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The Role of Lysogeny in the Microbial Ecology of Lake Matoaka

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by

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ABSTRACT

Viruses play important roles in aquatic ecosystems. They drive nutrient cycling and maintain a diverse microbial community. Temperate phages, or those viruses that have the ability to reproduce lysogenically, may also contribute to host survival and the genetic diversity of their hosts. The study of lysogeny in various ecosystems is valuable to understanding microbial communities. Information on freshwater ecosystems is of particular importance, as lysogeny in marine systems is more widely studied This study examined the occurrence of lysogeny in a hypereutrophic, freshwater lake in Williamsburg, VA for the months of November and December 2009 and February and March 2010. Mitomycin C was added to ambient lake water and inductions were measured as the increase in viral direct counts from control sample counts using epifluorescence microscopy. March water samples were the only to demonstrate significant prophage induction. Burst size ranged from 0 to 98.53 and lysogenic fractions ranged from 2.67% to 54.50%. No significant correlations were found between water quality data and biological data. Experiments were also carried out with different inducing agents on a lake water sample and *E. coli* (λ) lysogens, including mitomycin C, ultraviolet radiation, and an herbicide (SedgeHammer). Mitomycin C induced the most viruses in *E. coli* (λ), while the highest concentration of the herbicide induced the most viruses in the ambient lake water. The results of the study indicate that lysogeny is more prevalent in March, when nutrient concentrations are lower as a result of curly pondweed (*Potamogeton crispus*) growth. Finally, mitomycin C, ultraviolet radiation, and herbicide

were all shown to induce phage production from lysogenic cells, with the herbicide inducing the greatest number of prophages in environmental samples.

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INTRODUCTION

Viral Importance in Aquatic Ecosystems

Viruses play important roles in aquatic ecosystems. Viral abundance in these systems can exceed bacterial abundance by an order of magnitude (Wommack and Colwell, 2000). In the oceans, total viral counts are estimated at $4x10^{30}$ particles, while total prokaryotic counts are estimated at $1x10^{26}$ cells (Madsen, 2008). A significant portion of environmental prokaryotes are infected and lysed by viruses every day (Madsen, 2008). The quantity of infectious phages in aquatic ecosystems illustrates their relative importance, as numerous viral infections have an impact on the mortality of host species. From a community dynamics point of view, viruses control the abundances of other microbial species, including various groups of plankton in the ocean (Suttle, 2005). Thingstad's "kill the winner" (2000) hypothesis describes the process whereby the diversity of bacterial species is maintained because when one species begins to show dominance, viruses specific to that host proliferate as well, preventing any one species from outcompeting the others for an extended period of time.

Through lysis of microbial cells, viruses help to drive nutrient cycling and speed up the release of nutrients into the environment, which microbial organisms can uptake. This "viral shunt" is the virally-mediated movement of nutrients stored in bacteria to dissolved and particulate nutrient pools (Suttle, 2005). In the oceans, 10 to greater than 100% of bacterial standing stocks are lysed as a result of viral infection each day (Fuhrman, 1999, Weinbaurer *et al.*, 1995). Similar impacts apply to freshwater ecosystems. For example, in Sri Lankan tropical freshwater reservoirs, Peduzzi and

Schiemer found that 13.2% to 46.1% of the bacterial population was infected and would be killed through virus-mediated mortality (2004). The carbon cycle is altered by the viral shunt and the effects of virally-mediated mortality. The role of viruses in the carbon cycle is essential, as they add dissolved and particulate carbon to pools in the ocean (Suttle, 2005). Virus-mediated mortality of bacteria, phytoplankton, protozoa, and metazoa accounts for between 6% and 26% of dissolved organic carbon (DOC) in the oceans (Wilhelm and Suttle, 1999). This amount of carbon is therefore prevented from entering stores in the deep ocean or moving to higher trophic levels, as it is respired by surface layer bacteria. A result of diverting carbon from long-term storage to immediate uptake and use is that more CO_2 enters the atmosphere, possibly exacerbating climate change (Madsen 2008). These virus-driven processes must be included and considered in carbon balances and climate models in order to account for inputs and outputs from the system. Beyond the carbon cycle, prokaryotes involved in various nutrient cycle reactions, including nitrogen, phosphorus, and sulfur, can be infected and lysed by viruses, decreasing populations of these prokaryotes and, subsequently, their cycling activity (Fuhrman, 1999, Madsen, 2008).

Viral Replication and Induction

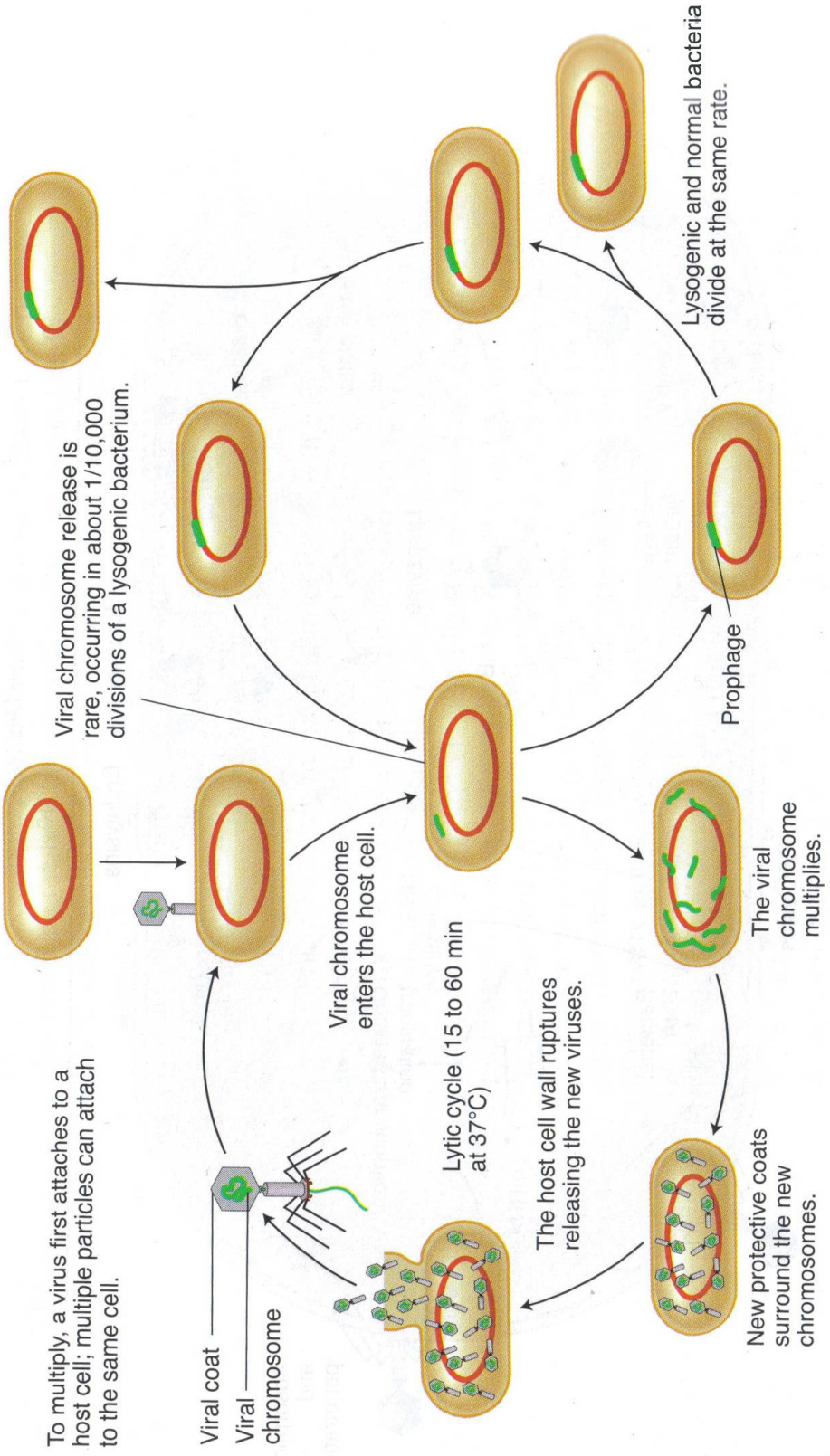
The modes through which viruses replicate strongly determine specific viral impacts within an ecosystem. All viruses replicate by first attaching to a host cell and injecting the genetic material into the host. Then, the host produces the proteins encoded by the viral genes. At this point, the specific steps involved in replication diverge, depending on the virus. For lytic viruses, or virulent phages, viral capsids are produced

within the cell and viral genomes are replicated and packed inside. Eventually, whole viruses lyse from the cell, killing the host and repeating this pattern known as the lytic cycle (Figure 1., left side; Madsen, 2008). However, some viruses have the ability to carry out both the lytic and a second life cycle called lysogenic replication. These viruses are called temperate phages. During the process of lysogeny, the expression of genes involved in the lytic cycle is silenced and the viral genome is integrated into the host's chromosome. The integrated virus genome is known as a prophage (Fig 1, right side).

For temperate phages, there are cellular cues that cause the viral genome to enter the lysogenic cycle. This process is best understood from studies with phage λ. Phage λ encodes a protein called cII that is degraded by host proteases when the cell has sufficient nutrients and is metabolically active. An active cell processes many proteins, which requires proteases. These proteases act upon cII as well. If the cell is under starving conditions, host proteases become inactive as there are not ample nutrients for the cell to manufacture all cellular products. In this case, enough cII may escape degradation by proteases and bind to promoters in the λ genome that control the expression of an integrase and a repressor. The integrase is responsible for recombination between the phage and host genomes, and the repressor prevents the transcription of nearly all lytic viral products. Therefore, when the host cell is under starved conditions, temperate phages enter lysogeny. When the host cell is under nutrient replete conditions, cII is degraded and the integrase and repressor are not produced. This type of sensing of host status is important because a starving cell is not able to supply the resources necessary to produce more virus particles, as would be required if the virus immediately entered the lytic cycle. In this way, lysogeny is a method for temperate phages to survive when a

lytic infection would be unproductive. After integration, lysogeny is stable and the prophage is inherited by all progeny of the host. This produces a population of lysogenic cells and is advantageous to the virus as a slow but effective way to replicate (Ptashe, 1987).

Figure 1. Lytic and lysogenic life cycles (Shors, 2009)



The prophage also has a mechanism by which it can be induced to enter the lytic cycle after lysogeny has been established. Certain environmental stimuli that cause DNA damage, such as ultraviolet (UV) light, act as inducing agents. Bacteria naturally produce a protein called RecA that is involved in repairing damaged DNA by cleaving the cellular repressor responsible for regulation of the repair system, or SOS response. When a prophage is present, however, RecA also degrades the prophage repressor. Consequently, if RecA degrades enough of the repressor molecule, phage genes are able to be transcribed by RNA polymerase. These genes encode for lytic growth, so that the viral genome is excised from the host chromosome and replication of the phage begins (Ptashe, 1987). Thus, the induction mechanism is a way for temperate phages to escape a dying host and survive. Occurrence of prophages can be measured in environmental samples by inducing lysogeny. This is done by applying an inducing agent to a sample containing potentially lysogenic bacteria,and microscopically observing the release of induced viruses. Mitomycin C and UV light, both of which cause DNA crosslinkage which hinders transcription by polymerase, are most commonly used as inducing agents. The difference in free viruses from control samples receiving no inducing agents and those receiving a dose is calculated as the number of inducible lysogens (Williamson *et al.*, 2008).

