objectives

The overall objective of this study is to measure the impact of viral infectivity on phototrophic microbes for biofuel applications.

- To measure the impact of viruses on the growth rate of phototrophic microbes in laboratory bioreactors
- To examine the presence and viral attachment to infected cells using Transmission Electron Microscopy (TEM)
- To perform field sampling for the detection of viruses (phages) in the environment

CHAPTER 2 - LITERATURE REVIEW

Background

Microalgae and Cyanobacteria. Oxygenic photosynthetic microalgae and cyanobacteria are an enormously varied, yet highly specialized group of microorganisms that can live in a wide variety of ecological habitats such as marine, freshwater, hyper-saline and brackish waters, with a range of temperature and pH, and unique nutrient availability conditions (Hu et al., 2008).

> "With over 40,000 species already identified and with many more yet to be identified, algae are classified in multiple major groupings as follows: cyanobacteria (Cyanophyceae), green algae (Chlorophyceae), diatoms (Bacillariophyceae), yellow-green algae (Xanthophyceae), golden algae (Chrysophyceae), red algae (Rhodophyceae), brown algae (Phaeophyceae), dinoflagellates (Dinophyceae) and 'pico-plankton' (Prasinophyceae and Eustigmatophyceae)." (Hu et al., 2008)

Cyanobacteria and microalgae have simple growth requirements, and use light, carbon dioxide and other inorganic nutrients efficiently. Cyanobacteria and microalgae are the only organisms known so far that are capable of both oxygenic photosynthesis and hydrogen production. Photobiological production of H2 by microorganisms is of great public interest because it promises a renewable energy carrier from nature's most plentiful resources: solar energy and water. They have been investigated to produce different feed stocks for energy generation like hydrogen (by direct synthesis in cyanobacteria), lipids for biodiesel and jet fuel production, hydrocarbons and isoprenoids for gasoline production and carbohydrates for ethanol production. Beyond that, the complete algal biomass can also be processed for syngas production followed or not by a fischer–tropsch process, hydrothermal gasification for hydrogen or methane production,

methane production by anaerobic digestion, and co-combustion for electricity production. Hence, cyanobacterial and microalgal systems could contribute to a sustainable bioenergy production. However different biotechnical, environmental and economic challenges have to be overcome before energy products from these systems can enter the market (Parmar et al., 2011).

Cyanobacteria. Cyanobacteria, also known as blue green algae, are oxygenic photosynthetic bacteria that play significant roles in global oxygen production, the nitrogen cycle, and biological carbon sequestration (Parmar et al, 2011). Cyanobacteria have the potential to be developed as excellent microbial cell factories that can harvest solar energy and convert atmospheric CO2 to useful products such as biofuels (Parmar et al., 2011). Cyanobacteria is an ancient phylum, with some fossil traces of cyanobacteria claimed to have been found from around 3.5 billion years ago, and they most likely "played a key role in the formation of atmospheric oxygen, and are thought to have evolved into present-day chloroplasts of algae and green plants" (Tamagnini et al., 2007).

Phototrophic Microbial Biofuels

Comparison to other Biofuel Feedstock's. If one looks at algae in comparison to other potential biofuel crops, it is evident that cyanobacteria and microalgae are the most practical crop to use as a source for biofuels. This is clearly demonstrated by Table 1, which shows that estimated areas required to meet the global oil demand for several different crops. A great example is the comparison of oil palm, one of the most productive oil crops, with microalgae.

Chisti, 2008 found that:

> "An average annual productivity of microalgal biomass in a well designed production system located in a tropical zone can be in the region of 1.535 kg m^{-3} d^{-1}. At this level of biomass productivity, and if an average oil content of 30% dry weight in the biomass is assumed, oil yield per hectare of total land area is ~123 m^3 for 90% of the calendar year. (About 10% of the year is unproductive, because the production facility must be shut down for routine maintenance and cleaning.) This amounts to a microalgal biodiesel yield of 98.4 m^3 (98,400 L) per hectare."

While oil palm only yield approximately 5,950 liters of oil per hectare, with a conversion rate of ~80%, yields ~4760 liters of biodiesel (Chisti, 2008). These results mean that the oil palm is over 20 times less efficient than microalgae on a per hectare basis. This difference in production yield can be further explained by the large variation in photosynthetic efficiencies between feedstocks. According to Parmar et al., 2011, "cyanobacteria and their superior photosynthesis capabilities can convert up to 10% of the sun's energy into biomass, compared to the 1% recorded by conventional energy crops such as corn or sugarcane, or the 5% achieved by algae." So it makes sense that cyanobacteria and algae have much more potential sources for biofuels.

Table 1

Comparison of Estimated Biodiesel Production Efficiencies from Vascular Plants and Microalgae

Biodiesel Feedstock	Area needed to meet global oil demand (10^6 hectares)	Area required as a percent of total global land	Area required as a percent of total arable global land
Cotton	15,000	101	757
Soybean	10,900	73	552
Mustard seed	8,500	57	430
Sunflower	5,100	34	258
Rapeseed/canola	4,100	27	207
Jatropha	2,600	17	130 (0)[a]
Oil palm	820	5.5	41
Microalgae (10 g/m^2/day, 30% TAG)	410	2.7	21 (0)[b]
Microalgae (50 g/m^2/day, 50% TAG)	49	0.3	2.5 (0)[b]

[a] Jatropha is mainly grown on marginal land.
[b] Assuming that microalgal ponds and bioreactors are located on non-arable land.
Note: Triacyglycerols (TAGs) are a storage lipid produced by photosynthetic plants that can be used to synthesize biodiesel fuels (Smith et al., 2010).

However, as of now, there is no clear best strain of algae for biofuel production. This is demonstrated by the large variety of species currently being researched for biofuel applications. Examples can be seen in Table 2. Note the high level of overlap in the

results. Also notice how there can be large variations even among related varieties of algae, as seen by the two different species of *Chlorella*.

Table 2:

Lipid Content and Productivities of Different Microalgae Species

Marine and freshwater microalgae species	Lipid Content (% dry weight biomass)	Lipid productivity (mg/L/day)	Volumetric productivity of biomass (g/L/day)	Areal productivity of biomass ($g/m^2/day$)
Ankistrodesmus sp.	24.0-31	-	-	11.5-17.4
Chlorella sp.	10.0-48.0	42.1	0.02-2.5	1.61-16.47/25
Chlorella pyrenoidosa	2.0	-	2.90-3.64	72.5/130
Dunaliella salina	6.0-25.0	116.0	0.22-0.34	1.6-3.5/20-38
Haematococcus pulvialis	25.0	-	0.05-0.06	10.2-36.4
Scenedesmus sp.	19.6-21.1	40.8-53.9	0.03-0.26	2.43-13.52
Spirulina platensis	4.0-16.6	-	0.06-4.3	1.5-14.5/24-51

Adapted from: (Mata et al., 2010)

Potential Limitations. As previously mentioned, it is understood that predators such as rotifers, protozoa and micro-crustaceans are prevalent in the environment and feed on the algal communities, potentially significantly decreasing yields (Kazamia et al., 2012).

> "From a biofuel production standpoint, high amplitude predator–prey oscillations can lead to algal biomass ‘‘crashes,’’ causing large and unpredictable reductions in biodiesel production. For example, microalgal biomass peaks as high as 0.33

> mg L^{-1} chlorophyll a were observed in a Luxembourg lagoon when Daphnia were rare in the water column; in contrast, microalgal biomass declined by more than two orders of magnitude to only 0.001–0.002 mg L^{-1} during periods of maximum Daphnia abundance and grazing intensity." (Smith et al., 2010)

Furthermore viruses could also have a large impact on the yield of algal biofuel production. A virally contaminated stock may get reduced yields, or it could potentially experience a complete population crash. Furthermore, contamination by other bacteria and algae can reduce yields through resource competition.

Additionally, the prime algal growing locations for the U.S. are located in the Southwest portion of the nation and are water scarce. If these regions were to be used as the primary source of algal fuels for the U.S., this could result in a drastic changes in water tables at regional levels as large amounts of water would be transported out of the region.

One study by Yang et al., 2011, quantified the water footprint and nutrients usages during microalgae biodiesel production. They found that it took 3726 kg water, 0.33 kg nitrogen, and 0.71 kg phosphate to produce 1 kg microalgae biodiesel if freshwater is used without recycling (Yang et al., 2011). "Recycling harvest water reduces the water and nutrients usage by 84% and 55%, respectively. Using sea/wastewater as culture medium de- creases 90% water requirement, and eliminates the need of all the nutrients except phosphate" (Yang et al., 2011). So using seawater or wastewater could lead to significant reduction in the amount of water needed, but also greatly increase chances of contamination.

Viruses in Aquatic Environments

According to Suttle, 2005, viruses exist wherever life is found, and are a major cause of mortality in the global ecosystem, impacting the composition of microbial communities and driving global geochemical cycles. Furthermore, they are a reservoir of the greatest genetic diversity on Earth, play an important factor in microbial evolution through mediation of gene transfer, and may be responsible for many of the differences in the genomes of closely related microbes (Sullivan et al., 2003; Suttle, 2005). Viruses can also move between marine and terrestrial reservoirs, necessitating the need to understand both marine and freshwater viruses (Suttle, 2005). As our understanding of the effects of viruses grows, it becomes increasingly evident that they play a major role in global processes.

Marine. Viruses are the most abundant biological entities in global ecosystems, with approximately 10^{30} viruses in the world's oceans; and they almost certainly infect all living things in the ocean, from bacteria to whales (Danovaro et al., 2011; Suttle, 2005). To put the prevalence of oceanic viruses in perspective, they are approximately 10 times more abundant than all the prokaryotes in the ocean combined, they have the equivalent carbon of 75 million blue whales, and if the viruses in the ocean were stretched end to end, they would span farther than the nearest 60 galaxies (Danovaro et al., 2011; Suttle, 2007). According to Danovaro et al., (2011) the, "viral abundance in marine waters ranges from about 10^{7} to 10^{10} L^{-1}, and 10^{7} to 10^{10} g^{-1} of dry weight in marine sediments." Furthermore, there are approximately 10^{23} viral infections every second in the ocean (Suttle, 2007).

Recent studies have shown that marine viruses play critical roles in shaping aquatic communities and determining ecosystem dynamics (Danovaro et al., 2011). Viruses are major pathogens of planktonic organisms and consequently are significant players in nutrient and energy cycling. Furthermore, there is good evidence that some viruses move between marine and terrestrial reservoirs. Recognizing that viruses play a major role in marine ecosystems has added a significant new dimension to the understanding of biological oceanographic processes (Suttle, 2007).

Freshwater. Although freshwater environments are more important than marine systems in terms of their influence on human activities, microbial communities (including viruses) in freshwater environments have received surprisingly little attention relative to their marine counterparts (Wilhelm et al., 2006)

Freshwater cyanophages are poorly characterized in comparison to their marine counterparts, however, the level of genetic diversity that exists in freshwater cyanophage communities is likely to exceed that found in marine environments, due to the habitat heterogeneity within freshwater systems. Many cyanophages are specialized for infecting a single host species or strain; however, some are less fastidious and have broad host range resulting in infection of a number of different genotypes of a single species or even multiple species from different genera. (Watkins et al., 2014)

Morphology.

