Using *Tetrahymena thermophila* to study the role of protozoa in the inactivation of viruses in water

by

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I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Ciliated protozoa, such as *Tetrahymena thermophila*, are ubiquitous in the aquatic environment, including in sewage treatment plants. Their ability to graze on particulates could influence water quality in a number of ways, one of which is through their interaction with viruses. The viruses could be bacteriophages or enteric viruses. Bacteriophages can influence water quality by influencing the size and diversity of bacterial populations through the lysis of fast-growing bacteria. This bacterial lysis will affect nutrient cycling in natural waters and effluent quality in sewage treatment, by releasing cell contents and providing slow-growing bacteria an opportunity to establish a stable population. Bacteriophages are also used as surrogates for studying enteric viruses, which are often more difficult to monitor in environmental samples than phage. As a result, studying how bacteriophages are inactivated is important to understanding how to enhance water quality.

To this end, I have examined the ability of *T. thermophila* to inactivate two types of bacteriophages: T4 and MS2. T4 is one of the largest phages; MS2, one of the smallest. The genome of T4 is a single linear molecule of double stranded DNA , making it quite resistant to natural decay. MS2 is a single stranded RNA phage, and susceptible to decay even at room temperature. This phage is sometimes used in environmental monitoring as a substitute for poliovirus. In this study, phage and ciliates were incubated together under different conditions and for varying times, after which the mixture was centrifuged through a step gradient to produce a top layer free of ciliates. Titre was assayed using the double-agar overlay. For T4, the titre in the top layer decreased as co-incubation time increased, but no decrease was seen if T4 were incubated with formalin-fixed *Tetrahymena*. The T4 titre associated with the pellet of

living ciliates was very low, suggesting that removal of the phage by *Tetrahymena* inactivated T4. In the case of MS2, there was a similar, although smaller, loss of infective phage attributed to viable ciliates. Natural decay of MS2 was detectable, but less than loss due to viable ciliate. MS2 titre when incubated with fixed-*Tetrahymena* was not found to differ from the no-ciliate control. When *Tetrahymena* were incubated with SYBR Gold-labeled T4 or MS2 phage, fluorescence was localized in structures that had the size and position of food vacuoles. Incubation of phage with ciliates that had been exposed to cytochalasin B or to 4 ^{o}C, conditions inhibiting food vacuole formation, resulted in much lower phage inactivation. When SYBR Gold-labeled phage were incubated with ciliates that had been exposed to these same food vacuole inhibiting conditions, no internal fluorescence was seen.

Results suggest the active removal of T4 from fluid by macropinocytosis, followed by digestion in food vacuoles. For MS2, inactivation by *Tetrahymena* also appeared to occur by macropinocytosis, however the role of micropinocytosis could not be discounted. Studying the inactivation of MS2 is more difficult because of the natural decay of the phage over time in a saline solution. Thus, whether ciliate virophagy is selective or general in regards to viruses, remains unanswered. Either way, phage inactivation by *T. thermophila* could be occurring in natural waters and sewage treatment plants, and contributing to water quality.

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General Introduction

As humanity's ecological footprint increases, there is a growing need to reduce the negative impacts that humans impose on the Earth's natural resources. Water is one such resource; it is integral to our survival, but maintaining suitable sources for consumption, or other human uses, has become complicated and has necessitated the development of methods for dealing with various contaminants. Biological wastes, resulting from animal and human excrement, are common origins for a great deal of water contamination, and pathogenic bacteria and viruses originating from these wastes are referred to as *enteric* pathogens. These microbial pathogens pose significant threats to public health, with 250 million people suffering from water-borne disease yearly (Toze, 1999). Aside from enteric pathogens, most surface waters are populated by wide ranges of native bacteria, protozoa, and viruses whose interactions are essential to the proper functioning of their ecosystem. Of all the microbes inhabiting aquatic environments, viruses are the most abundant, present at levels on the order of 10^{10} l^{-1} and at least a portion of the viral community is pathogenic to humans (Fuhrman, 1999). Untreated wastewater may contain viral pathogen titres exceeding 10^{3}-10^{4} l^{-1} (Grabow, 2001) and may include poliovirus, enterovirus, hepatitis A, adenovirus, reovirus, Norwalk virus, rotavirus, F-specific bacteriophage, and astrovirus (US EPA, 1992). Thus, understanding the biology of viral inactivation is critical to the development of reliable and effective methods for reducing and removing viral pathogens in water.

A. Viruses

Viruses are non-cellular entities that infect their host organism, hijacking host gene replication systems for the reproduction of viral genetic material. The viral genome may be

comprised of single-stranded or double-stranded DNA or RNA. The viral genome is enclosed by a proteinaceous shell referred to as a *capsid*, which may have an icosehedral or helical morphology. Some viruses are more complex, and are housed within a lipid bilayer *envelope*, which houses essential viral glycoproteins along with the capsid and genome (Flint *et al.,* 2000). Organisms from all kingdoms are subject to viral infection and, generally, viruses are specially adapted to a limited number of host organisms.

Regardless of host, viruses have considerable variability in physiochemical properties. Both size and shape may vary considerably from less than 20 nm to 300 nm diameter (Flint *et al.,* 2000). The presence of a capsid or thick envelope will also change the properties of a virus and increase its size, resulting in very complex viruses. Complexity of a virus, however, does not correspond to complexity of the host organism. *Poliovirus*, the virus causing polio in humans, belongs to the *picornavirus* family, has a non-enveloped capsid and a simple icosehedral shape. Its genome is a single strand of RNA and the virus is approximately 20 nm in diameter, making it one of the smallest and simplest human pathogens (Flint *et al.,* 2000). Poliovirus also bears a large degree of similarity to MS2 bacteriophage (Figure I.1). MS2 is an *enteric coliphage*; a phage originating in the gut of animals and infecting *Escherichia coli* C3000, and is a common lab surrogate for poliovirus to the point that the two may be indistinguishable under the electron microscope (Grabow *et al.*, 1999). MS2 is also a common virus used in the modeling of pathogen dispersal in the environment, as it shows a high affinity for adsorption to solids. This is due to a low isoelectric point (pI), or the pH at which the capsid exhibits a neutral charge (Dowd *et al.*, 1998). MS2 has a pI of 3.9, much lower than that of other model bacteriophage, such as T4, whose pI is 4.2 and is much less-likely to adsorb to surfaces (Sakoda *et al.*, 1997). Bacteriophage T4 (Figure I.2) is also an *enteric coliphage,*

but shows much different physiochemical properties from MS2. Aside from pI, its morphology is much different. Its icosehedral head (~ 85 nm in diameter) region has a large protruding tail (~200 nm in length) and its genome is encoded on a double-stranded DNA genome (Flint *et al.,* 2000).