Importance of Lysogeny and Previous Studies on Aquatic Ecosystems

While lytic infections in the environment drive nutrient cycles and maintain community diversity through bacterial mortality, lysogenic infections in the environment have different effects. Viral infection is often thought of in terms of benefits to the virus

as it reproduces at the expense of the host, but lysogenic infections may also benefit the host. One study (Paul, 2008) discovered transcriptional regulatory and repressor-like proteins in marine prophages. It was proposed that the repressor and regulator genes of marine temperate phages directly affect host metabolism through repression of host genes. The host may therefore receive protection from low nutrient conditions by having a slower metabolism. Without this type of metabolic suppression, low-nutrient niches would probably be unavailable to fast-growing bacteria. With metabolism repression, however, nutrients in the environment are not depleted as quickly and the host population can survive for a longer period of time on the same amount of nutrients.

Viruses are genetically diverse (Suttle, 2005) and, by means of lysogeny, also promote horizontal gene transfer which alters the genetic diversity of bacterial hosts (Paul, 2008). It is possible for temperate phages to engage in transduction, which occurs when the excision of the phage genome erroneously includes a host gene or genes and these are transferred to another host upon genome integration. Moreover, some temperate phages carry virulence genes that cause their hosts to become pathogenic when the phage genome is integrated. This is known as lysogenic conversion of the host (Shors 2009). Thus, lysogeny can have subtle but often important impacts on host fitness and evolution.

Factors that determine whether or not lysogeny is widespread in an ecosystem have varied among studies, but include nutrient levels and availability, host abundance and density, temperature, and primary productivity (Choi *et al.*, 2009, Weinbauer, 2003, Williamson *et al.*, 2002). Lysogeny has been studied in both freshwater and marine systems (Bettarel *et al.*, 2006, Choi *et al.*, 2009, Jiang and Paul, 1996, Paul, 2008, Tapper and Hicks, 1998, Weinbauer, 2003, Williamson *et al.*, 2002), although more research has

been done in marine systems. In the Baltic and Mediterranean Seas, the lysogenic fraction (LF, proportion of bacteria carrying prophage) ranged from 0% to as high as 84% in deep water (Weinbaurer *et al.*, 2003), presumably where bacterial abundance and nutrient availability are low. In the saline Tampa Bay estuary, lower host growth, temperature, primary productivity, rainfall, and nutrient conditions in the winter months promoted a higher LF and the opposite conditions promoted a lower LF in the summer. Although the range of LFs was great due to large standard deviations, average values were between 27.6% and 45.9%, depending upon the burst size. It was proposed that the higher temperatures and exposure to UV light had already induced many temperate phages, causing lower LF percentages in the summer months (Williamson *et al.*, 2002). In the estuarine systems of the Gulf of Mexico and Mamala Bay, Oahu, LF was reported to be between 10.5% and 38% (Jiang and Paul 1996). The lysogenic fraction in marine systems appears to vary greatly (0-84%, Weinbaurer *et al.*, 2003), but is dependent upon the unique environmental conditions, such as high UV exposure or varying temperatures.

In a comparison of freshwater, brackish and marine systems, 42% of bacteria in marine samples were found to be inducible, but only 23% of bacteria were inducible lysogens in warmer freshwater (Choi *et al.*, 2009). The freshwater study site had the highest bacterial and viral abundance, leading to the lower LF as a result of adequate nutrients and bacterial density to support more lytic infections. Although a significant relationship was not found between environmental factors and lysogeny, it was shown that nutrient levels perhaps have been overemphasized as one of the factors influencing the choice between lytic and lysogenic growth. A Bayesian analysis showed that nutrient levels are not a direct factor in predicting the lysogenic fraction. Rather, bacterial density

and metabolism have significant influences on lysogenic fraction; but these in turn are affected by nutrient levels. In this way, nutrient concentrations indirectly influence lysogeny (Choi *et al.*, 2009).

A study of seven eutrophic or hypereutrophic West African lakes, ranging from freshwater to hypersaline bodies of water, found LFs lower than 8%, concluding that lysogeny was not prevalent in these systems (Bettarel *et al.*, 2006). This supports the findings of the Choi *et al.* study (2009), since the high concentrations of nutrients in the West African lakes most likely support a high bacterial density and sufficient metabolic rate to deter lysogenic infections. In Lake Superior, the LF ranged from 0.1-7.4%, results that seem to agree with other freshwater systems. However, the LF was higher at the surface, rather than the subsurface, which, considering the factors thought to influence lysogeny, was contrary to the authors' hypothesis. UV light was expected to have already induced any lysogens residing in the surface waters (Tapper and Hicks, 1998). In general, freshwater systems appear to have lower LFs than marine systems (0.1-23%, Bettarel *et al.*, 2006, Choi *et al.*, 2009).

Research Objectives

From these studies, certain trends can be seen in the occurrence of lysogeny and the factors that influence it in aquatic ecosystems. However, most studies of lysogeny have focused on trends in marine systems. It was the goal of this study to provide data on lysogeny in a freshwater system. This study examined the importance of lysogeny and its temporal variation in the microbial ecosystem of the hypereutrophic, freshwater Lake Matoaka during the winter and spring. Water quality measurements (pH, temperature,

and chlorophyll a) were correlated with incidence of lysogeny. These data address questions of prevalence and parameters associated with lysogeny.

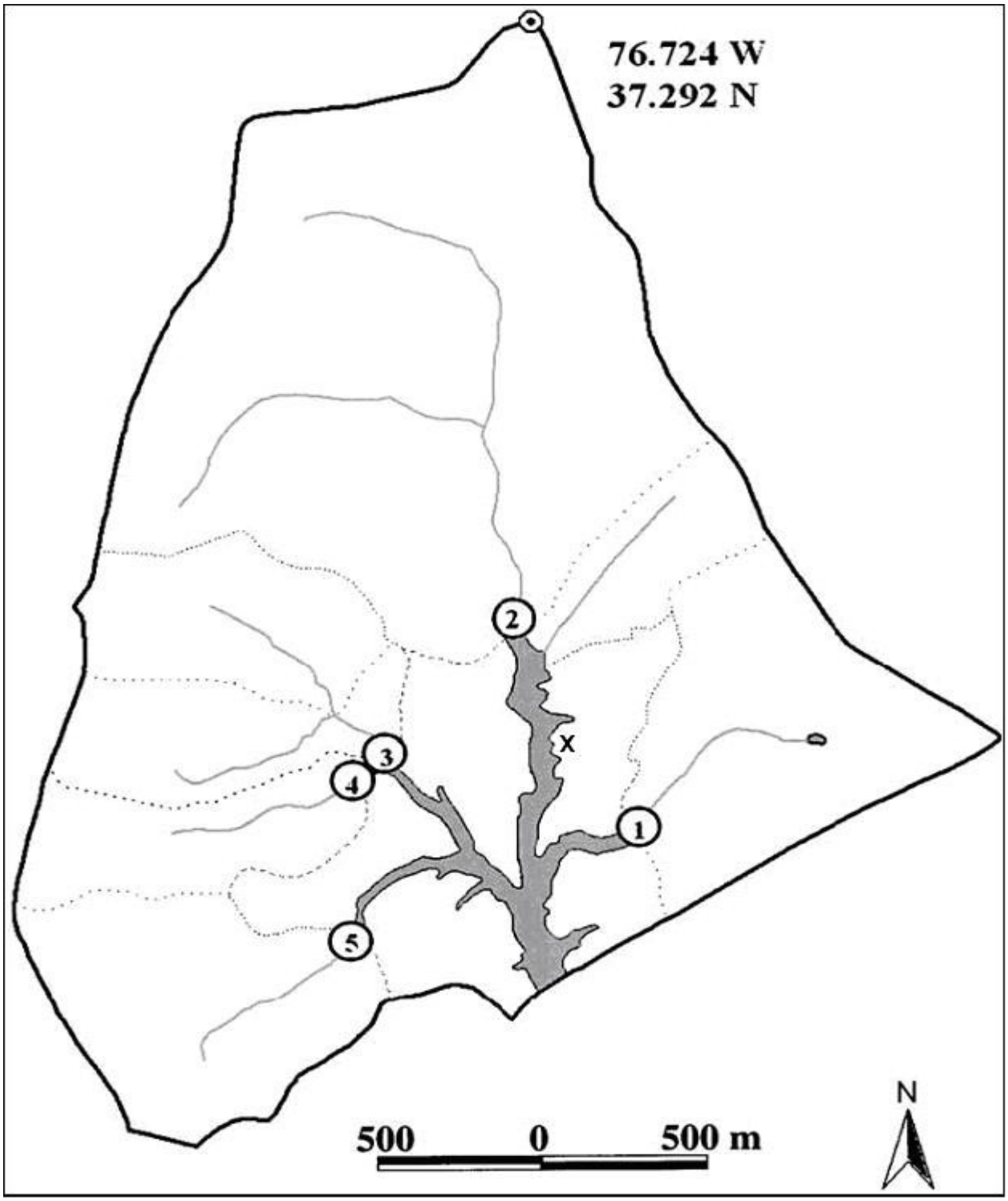
In assessments of lysogeny within microbial communities, the question of what anthropogenic compounds cause induction in nature has not been completely resolved. Danovaro and Corinaldesi (2003) found that sunscreen and solar oil cause induction, and Jiang and Paul (1996) found that hydrocarbons are inducing agents as well. This research also investigated possible causes of induction in Lake Matoaka bacterial communities using UV irradiation and herbicide exposure. As samples were taken from surface waters, UV radiation was expected to be encountered in the environment. The herbicide applied to samples was chosen because it is known to be used in the Lake Matoaka watershed. The objective of these inducing agent experiments was to ascertain the role of environmentally-relevant stimuli in the induction of lysogeny. The results of this study add to the growing body of work on viruses in freshwater systems, in addition to a general understanding of lysogeny in all ecosystems.

METHODS

Site Description

Lake Matoaka is a hypereutrophic man-made lake located in Williamsburg, VA. The lake was created by damming the natural riparian-forested wetland between 1720 and 1730 and was increased in size in 1930 by increasing the spillway height 1 m. Lake Matoaka is now 16 ha and has a 600 ha watershed. A total of five streams discharge water into the lake, three that come from forested and two that travel through human development (Pensa and Chambers, 2004).

Map of Lake Matoaka watershed with numbers indicating streams and an x indicating the sample site (Pensa and Chambers, 2004)



Sample Collection

Water samples were taken from the dock of the Keck Environmental Laboratory on Lake Matoaka in November and December 2009, and February and March 2010 near the end of each month. A 250 mL polycarbonate Nalgene bottle was used for collection by rinsing and then filling it with lake surface water. Induction experiments were set up within two hours of collection from the lake.