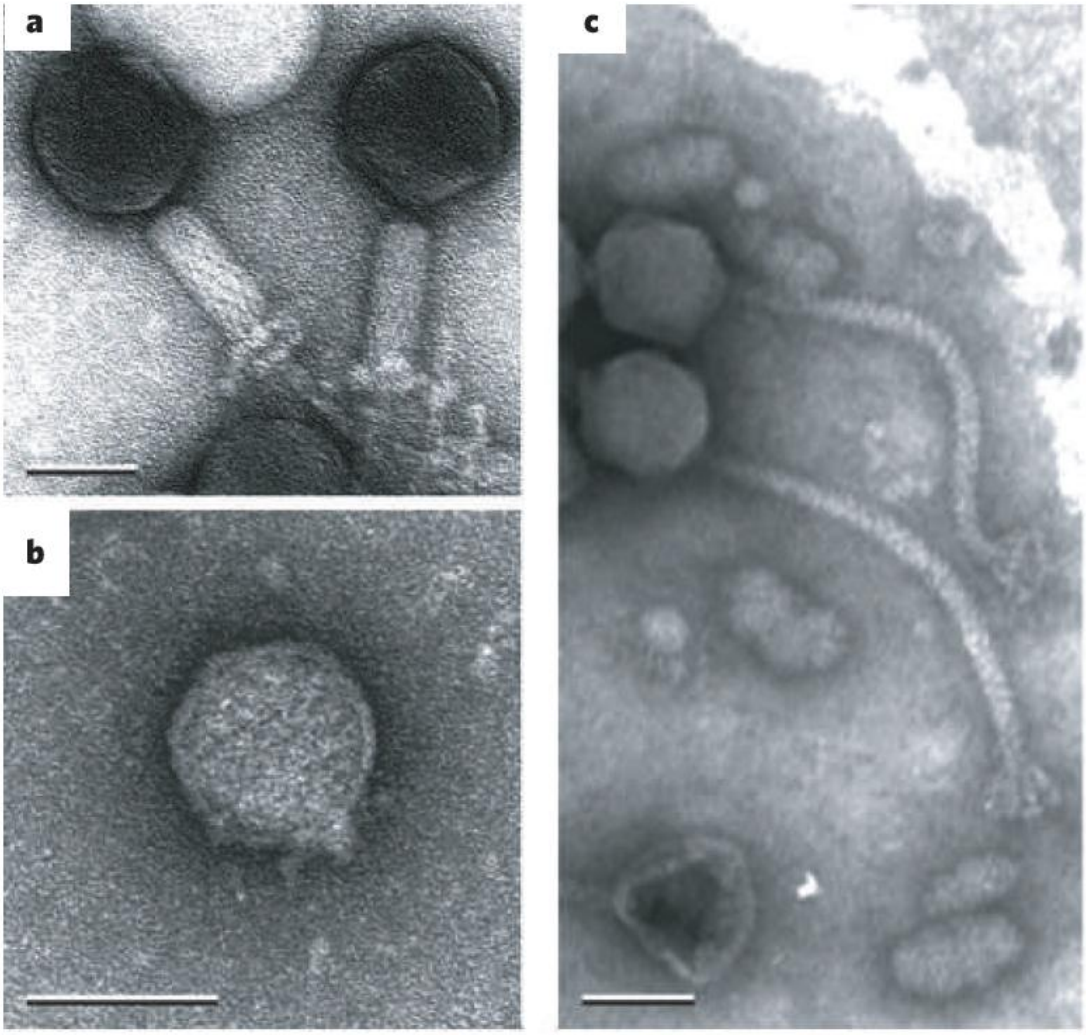


Figure 3. "The three families of tailed dsDNA viruses (phages) that infect bacteria. (a) Myoviruses are often the most commonly isolated phage from natural marine viral communities. They have contractile tails, are typically lytic and often have relatively broad host ranges. (b) Podoviruses have a short non-contractile tail, are also typically lytic and have very narrow host ranges. They are less commonly isolated from seawater. (c) Siphoviruses have long non-contractile tails. They are frequently isolated from seawater, often have a relatively broad host range, and many are capable of integrating into the host genome. Scale bar, 50 nm." (Suttle, 2005)

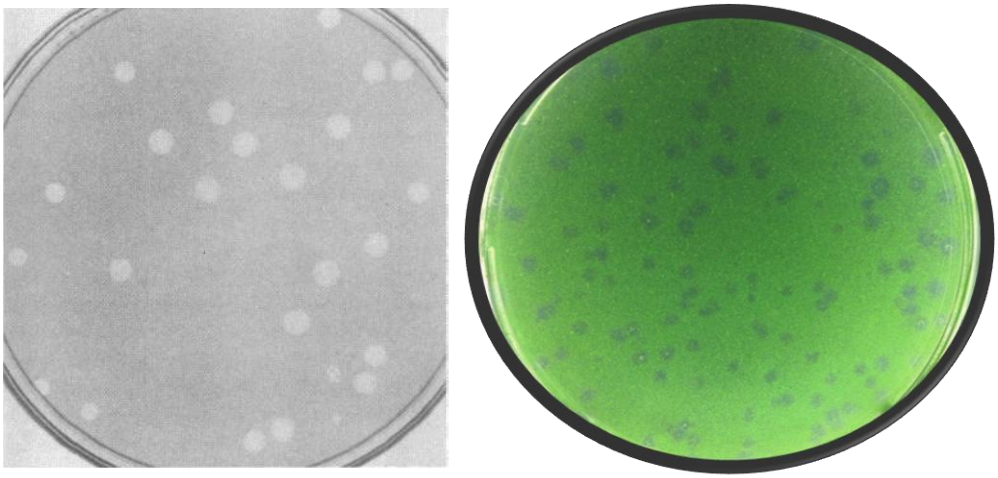
Specificity and Sensitivity of Viral Infectivity. It has been found that most viruses in seawater seem to be infectious (Wilhelm et al., 1998), and "some can remain

infectious in sediments for long periods, from decades to a hundred years or more" (Suttle, 2005). Rate of infectivity can vary greatly depending on the conditions. Suttle et al., 1994 found that as low as 1% of viral collisions with of *Synechococcus* DC2 resulted in infection in near shore waters, but that almost all collisions in offshore waters resulted in infection. Viruses are known to infect every major algal phylum, causing cellular lysis upon completion of each virus life cycle (Lawrence, 2005). Some viruses infect a single host species or strain; however, some are able to infect a number of different host genotypes within the same species or even hosts from different genera (Watkins et al., 2014).

Detection Methodologies. Five methods are used to estimate the abundance of viruses in aquatic samples: plaque assays (PAs); most-probable- number assays (MPNs); transmission electron microscopy (TEM); epifluorescence microscopy (EfM); and flow cytometry (FC). Which procedure is used depends on the question being addressed and the accuracy and sensitivity that is required (Suttle, 2007).

Culture Based Techniques. The detection and quantification of viral contamination can be measured through an algal plaque assay procedure adapted from Van Etten et al., 1991. A plaque assay is a standard method to determine viral concentrations in a sample. It is a double layer agar assay, where the bottom agar contains growth media, and the top agar hosts a microbial lawn that is infected with viruses. The viruses lyse infected cells causing circular clear zones (plaques) to form. Each clear zone is counted as one plaque forming unit (PFU), with the viral concentrations being recorded as PFUs/volume. Plaques may be visualized as early as 24

hours after plating, or they may take several days to appear. This is dependent on the host, virus, and growth conditions. Examples of a plaque assay can be seen below in Figure 4.



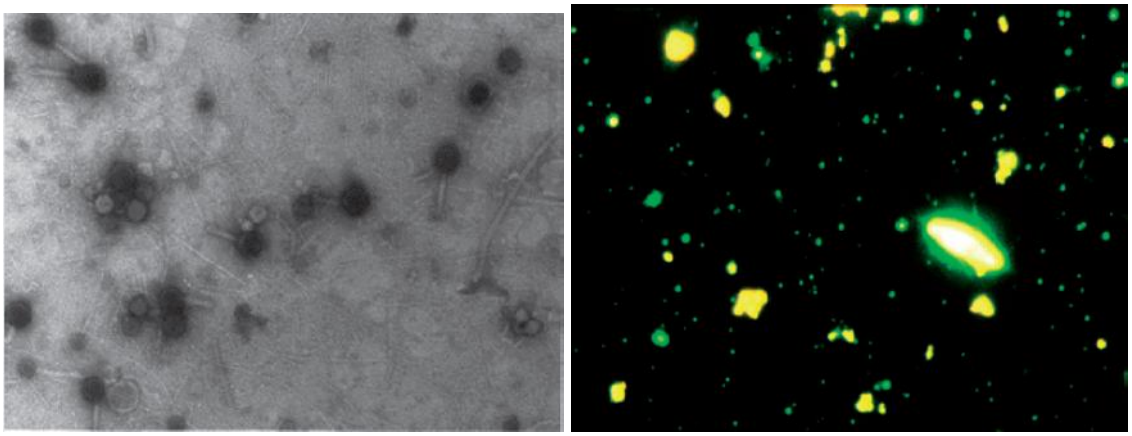
a b

Figure 4: Plaque assay of (a) PBCV-1 virus on a lawn of *Chlorella* strain NC64A (Van Etten et al., 1991). (b) AZ-TTL3

MPNs are used for cells that are cultivable, but which cannot be grown on solid substrates, and use a series of dilutions, with ten or more replicates at each dilution. The replicates in which no growth, or growth followed by cell lysis, occurs are assumed to contain at least one infectious virus. The number of replicates at each dilution in which lysis occurred can be used to calculate the number of infective units in the original sample. PAs and MPNs are the only methods that can be used to directly determine the abundance of infectious viruses, and they can also be used to obtain and purify specific

viral isolates. However, these methods provide no information on the total abundance of viruses in a sample. (Suttle, 2007)

Molecular Techniques.



a b

Figure 5. (a) Shows a transmission electron micrograph of a natural virus community and (b) shows an epifluorescence micrograph of a seawater sample that has been stained with YO-PRO-1 (Suttle, 2007)

> "TEM is the only method that provides data on both the abundance and morphology of virus-like particles (a). The viruses must be concentrated from seawater, deposited on a supporting grid and stained with an electron-dense material, such as uranyl acetate. This approach has the advantage that particles that resemble viruses can be identified and quantified. However, there are many technical aspects that are involved with concentrating, staining and visualizing the viruses, which can lead to variable and inaccurate estimates of the total abundance. The TEM approach has largely been superceded by EfM, except where data on the morphology of the virus particles are required.
>
> EfM is currently the most widely used approach for estimating the total abundance of virus particles. In this method, the viruses are concentrated on a membrane filter, their nucleic acids are stained with a brightly fluorescent dye and the abundance of viruses is estimated by EfM (b). The first estimates of viral abundances that were made by EfM used DAPI (4′, 6-diamidino-2-phenylindole), although the fluorescence was near the limit of detection for many microscopes. Subsequently, a new generation of brightly fluorescent dyes, such as YO-PRO11 and SYBR Green , have made accurate and high-precision counts routinely obtainable. However, many estimates have been derived from samples that were

> inappropriately preserved for EfM, and consequently much of the data in the literature are underestimates.
>
> Most recently, FC has been used to estimate viral abundances. This accurate high-throughput method also allows the quantification of subpopulations of viruses that differ in their characteristics of fluorescence and light scattering FC allows large numbers of samples to be analysed quickly, which should begin to supply us with a synoptic picture of the distribution and abundance of viruses in the sea.
>
> There is now high confidence in the estimates of the abundance of free double-stranded DNA viruses that are provided by EfM and FC. However, even our current estimates are too low because of the presence of RNA and single-stranded DNA viruses that occur in the sea but that cannot be resolved using the currently available methods. In addition, viruses that are attached to particles can be abundant, but are difficult to quantify by EfM and will be missed by FC. Despite these caveats, our ability to accurately quantify viruses in aquatic samples has improved vastly over the past 15 years." (Suttle, 2007)