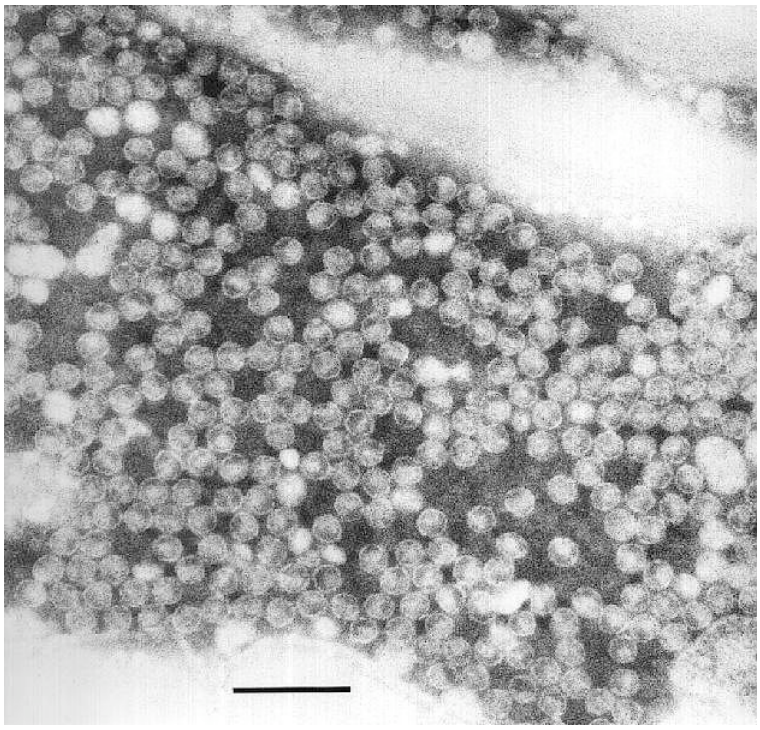

Figure I.1 External Morphology of Enterobacteriophage MS2

Micrograph originally produced by Dr. Hans Ackermann of the Université Laval, using a Philips EM 300 electron microscope operating at 60 kV. The bar represents 100 nm. Image freely available online at ICTVdb (2006).

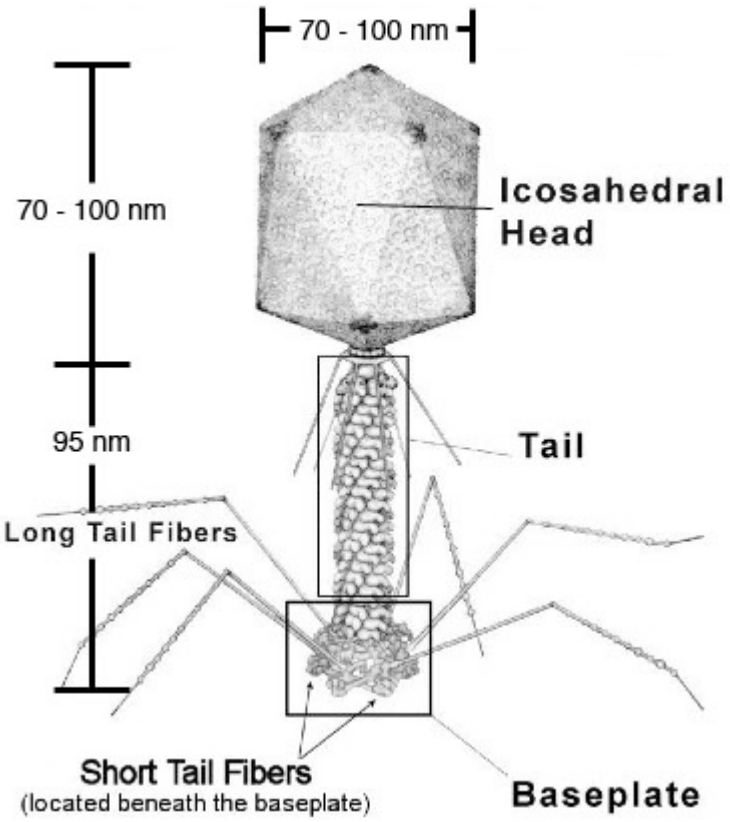

Figure I.2 External Morphology of Enterobacteriophage T4

Representation of the morphology and size of the bacteriophage T4 virion, where the long tail can be seen to extend from the head region. This image was originally adapted from Petr Leiman of Purdue University (Mesyanzhinov *et al.,* 2004).

B. Ciliated protozoa

Ciliated protozoa are found in both natural and man-made ecosystems. The naturally found, microphagous ciliate *Tetrahymena thermophila* is frequently used in ciliate studies, and holds many advantages over other protozoa. They are easily grown axenically in inexpensive medium and will generally achieve sufficient density for experimentation overnight (Fenchel, 1980). They have a pear shape, measuring about 50 μm in length and 30 μm in width (Figure 0.3), and are very motile, thanks to numerous parallel rows of cilia covering the majority of the cell surface; a schematic of ciliate structure is presented as figure 0.3 (Hill, 1972). *Tetrahymena* is one of the most studied ciliates, resulting in a very well understood cellular system and digestive pathways (Nilsson, 1976). At the cell surface, *micropinocytosis* will ingest liquid and produce micropinosomes (0.07 – 0.1 μm diameter) in a process that is poorly understood (Bishop, 1997). Material entering the buccal cavity will be moved toward the base of this mouth region by the action of oral cilia, and be ingested by either *phagocytosis* (large particulates) or *macropinocytosis* (liquid). Here, a membrane fusion will produce an enclosed food vacuole (~5 μm diameter) or a macropinosome (0.5 – 2 μm diameter) (Bishop, 1997). The vacuole will then leave the oral region (cytopharynx) and will fuse with other endosomes, and the lysosome, at which point digestion will occur (Nilsson, 1976). Following digestion, indigestible material will be moved to, and expelled from the cytoproct. There is some controversy as to whether expelled material is enclosed by a membrane. However, Brandl et al (2005) recently presented evidence that *Tetrahymena* released *Salmonella enterica* enclosed within vesicles. The progression for a single food-vacuole from formation to egestion will take about 2 hours (Nilsson, 1972). Although *T. thermophila* requires a nutrient-rich medium to proliferate, they may be maintained in a minimal inorganic salt buffer over a number of days;

this is essential for many studies involving viruses. Additionally, they have been isolated from sewage plants and thus studying their interactions with viruses will be biologically relevant (Curds, 1973).