Induction Assays

Water samples were transferred to sterile 50 mL polypropylene tubes. For the months of November and December, 25 mL of lake water was placed into each of four tubes with two designated as controls and two as treatments. For the months of February and March, 20 mL of lake water was placed into each of eight tubes, with four designated as controls and four as treatments. The treatments received mitomycin C aliquots at a final concentration of 0.5 µg mL^{-1} (Williamson *et al.* 2007;Williamson *et al.*, 2002). For March samples, six additional treatments were tested to determine induction response to other potential inducing agents. Two replicates (20 mL each) received a dose of 120 mJ cm^{-2} UV using a Spectrolinker XL-1000 UV crosslinker (Spectronics Corp.) (Williamson *et al.*, 2008). Four replicates (20 mL each) received aliquots of SedgeHammer (halosulfuron-methyl; $C_{13}H_{15}C1N_6O_7S$; Gowan Company, LLC) that is widely used on the College campus as an herbicide to control nutsedge. Two replicates received SedgeHammer aliquots at a final concentration of 0.05 µg mL^{-1} and two received aliquots at a final concentration of 0.005 µg mL^{-1} (Jiang and Paul, 1996). Two aliquots (20 mL

each) were unamended and served as controls. Controls and treatments were incubated at room temperature in the dark for 24 hours.

Enumeration of Bacteria and Virus Particles

After incubating water samples overnight (18-24 hours) to allow for induction, aliquots were filtered through Whatman 0.02 µm Anodisc filters under vacuum. Filters were stained with 2.5 X SYBR Gold for 15 minutes in the dark. The SYBR Gold was pulled through using a vacuum and the Anodisc was removed from the holder and allowed to dry. Anodiscs were mounted on glass slides with 10 µL of Antifade and a cover slip was placed on top, Antifade-side down. One Anodisc was prepared per replicate tube for all controls and treatments. Anodiscs were viewed under blue excitation with a FITC filter. Using epifluorescence microscopy and MetaMorph (Olympus) software, 10 pictures of each Anodisc were taken in random locations on the filter at 100X magnification. MetaMorph (Olympus) was used to enumerate viral particles and bacteria using average sizes for each. Average values for viruses and bacteria were calculated on a per-milliliter basis for each sample. Inductions were considered as the occurrence of a significant difference ($p \leq 0.05$) between counts of viruses in the control and treatment. Bacterial mortality was not used to measure incidence of lysogeny (Williamson *et al.*, 2002).

Generating λ Lysogens

Eschericia coli C600 cells (ATCC 23724) were grown to log phase in tryptic soy broth (TSB) at 37° C with aeration. A 100 µL aliquot of culture was added to 10mL of

0.02M $MgSO_4$. The cells were then incubated at 37° C with aeration for an hour to produce starved cells. A 100 µL aliquot of concentrated λ phage stock (ATCC 23724-B2) (ca. 10^6 pfu ml^{-1}) was added to the tube and incubated at room temperate overnight. Using aliquots of the λ –infected *E. coli*, six serial ten-fold dilutions were made in 0.02M $MgSO_4$ and plated on separate TSB plates which were incubated overnight at 37 ° C (Usharanjan and Skalka, 1976). Ten colonies were picked at random for confirmation of lambda prophage. PCR was carried out with ten colonies and a negative control using forward and reverse λ primers (forward: 5'-GAG GTA CCA GCG CGG TTT GAT C-3', reverse: 5'-ACT CGT CGC GAA CCG CTT TC-3') (PCR reagents for each reaction: 1 µL of each primer diluted to 50 pm μL^{-1}, 5 µL of 10X PCR buffer, 4 µL of 0.2 mM dNTPs, 0.5 µL of Taq polymerase, 1 µL of *E. coli* colony suspended in sterile water). The thermocycler was set to run at 95 ° C for 1 minute and repeat the following sequence 25 times: 95 ° C for 1 minute, 55 ° C for 1 minute, 72 ° C for 1 minute. The thermocycler then ran at 72 ° C for 5 minutes. Agarose gel electrophoresis (0.8%) was used to analyze PCR products and the gel was stained with SYBR Safe for imaging. Bands located at 500 bp indicated λ lysogens (Powell *et al.*, 1994).

Comparison of Inducing Agents on the *E.coli* (λ) System

One of the PCR-confirmed *E.coli* (λ) colonies was incubated overnight at 37 ° C in 20 mL of TSB. A 100 µL aliquot of the overnight culture was added to 300 mL of TSB and incubated at 37 ° C for 8 hours. Ten 20 mL aliquots of the exponential phase culture were added to separate 50 mL tubes. Two aliquots were controls while the rest were treatments. The treatment samples were given the same concentrations and amounts of

the treatments as the March lake samples. Two samples were treated with mitomycin C, two with UV irradiation, two with the higher concentration of Sedgehammer, and two with the lower concentration of SedgeHammer. Bacterial cells and virus particles were enumerated as described above.

Calculations

The average number of viruses and bacteria per field of view was calculated from the 10 fields used for pictures. Viruses and bacteria per mL were calculated using the following formulae and constants:

$$\text{Eq. 1. VLP mL}^{-1} = \frac{\overline{\text{VLP}_f} \times (A_{disc}/A_{field})}{\text{volume}}$$

VLP mL^{-1} is the number of virus-like particles (or BC for bacterial cells); VLP_f is the average number of virus-like particles (or BC for bacterial cells) per field; A_{disc} is the area of the Anodisc; A_{field} is the area of the viewing field; And volume is the volume of aliquot filtered (in mL).

$$\text{Eq. 2. BC mL}^{-1} = \frac{\overline{\text{BC}_f} \times (A_{disc}/A_{field})}{\text{volume}}$$

BC mL^{-1} is the number of bacterial cells; BC_f is the average number of bacterial cells per field; A_{disc} is the area of the Anodisc; A_{field} is the area of the viewing field; And volume is the volume of aliquot filtered (in mL).

After obtaining counts per mL, these numbers were used in the following calculations:

Induced burst size (B_z) is the average number of viruses produced per cell upon lysis. An assumed B_z of 50 was used for separate calculations based on averages of eutrophic freshwater systems (Parada *et al.*, 2006).

Eq. 3. $B_z = (V_I - V_C) / B_C - B_I)$ (Williamson *et al.*, 2002)

Lysogenic fraction (LF) is the percentage of bacteria with prophages.

Eq. 4. $LF = [(V_I - V_C) / B_z] / B_C \times 100$ (Williamson *et al.*, 2002)

In Eqs. 3 and 4, V_I is the number of viruses mL^{-1} in the induced sample after 24hr. V_C is the number of viruses mL^{-1} in the control after 24hr. B_I is the number of bacteria mL^{-1} in the treatment after 24hr. B_C is the number of bacteria mL^{-1} in the control after 24hr. Each of these four values were averaged from the replicates within each experiment.

For samples with multiple treatments, different variables were used to indicate the specific agent used: V_{mitc} and B_{mitc} denote mitomycin C as an inducing agent; V_{uv} and B_{uv} denote UV as an inducing agent; $V_{[0.05]}$ and $B_{[0.05]}$ indicate SedgeHammer at 0.05 µg mL^{-1} was used as an inducing agent; and $V_{[0.005]}$ and $B_{[0.005]}$ indicate SedgeHammer at 0.005 µg mL^{-1} was used as an inducing agent.

Statistical analysis

Statistical analyses were performed using Prism 5 (GraphPad). The data were tested for normality and those with low p-values were either logarithm- or reciprocal-

transformed to obtain normal distributions. The transformation that produced a data set closest to a normal distribution was used for a one-way ANOVA (95% confidence interval) with a Tukey post-test. For example, the probability plot of V_C for the months of November, December, February, and March yielded a p value of 0.024. The log of V_C yielded p=0.152 and the reciprocal values yielded p=0.313. The reciprocals were used for V_C because this transformed the data closer to a normal distribution than the log of the values.

As a result of the large number of correlations calculated between data, the Bonferroni correction was used to calculate the p-value necessary to demonstrate a significant difference between data. This p-value was 0.00238, requiring a 99.76% confidence interval for correlations to be significant.

RESULTS

Monthly Sampling and Induction with Mitomycin C

The trend for temperature across the four months of sampling period was a decrease from November to December, an increase from December to February, and a decrease from February to March (Fig. 3). Chlorophyll a increased steadily from November to February and decreased slightly from February to March (Fig. 3). pH increased slightly over the four sampling months but maintained a small range around a neutral pH (Fig. 3).

The November, December, and February samples had fewer viruses in the ambient lake water (controls) than the March samples by an order of magnitude, and fewer bacteria by almost an order of magnitude (Fig. 4). The minimum viral direct count (VDC) of ambient lake water was 2.98×10^5 virus mL^{-1} from December and the maximum was 4.05×10^6 virus mL^{-1} from March (Fig. 4). The minimum bacterial direct count (BDC) of ambient lake water was 1.28×10^5 bacteria mL^{-1} from December and the maximum BDC was 4.31×10^5 bacteria mL^{-1} from March (Fig. 4). The trend is an increase in both bacterial cells and virus particles in the spring compared with the winter.

The virus-to-bacterium ratio (VBR) increased from November to December due to a drop in BDC and continuation of a similar VDC. In February, the VBR decreased because the BDC had a greater increase than the VDC, although both increased. In March, the VBR decreased from February because the BDC increased more rapidly than the VDC again, although both counts increased (Fig. 4).

Using an unpaired two sample t-test, the differences between viral enumerations of controls and samples induced with mitomycin C were not significant for November (Fig. 5), December (Fig. 6), or February (Fig. 7) (November: $p = 0.718$, December: $p = 0.934$, February: $p = 0.452$), but were significant for March (Fig. 8) ($p = 0.042$). After transforming the data, no significant difference was observed between V_C, V_I, B_C, or B_I of the four sampling months, except between the V_C of December and March ($p=0.0309$). This means that December VDC differed significantly from March VDC.

Bacterial mortality (differences between bacterial enumerations of controls and induced samples) was not found to be significant in any of the samples (Figs. 5, 6, 7, and 9) (November: $p = 0.869$, December: $p = 0.369$, February: $p = 0.863$, March: $p = 0.135$).

The calculated B_z and LF for each month were highly variable (Table 1). The minimum B_z was -0.15 (assumed value of 0 since negative burst size is illogical) in December, while the maximum was 98.53 in February. February had the highest temperature and chlorophyll a measurements, as well as the highest B_z. December had the lowest temperature and lowest B_z. VDC and BDC were highest in March, although March, November, and December had relatively low burst sizes compared with February. VBR was highest in December and this month had the lowest B_z, although February had the second highest VBR and the highest B_z.