Impact of Viral Infectivity on Microbial Growth. Suttle (1994) found that, "about 20% of marine heterotrophic bacteria are infected by viruses and 10-20% of the bacterial community is lysed daily by viruses. The effect of viruses on phytoplankton is less certain, but ca. 3% of *Synechococcus* biomass may be lysed daily. The fraction of primary productivity this represents depends upon the relative biomass and growth rate of *Synechococcus*. Virus enrichment experiments suggest that the productivity of eukaryotic phytoplankton would be ca. 2% higher in the absence of viruses. Overall, probably about 2-3% of primary productivity is lost to viral lysis. There is considerable variation about these estimates; however, they represent a starting point for incorporating viral-mediated processes into aquatic ecosystem models"

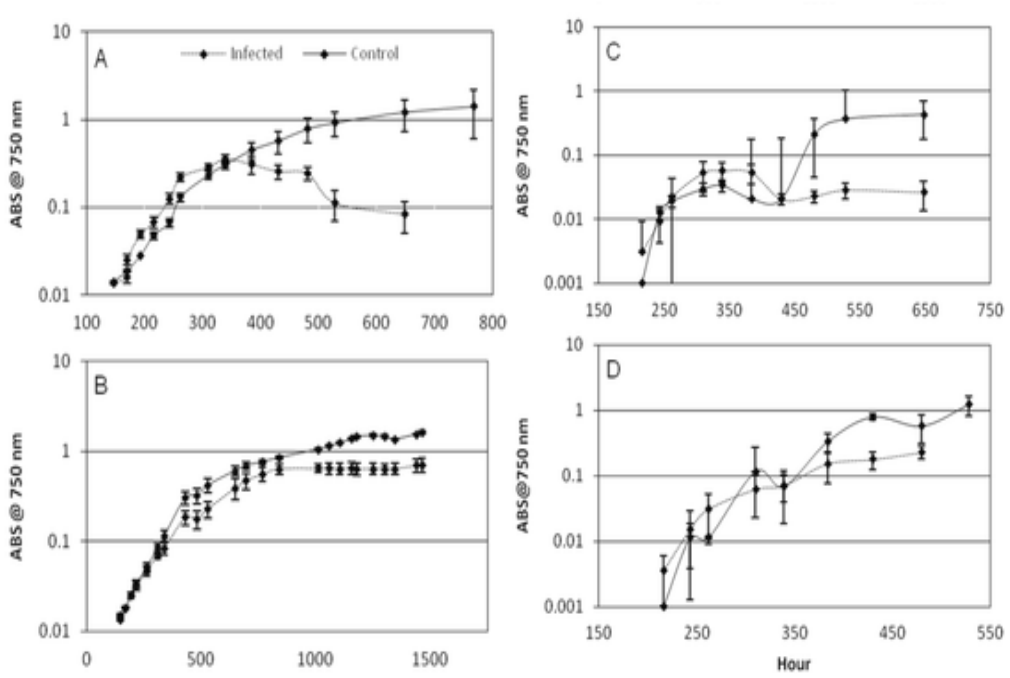


Figure 6. Lysis profiles generated by infecting five strains of cyanobacteria with ϕMHI42, as determined by measuring absorbance at 750 nm. (a) *M. aeruginosa* BC84/1. (b) *M. aeruginosa* 1450/8. (c) *Planktothrix agardhi* 137. (d) *Planktothrix rubescens* 9316. Adapted from (Watkins et al., 2014)

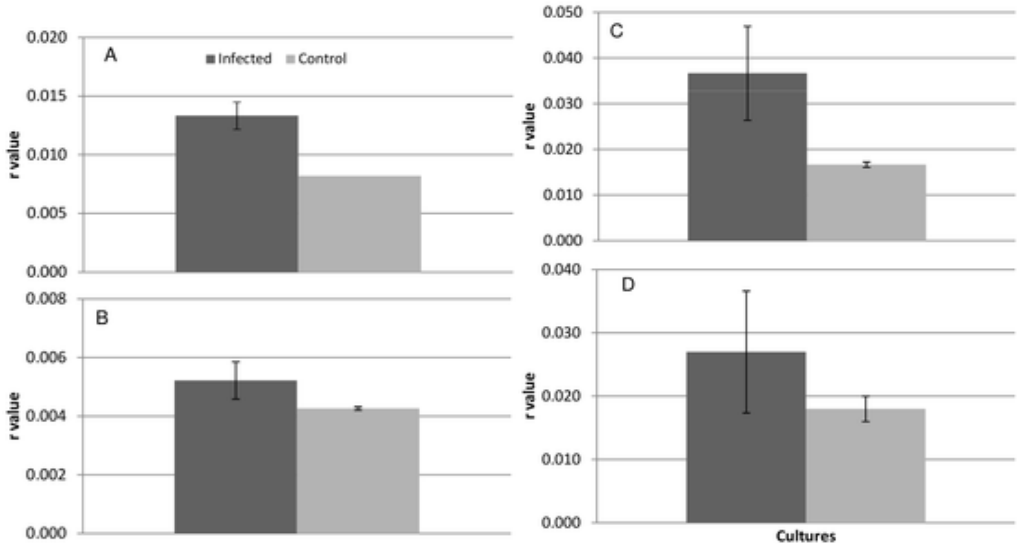


Figure 7. Statistical analysis based on mean r values for the replicates cultures of each strain infected by ϕMHI42. (a) M. aeruginosa BC84/1. (b) M. aeruginosa 1450/8. (c) Planktothrix agardhii 137. (d) Planktothrix rubescens 9316. Adapted from (Watkins et al., 2014)

In Figures 6 and 7 virus ϕMHI42 can be seen to impact the growth of four different cyanobacteria, ultimately causing a reduction in growth. The authors found that:

> "That four different strains of cyanobacteria can exhibit different responses to infection by the same cyanophage, is likely to be related to the concepts of 'ecotypes' and niche separation in the environment. The existence of ecotypes has been clearly demonstrated in cyanobacteria, where strains with similar or identical 16S rRNA gene sequences can demonstrate considerable differences in phenotype. In part, the niche occupied by particular ecotypes can be defined by the composition of phage community, i.e. resistance or susceptibility of individual cyanobacterial lineages to phage communities will determine whether or not they are able to grow at that location. The interaction between the host and any infecting phage is dynamic and often described as an ''arms race'' where the level of exposure to the host and to other phages infecting the same host may all play a role in phage infection dynamics, which in turn will influence the evolution of host defence against phage attack. How the host response to attack by phage is likely to have developed is less clear, and further investigation is warranted to assess the presence of host restriction modification enzymes or CRISPR/cas systems in resistant strains. The presence and overgrowth of contaminating bacteria in response to lysis of the dominant member of a non-axenic culture highlights further difficulties associated with examining cyanobacterial infections and subsequent physiological aspects of phage characterisation. Cyanobacteria obtained from culture collections are rarely axenic, and growth conditions selecting against the growth of the cyanobacterium will often result in rapid growth of the contaminants. Non-axenic overgrowth was observed in three of the four cyanobacterial cultures used for lysis profiles, P.agardhii being the only culture not demonstrating this trait.
>
> The instability observed during the infection cycle of M. aeruginosa 1450/8 may have resulted solely from the overgrowth by the normally background-level bacterial contaminants present in these non- axenic cultures, however, it may also relate to the unstable nature of the type of phage growth that allows the simultaneous survival of the host and the production of viable virions. Retardation of growth in M. aeruginosa 1450/8 in response to infection, as opposed to acute lysis, may suggest a state of chronic infection or pseudo- lysogeny (Watkins et al., 2014)."

CHAPTER 3 - IMPACT OF VIRAL INFECTIVITY ON ALGAL AND CYANOBACTERIA CELLS IN BIOREACTORS

Abstract

Research in microbial biofuels has dramatically increased over the last decade. The bulk of this research has focused on increasing the production yields of cyanobacteria and algal cells and improving extraction processes. However, there has been little to no research on the potential impact of viruses on the yields of these phototrophic microbes for biofuel production. Viruses have the potential to significantly reduce microbial populations and limit their growth rates. It is therefore important to understand how viruses affect phototrophic microbes and the prevalence of these viruses in the environment. For this study, phototrophic microbes were grown in a bioreactor consisting of 22 inch long and 1.5 inch in diameter glass tubes with continuous aeration. In addition, miniaturized bioreactors were developed to increase replicates and minimize use of reagents. Detection and quantification of viruses of both environmental and laboratory microbial strains were measured through the use of a plaque assay. Several environmental samples were taken from Tempe Town Lake (Tempe, AZ) and all the samples tested positive for viruses. Virus free phototrophic microbes were obtained from plaque assay plates by using a sterile loop to scoop up a virus free portion of the microbial lawn and transferred into a new bioreactor. Isolated cells were confirmed virus free through subsequent plaque assays. Viruses were detected from the bench scale bioreactors of cyanobacteria *Synechocystis* sp. PCC 6803 and the environmental samples. Plaques from the environmental samples were found to be significantly larger than those

from the laboratory bioreactor. Viruses were consistently present through subsequent passage in fresh cultures; demonstrating viral contamination can be a chronic problem. In addition TEM was performed to confirm viral attachment to cyanobacterial cells and to characterize viral particles morphology. Electron micrographs confirmed viral attachment and that the viruses detected were all of a similar size and shape. Particle sizes were measured to be approximately 50-60 nm. Cell reduction was observed as a decrease in optical density, with a transition of color in the bioreactors from a dark green to a yellow green color. Phototrophic microbial viruses were demonstrated to persist in the natural environment and to cause a reduction in algal populations in the bioreactors. Therefore it is likely that viruses could have a significant impact on microbial biofuel production by limiting the yields of production ponds.

Objectives

The overall objective of this study is to characterize the impact of viral infectivity on phototrophic microbes for biofuel applications. More specifically;

- To identify wild type cyanophages capable of infecting phototrophic microbes
- To characterize cyanophages isolates using virological techniques
- To measure the impact of viruses on the growth rate of phototrophic microbes in laboratory bioreactors
- To examine the presence and viral attachment to infected cells using Transmission Electron Microscopy (TEM)

Materials and Methods

Phototrophic Microbes' Growth Conditions. Phototrophic Microbes were grown in a bioreactor consisting of 22 inch long and 1.5 inch diameter glass tubes with an aeration tube (~5 mm diameter) reaching to the bottom of the reactor glass tube. All the components were autoclaved/sterilized before assembly of each of bioreactor. The open end of each glass tube was plugged using stacked layers of cheese cloth for controlling aerosolization and also to minimize chances of contamination (Figure 8). All the bioreactors were incubated at room temperature and operated under continuous light and aeration. For aeration air was filtered through bubble humidifiers containing autoclave sterilized DI water and the flow was controlled using air flow regulators used in aquariums.