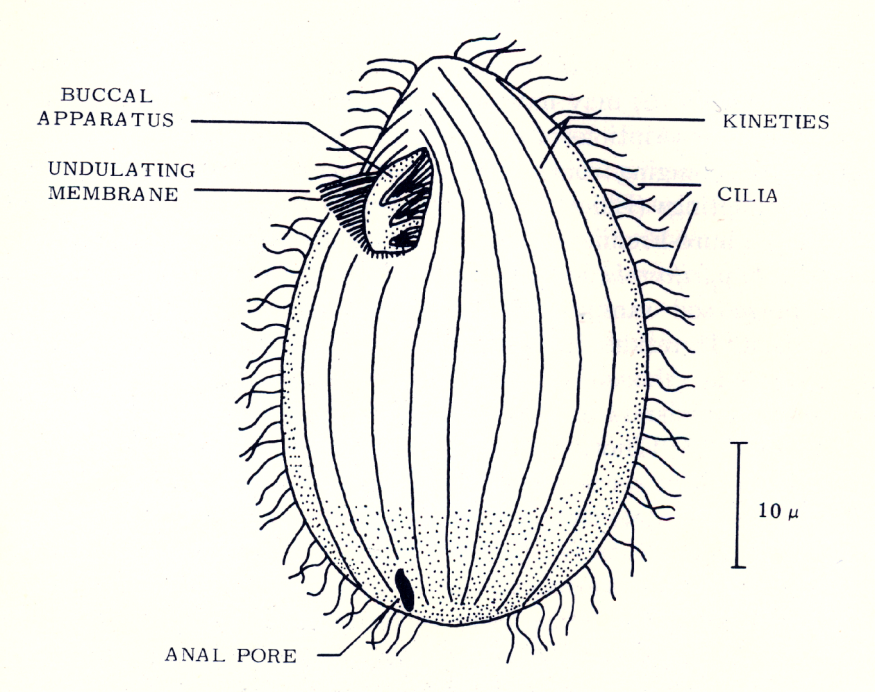

Figure 0.3 Morphology of *Tetrahymena thermophila*

External morphology of the grazing ciliate *T. thermophila.* Areas of interest include the *undulating membrane*, which will direct particles into the *buccal apparatus* or mouth region, where material can be internalised. The *anal pore* is the site at which indigestible material will be expelled, following the digestive cycle. Rows of surface *cilia* will move the ciliate through the medium. Adapted from Hill (1972).

C. Man-made ecosystems

Man-made ecosystems are those that are designed, stocked, and monitored by man, and that would not normally be found in the environment. Sewage treatment plants utilize man-made ecosystems in secondary treatment of sewage, where much of the bacterial contaminant removal occurs. There are two methods of secondary treatment; percolating (or biological) filters (Figure I.4) and activated-sludge (Figure I.5), and both involve complex microbial environments commonly involving biofilm and floc producing microbes in the reduction of harmful microbe numbers. Curds (1992) provides an excellent overview of the two processes. Percolating filters use large, circular vessels lined with a mineral medium (such as stones or gravel), whereupon a complex microbial milieu of bacteria, protozoa, algae, macro-invertebrates, and fungi grow. This creates a large, aerated surface on which bio-films will form, and over which sewage will flow. Small particulates from the sewage will be formed by bacteria into floc that will settle on the vessel bottom where they may be degraded by bacterial and fungal extracellular enzymes or be consumed by macro-invertebrates or protozoa. Additionally, nitrifying bacteria present in the lower regions of the filters will oxidize ammonia. Finally, settled sewage is removed by rotating arms, preventing overflow. The activated-sludge treatment involves seeding of new sewage with well-aerated sludge from past treatment that is maintained at upper-mesophilic temperatures (30 ^{o}C - 40 ^{o}C) in large aeration tanks. This well-aerated sludge, referred to as *activated sludge*, will promote growth of heterotrophic bacteria in the new sludge. Dissolved oxygen in the system must be monitored and maintained to allow nitrification. After several hours of aeration and mixture, activated sludge will overflow into a large separation tank, where solids will settle and be removed as waste (with some of this material returning to the original aeration tank to seed new sludge).

Activated sludge biota differs from that of percolating filters, and is generally populated by nematodes, rotifers and annelid worms in small numbers, with a higher number of floc-producing bacteria than percolating filters, and much higher numbers of protozoa with abundances of up to 50,000 cells per mL of mixed liquor being recorded (Curds, 1992).

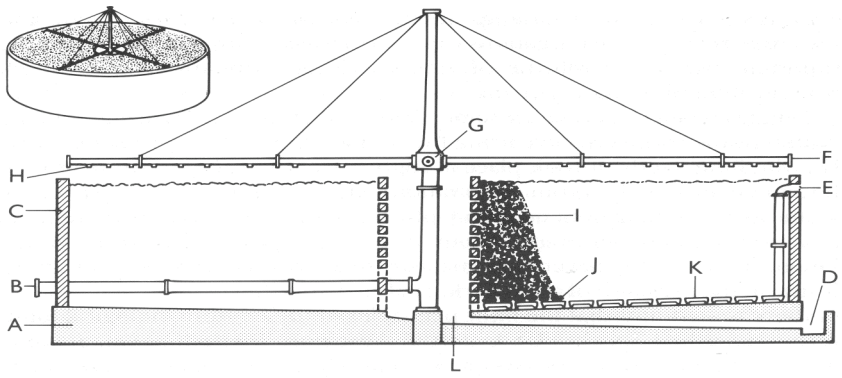

Figure I.4 Sectional View of a Standard Biological Filter

A foundation, **B** feed pipe, **C** retaining wall, **D** effluent channel, **E** ventilation pipe, **F** distributor arm, **G** rotary seal, **H** jets, **I** mineral medium bed, **J** medium base, **K** filter tiles, **L** central well for effluent collection. Microbes will flourish on the mineral bed, creating bio-films, while motile protozoa or nematodes will live along this bacterial surface. Over this extensive surface area, sewage will be passed. Floc will be formed by resident bacteria and will settle to the vessel bottom. Along with flocculation, bacterial and fungal enzymes and grazing invertebrates will remove waste. At the lower regions of the bioreactor, nitrifying bacteria will oxidize excess ammonia to nitrate. Ultimately, the rotating arms will move settled sewage to the central well where it will be moved to the next step in the treatment process. Figure adapted from Curds and Hawkes (1983).