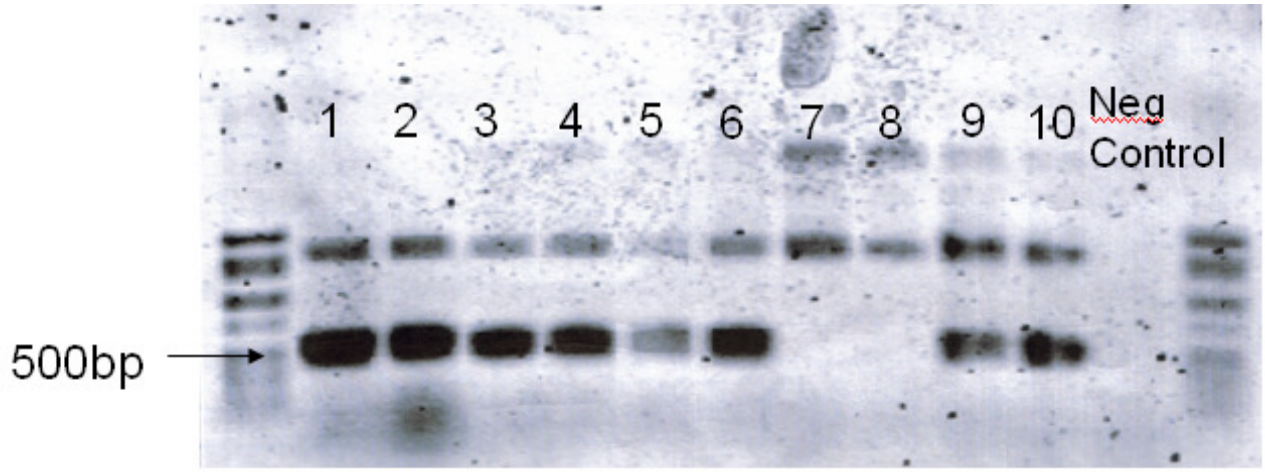
The minimum LF was 2.67% in February, and 54.50% in December (Table 1). March's LF was also distinctly higher than November and February, with 39.27% of the bacterial population carrying a prophage. VDC and BDC from March were highest and March had a high LF compared to November and February. There was no apparent trend between LF and temperature, chlorophyll a, pH, or VBR.

Inducing Agent Experiments

Bacteria in March water samples induced with mitomycin C, UV, and SedgeHammer at 0.05 µg mL^{-1} showed significant increases relative to the VDC of controls, (Fig. 8) (MitC: $p = 0.042$, UV: $p = 0.024$, SedgeHamer: $p = 0.010$) while VDC of samples induced with SedgeHammer at 0.005 µg mL^{-1} were not significantly different from controls ($p = 0.061$). SedgeHammer at 0.05 µg mL^{-1} had the highest average VDC, followed by UV, mitomycin C, and SedgeHammer at 0.005 µg mL^{-1}. Bacterial mortality was not significant for any of the induction treatments in the March water samples (Fig. 9) (MitC: $p = 0.135$, UV: $p = 0.645$, $SH_{[0.05]}$: $p = 0.605$, $SH_{[0.005]}$: $p = 0.316$). Mitomycin C induction caused the highest level of bacterial mortality, followed by SedgeHammer at 0.005 µg mL^{-1}, SedgeHammer at 0.05 µg mL^{-1}, and UV, which caused the least bacterial mortality.

In order to test potential prophage induction activity of specific agents, the well-characterized E. coli (λ) system was used for induction trials. E. coli was infected by λ under conditions favoring lysogeny, and PCR confirmed that eight of the ten colonies picked at random were λ lysogens (Fig. 2).

Figure 2. λ Lysogen Gel



One of the confirmed colonies was grown in broth and used for subsequent induction assays. VDCs in *E. coli* (λ) inductions were not significantly different from VDCs in controls (mitC: $p = 0.371$, UV: $p = 0.829$, $SH_{[0.05]}$: $p = 0.791$, $SH_{[0.005]}$: $p = 0.968$). Although *E. coli* (λ) VDCs were not significantly different from controls, mitomycin C induced the highest average VDC, followed by SedgeHammer at 0.05 µg mL^{-1}, UV, and SedgeHammer at 0.005 µg mL^{-1} (Fig. 6). Bacterial mortality for *E. coli* (λ) inductions was also not significant (MitC: $p = 0.181$, UV: $p = 0.781$, $SH_{[0.05]}$: $p = 0.701$, $SH_{[0.005]}$: $p = 0.881$). Mitomycin C induction caused the largest bacterial mortality, followed by SedgeHammer at 0.05 µg mL^{-1}, SedgeHammer at 0.005 µg mL^{-1}, and UV (Fig. 7).

Environmental water quality data and correlations

Pearson correlations were performed to determine potential relationships between lysogenic fraction and water quality data collected from Lake Matoaka during sample months, as well as biological data such as viral and bacterial abundance (Table 3). In addition to these parameters, data were checked for normality and transformations were

made on data that did not fit a normal distribution. The transformed data were also used in the Pearson correlation. Using the Bonferroni correction, the cutoff for significance was p = 0.00238. Temperature was the most important factor impacting lysogeny in Lake Matoaka. Although not significant with the Bonferroni correction (p = 0.034), temperature and burst size may have a slight positive relationship. After transforming the temperature values, p=0.15, which does not indicate significance. V_c and B_c (p= 0.012) were not significantly correlated, but the p-value hints at a positive relationship that may be determined with further sampling. However, the transformed data yielded a p-value of 0.204, perhaps negating the relationship seen in the raw data. LF had no significant correlation with the other measurements, but LF calculated with an assumed B_z of 50 may have a positive relationship with V_c after transforming V_c (p = 0.03)

DISCUSSION

Seasonal Trends in Lysogeny in Lake Matoaka

The results indicate that there are seasonal trends in lysogeny in Lake Matoaka. Comparing inductions with mitomycin C, samples collected in November, December, and February showed little to no induction (Fig. 5, 6, and 7), while March samples demonstrated significant induction (Fig. 8 and 9). Other studies (Choi *et al.*, 2009, Weinbauer, 2003, Williamson *et al.*, 2002) indicated that nutrient concentration, host abundance, temperature, and primary productivity have affects on lysogeny. Increases in nutrient concentration, temperature, bacterial density, and primary productivity usually occur in the spring and summer months and often indicate lower numbers of lysogens in aquatic ecosystems. The results from Lake Matoaka indicating that lysogeny is more prevalent in a spring month seem counterintuitive, as nutrient levels, and therefore the proteases that break down CII or its equivalent protein, would be expected to be lower in the winter and higher in the spring. However, a possible explanation for the higher LF in March rather than winter months, is that Lake Matoaka, although hypereutrophic, had an overgrowth of curly pondweed (*Potamogeton crispus*) during the March sampling. Many ambient nutrients in the lake have been absorbed by this plant to facilitate growth. Thus, the nutrient concentrations of the lake are actually lower during the time of this bloom. In this way, Lake Matoaka's increase in lysogens in March matches the results of other studies, and further supports the idea that nutrient availability is a key driver of lysogeny in aquatic bacteria.

The VBR was relatively small throughout the sampling months. The lowest VBR was 0.41 observed in November and the highest was 2.33 observed in December. This suggests that the majority of viruses in Lake Matoaka are bacteriophages since the ratio stays consistently low. VBR provides data about VDCs and BDCs of a system. A low VBR (10 or below) means that counts of viruses and bacteria have a difference of an order of magnitude or less. This ratio is observed in ecosystems with mainly bacteriophages because populations of viruses that infect bacteria fluctuate in numbers with the bacterial population. Similar ranges were found using count data from electron microscopy as well as epifluorescence light microscopy (Wommack and Colwell, 2000). A VBR of 0.41-2.33 is within the range of what is considered normal for bacteriophages. A VBR higher than 10 indicates that either viruses are reproducing more quickly than bacteria or bacteria are being removed from the system at a faster rate than they are reproducing.

Based on a review of the literature, freshwater burst size ranged from 17-121, and from 16-50 in marine waters. However, these studies often employed the frequency of visibly infected cells method (Wommack and Colwell, 2000). This method counts all visibly infected cells, and can not distinguish whether they are temperate or virulent infections. As result, these studies are not as accurate at measuring lysogeny specifically. The burst sizes for all 4 monthly samples were either lower than or within the range of 17-121 observed by Wommack and Colwell for freshwater systems (Table 1), which, if the frequency of visibly infected cells method overestimates lysogeny, fits into the findings of these studies.

The lysogenic fraction for each monthly sample was generally not as high as seen in marine environments (Weinbaurer *et al.*, 2003, Williamson *et al.*, 2002). The exception to this is the December sample LF (54.50%). However, this is most likely an artificially high LF because the burst size was less than one and LF calculations divide by this number (Eq. 3). The burst size could have been low due to sampling error, a result of the small number of lysogens, or due to the nature of calculating a mean value. As burst size is calculated as an average value for the sample community, this average burst size necessarily factors in both high induction and non-induction without distinction.

The LFs for the remaining samples were more in agreement with those from other freshwater studies (0.2 to 23%; Bettarel *et al.*, 2006, Choi *et al.*, 2009, Tapper and Hicks, 1998). While there are exceptions, the general trend is that freshwater LFs are lower than marine LFs based on studies of lysogeny using mitcomycin C as an induction agent (Choi *et al.*, 2009, Batteral *et al.*, 2006, Tapper and Hicks, 1998, Weinbaurer *et al.*, 2003, Williamson *et al.*, 2002). This indicates that, like other freshwater systems, lysogeny does not seem to play a significant role in the microbial ecology of Lake Matoaka. Even in March, when there was a significant level of induction, the LF (39.27%) was not nearly as high as, for example, the deep waters of the Baltic and Mediterranean Seas (up to 84%, Weinbaurer *et al.*, 2003).

One way to avoid problems stemming from calculated burst sizes is to use a standard assumed burst size (Williamson et al., 2002, Williamson et al. 2007). However, such assumed burst sizes are based on frequency of visibly infected cells and thus, may create different problems since these assumptions may not apply to lysogenic infections (as mentioned above). In spite of these potential problems, LF was computed using an

assumed burst size of 50 based on an average burst size of mesotrophic to eutrophic freshwater aquatic systems found in the review by Parada *et al.* (2006) (Table 1). However, assuming a uniform burst size, when it has been shown to be highly variable in the environment, mostly likely does not provide an accurate depiction of burst size (Williamson *et al.*, 2008). Thus, in spite of high variability, the values based upon individually calculated B_z are probably more realistic.

Induction typically results in lysis of the host cell as induced viruses are released. Although, compared to controls, bacterial mortality in induced samples was not significant (Figs. 5, 6, 7, 9). A possible explanation is that the amount of inducing agent applied was not at a high enough concentration to fully kill cells. This may actually have stimulated a reproductive response in bacteria in order to prevent the decimation of the populations. As a result, noted increases in VDC are more accurate measurements of lysogens (Williamson *et al.*, 2002).

Factors influencing lysogeny are poorly understood. The individual influence of factors such as temperature, nutrients, and bacterial abundance is difficult to determine (Choi *et al.*, 2009). This is exemplified by large variances in the data and the lack of significant difference between VDCs and BDCs for the four monthly samples. Large standard deviations associated with the data (Tables 1, 2) have been seen in other studies (e.g., Williamson *et al.*, 2002). This is most likely due to the nature of Lake Matoaka and aquatic ecosystems general. These are complex ecosystems, making it difficult to tease out trends from highly variable numbers, particularly when combined with infrequent monthly sampling. Environmental data is often less consistent than data produced in the lab. In order to draw firmer conclusions about the prevalence of lysogeny and its seasonal

trends in the lake, monthly sampling should be continued year-round. Three of four samples were taken at the end of fall and the winter months, so it is difficult to extrapolate what the March values mean for the rest of spring and summer. More than two replicates should also be aliquoted each sample month because there is more likely to be sampling error with fewer replicates (December enumeration, Fig. 3). Furthermore, although monthly sampling was conducted, microbial communities are dynamic and can change composition quickly (Fuhrman, 1999, Thingstad, 2000, Weinbaurer *et al.*, 1995) It was assumed that monthly samples were representative of the general trends in the month, but each sample is actually a representation of the lake's surface layer community at the specific time of sampling. Future studies could sample more frequently in addition to year round to determine whether these results are reproducible. A study could demonstrate whether the results in March are reproducible with curly pondweed growing in the lake again.