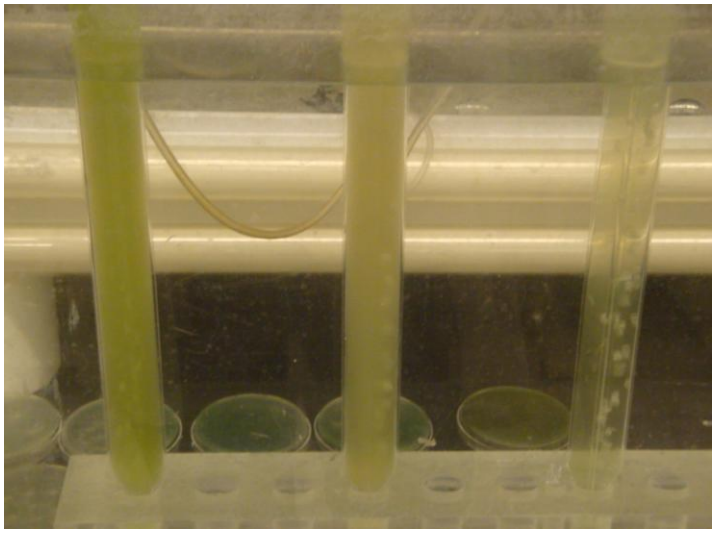


Figure 8. Bioreactor

Additionally miniaturized bioreactors were assembled to increase the number of replicates and minimize use of reagents (Figure 9)

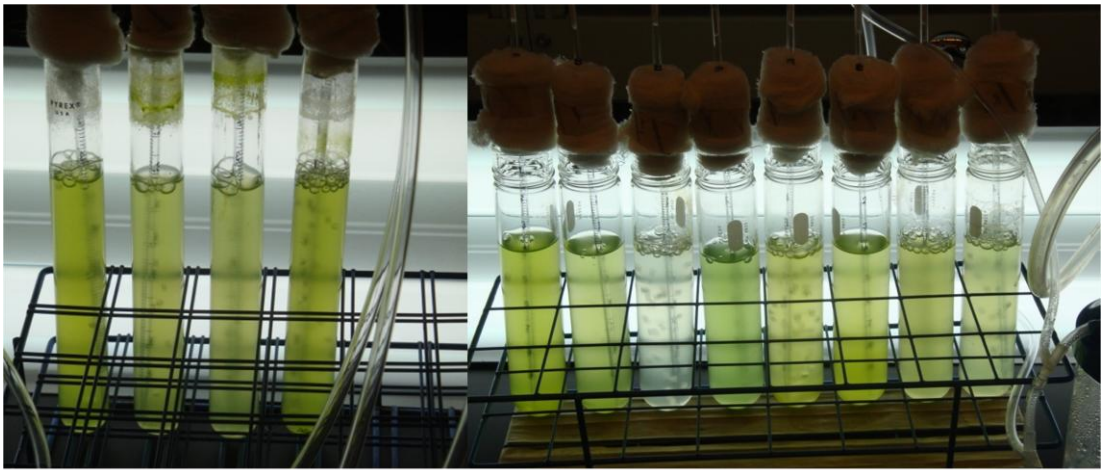


Figure 9. Miniaturized bioreactors. (a) Culture volumes up to 50mL. (b) Culture volumes up to 100mL.

BG-11 Growth Media, ATCC Medium 616. In order to make BG-11 several stock solutions were prepared according to the composition given in Tables 3, 4 and 5. Briefly, to make 1L of trace mineral stock solution, 900 ml of deionized (DI) water was taken in a 2L glass flask. All the ingredients were added to the flask in the order shown in Table 3. Each component was mixed until completely dissolved before adding the next ingredient. Additional DI water was added to bring the final volume of the trace mineral stock solution to 1L. Similarly, to make 1L of BG-11 Stock solution (100X) (without iron, phosphate and carbonate), 850 mL DI water was taken in a 2L glass flask and sodium nitrate magnesium sulfate, calcium chloride, citric acid and NaEDTA were added in the amounts shown in Table 4. After dissolving all the salts, 100mL of the trace mineral stock solution was added and completely mixed. Finally, additional DI water was added to bring the final volume of the 100X BG-11 stock solution to 1L. The flasks were sealed with aluminum foil and labeled with autoclave tape. The stock solution was sterilized using a validated autoclave at $1kg/cm^2$ (15psi), 121°C, for 15 minutes. The solutions were allowed to cool before transferring to labeled sterilized sealed containers for storage.

The working solution of BG-11 was prepared according the composition shown in Table5. Briefly, 1mL each of the stock solutions of ferric ammonium citrate, sodium carbonate, and potassium phosphate was taken in 1L graduated cylinder. Then 10 mL of the 100x BG-11 stock solution was added and mixed by swirling movement. Finally, additional DI water was added to bring the final volume of the BG-11 working solution to 1L. The final pH was adjusted to 7.1 after autoclaving using 1N HCl.

Table 3:

Composition of the Trace Minerals Stock Solution

The following reagents were added to 1 liter of deionized water	
Reagents	Amount added
H_3BO_3 (Boric Acid)	2.86 g
$MnCl_2 \cdot 4H_2O$ (Manganese Chloride)	1.81 g
$Na_2MoO_4 \cdot 2H_2O$ (Sodium molybdate)	0.22 g
$ZnSO_4 \cdot 7H_2O$ (Zinc sulfate)	0.39 g
$CuSO_4 \cdot 6H_2O$ (Cupric Sulfate)	0.079 g
$Co(NO_3)2 \cdot 6H_2O$ (Cobalt nitrate)	0.049 g

Table 4:

Composition of 100x BG-11 Stock Solution without Iron, Phosphate, or Carbonate

Recipe

The following reagents were added to 850 mL of deionized water	
Reagents	Amount added
$NaNO_3$ (Sodium nitrate)	149.6 g
$MgSO_4 \cdot 7H_2O$ (magnesium sulfate)	7.5 g
$CaCl2 \cdot 2H_2O$ (Calcium chloride)	3.6 g
Citric acid	0.6 g
NaEDTA (0.25 M, pH 8.0)	1.12 mL
Trace Minerals	100 mL

Table 5:

Composition of BG-11 Liquid Medium Working Solution

Chemical Solution	Amount added
1000x Ferric ammonium citrate (0.6 g $C_6H_8O_7$.xFe.xNH_3 per 100 mL H_2O)	1 mL
1000x Na_2CO_3 (Sodium carbonate) (2.0 g Na_2CO_3 per 100 mL H_2O)	1 mL
1000x K_2HPO_4 (Potassium Phosphate dibasic) (3.05 g K_2HPO_4 per 100 mL H_2O)	1mL
100x BG-11 no iron, phosphate, or carbonate	10 mL
Deionized Water	1 L

Table 6:

For BG-11 Solid Agar Plates

Sodium Thiosulfate anhydrous	1 mL/L	31.7 g/200 mL dH2O	1 mM
Difco Bacto-agar	15.0 g/L		

Environmental Sampling. A minimum of 1 L of environmental water sample was collected in autoclave sterilized polycarbonate 1 L Nalgene bottles. The 1 L samples were concentrated by centrifugation at 3000 rpm (1863x g) for 10 minutes. Samples were centrifuged using the SLC-4000 rotor in a Sorvall RC 5C centrifuge. Excess water was then removed carefully not to disturb the pellet. Centrifugal concentration was continued until the desired total volume was achieved. The pellet was then resuspended. BG-11

medium was added if additional fluid was necessary to bring the total volume to the desired level. The concentrated sample was then analyzed using a plaque assay.

Plaque Assay. A plaque assay is a standard method to determine viral concentrations in a sample. A microbial lawn was grown on a BG-11 solid agar plate and infected with viruses. Viruses lyse infected cells causing circular clear zones (plaques) formation. Each clear zone is counted as one plaque forming unit (PFU). It takes approximately 3-7 days for plaques to be visualized. Concentrations are recorded as PFUs/volume. BG-11 growth media was used to make both the top and bottom agars. The plates consisted of 15 mL of BG-11 with 1.5% agar by weight. The top agar consisted of 5 mL BG-11 with 0.7% agar by weight. Six hundred μL of concentrated cyanobacteria or algae and 100 μL of the viral stock were added to the molten top agar, mixed and poured onto the bottom agar. The cyanobacteria or algae cells in concentrated samples spread evenly on bottom agar forming a uniform microbial lawn providing host population for the viruses in sample. Plates were incubated at 25°C under continuous direct florescent light. Under favorable conditions, the host population (algae or cyanobacteria) start to die/lyse as a result of virus infection, which is manifested by the plaque formation.

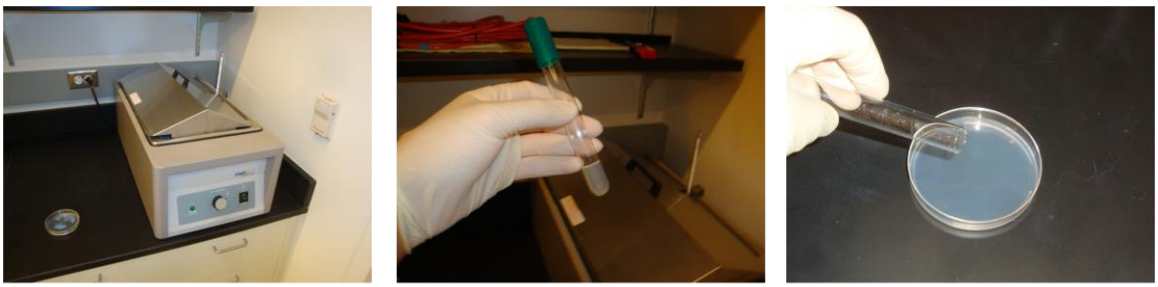


Figure 10. Plaque Assay Procedure. (a) Water bath at 50°C. (b) Top agar. (c) Pouring mixed top agar into plate.

Virus Isolation. Viruses were isolated by scooping plaques from a positive plate using a sterile loop. The loop was flame sterilized, and allowed to cool before being used. Recovered plaques were placed into 1.5 mL autoclave sterilized microcentrifuge tubes containing 0.5x PBS. The samples were then vortexed for 10 seconds to suspend the viruses. The plaques were suspended in the PBS solution for 30 minutes to allow viruses to diffuse out of the agar. The samples were then purified through centrifugation. The samples were centrifuged at 10,000x g for 3 minutes at 4°C using a Sorvall Biofuge primo R centrifuge. Then 900 μL of the supernatant was removed, taking care not to disturb the pellet, and transferred to a new sterile microcentrifuge tube before being centrifuged again. Then 800 μL of the supernatant was removed and placed into new sterile 1.5 mL microcentrifuge tube for storage in a 4°C refrigerator.

Microbial Isolation. Virus free microbial cells were obtained from positive plaque assay plates by using a sterile loop to scoop up a virus free portion of the microbial lawn and transferred into a new bioreactor for culturing. Once the culture had matured, it was confirmed virus free through subsequent plaque assays

Growth Rate Measurement. Growth rate measurements were obtained through measuring the optical densities of the cultures using a Hach DR5000 spectrophotometer (Loveland, Colorado) at the 730 nm wavelength using 1 mL samples placed in 1.5 ml cuvettes. Acceptable readings were between 0 to 1 absorbance units (AU). If the absorbance levels were too high then the substrate was diluted using BG-11 media to bring it into an acceptable level. If they were too low, then the sample was concentrated through centrifugation. The growth media BG-11 was used as the blank for these tests.

A dry weight calibration curve was constructed for the absorbance range of 0.1 to 1 for *Synechocystis* sp. PCC 6803. A culture of *Synechocystis* was diluted to obtain 5 different samples, 8 mL each, with the absorbances of ~0.1, 0.325, 0.525, 0.725 and 1 AU. Whatman Glass Microfiber Filters GF/C (47 mm diameter) were used for this test. Each filter was weighed before use. Five mL of each sample was then passed through an assigned filter and allowed to dry overnight in a desiccator. Twenty four hours later, the filters were reweighed. The dry weight was calculated by subtracting the initial filter weight from the final filter weight. The dry weights were then compared graphically to their corresponding absorbance's to obtain the dry weight calibration curve as (Figure 15).

Fluorescence Excitation-Emission Matrix Spectroscopy (EEM). EEM measurements were conducted using a Horiba Aqualog device (Kyoto, Japan). Three mL samples were loaded into a 3.5 mL quartz cuvette for testing. The cuvette was stored in a 10% HCl acid solution, and was washed clean with nanopure water before each use. The excitation range was set to 200 – 500 nm with a step size of 3 nm. The emissions range measured was set to 200 – 600 nm with a step size of 1 nm. The data recorded was run through the inner-filter effect correction factor and 2nd order Rayleigh Masking, before being graphed as a contour map by the devices software.