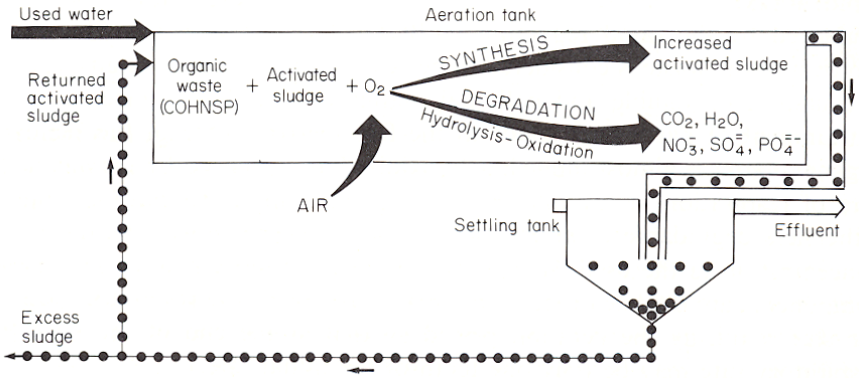

Figure I.5 Schematic of an Activated Sludge System

Sewage and activated sludge from previous runs of the system are mixed (producing *mixed liquor*) and added to an aeration tank. During aeration, aerobic microbes will thrive, producing more activated sludge and this high biomass (largely floc) is continuously mixed to allow contact of the floc with sewage and removal of metabolic wastes of the floc-inhabiting bacteria. After aeration, mixed liquor is moved to a settling tank, where sludge settles from the purified liquor. Purified liquor is moved to additional treatment, while the settled activated sludge is partially recycled, with the excess sludge being removed from the system. Figure adapted from Curds and Hawkes (1983).

D. Viral inactivation

Viral inactivation is a process by which viruses lose the capacity to reproduce. This has been studied for several reasons. Research has been most intensive for the purpose of assuring the safety of human blood plasma products and of inactivated vaccines. Treatments that have been used to inactivate viruses include pasteurization, dry heat, vapour heat, solvent/detergent and low pH. Another reason for studying viral inactivation has been in the treatment of sewage and animal waste. A wide variety of treatments have been used, including ozonation, gamma irradiation, chlorination, heating and drying (Turner and Burton, 1997). Finally, as the ecological importance of viruses has been increasingly recognized, the inactivation of viruses in natural environments has begun to be considered (Weinbauer, 2004). For natural and man-made ecosystems, such as sewage treatment plants, the biological inactivation of viruses has begun to be explored (Kim and Unno, 1996; Miki and Yamamura, 2005). Viral inactivation in these environments is briefly reviewed below.

E. Viral inactivation in sewage treatment

Due to the low infectious dose, common to many viral pathogens, various methods of water treatment have been designed in an attempt to completely eliminate the risk posed by these viruses. Treatment facilities will vary, and selection of suitable protocols will be dependent on factors such as origin of the influent water, nature of the contamination, and intended use of effluents (Curds, 1992). Generally, treatment protocols will focus on chemical or bacterial contaminants, but it is also important to reduce viral contamination in wastewater, as it has been shown that this will greatly reduce the viral load of environmental water downstream of treatment plants (Kim and Unno, 1996).

The method by which viruses are inactivated has not been completely elucidated; however there are various factors contributing to inactivation. Flocculation, the formation of floc by floc-producing bacteria, will reduce organic matter in the sludge, but will also provide a solid medium to which some viruses may adsorb. Once this floc settles and is mechanically removed from the sewage, it effectively removes any adsorbed virus as well (Curds, 1992). Aside from adsorption, floc-inhabiting bacteria are believed to release virucidal agents, contributing to the reduction of viral load (Knowlton and Ward, 1987). The impact of grazing ciliates and flagellates, present both on floc and free-living, is still not fully understood (Kim and Unno, 1996). However, metabolic functions carried out by sludge microbes will result in the maintenance of mesophilic temperatures that partially contribute to the inactivation of RNA viruses, through damage to their genomes, which they do not have the capacity to repair (Turner and Burton, 1997).

Numerous studies have been done comparing different methods of treatment for effectiveness of viral inactivation; the variability between these processes seems undeniable (Tree *et al.*, 1997; Tree *et al.*, 2003; Arraj *et al.*, 2005; Duran *et al.*, 2003; Collivignarelli *et al.*, 2000). In one study comparing inlet and outlet titres for a variety of viruses (including adenovirus, hepatitis A, and F-specific bacteriophage) in three treatment plants, each utilizing different processes of treatment, there existed wide variation in the degrees of viral reduction and that, despite current practices, large numbers of virus were being released to the environment (Myrmel *et al.*, 2006). Thus, the need for a better understanding of the intra-species interactions taking place in biological treatment systems becomes a necessity to developing better treatment practices, and two prime candidate species for further investigation are the bacteriophage and the free-swimming, grazing ciliates.

F. Viral inactivation in natural ecosystems

The influence of *bacteriophage*, or viruses infecting solely bacteria, on their respective eco-systems has been reviewed by Fuhrman (1999), and their effects on a treatment environment are diverse and considered essential to the proper functioning of an activated sludge system (Hantula *et al.*, 1991). Bacteriophage will limit proliferation of their host bacteria, acting as a method of control over bacterial number. This lysis of bacteria will increase levels of dissolved organic matter, which will then be available to newly growing bacteria. This limits the amount of organic matter available to higher trophic levels, creating a "viral loop." Further, bacteria with higher growth rates will make use of nutrients more quickly ("winning" the race to use them), but will also be more commonly infected by bacteriophage. Lysis of these bacteria will aid in preventing the exhaustion of available nutrients, and allow slower growing bacteria to co-exist. This is especially important to less stable stages of sewage treatment, such as nitrification, which require the presence of certain bacterial species that are slow growing (Wanner *et al.,* 2000). The increased rate of infection of nutritional "winners" prevents competitive exclusion, where one species would out-compete other species that share the same ecological niche, but do not show the same level of environmental fitness (Thingstad and Lignell, 1997). This is the basis of the 'kill the winner' hypothesis, and it may be expanded upon to include the free-swimming ciliates known to proliferate in treatment systems and in other environmental waters.