Comparison of Inducing Agents

In addition to exploring seasonal trends in lysogeny in Lake Matoaka, this project also assessed prophage induction using different inducing agents. This experiment intended to make the results environmentally relevant to the actual conditions of Lake Matoaka. Mitomycin C is a laboratory standard known to cause induction. However, mitomycin C is unlikely to be found in most environments. UV light and anthropogenic chemicals such as herbicides are much more likely to be encountered by lysogens in the environment. The herbicide used was SedgeHammer, a compound used to eliminate nutsedge on the campus grounds of The College of William and Mary, which is part of

the Lake Matoaka watershed. SedgeHammer works by inhibiting the acetolactate synthase (ALS) enzyme. ALS (or acetohydroxy acid synthase, AHAS) is a group of enzymes that undergo the first catalysis in the biosynthetic pathway to produce the amino acids isoleucine, valine, and leucine. ALS enzymes are found in most bacteria (Chipman et al., 1998).

It has been shown that *E. coli* cannot grow when ALS enzymes, of which E. coli has two, are not present and a carbon source is provided (Dailey and Cronan, 1986). The inhibition of ALS prevents the production of three amino acids, eventually stopping cell activities like DNA replication. SedgeHammer is used to treat both purple and yellow nutsedge, both of which are found in Virginia. These species use the C4 carbon fixation pathway. Yellow nutsedge is hardier than purple nutsedge under cold and low moisture conditions, although both species prefer high moisture content. At least a 12 hour photoperiod is required for growth of rhizomes and shoots (Bendixen and Nandihalli, 1987), indicating that these species are a weed problem between the two equinoxes in March and September. SedgeHammer is probably most often applied to campus grounds during these months and may be found at higher concentrations in Lake Matoaka at this time. Future studies could measure the concentration of SedgeHammer in the lake, and, therefore, its environmental relevance, using high performance liquid chromatography.

In order to determine the potential inducing power of each agent, experiments were performed using the well-characterized inducible system *E. coli* (λ). These experiments were designed to act as a comparison group for the same treatments applied to lake water. However, VDC in the two control replicates varied greatly, causing a large standard deviation about the mean used to compare the induction of λ prophages (Fig.

10). If more time were allotted for this study, this experiment would be repeated with certain modifications. Instead of keeping the cells suspended in broth for the duration of the experiment, at t = 0 (just prior to adding inducing agent) cells would be pelleted down and then resuspended in fresh media so as to remove any free λ phages in the medium. It is possible that this wide variation in the baseline count of λ caused the lack of significant difference between the controls and treatments (Fig. 10).

While mitomycin C induced the greatest numbers of viruses in *E. coli* (λ), SedgeHammer at a concentration of 0.05 μg mL^{-1} caused the largest increase in VDC in the environmental sample. As has been shown previously with induction of ALS, bacterial cells eventually die from an inability to create three amino acids. This indicates that RecA is most likely activated during this time, causing induction of the prophage. UV light also significantly induced prophage in environmental samples; however, future studies could measure the amount of UV radiation absorbed by Lake Matoaka in order to determine whether the laboratory application of radiation was similar in strength. The lowest concentration of SedgeHammer actually appeared to cause a decrease in average VDC in the lake water, although the reason for this is unknown. This demonstrates that all three agents tested were able to induce prophage, although concentration plays a role.

Bacterial mortality of *E. coli* cells was highest due to mitomycin C, but this trend was not observed as strongly in environmental samples (Figs. 9, 11). This may be due to the DNA repair abilities of species of bacteria in the lake. UV light caused no apparent bacterial mortality in lake water, possibly because bacteria living in the surface waters of Lake Matoaka during the month of March may be more fit to deal with strong UV radiation and the DNA repair that must occur as a result.

Other studies have been conducted on environmental pollutants as inducing agents. Jiang and Paul (1996) found that the application of aliphatic and aromatic hydrocarbons induced lysogeny in indigenous marine samples. Danovaro and Corinaldesi (2003) showed that sunscreen and sun oil induce prophage in marine samples as well. Testing environmental pollutants as inducing agents is a valuable area of study as we obtain a greater understanding of the importance and role of temperate phages in aquatic systems. This study's work with the herbicide SedgeHammer adds to our knowledge of environmentally applicable inducing agents.

Correlations of Environmental Data and Lysogeny

In order to determine potential drivers of lysogeny in Lake Matoaka, Pearson correlations were performed, testing for relationshops between LF and all measured parameters in the Lake. P-values were used to determine the significance of any correlations and were considered significant using the Bonferroni correction ($p \leq 0.00238$) (Table 3). However, no data in the present study met this requirement. The burst size and temperature and VDC and BDC had small p-values, however, indicating that burst size and temperature may increase together, and ambient viral abundance and bacterial abundance may do the same. After transforming the data for normality, these slight relationships were no longer seen. These weak relationships might be made strong upon further sampling. However, based on the lack of any significant relationships, it appears that something other than pH, temperature, chlorophyll a, viral abundance, or bacterial abudance is driving lysogeny in Lake Matoaka, represented by the lysogenic fraction. Choi *et al.* (2009) found that higher bacterial abundance caused a higher LF.

However, data from the present study did not support that conclusion. Although nutrient data was not obtained for sampling months, it is possible that adding nutrient data to the Pearson correlation would demonstrate a relationship between lysogeny and nutrients.

Conclusions

Lake Matoaka is hypereutrophic, shallow, freshwater lake that is comparable to many temperate lakes around the world. Studying Lake Matoaka may illuminate trends in similar ecosystems. The monthly samples in this study suggest that lysogeny does not play a large role in the microbial ecology of Lake Matoaka in the winter. Lysogens in the lake are more prevalent in March, when there is actually drop in nutrients. This provides additional rationale for the inclusion of nutrients levels in all future studies. The low apparent incidence of lysogeny in the winter is most likely due to the fact that Lake Matoaka is hypereutrophic, providing ample nutrients for bacteria and, therefore, efficient lytic infections. Moreover, UV and an herbicide were shown to cause induction in the lake when lysogeny was more prevalent (39.27% LF) than the percentages demonstrated in the winter (Table 1). If UV and SedgeHammer are common in the environment, these may cause induction in the lake before samples are taken, thereby decreasing the amount of detectable lysogeny. Based on Pearson correlations and t-tests, higher temperatures correlate with a decrease the incidence of lysogeny in the lake, probably due to temperature-dependent increases in other measurable parameters like bacterial density. However, the LF in March (39.27%) seems to show that low nutrient levels override the effects of a higher temperature. Overall, it seems as though lysogeny is not an important facet of the microbial ecology of Lake Matoaka.

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TABLES

Table 1. VDC, BDC, burst size, and lysogenic fraction for November, December, February, and March

	$V_{induced}$ [a] ($X 10^5$)	SD [b] ($X 10^5$)	B_{lysed} [c] ($X 10^4$)	SD ($X 10^4$)	B_z	SD	LF (%)	SD (%)	LF assuming $B_z = 50$ (%)	SD (%)
Nov-09	0.82	1.74	1.24	5.95	6.65	94.60	16.63	94.60	2.21	4.91
Dec-09	-0.11	1.15	6.97	4.56	-0.15	-1.65	54.50	825.00	-0.17	-1.80
Feb-10	1.25	2.61	0.13	1.22	98.53	964.00	26.70	26.60	5.23	10.90
Mar-10	17.30	11.10	16.90	14.50	10.23	10.90	39.27	50.50	8.04	5.78

V_C, V_I in viruses mL^{-1}; B_C, B_I in bacteria mL^{-1}; B_z in number of viruses

[a].$V_{induced}$ is $V_I - V_C$. [b] SD is standard deviation. [c] B_{lysed} is $B_C - B_I$.

Table 2. Comparison of inducing agents in *E.coli* (λ) and March samples

		$V_{induced}$ (X 10^5)	SD (X 10^5)	B_{lysed} (X 10^4)	SD (X 10^4)	B_z	SD	LF (%)	SD (%)	LF assuming B_z = 50 (%)	SD (%)
E. coli	MitC	2639.33	1742.31	6644.59	1946.17	3.97	72.20	76.25	76.40	6.06	4.23
	UV	415.50	1507.59	-689.69	1923.35	-6.02	-27.60	-7.92	-46.30	0.95	3.47
	Sedge Hammer [0.05]	519.79	1528.73	975.66	1973.89	5.33	19.00	11.20	51.80	1.19	3.52
	Sedge Hammer [0.005]	74.02	1494.35	437.37	2315.30	1.69	35.30	5.02	146.00	0.17	3.43
March	MitC	17.33	11.05	16.94	14.45	10.23	10.90	39.30	50.50	8.04	5.78
	UV	20.12	7.87	-4.34	14.48	-46.35	-156.00	-10.06	-34.20	9.33	4.79
	Sedge Hammer [0.05]	27.03	7.80	5.08	14.67	53.20	154.00	11.78	34.60	12.50	5.52
	Sedge Hammer [0.005]	-15.03	8.12	9.96	14.36	-15.10	-23.20	23.09	38.50	-6.97	-4.42

V_C, V_I in viruses mL^{-1}; B_C, B_I in bacteria mL^{-1}; B_z in number of viruses

Table 3. Pearson correlations and p-values of Lake Matoaka water data for November, December, February, and March.