Results and Discussion

Repeat sampling of field sites demonstrated that viruses persist in the natural environment. Based on the plaque morphology two types of cyanophages were identified in the lab samples and designated as AZ-CP1 and AZ-CP 2. These isolates caused a

reduction in phototrophic microbial populations in the laboratory bioreactors. Additional isolates of algal phages were detected from Tempe Town Lake and designated as AZ-TTLV. In Figure 11, plaques can be seen growing in these environmental samples, indicating viral presence in the water samples.

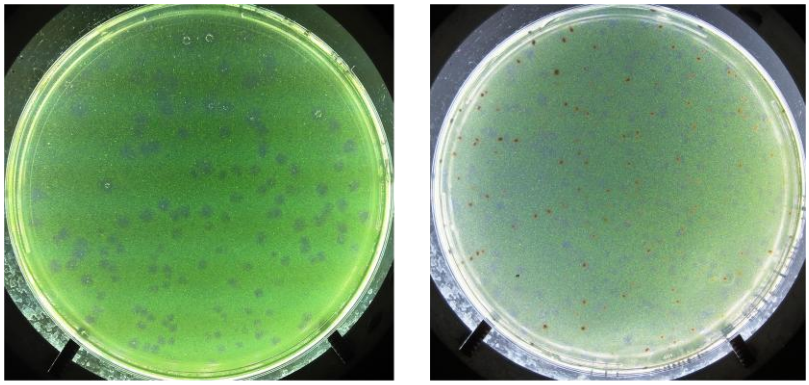


Figure 11. Tempe Town Lake Samples collected on different dates tested positive for algal phages. (a) 11/8/2012. (b). 2/27/13.

Furthermore, viral contamination was detected throughout the year, over the course of several samplings. A virus infected *Synechocystis* PCC 6803 was also detected and isolated. In Figure 12, (a) shows a high number of viruses present on a culture plate of *Synechocystis*, and (b) is another plate of *Synechocystis* infected with the isolated virus AZ-CP2.

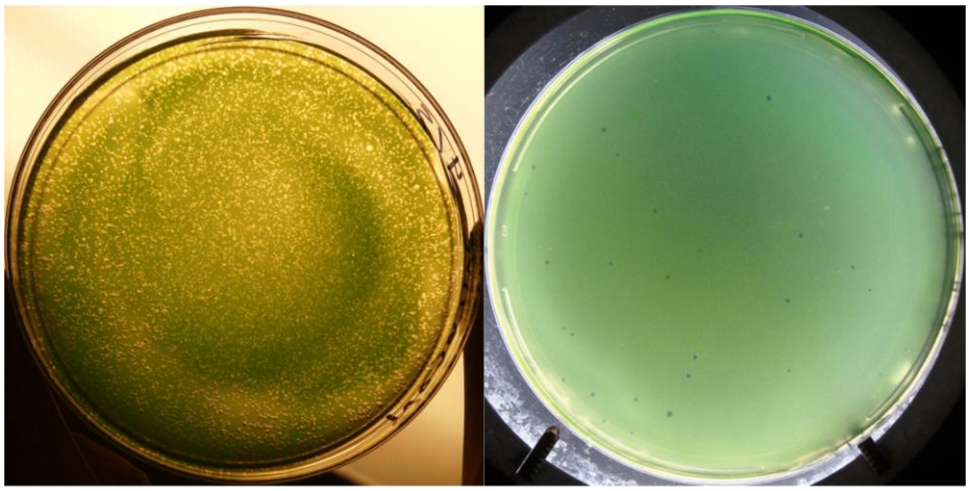


Figure 12. Infected *Synechocystis* PCC 6803 (a) Spiked with AZ-CP1. (b) Spiked with AZ-CP1.

Cyanophages were further characterized by documenting the plaque progression pattern. In Figure 13, a plaque was examined over the course of several days under a light microscope at 100x magnification. The plaque size increases with time, as the virus spreads outward from the center of the plaque.

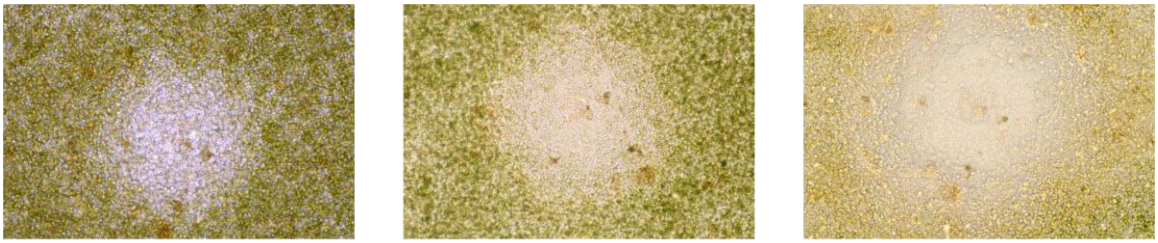


Figure 13. Plaque Progression of AZ-CP2 in host Synechocystis PCC 6803 at 100x magnification. (a) Day 4. (b) Day 5. (c) Day 6.

Different concentrations of top agar were evaluated, to examine the impact of percent of agar in plaque formation size. The top agar concentration levels tested were

0.5%, 0.7%, 1% and 1.5% agar by weight. For the *Synechocystis* PCC 6803 tests conducted with viruses AZ-CP1 and AZ-CP2 there was no discernible difference in plaque size. These findings were mirrored in the Tempe Town Lake sample experimental results. The results are demonstrated in Figure 14, where the plaque is actually larger in the higher percentage agar plate. Whereas initially it was thought that higher agar levels would result in smaller plaque sizes, due to increased resistance to viral transmission through the denser agar. However, the size differences were found to be negligible, and could not be distinguished from normal plaque size variation.

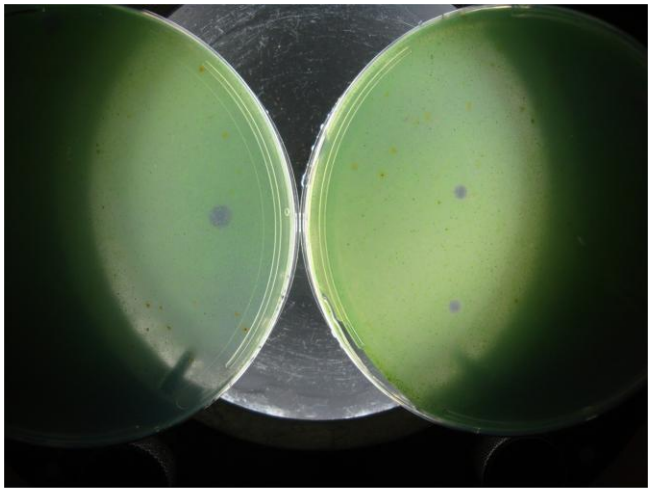


Figure 14. Impact of varying agar concentration on plaque formation of AZ-TTLV (a) 1% agar. (b) 0.5% agar

After initial characterization of AZ-CP1 and AZ-CP2 phages, additional experiments were performed to study the impact of cyanophages' infection on the growth and yield of phototrophic microbes commonly used for biofuel production. The results of the dry weight calibration curve conducted for *Synechocystis* PCC 6803 are summarized

in Figure 15. The data is linear as seen by the high R^2 value of the calculated linear regression lines, and therefore provides an accurate means of converting the values between UV absorbance and dry weight for the ranges of 0.1 to 1 AU.

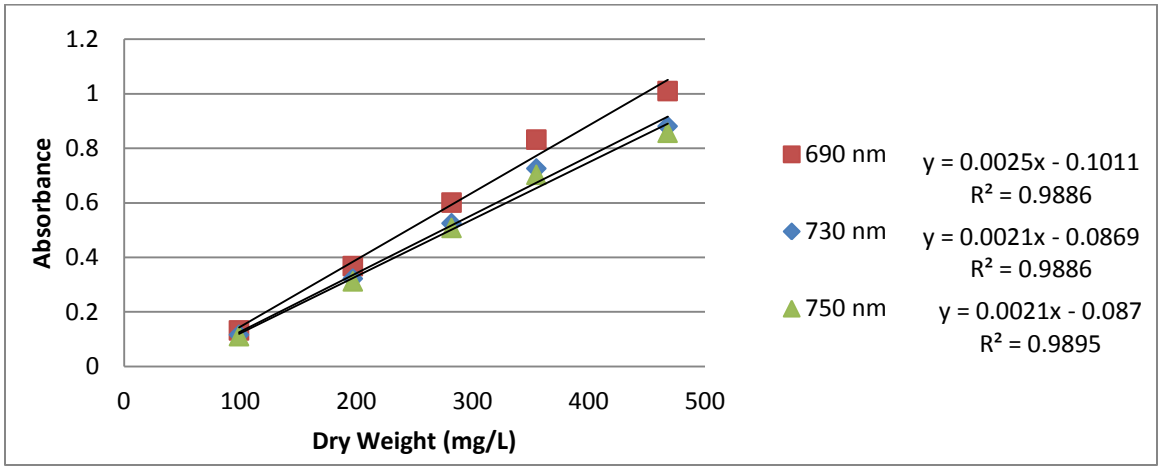


Figure 15. Dry Weight Calibration Curve of *Synechocystis* PCC 6803

The results for the first non-aerated growth rate comparison of virally infected and virus free *Synechocystis* PCC 6803 are shown in Figure 16. All the experiments were performed in triplicate and each data point is the average of three replicates. The virally infected cultures unexpectedly had higher initial growth rates than the virus free cultures. This is most likely due to the viruses tweaking the host metabolism, by up regulating it in order to increase viral replication just after initiation of the infection process. This may have caused the virally contaminated cultures to experience higher initial growth rates until viral replication kicks into full swing. Once the host cell machinery is hijacked for viral reproduction it results in the consumption of most cell metabolites/reservoirs for synthesis of new virus particles. Increasing cell lysis of the virally infected cultures further reduces *Synechocystis* PCC 6803 growth with elapsed time after infection.

However, both cultures' microbial densities seem to plateau rather quickly. This may have been due to a lack of nutrients. The reactors were kept sealed except for sampling. This may have caused the reactors to become depleted of CO_2, robbing the cyanobacteria of a carbon source required for routine cell metabolism and replication.

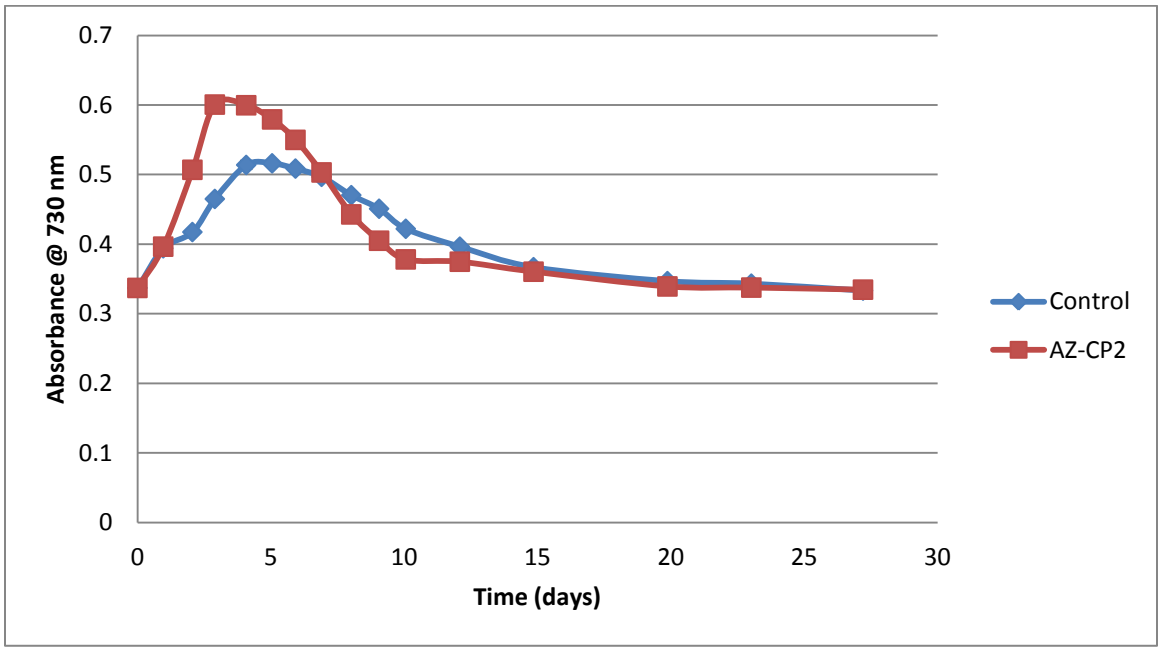


Figure 16. Growth Rate Comparison of *Synechocystis* PCC 6803 Infected with AZ-CP2 Phage (Non-aerated)

In order to ensure that nitrogen was not the limiting growth nutrient in the system, a nitrogen balance was calculated for the control bioreactors.