The interaction between phage, bacteria and ciliates could be very complex. Free-living, grazing ciliates will certainly be ingesting bacteria that may, coincidently, be infected by bacteriophage, however there has been little definitive proof that these protozoan are grazing upon free viral particles (Gonzalez and Suttle, 1993), let alone whether their presence

would significantly impact viral number (Frankel, 2000). Further, the fate of any virus that may interact with a protozoan, be it through ingestion or surface adsorption, remains uncertain and in dispute. In a study by Kim and Unno (1996), removal of the human pathogen poliovirus was investigated in the presence of a complex mixture of floc-producing bacteria and either a ciliate (*Tetrahymena pyriformis*), stalked rotifer (*Philodina erythrophthalma*), or a detritus-consuming annelid (*Aeolosoma hemprichi*); all isolated from activated sludge. They concluded that detritus-feeding species would steadily remove virus from the environment due to the ingestion of floc and other solids to which virus had adsorbed to, but the impact of filter-feeding and grazing organisms had a much larger effect on viral reduction. Unfortunately, this group did not strive to investigate the cellular mechanism involved in this removal nor what would ultimately happen to the virus once removed from the environment by these microbes. *Legionella pneumophila* is the bacteria responsible for Legionnaires' disease and is known to replicate within amoeba after being phagocytized. It may remain viable upon expulsion from the protozoan cytoproct (Barker and Brown, 1994). There have also been scattered accounts of similar activity occurring within other protozoa (Curds, 1992). This ability for bacteria to survive protozoan ingestion is a noted evolutionary advantage and has been discovered to involve bacteriophage as well. Using electron microscope counts, Clarke (1998) found that when lake water having a high level of bacteriophage activity ($\sim 10^9$ ml^{-1}) was combined with naturally-occurring bacteria and various species of ciliates and heterotrophic flagellates, bacteria remaining intact within protozoan food vacuoles were 25 times more likely to have been infected with bacteriophage, and that approximately 10% of the visible bacteriophage therein were surviving digestion and being released back to the environment. The possibility that grazing organisms, which are generally much more resistant to current chemical anti-virals

(King *et al.*, 1988), could protect virus from exposure to these chemicals and release infective viral particles after a certain period of time (possibly away from these anti-virals) is one that must be considered. It is these kind of intraguild interactions that are largely unknown and require a great deal more study.

The various intra-species interactions occurring in aquatic environments influence water greatly, and are incredibly complicated. Sewage treatment is certainly no exception, where microbes are present in large numbers, and in many cases, certain organisms are required for remediation processes that can easily be out-competed by faster growing organisms or through predation. Bacteriophage have been shown to aid in determining community composition in this environment. Further, protozoa have been suggested to modify not only bacterial populations, but those of bacteriophage and, potentially, those of pathogenic virus as well. However, these interactions are far from well-understood, and a great deal more investigation is required. The establishment of mixed cultures of protozoa and viruses would be required. Assessing the impacts of any interactions would require an efficient manner of separating the two organisms, enumerating changes to the viral population, and determining their ultimate fate following any interaction.

For this project, my goal was to investigate the influence of *Tetrahymena thermophila* on bacteriophage when the two were maintained in co-culture, to determine whether the ciliates actively affect a viral population. The influence of living ciliates was compared to that of formaldehyde-fixed cells and to "conditioned media," in which Tetrahymena had been previously grown but then removed for the analysis. Once the effect of living ciliates had been established, I then attempted to probe and identify the cellular mechanisms responsible. One approach was to use techniques designed to affect the normal ingestion mechanisms of

Tetrahymena, to observe what effect this had on viral inactivation. A second approach was to use fluorescence microscopy to visualize the phage-ciliate interaction. My aim was to further understanding of the potential role of grazing ciliates as regulators of bacteriophage numbers in the environment, when the two are in close proximity. A variety of ciliates are present in aquatic environments and their influence could potentially aid in regulating viral numbers and may undermine treatment practices when sewage is processed prior to release to the environment.

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Chapter 1

The fate of bacteriophage T4 when mixed in culture with the grazing ciliate, *Tetrahymena thermophila*.

1.1 Abstract

The ability of a ciliate to inactivate bacteriophage was studied because these viruses are known to influence the size and diversity of bacterial populations, which in turn affect nutrient cycling in natural waters and effluent quality in sewage treatment, and because ciliates are ubiquitous in aquatic environments, including sewage treatment plants. *Tetrahymena thermophila* was used as a representative ciliate and we used T4, as a model bacteriophage. T4 titre was monitored on *Escherichia coli* B in a double-agar overlay assay. T4 and the ciliate were incubated together under different conditions and for varying times after which the mixture was centrifuged through a step gradient, producing a top layer free of ciliates. The T4 titre in this layer decreased as co-incubation time increased, but no decrease was seen if phage were incubated with formalin-fixed *Tetrahymena*. The T4 titre associated with the pellet of living ciliates was very low suggesting that removal of the phage by *Tetrahymena* inactivated T4. When *Tetrahymena* were incubated with SYBR Gold-labelled phage, fluorescence was localized in structures that had the shape and position of food vacuoles. Incubation of the phage and ciliate with cytochalasin B or at 4 °C impaired T4 inactivation. These results suggest the active removal of T4 bacteriophage from fluid by macropinocytosis, followed by digestion in food vacuoles. Such ciliate virophagy may be a mechanism occurring in natural

waters and sewage treatment and the methods described here could be used to study the factors influencing inactivation and possibly water quality.

1.2 Introduction

The potential to study protozoa and viral interactions in the lab relies on the ability to safely and efficiently maintain these organisms throughout the course of a study. The selected protozoan was the ciliate *Tetrahymena thermophila*, a grazing ciliate that has been found in various natural and man-made aquatic environments. They have been used extensively due to their ability to be cultured axenically in the laboratory, and the absence of any health risks to the researcher. They are regarded as models for ciliated protozoa. Although bearing the designation "*thermophila*," they have shown favourable growth rates at room temperature (22 ^{o}C), which facilitates work with various commonly used viruses, as these are generally unstable at higher temperatures (Nilsson, 1976).

Studying viruses in the lab holds certain difficulties, not witnessed with other organisms. Obviously, the ability to study the relationships that occur between *T. thermophila* and environmental isolates of enteric pathogens or other viruses commonly found in sewage treatment would provide a more accurate idea of what will actually occur in the environment. However, working with human pathogens requires stringent safety protocols and puts the researchers at risk Additionally, host cells of many human pathogens are unculturable by current methods or require expensive media, thus studying viral activity requires the establishment of more workable model viruses, such as those infecting bacteria (Arraj *et al.*, 2004). Bacteriophage, or viruses infecting bacteria exclusively, were discovered independently by Twort, in 1915 and d'Herelle, in 1917 (Pelczar *et al.*, 1988). Bacteriophage

quickly became a lab surrogate for viral activity because they are safe, relatively easy to work with, and viral titres can be assessed overnight. Also, some bacteriophage show various similarities to human pathogens. For example, many bacteriophages show the same degrees of resistance to wastewater treatment, or rate of inactivation, as those recorded with human pathogens (Grabow, 2001). Certain classes of bacteriophage are also a useful model for enteric pathogens; enteric phages are consistently found in sewage, and their presence shows a strong correlation with the presence of human enteric pathogens (Grabow *et al.*, 1999). Thus, phage are an important lab surrogate for the study of human pathogenic virus behavior.