	Chl a	Temp	pH	Vc	Bc	VBR	LF	LF assuming Bz = 50 (%)	Bz	Reciprocal temp	log VBR	Reciprocal Vc	log Bc
Chl a		0.50	0.88	0.42	0.29	0.80	0.30	0.60	0.67	-0.35	0.72	-0.74	0.18
		0.50	0.12	0.58	0.71	0.20	0.70	0.40	0.33	0.65	0.28	0.26	0.82
Temp	0.50		0.30	-0.19	-0.23	0.85	-0.59	0.37	0.97	-0.95	0.79	-0.31	0.04
	0.50		0.70	0.81	0.77	0.15	0.41	0.63	0.03	0.05	0.21	0.69	0.96
pH	0.88	0.30		0.80	0.71	0.75	0.20	0.87	0.40	-0.28	0.77	-0.96	0.62
	0.12	0.70		0.20	0.29	0.25	0.80	0.13	0.60	0.72	0.23	0.04	0.38
Vc	0.42	-0.19	0.80		0.99	0.32	0.18	0.84	-0.17	0.06	0.45	-0.87	0.87
	0.58	0.81	0.20		0.01	0.68	0.82	0.16	0.83	0.94	0.55	0.13	0.13
Bc	0.29	-0.23	0.71	0.99		0.25	0.08	0.82	-0.25	0.06	0.41	-0.82	0.92
	0.71	0.77	0.29	0.01		0.75	0.92	0.18	0.75	0.94	0.59	0.18	0.08
VBR	0.80	0.85	0.75	0.32	0.25		-0.33	0.75	0.88	-0.82	0.98	-0.75	0.40
	0.20	0.15	0.25	0.68	0.75		0.67	0.25	0.12	0.18	0.02	0.25	0.60
LF	0.30	-0.59	0.20	0.18	0.08	-0.33		-0.24	-0.36	0.78	-0.42	0.01	-0.33
	0.70	0.41	0.80	0.82	0.92	0.67		0.76	0.64	0.22	0.58	0.99	0.67
LF assuming Bz = 50 (%)	0.60	0.37	0.87	0.84	0.82	0.75	-0.24		0.34	-0.49	0.86	-0.97	0.89
	0.40	0.63	0.13	0.16	0.18	0.25	0.76		0.66	0.51	0.14	0.03	0.11
Bz	0.67	0.97	0.40	-0.17	-0.25	0.88	-0.36	0.34		-0.85	0.77	-0.34	-0.06
	0.33	0.03	0.60	0.83	0.75	0.12	0.64	0.66		0.15	0.23	0.66	0.94
Reciprocal temp	-0.35	-0.95	-0.28	0.06	0.06	-0.82	0.78	-0.49	-0.85		-0.83	0.37	-0.28
	0.65	0.05	0.72	0.94	0.94	0.18	0.22	0.51	0.15		0.17	0.63	0.72
log VBR	0.72	0.79	0.77	0.45	0.41	0.98	-0.42	0.86	0.77	-0.83		-0.82	0.58
	0.28	0.21	0.23	0.55	0.59	0.02	0.58	0.14	0.23	0.17		0.18	0.42
Reciprocal Vc	-0.74	-0.31	-0.96	-0.87	-0.82	-0.75	0.01	-0.97	-0.34	0.37	-0.82		-0.80
	0.26	0.69	0.04	0.13	0.18	0.25	0.99	0.03	0.66	0.63	0.18		0.20
log Bc	0.18	0.04	0.62	0.87	0.92	0.40	-0.33	0.89	-0.06	-0.28	0.58	-0.80	
	0.82	0.96	0.38	0.13	0.08	0.60	0.67	0.11	0.94	0.72	0.42	0.20	

The top value is the Pearson correlation coefficient, *r*, and the bottom value is the p-value.