Total g N per L BG-11 = 0.04063 g

Total g N per 50 mL reactor = 0.002031 g

Table 6:

Synechocystis sp. PCC 6803 Nitrogen Utilization Rate

		Day		
Variable	Unit	0-3	3-9	9-12
Biomass production rate	g DW / L day	0.116	0.744	0.760
Nitrogen utilization rate	g N / L day	0.015	0.096	0.098

Adapted from: (Kim et al., 2011)

From the dry weight calibration curve we have the following equation:

$$y(Abs) = 0.0021x\left(\frac{mg\ DW}{L}\right) - 0.0869$$

$$x\left(\frac{mg\ DW}{L}\right) = \frac{y(Abs) + 0.0869}{0.0021}$$

$$x\left(\frac{g\ DW}{L}\right) = \frac{y(Abs) + 0.0869}{0.0021} \times \frac{1\ g}{1000\ mg}$$

Dry weight for days 0 and 3 were calculated:

$$Day\ 0\ DW = \frac{0.337 - 0.0869}{0.0021 \times 1000} = 0.2019\ \frac{g\ DW}{L\ day}$$

$$Day\ 3\ DW = \frac{0.4649 - 0.0869}{0.0021 \times 1000} = 0.2628\ \frac{g\ DW}{L\ day}$$

$$Bimass\ production\ rate = \frac{0.2628\ - 0.2109}{2.918\ days} = 0.02087 \frac{g\ DW}{L\ day}$$

$$Average\ of\ N\ utilization\ rates\ from\ literature = \frac{0.015 + 0.096 + 0.098}{0.116 + 0.744 + 0.760}$$

$$= 0.1284 \frac{g\ N/_{L\ day}}{g\ DW/_{L\ day}}$$

$$N\ utilization\ rate = 0.1284 * 0.02087 = 0.002680 \frac{g\ N}{L\ day}$$

$$N\ utilization\ rate\ per\ reactor = 0.002680 \frac{g\ N}{L\ day} \times \frac{1\ L}{1000\ mL} \times 50\ mL = 1.34 \times 10^{-4} \frac{g\ N}{day}$$

$$Days\ it\ would\ theoritcally\ take\ to\ run\ out\ of\ N\ at\ this\ rate = \frac{0.002031\ g\ N}{1.34 \times 10^{-4} \frac{g\ N}{day}}$$

$$= 15.16 days$$

The peak population density was recorded at day 6 for the control reactors, which is before the calculated depletion of nitrogen in the reactors. Therefore, nitrogen is not the limiting nutrient for the growth of *Synechocystis* in the bioreactors. Instead the limiting factor is most likely carbon as previously stated.

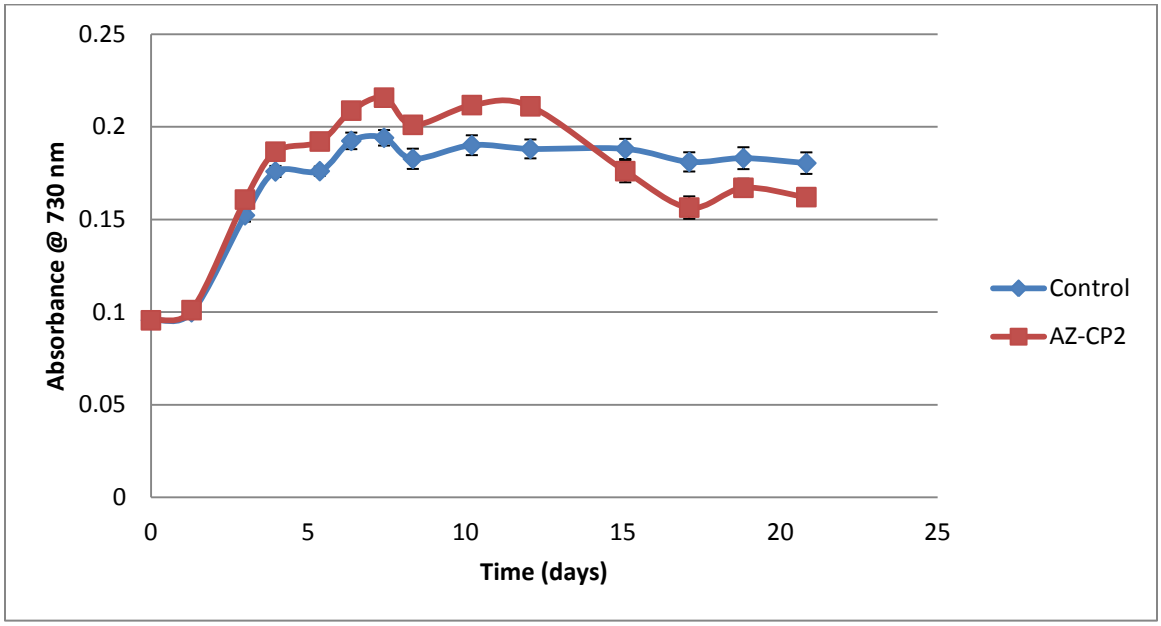


Figure 17 Growth Rate Comparison of *Synechocystis* PCC 6803 Infected with AZ-CP2 Phage (Non-aerated)

In Figure 17, the non-aerated growth rate comparison was repeated with a few changes:

- The bioreactors' caps were left partially open for 30 minutes every day to allow for air exchange, in an attempt to prevent CO_2 depletion.
- The initial starting concentration of *Synechocystis* PCC 6803 was reduced in a further attempt to decrease any potential nutritional limitations for limiting impact on the growth rates.

The results were similar to the initial growth rate experiment, with the virally infected samples initially growing faster than the non-infected samples, but ultimately seeing a decrease in microbial population with time as the viral infections resulted in cell lysis.

The results for the aerated growth rate comparison are presented in Figure 18. All the experiments were performed in multiple replicates and each data point is the average of four replicates. The growth rates for both cultures remained relatively similar for approximately 1 week. Afterwards, the virally infected culture of phototrophic microbes exhibited reduced growth rates relative to the virus free culture. This could mean that this particular virus may have a latent period of approximately 1 week under these growth conditions. After 22 days the yield for the virally infected culture was 13.4% less than the yield for the virus free culture. That is a very significant reduction, and it could greatly reduce yields for biofuel applications if similar results were to be observed in large scale growth conditions.

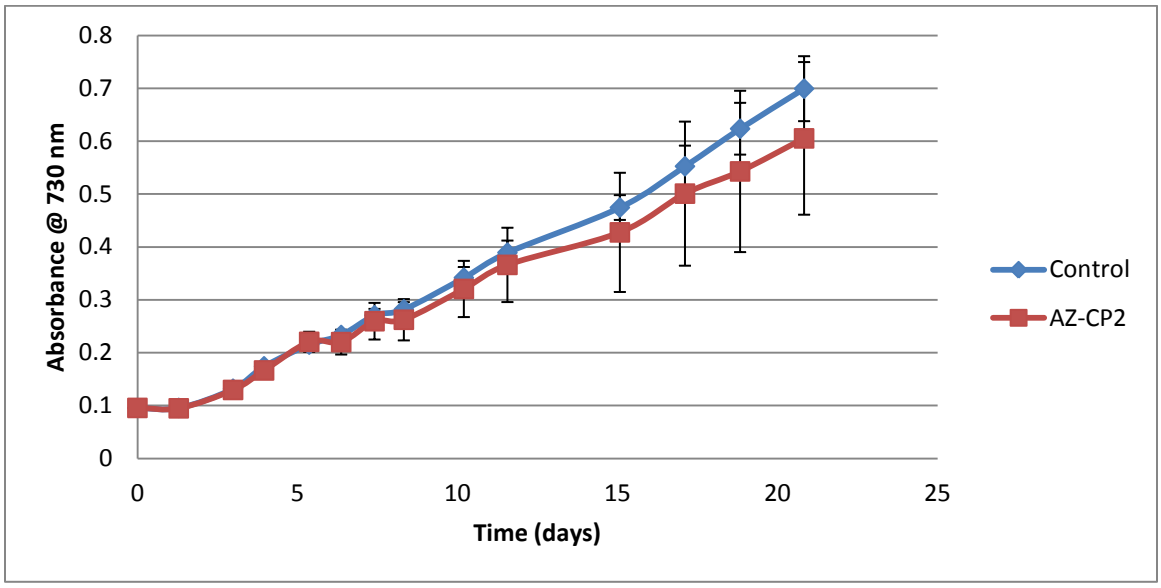


Figure 18. Growth Rate Comparison of *Synechocystis* PCC 6803 Infected with AZ-CP2 Phage (Aerated)

After conducting an unpaired *t* test on the data in Figure 18, it was found that the difference between the control and infected samples was not statistically significant. This was due the high variability within the replicates of the infected samples. For unknown reasons, two of the infected samples had results similar to those of the controls. These two replicates were considered to be outliers and removed from the infected average (Figure 19). The new graph clearly shows that the growth rate of *Synechocystis* sp. PCC 6803 were severely impacted by the viral infection of AZ-CP2, and ultimately experienced a ~48% reduction in cell numbers when compared to the control samples.