Enumeration of bacteriophage using electron microscopy is regarded as quite accurate, but counting many different samples is very expensive and time-consuming (Ewert and Paynter, 1980). The traditional double-agar overlay, originally designed by Adams in 1959, remains one of the most widely used methods for phage enumeration due to its relative accuracy, cost, and ability to produce results quickly (ISO, 1995). The incubation of a small volume of a given sample, added to molten agar along with bacterial host culture, will produce zones of clearing (plaques) on the resulting bacterial lawn; theoretically, each individual plaque will indicate the presence of a single infectious phage particle.

For this study of a phage-protozoan relationship, the commonly used enterobacteriophage T4 was selected. T4 is a coliphage (i.e. phage infecting *Escherichia coli*), which has a single strand of linear, double-stranded DNA encoding its genetic information. It is regarded as the basic model for all DNA viruses due to its large, complex genome, which shows similarities to various other viruses including the human herpes simplex virus (Wagner and Hewlett, 1999). It is the one of the largest bacteriophage, having a non-enveloped, tailed icosehedral shape and measuring approximately 0.2 μm in length, with a head diameter of

0.07-0.10 μm (Wagner and Hewlett, 1999). The large size of this phage may hold some advantages when visualization is required, as it may be more easily viewed using epifluorescence microscopy.

To investigate *T. thermophila's* effects on a viral population, mixture of the two organisms will require an absence of suspended solids and nutrients, as viruses easily adsorb to any particulates (Sakoda *et al.*, 1997). In a medium devoid of nutrients, however, *T. thermophila* will quickly cease the production of food vacuoles (Nilsson, 1976). Thus, if the ciliate would normally remove any virus from the environment using either phagocytosis or pinocytosis, mixture in a minimal or starvation medium could prevent this from happening. However, even in a lack of nutrients, the presence of particles in the medium has been found to induce the production of food vacuoles (Nilsson, 1976). Although T4 phage may fall below the size limit for particles known to stimulate food vacuole production, it is possible that multiple phages may clump, forming a particle large enough to cause viral removal. Conversely, *T. thermophila* may take a more indirect route towards phage. Many microbes within wastewater biota are known to release antiviral metabolites into the environment, which may influence viral numbers. It is possible that the mixture of the two selected organisms may result in the release of antiviral components to the environment, or even that these compounds are produced constitutively by the ciliate. Finally, the possibility that no interaction will occur between the two, must be considered. Due to the variety of organisms interacting in sewage treatment, it is possible that only certain species will interact with viruses, or only with certain viral types. Regardless, investigating any interaction will yield a greater appreciation for the complexity of such mixed ecosystems.

1.3 Materials and Methods

1.3.1 T4 Bacteriophage and Bacterial Host

The viral model used for study was T4 bacteriophage (ATCC®: 11303-B4™) and the phage stock was maintained at 4°C in 3% (w/v) trypticase soy broth (TSB) (Becton-Dickinson, Sparks, MD). Although not required by the phage, the nutrient-rich TSB medium is required to sustain the bacterial host used for enumeration. Bacteriophage require a bacterial host in order to replicate. This is beneficial because phage used in phage-protozoa mixtures will not be expected to replicate, although in order to carry out the method of enumeration, a bacterial host must be added. The T4 host strain is *Escherichia coli* B (ATCC®: 11303™), and prior to use in experimentation it was grown overnight in TSB medium at 37°C.

Phage stock was produced using a modified "bulking" method, originally described by Einsenstark (1967). Log-phase bacterial host culture (1 ml) was combined with 100 µl of phage stock and the double-agar overlay was performed (as described in section1.3.2). Following overnight incubation at 37 °C, the top agar layer was scraped into a 50 ml capped test tube. A small volume of TSB medium (1-3 ml) was added to facilitate the removal of this agar. This was centrifuged at 400 x g for 10 minutes, and the supernatant was passed through a 0.2 µm syringe-filter, twice. This stock may be enumerated using the double-agar overlay. Phage stock was stored at 4 °C in a plastic, capped test tube.

1.3.2 Enumerating bacteriophage in a sample

Assessment of the phage titre was done using the double-agar overlay assay. This process, developed by Adams (1959), involves mixture of 1 ml of phage dilution, 100 µl of

host bacteria, and 3 ml of "top" agar, which is then poured over Petri plates that had been coated with approximately 5 mL of "bottom" agar. Top agar consisted of 3% (w/v) TSB, 0.7% (w/v) agar (Fisher Scientific, Fair Lawn, NJ), and 0.03% (w/v) $CaCl_2 \bullet 2H_2O$ (Sigma, St. Louis, MO), while bottom agar differed only in its agar content [1.5% (w/v) agar]. Plates were incubated overnight at 37 ^{o}C, to allow for the formation of plaques, or zones of clearing in the bacterial lawn. Each of these plaques theoretically represents the presence of one infectious phage particle. Serial dilutions of phage stocks were performed and plates containing between 20-200 plaques were counted. For every assessment of titre, all samples were plated in duplicate.

1.3.3 Tetrahymena thermophila

Tetrahymena thermophila starter cultures are available from the American Type Culture Collection (ATCC), however the lab culture I used came from the University of Guelph, as it was originally donated by Dr. Denis H. Lynn (Department of Zoology, University of Guelph, Guelph, ON, Canada, N1G 2W1). The method used for culturing *Tetrahymena thermophila* was adapted from Gilron *et al* (1999). Batch cultures were maintained at 22^{o}C in 10 ml of proteose peptone, yeast extract-enriched medium (PPYE), composed of 0.125% (w/v) dextrose (Sigma), 0.5% (w/v) proteose peptone (Becton-Dickinson) and 0.5% (w/v) yeast extract (Becton-Dickinson) dissolved in MilliQ water. Each batch culture was started, maintained, and sub-cultured every 7-14 days. In preparation for experimentation, 1 ml of the *T. thermophila* batch culture was added to 10 ml of fresh PPYE in order to maintain a constant supply of healthy ciliate stock. The remaining 9 ml were added to 50 ml of fresh PPYE in a 75cm^2 non-tissue culture treated flask and kept at 22^{o}C, on an orbital shaker (50 RPM)

overnight. Enumeration of ciliate cultures was performed using a Z2 Coulter Particle Counter and Size Analyzer (Coulter, Luton, UK), using an upper particle limit diameter of 50 μm and a lower limit diameter of 20 μm, the average size of the ciliate (Nilsson, 1976). Prior to enumeration, preparation of *T. thermophila* populations for counting involved combining 19 ml of the isotonic IsoFlow Sheath Fluid solution (Coulter), 500 μl of *T. thermophila* culture, and 500 μl of 10% neutral, buffered Formalin (Sigma). Four counts were done per sample and these values were added then multiplied by 20 to convert to cells/ml.