FIGURES

Figure 3. Seasonal Variation in Chlorophyll a, Temperature, and pH

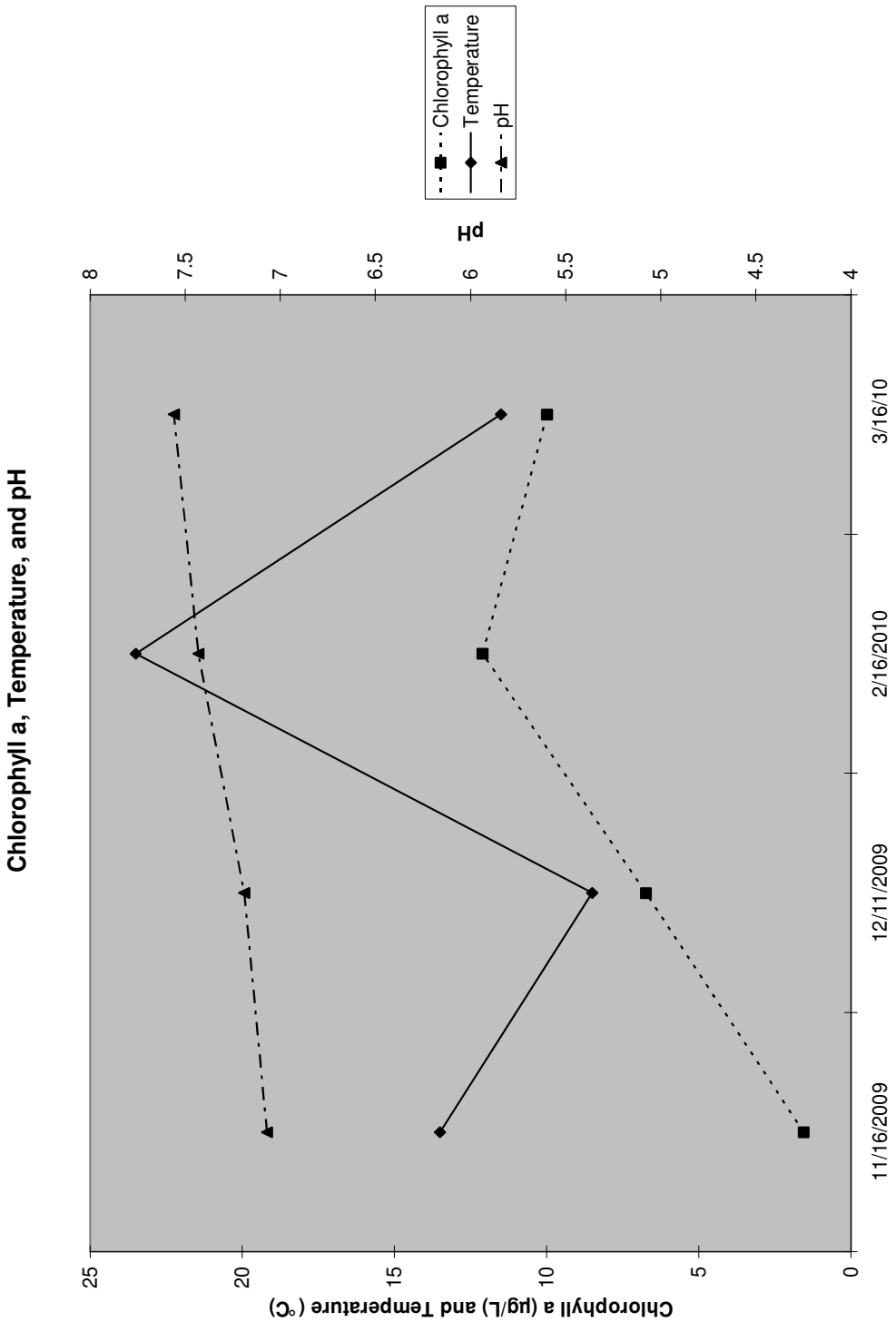

Figure 4. Seasonal Variation in BDC, VDC, and VBR

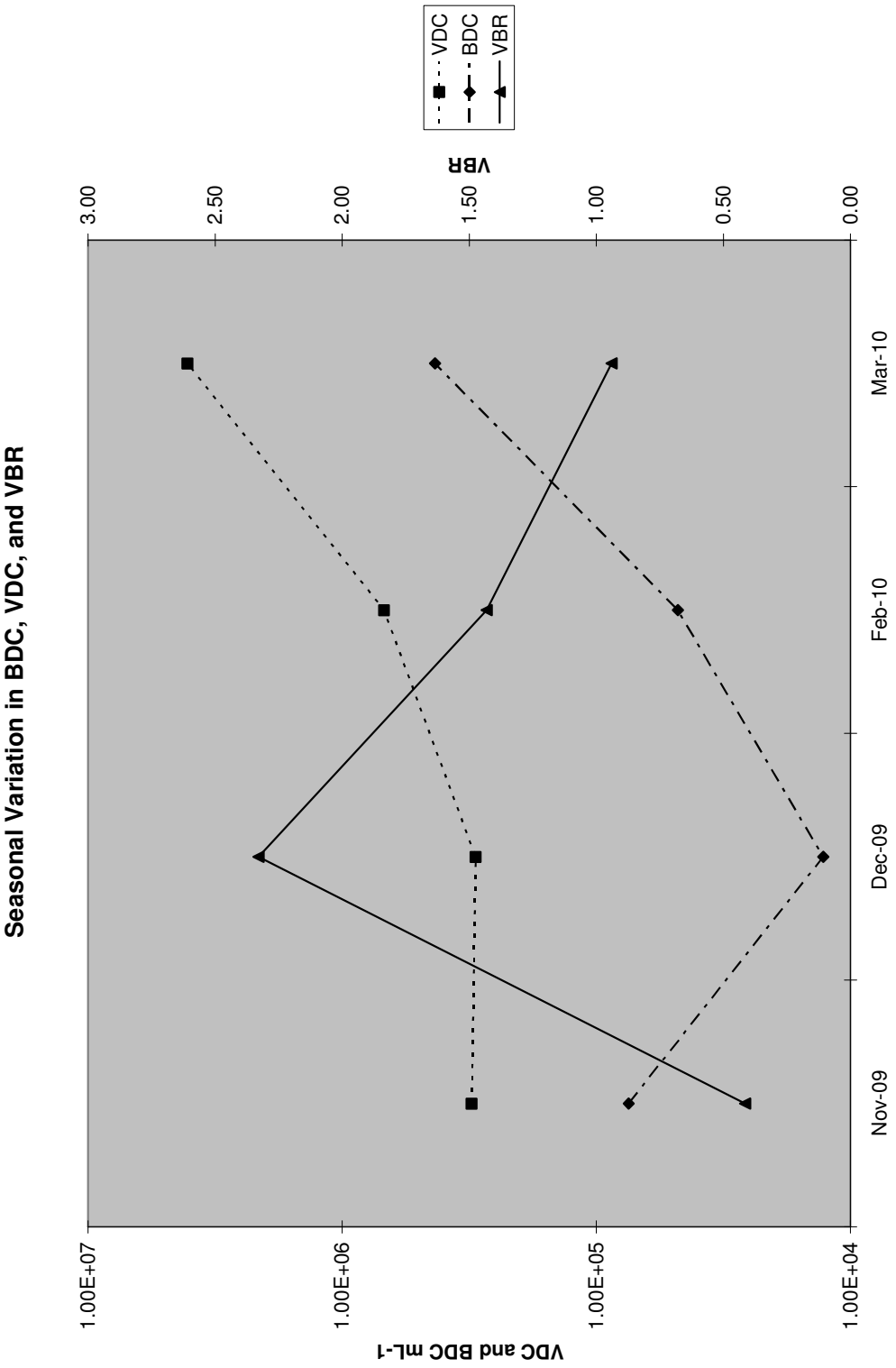

Figure 5. November enumeration of VDC and BDC

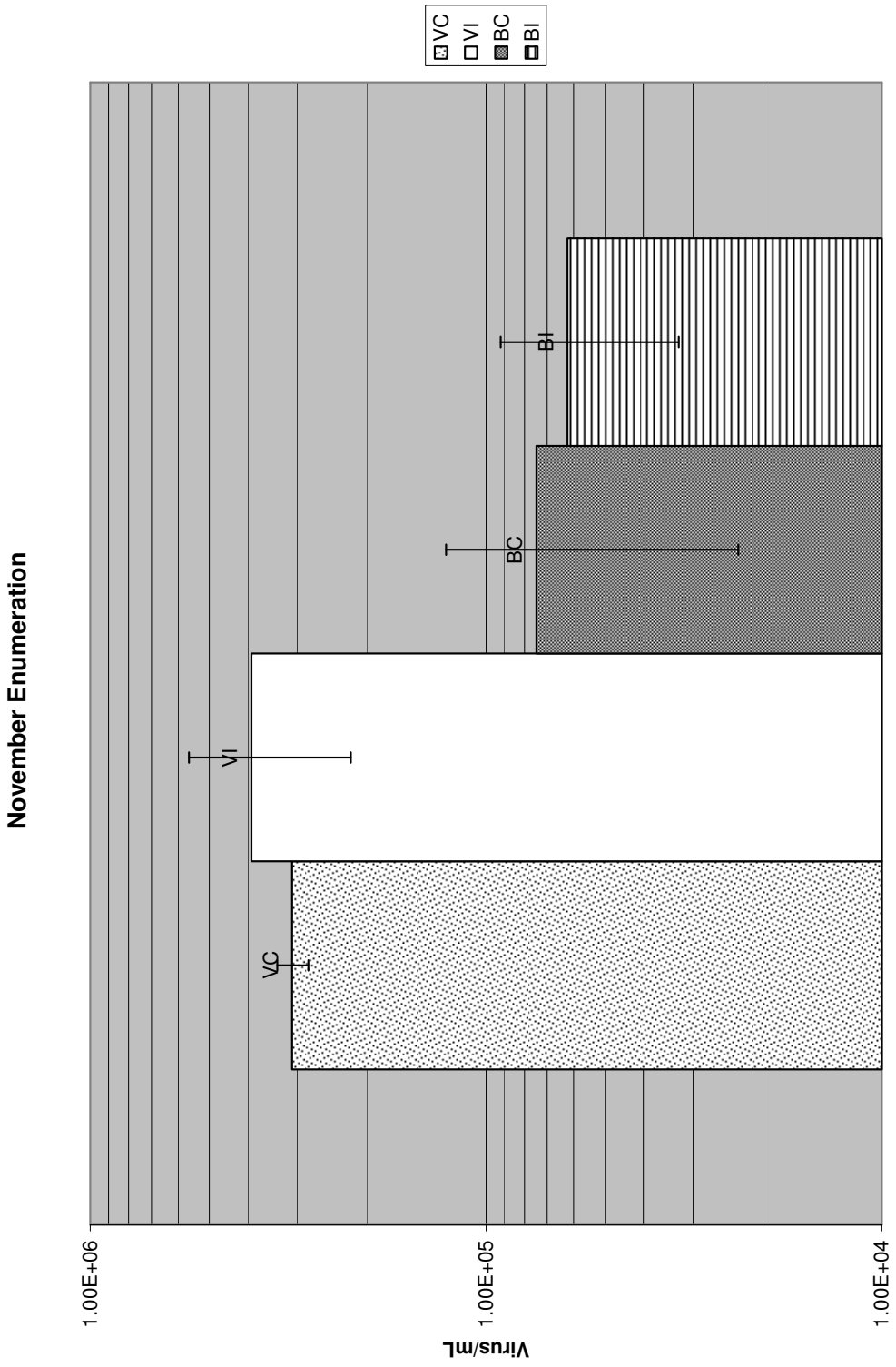

Figure 6. December enumeration of VDC and BDC

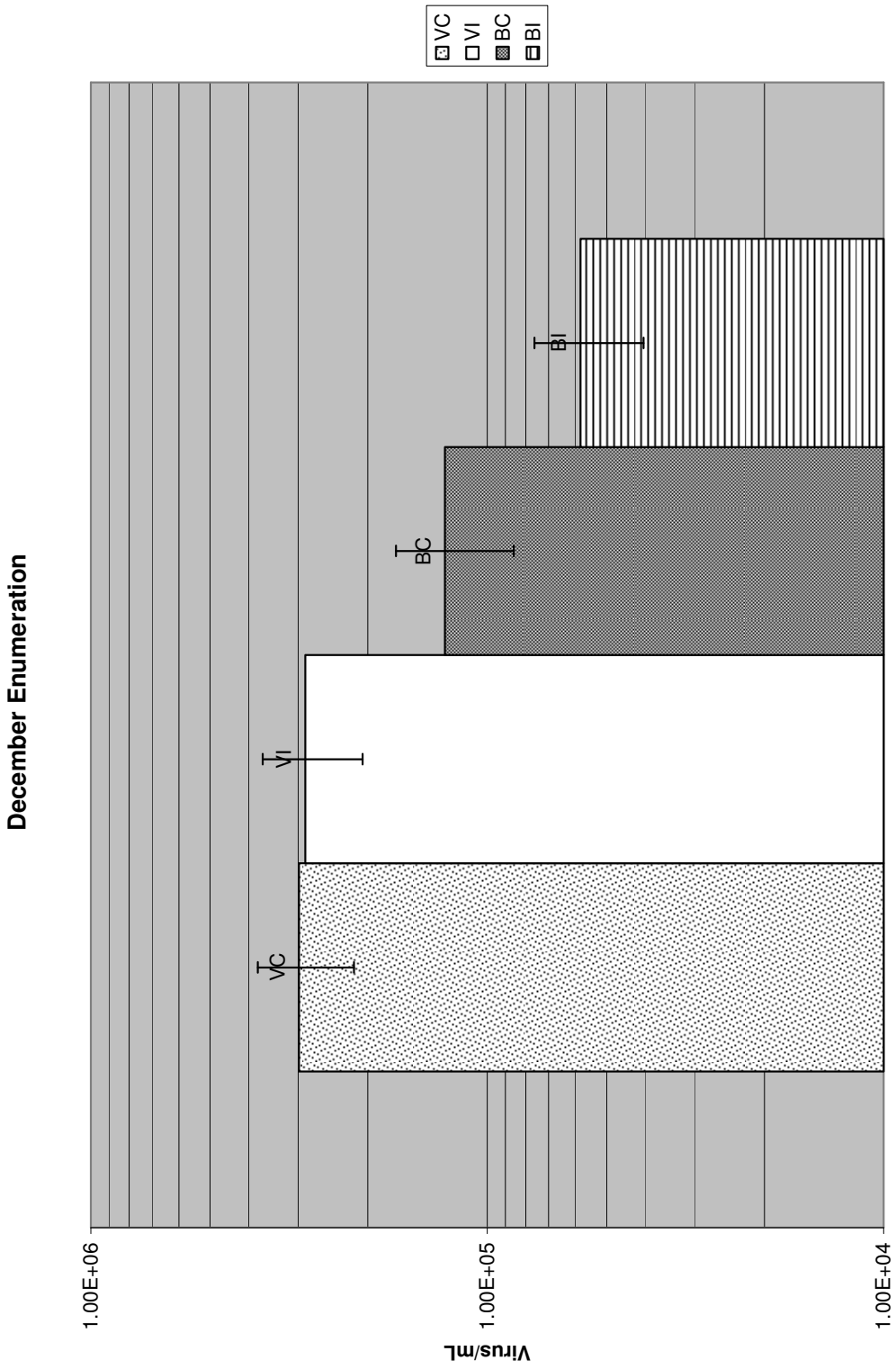