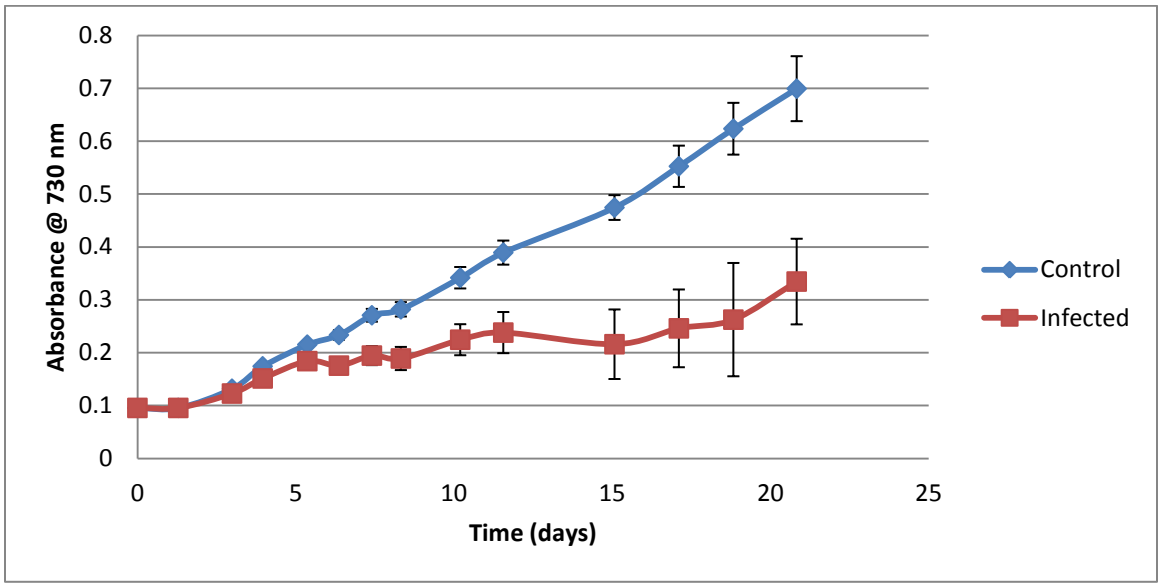


Figure 19. Growth Rate Comparison of *Synechocystis* PCC 6803 Infected with AZ-CP2 Phage without the Outliers Data Points (Aerated)

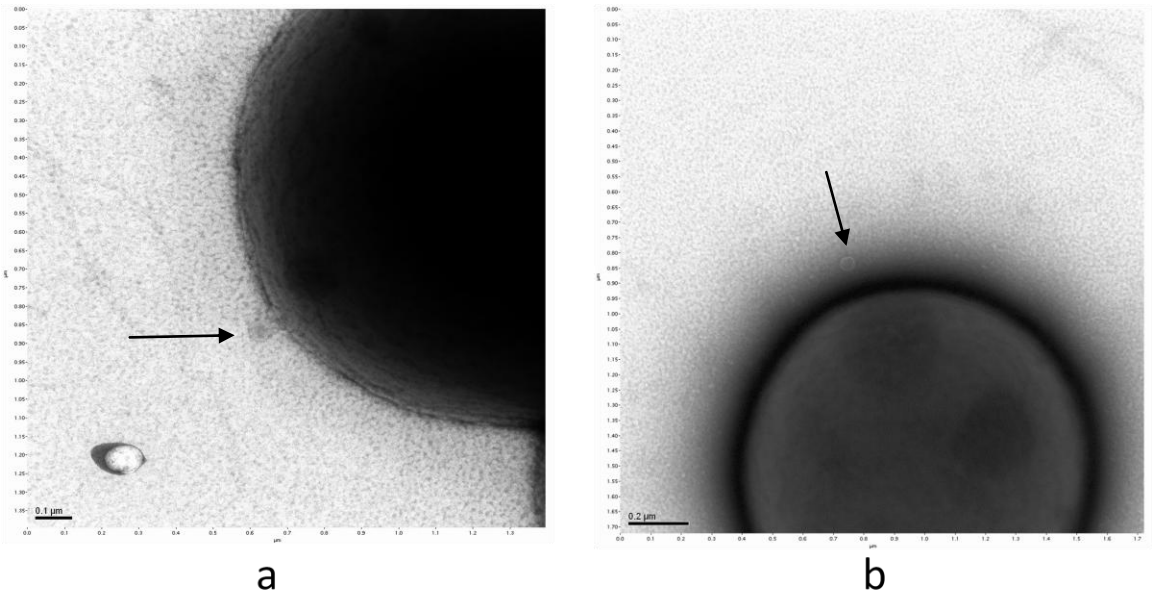


Figure 20. Transmission Electron Microscopy of *Synechocystis* PCC 6803 and AZ-CP2 Viral Attachment for in a and b

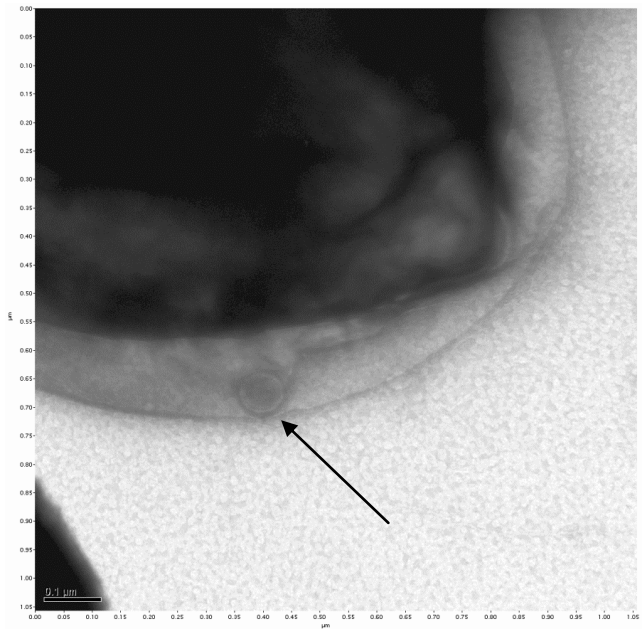


Figure 21. Transmission Electron Microscopy of *Synechocystis* PCC 6803 and AZ-CP2 Viral Attachment

In the initial experiment, the cyanophage isolates were culturally characterized and their impact on phototrophic microbes was evaluated. Thereafter, transmission electron microscopy was performed to further characterize these cyanophages. Figures 20 and 21 are TEM images of AZ-CP2 virus and *Synechocystis* PCC 6803. Figure 20a shows viral attachment of AZ-CP2 to the host *Synechocystis*, and Figure 20b shows viral association with host *Synechocystis*. In Figure 20, it is believed that AZ-CP2 is attached to the top of a *Synechocystis* cell. The virus AZ-CP2 was observed to be 60-70 nm in size, and icosahedral in shape, possible belonging to the Podovirus family.

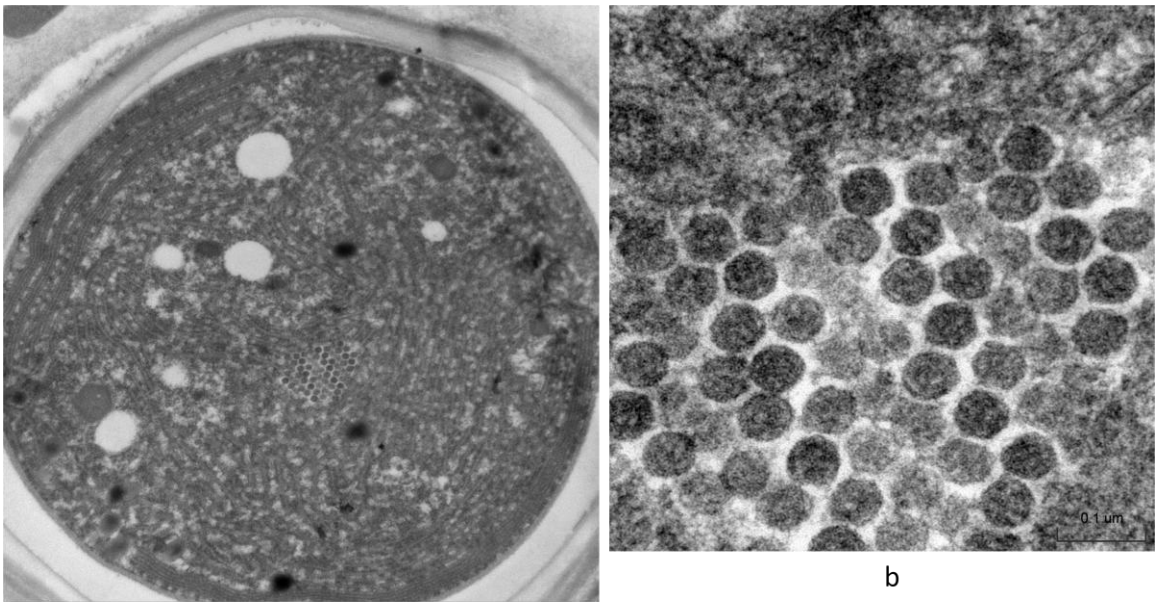
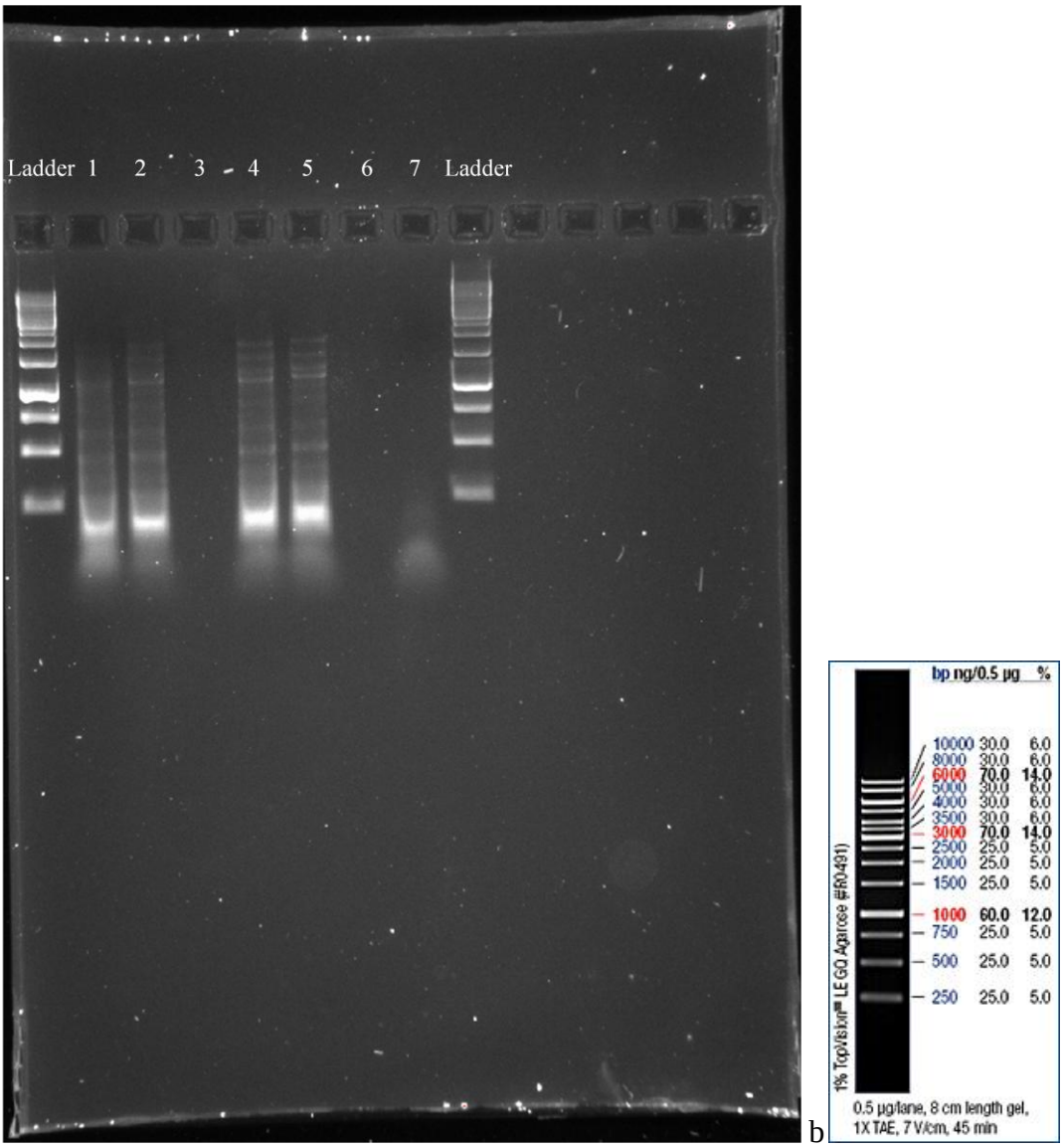


Figure 22. TEM of Virally Infected Cyanobacteria (a) 15K magnification. (b) 120K magnification. Courtesy of David Lowry at Arizona State University (Lowry, 2010)

Figure 22 illustrates thin section transmission electron micrographs of lab cultured cyanobacteria. These micrographs were taken initially to observe the inner structures of these cyanobacterial cells; however it was discovered that they were virally infected. This shows that viral contamination from unknown viruses is possible in a lab environment. This contamination may have occurred from previously dormant viral DNA becoming activated due to some unknown environmental factors or possible viral transmission to the bioreactor.



a b

Figure 23. PCR of AZ-CP1 and AZ-CP2. (a) Ladder: 1kbp, Lane 1: Sample 1 (15 ul of target in 50ul PCR reaction), Lane 2: Sample 1 (10 ul of target in 50ul PCR reaction), Lane 3: Blank, Lane 4: Sample 2 (15 ul of target in 50ul PCR reaction), Lane 5: Sample 2 (10 ul of target in 50ul PCR reaction), Lane 6: Blank, Lane 7: Negative Control, and Ladder: 1kbp. (b) Ladder: 1kbp

Based on the morphological and cultural conditions a set of primers were selected to perform polymerase chain reaction for the molecular characterization of the AZ-CP1 and AZ-CP2 viruses. Figure 23 is the result of PCR amplification attempts for the

cyanophages AZ-CP1 and AZ-CP2. No distinct patterns were observed, therefore, viral characterization using the amplification assay was not successful.