1.3.4 General Protocol for Bacteriophage/Protozoan Co-incubation

In order to focus on the interactions occurring between ciliate and phage, a physiologically balanced simple salt solution designed by Osterhout (1906) to maintain an isotonic environment was the preferred medium. Thus, prior to mixture with phage, ciliates required transfer to this simple salt medium. Following enumeration, *T. thermophila* culture was centrifuged for 10 min at 400 x g, and the supernatant was removed. Pelleted ciliates were washed twice with Osterhout's solution and were re-suspended in fresh Osterhout's solution for use in experimentation. T4 bacteriophage stock was added as required to produce the desired final titre, which varied with the proposed experiment. Co-incubations were maintained in 15ml capped, plastic test tubes at 22^{o}C (unless otherwise stated) on an orbital shaker (50 RPM). The titre of the phage remaining in the medium, unassociated with *Tetrahymena*, was determined as described above.

1.3.5 Step-gradient Separation (SGS) of the Bacteriophage/Protozoan Mixture

To accurately assess numbers of bacteriophage unassociated with ciliates and remaining in media, phage must be separated from ciliates in order to perform the double-agar overlay. Ciliate-associated phage may remain infective and because they are concentrated to a small area (i.e. at/in the ciliate), multiple phages would produce only a single plaque. Unassociated phage were separated from protozoans using a novel technique of step-gradient separation adapted from techniques used for blood cell separation (Figure 1.1). Samples of phage-ciliate mixtures were added to sterile, capped test tubes. To this, a sterile Pasteur pipette was used to add 1 ml of Histopaque-1119 (Sigma-Aldrich, St. Louis, MO) for every 1 ml of sample, beneath the protozoan-virus mixture, creating a second denser, liquid phase. This was centrifuged at 400 x g for 10 min, separating the ciliates from any unassociated phage by forcing them to pellet. The supernatant (referring to the Osterhout's phase above the dense liquid Histopaque phase) was removed and diluted with TSB as required for use in the double-agar assay. After overnight incubation at 37^{o}C, plaques were counted to assess titre.

Since T4 phage would be maintained in Histopaque-1119 for a period (albeit, a very short one), it was necessary to determine if this sucrose solution would have any adverse effect on phage infectivity. In order to do this, T4 phage was added to Histopaque-1119 at a final titre of 10^5 pfu/ml and after 24 hours the titre of the sample was assayed with the double-agar overlay. Additionally, the efficiency of SGS for isolating bacteriophage was tested by inoculating a sample of Osterhout's solution with T4 phage (10^5 pfu/ml) and immediately plating the pre-separation sample to confirm the titre, followed by the step-gradient separation (SGS), and finally plating the supernatant along with an undiluted sample of the Histopaque-1119 phase.

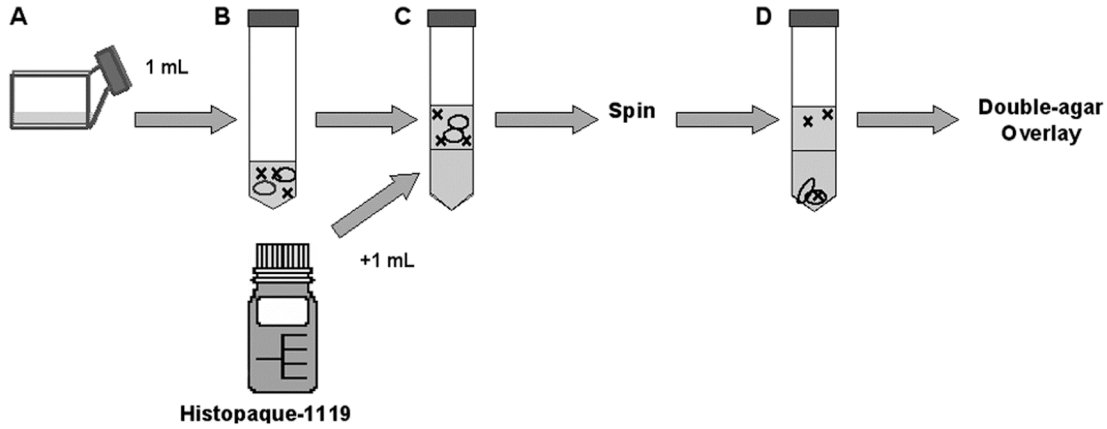

Figure 1.1 Experimental protocol for evaluating *Tetrahymena*-phage interactions

(A) Experiments began by adding T4 bacteriophage to *T. thermophila* in medium, usually Osterhout's solution, and incubating this culture in 75 cm^2 tissue culture flasks that were maintained on an orbital shaker (50 RPM) at room temperature. **(B)** At various times afterwards a 1ml sample of the culture was transferred to a 15ml sterile plastic centrifuge tube. **(C)** A long glass Pasteur pipette was used to layer 1 ml of Histopaque under this sample after which the tube was centrifuged at 400 x g for 10 minutes at room temperature. **(D)** After centrifugation, a pellet was observed as well as a distinct top layer (the medium) and a distinct bottom layer (the Histopaque) was evident. The distribution of *Tetrahymena*, which are indicated as ovals in the diagram, was monitored by phase contrast microscopy and the distribution of phage, which are indicated by an x, by double-agar overlay. After the step-gradient centrifugation, all the *Tetrahymena* were found as a pellet, regardless of the experiment. By contrast, the phage remaining unassociated with the ciliates would remain in the less dense, Osterhout's supernatant.

1.3.6 T4 Phage Infectivity in Media Commonly Used With Ciliates

Prior to co-incubation of phage with live ciliates, T4 infectivity was assessed in various media that would potentially be used to ensure that there were no media-associated factors influencing any phage-ciliate interaction that may be found. For this assay, ten ml of both Osterhout's solution and PPYE were inoculated with T4 stock to produce a final titre of 10^5 pfu/ml, in the absence of protozoa. All samples were kept in capped, plastic test tubes at 22°C. At 0, 4, 24, 48, and 72 h, samples were serially diluted using TSB and plated using the double-agar overlay. After overnight incubation at 37°C, plaques were counted to assess viral titre. Sterile Histopaque-1119 was also tested for effects on viral infectivity in the same manner, with the time frame being limited to testing at 0, 4, and 24 h.