Figure 7. February enumeration of VDC and BDC

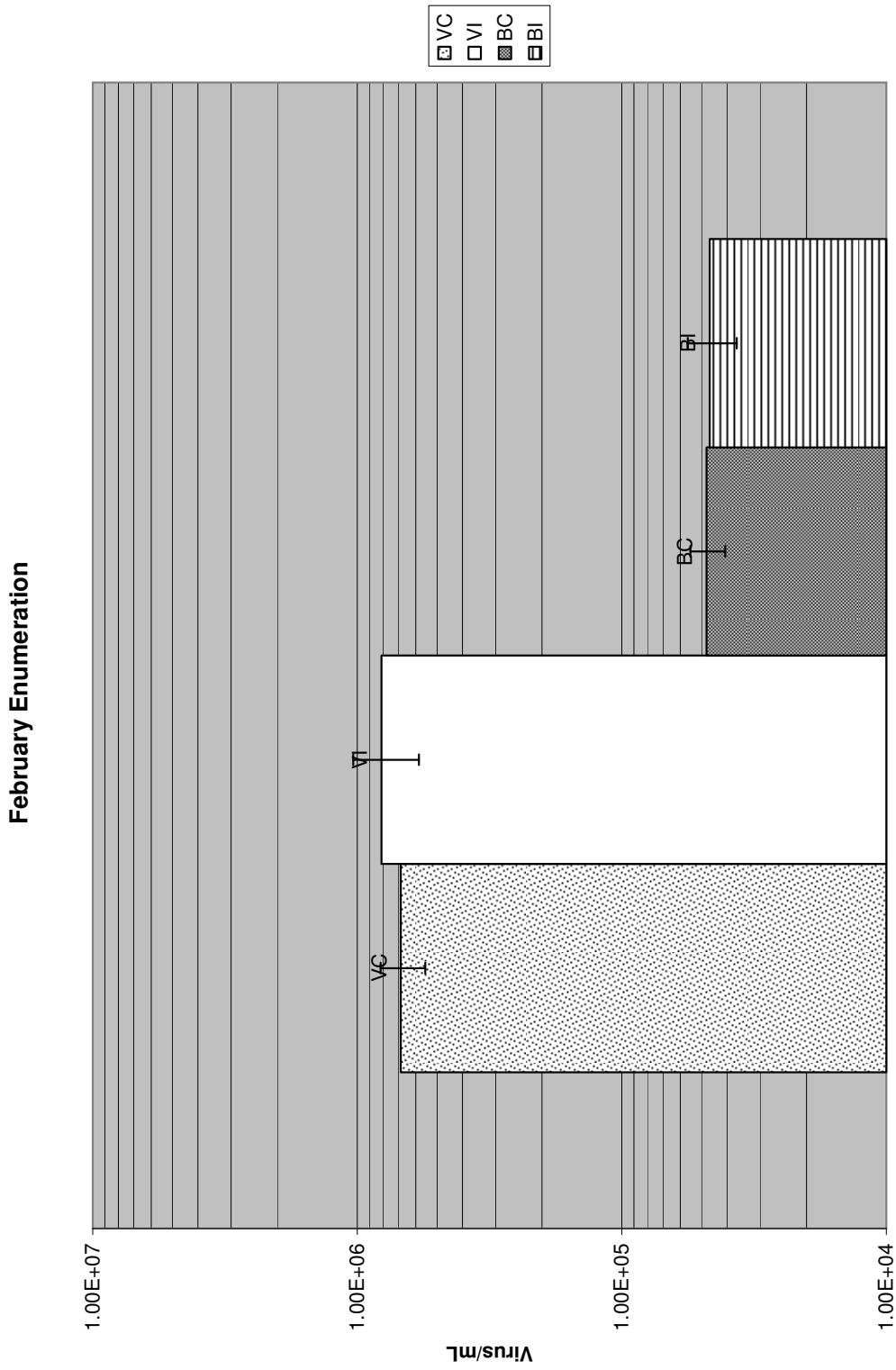

Figure 8. March enumeration of VDC

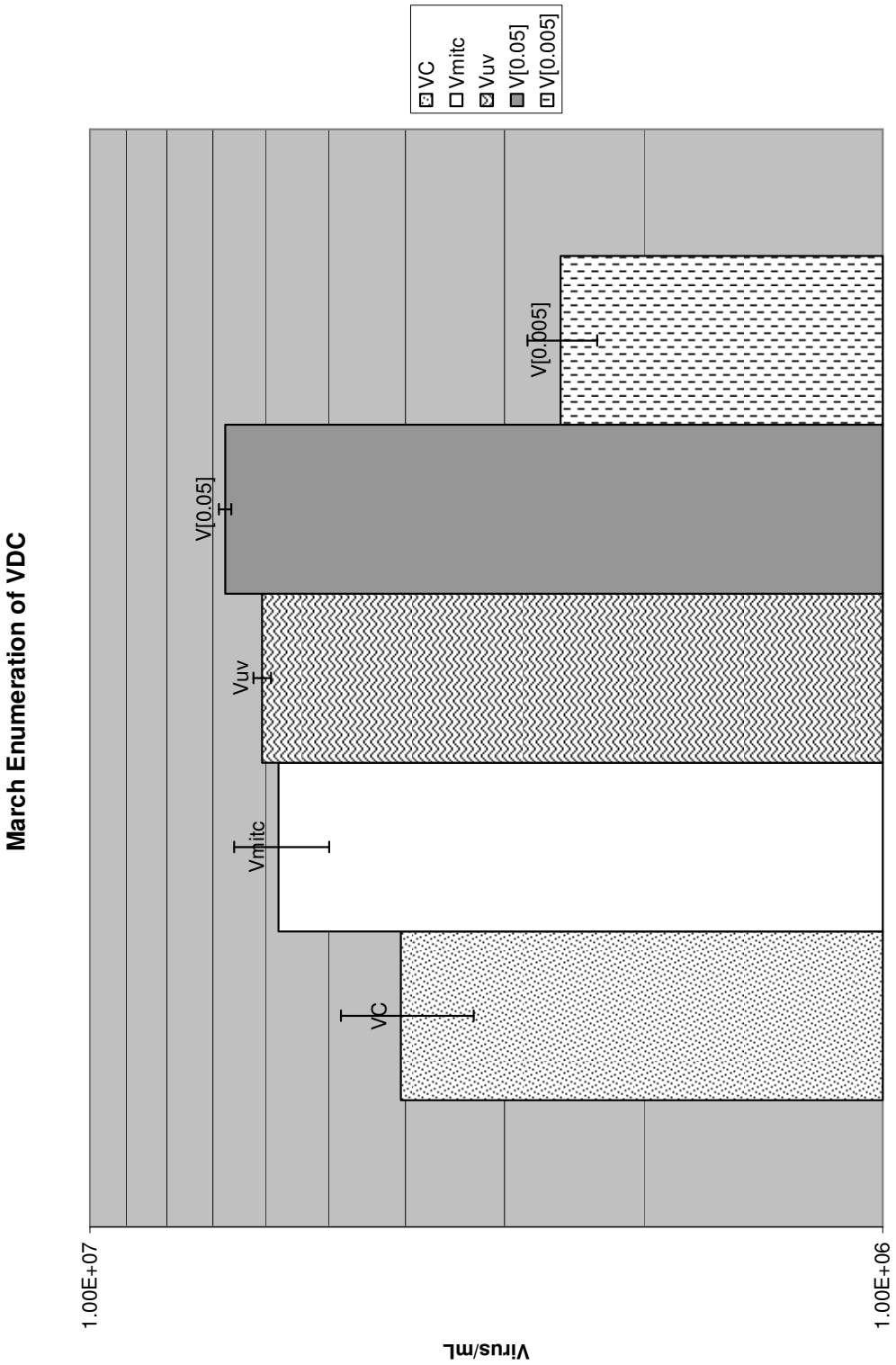

Figure 9. March enumeration of BDC

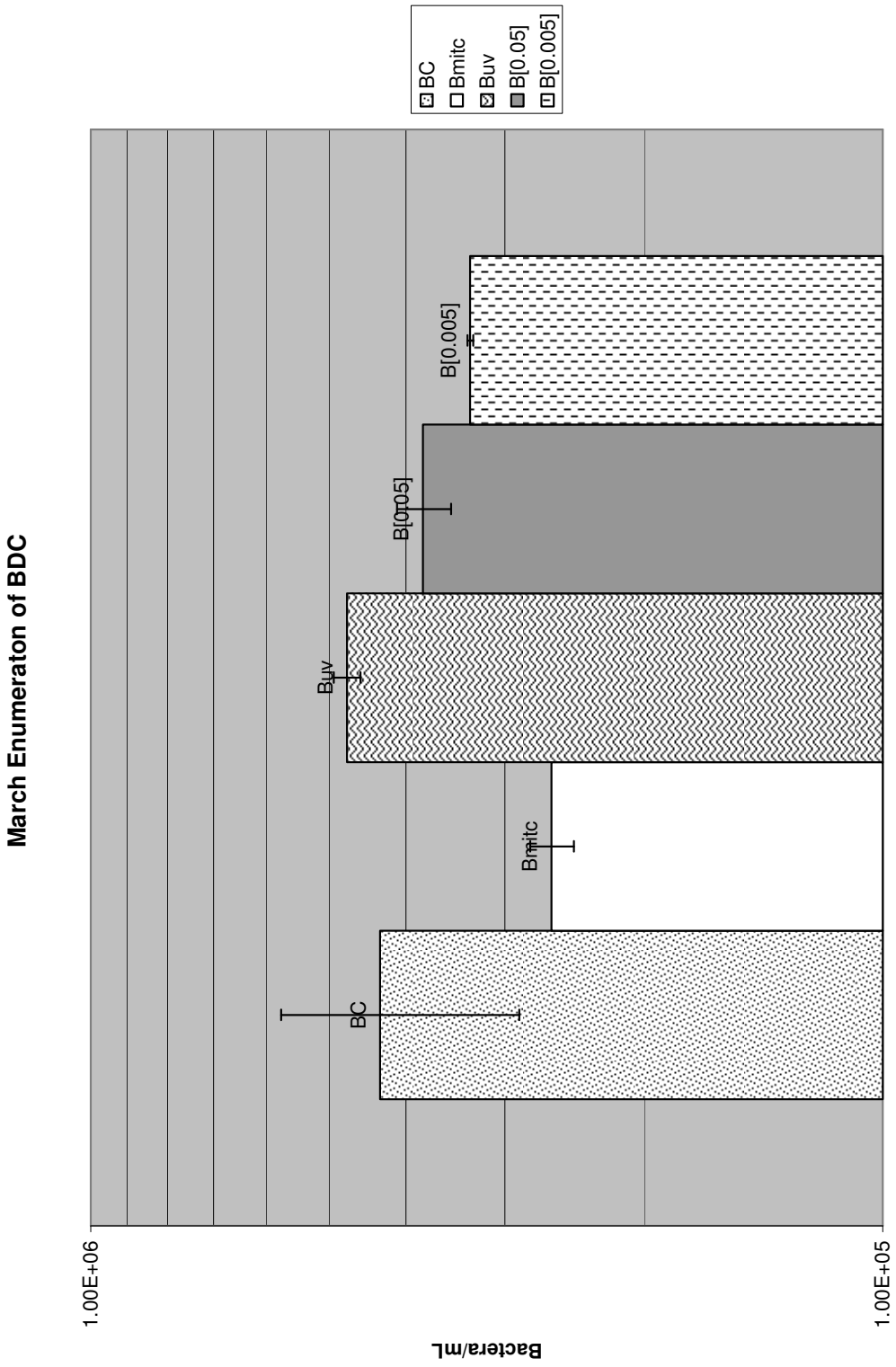

Figure 10. *E. coli* (λ) enumeration of VDC

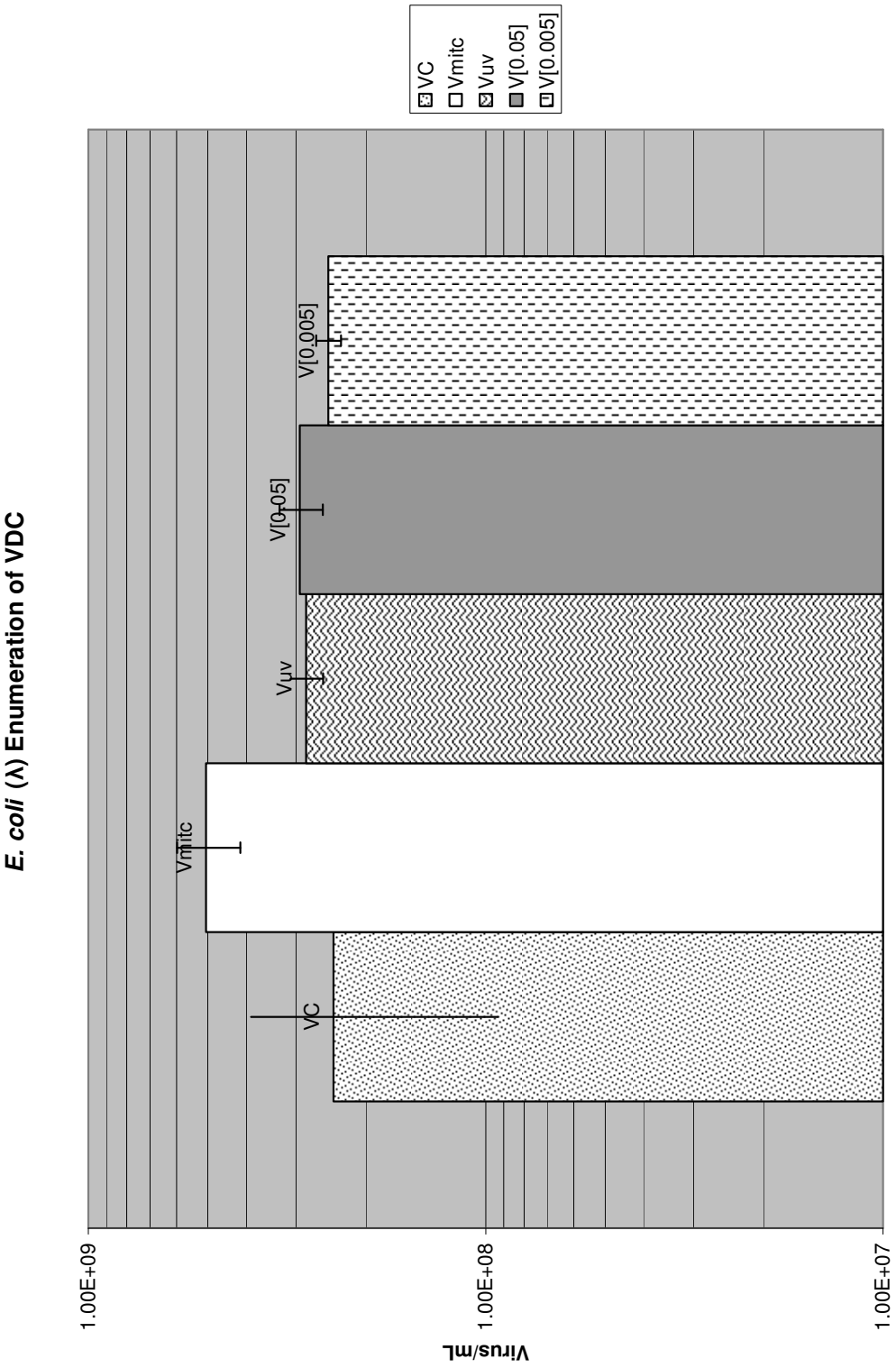

Figure 11. *E. coli* (λ) enumeration of BDC