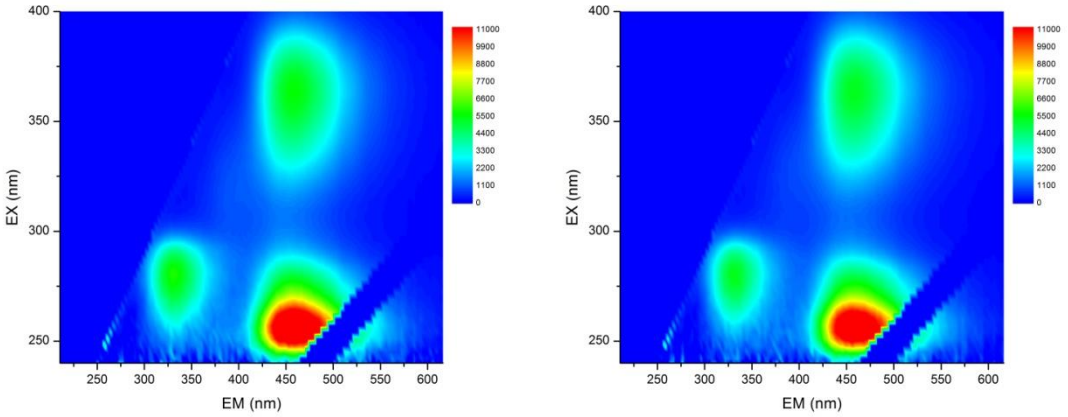


Figure 24. EEM of *Synechocystis* PCC 6803 Samples (a) Control. (b) Virally Infected with AZ-CP2 Phage

Additional experiments were performed to identify any specific metabolic products of virally infected phototrophic microbes. The objective was to use EEM for identifying infectivity biomarkers. Figure 24 are two examples of 3D contour plots derived from EEM data taken over the course of growth rate comparison studies. It was hoped that the virally infected samples of *Synechocystis* PCC 6803 would have an additional region(s) of fluorescence and activity due to the presence of cell metabolites from the infection by AZ-CP2 cyanophage. If different patterns were observed in any of the region(s) EEM could be a fast and inexpensive method of detecting viral infectivity in a bioreactor. However, no such region was observed. Further testing would be required to determine if this technology would be useful in the rapid detection of other algal viruses.

CHAPTER 4 - CONCLUSIONS AND CONSIDERATIONS

Algal biofuels are the most promising alternative to petroleum based fuels. This is due to their high yields, lack of competition with traditional agriculture, and their sustainability. Furthermore, they allow for the continued use of the current infrastructure, such as gas stations, and do not require the retrofitting of current automobiles. However, there are limiting factors associated with biofuels. For instance, contamination of large scale algal ponds could greatly impact theirs yields, affecting the current projections of required land to meet fuel demands. This could possibly be mitigated with the use of a combination of the closed photobioreactor and open pond; however such practices may minimize potential contamination but not eliminate the possibility of infection.

Viruses were demonstrated to persist in the natural environment and under laboratory conditions resulting in a reduction in phototrophic microorganism populations in the bioreactors. Therefore it is likely that viruses could have a significant impact on microbial biofuel production by limiting the yields in a production pond. Further research on the effects that viruses have on algal yields is required. If they are found to markedly impact production of algae, then remediation strategies and methods must be developed. Examples could include antiviral polypeptides and viral filters. Some species, like cyanobacteria, may even be able to make use of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) to provide an acquired immunity to the algal phages.

Additionally, the prime algal growing locations for the U.S. are located in the Southwest portion of the U.S. which are water scarce. If these regions were to be used as the primary source of algal fuel production in the U.S., water scarcity and water quality

will be new major challenges for the region. Perhaps this technology could prove to be an economic boom for other regions or countries. There are large amounts of capital required to transition from petroleum to algal biofuels. However, this could be spread out over several years if the algal fuels are blended with gasoline in a similar practice to that of ethanol. This transition would occur more rapidly if algal biofuels became economically viable. Spurring the need for further research into the efficiency of algal production and processing.

REFERENCES

Allenby, B. (2012). *The Theory and Practice of Sustainable Engineering*. (K. R. Marcia Horton, Holly Stark, Ed.) (First., pp. 184–185). Upper Saddle River, NJ: Pearson Higher Ed.

Chisti, Y. (2008). Biodiesel from microalgae beats bioethanol. *Trends in Biotechnology*, *26*(3), 126–31. doi:10.1016/j.tibtech.2007.12.002

Danovaro, R., Corinaldesi, C., Dell'anno, A., Fuhrman, J. a, Middelburg, J. J., Noble, R. T., & Suttle, C. a. (2011). Marine viruses and global climate change. *FEMS Microbiology Reviews*, *35*(6), 993–1034. doi:10.1111/j.1574-6976.2010.00258.x

Greene, D. L., Hopson, J. L., & Li, J. (2006). Have we run out of oil yet? Oil peaking analysis from an optimist's perspective. *Energy Policy*, *34*(5), 515–531. doi:10.1016/j.enpol.2005.11.025

Hu, Q., Sommerfeld, M., Jarvis, E., Ghirardi, M., Posewitz, M., Seibert, M., & Darzins, A. (2008). Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *The Plant Journal : For Cell and Molecular Biology*, *54*(4), 621–39. doi:10.1111/j.1365-313X.2008.03492.x

Kazamia, E., Aldridge, D. C., & Smith, A. G. (2012). Synthetic ecology – A way forward for sustainable algal biofuel production? *Journal of Biotechnology*, *162*(1), 163–169. doi:10.1016/j.jbiotec.2012.03.022

Kim, H. W., Vannela, R., Zhou, C., & Rittmann, B. E. (2011). Nutrient acquisition and limitation for the photoautotrophic growth of Synechocystis sp. PCC6803 as a renewable biomass source. *Biotechnology and Bioengineering*, *108*(2), 277–85. doi:10.1002/bit.22928

Li, Y., Horsman, M., Wu, N., Lan, C. Q., & Dubois-calero, N. (2008). Biofuels from Microalgae. *Biotechnology Progress*, *24*(4), 815–820. doi:10.1021/bp.070371k

Lowry, D. (2010). *Y-cell-15k*.

Mata, T. M., Martins, A. a., & Caetano, N. S. (2010). Microalgae for biodiesel production and other applications: A review. *Renewable and Sustainable Energy Reviews*, *14*(1), 217–232. doi:10.1016/j.rser.2009.07.020

Parmar, A., Singh, N. K., Pandey, A., Gnansounou, E., & Madamwar, D. (2011). Cyanobacteria and microalgae: a positive prospect for biofuels. *Bioresource Technology*, *102*(22), 10163–72. doi:10.1016/j.biortech.2011.08.030

Smith, V. H., Sturm, B. S. M., Denoyelles, F. J., & Billings, S. a. (2010). The ecology of algal biodiesel production. *Trends in Ecology & Evolution*, *25*(5), 301–9. doi:10.1016/j.tree.2009.11.007

Stephens, E., Ross, I. L., King, Z., Mussgnug, J. H., Kruse, O., Posten, C., … Hankamer, B. (2010). An economic and technical evaluation of microalgal biofuels. *Nature Biotechnology*, *28*(2), 126–8. doi:10.1038/nbt0210-126

Sullivan, M. B., Waterbury, J. B., & Chisholm, S. W. (2003). Cyanophages infecting the oceanic cyanobacterium Prochlorococcus. *Nature*, *424*(6952), 1047–51. doi:10.1038/nature01929

Suttle, C. A. (1994). The Significance of Viruses to Mortality in Aquatic Microbial Communities. *Microbial Ecology*, *28*(2), 237–243.

Suttle, C. A. (2005). Viruses in the sea. *Nature*, *437*(7057), 356–61. doi:10.1038/nature04160

Suttle, C. A. (2007). Marine viruses--major players in the global ecosystem. *Nature Reviews. Microbiology*, *5*(10), 801–12. doi:10.1038/nrmicro1750

Suttle, C. A., Chan, A. M., Sutile, C. A., & Chan, A. M. Y. M. (1994). Dynamics and Distribution of Cyanophages and Their Effect on Marine Synechococcus Dynamics and Distribution of Cyanophages and Their Effect on Marine Synechococcus spp . t, *60*(9).

Tamagnini, P., Leitão, E., Oliveira, P., Ferreira, D., Pinto, F., Harris, D. J., … Lindblad, P. (2007). Cyanobacterial hydrogenases: diversity, regulation and applications. *FEMS Microbiology Reviews*, *31*(6), 692–720. doi:10.1111/j.1574-6976.2007.00085.x

U.S. Energy Information Administration (EIA). (2012). Nonrenewable Sources. Retrieved from http://www.eia.gov/energyexplained/index.cfm?page=nonrenewable_home

U.S. Energy Information Administration (EIA). (2014a). Annual Energy Outlook 2014 with projections to 2040.

U.S. Energy Information Administration (EIA). (2014b). Despite decline in some regions, world oil consumption still seen rising. Retrieved January 11, 2014, from http://www.eia.gov/todayinenergy/detail.cfm?id=17931

U.S. Energy Information Administration (EIA). (2014c). What are the products and uses of petroleum? Retrieved January 11, 2014, from http://www.eia.gov/tools/faqs/faq.cfm?id=41&t=6

UC San Diego. (2014). Why Algae? Retrieved from http://algae.ucsd.edu/about/why-algae/

Van Etten, J. L., Lane, L. C., & Meints, R. H. (1991). Viruses and viruslike particles of eukaryotic algae. *Microbiological Reviews*, *55*(4), 586–620. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=372839&tool=pmcentrez&rendertype=abstract

Watkins, S. C., Smith, J. R., Hayes, P. K., & Watts, J. E. M. (2014). Characterisation of host growth after infection with a broad-range freshwater cyanopodophage. *PloS One*, *9*(1), e87339. doi:10.1371/journal.pone.0087339

Wilhelm, S. W., Carberry, M. J., Eldridge, M. L., Poorvin, L., Saxton, M. a, & Doblin, M. a. (2006). Marine and freshwater cyanophages in a Laurentian Great Lake: evidence from infectivity assays and molecular analyses of g20 genes. *Applied and Environmental Microbiology*, *72*(7), 4957–63. doi:10.1128/AEM.00349-06

Wilhelm, S. W., Suttle, C. A., Ralph, J., & David, L. (1998). Measurements of DNA damage and photoreactivation imply that most viruses in marine surface waters are infective, *14*, 215–222.

Yang, J., Xu, M., Zhang, X., Hu, Q., Sommerfeld, M., & Chen, Y. (2011). Life-cycle analysis on biodiesel production from microalgae: water footprint and nutrients balance. *Bioresource Technology*, *102*(1), 159–65. doi:10.1016/j.biortech.2010.07.017