1.3.7 The Influence of Tetrahymena-conditioned Media on Phage Infectivity

Along with sterile medium, medium which *T. thermophila* had previously conditioned was tested for effect to phage titre. This conditioned medium had sustained a ciliate culture for 14 days and was then syringe filtered. This was done to examine whether or not the ciliates were constitutively releasing antiviral metabolites into the environment. For this assay, ten ml of both Osterhout's solution and PPYE (protozoan-conditioned) were inoculated with T4 stock to produce a final titre of 10^5 pfu/ml, in the absence of protozoa. Conditioned PPYE was obtained by centrifuging two-week old *T. thermophila* cultures at 400 x g for 10 min. Conditioned supernatant was collected and passed through a 0.2µm syringe-filter. All samples were kept in capped, plastic test tubes at 22°C. At 0, 4, 24, 48 h, samples were serially diluted using TSB and plated using the double-agar overlay. After overnight incubation at 37°C, plaques were counted to assess viral titre.

1.3.8 Assessment of T4 Infectivity in the Presence of T. thermophila

Through the co-incubation of phage and *T. thermophila* cultures, any interaction taking place could be observed. However, discerning between an active interaction (such as phagocytosis or pinocytosis) or a passive adsorption of the phage to the ciliate would be required. In an attempt to discern which, if any would occur, both a sample of viable ciliates and formalin-fixed ciliates were assessed for effects on phage titre. Both viable and fixed *T. thermophila* samples consisted of an overnight culture (35,000 cells/ml final density), transferred to a 15ml test tube. For the fixed sample, 1 ml of 10% neutral, buffered formalin was added for every 3 ml of sample and allowed to sit for 10 min; viable samples were amended with Osterhout's solution in place of formalin. To each of the two samples, T4 phage stock was added to produce a final titre of 10^5 pfu/ml. At 0, 4, 24, 48, 72, and 96 h, the SGS was performed and phage infectivity was assessed by the double-agar overlay. A 1,000 fold dilution of the SGS supernatant was assayed at 0 and 4 h, with decreasing dilutions being plated as phage infectivity decreased.

1.3.9 Saturation of the Rate of Virophagy

The initial ratio of *T. thermophila* to T4 phage was altered in the attempt to saturate the ciliate's ability to inactivate. Using an enumerated, overnight *T. thermophila* culture, ciliates were transferred to Osterhout's solution to produce a final density of 35,000 cells/ml. T4 phage stock was used to produce samples with a final titre of 10^5, 10^6, 10^8, 10^9, and 10^{10} pfu/ml. At 0, 4, 24, and 48 h, the SGS was performed and change in titre was assayed using the double-agar overlay method.

1.3.10 Pellet Assay

In further attempt to discern between surface adsorption and phagocytic or pinocytic interaction between phage and ciliates, an assessment of intracellular titre of ciliates was conducted. An overnight culture of *T. thermophila* (35,000 cells/ml) was suspended in Osterhout's buffer and inoculated with T4 phage (10^5 pfu/ml). At 0, 4, and 24 h, 2 ml of sample were chilled on ice. An SGS was performed to isolate the ciliates and they were resuspended in 1 ml 20 mM Tris-HCl and returned to ice to chill. To this, was added 2 x lysis buffer (1.4 M sucrose, 4 mM EDTA, 2% Triton X-100) in a 1:1 ratio to the Tris-HCl volume, and the sample was vortexed for one minute. The lysed sample was then plated and any phage, which may have recently been internalized, should be detectable using the double-agar overlay.

As there has been evidence to suggest that viruses may survive the digestive process, resulting in the release of infective particles (Clarke, 1998), a method was devised to test whether *T. thermophila* were releasing infective phage. Ciliates, which were maintained in the presence of T4 phage for 24 hours, were removed through centrifugation (400 x g for 10 min), washed twice with fresh Osterhout's solution, and resuspended in fresh Osterhout's. Twenty-four hours following this transfer to fresh buffer, ciliates were pelleted using the SGS method. The viral titre of the supernatant was assayed to determine if any new phage were released to the medium. Additionally, pelleted ciliates were isolated they were resuspended in 1 ml 20 mM Tris-HCl and placed on ice to chill. Lysis of the cells was carried out through the addition of 2 x lysis buffer in a 1:1 ratio to the Tris-HCl volume, and the sample was vortexed for one minute. Plating the internal contents of these cells would determine the fate of any internalized phage.

1.3.11 Epifluorescence of T4 Phage

Epifluorescence microscopy has become an invaluable process in cell biology and microbiology, with SYBR Gold used as a dye sensitive enough to label various bacteriophages by labeling double or single stranded DNA or RNA (Mosier-Boss *et al.,* 2003). SYBR Gold nucleic acid stain (Invitrogen, Eugene, OR) was used as a means of fluorescently labeling T4 phage, in the hope of visualizing labeled-phage either adsorbed to the ciliate surface or localized intercellularly. Since phage reproduction requires the lysis of host bacteria, the presence of host cellular remnants (including its genome) in a prepared phage suspension seems inevitable and could potentially become labeled by SYBR Gold. In an attempt to limit remnants of the phage replication process, prior to SYBR Gold labeling, T4 stock was treated with RNase-free, DNase I (100 U/ml) for 10 minutes. SYBR Gold stock dye solution (provided as a 10,000 x concentrate) was diluted and added to the phage stock, resulting in a 2.5 x dye solution and allowed to sit in the dark for 10 min (Mosier-Boss *et al.*, 2003). The labeled-phage solution was then passed through a 100,000 MW Centricon® concentrator (Fisher) and centrifuged at 2000 x g for 5 min to remove unbound dye. The labeled-phage solution was rinsed twice by passing 1 ml of Osterhout's solution through the concentrator at 2000 x g for 5 min. As a negative control, a solution (consisting of Osterhout's solution, SYBR Gold dye solution, and DNase-free RNase I) was also passed through a fresh concentrator and rinsed twice with Osterhout's solution. In both cases, the concentrate was returned to its original volume using Osterhout's solution. The labeled-phage solution was diluted as required and added to *T. thermophila* (35,000 cells/ml), which had previously been suspended in Osterhout's solution, to produce samples of 10^{10}, 10^{9}, and 10^{8} pfu/ml. A control sample was also produced, consisting of *T. thermophila* suspended with the T4-free dye

solution. Samples were kept in the dark for 2 h, after which 60 µl were moved to a microcentrifuge tube and centrifuged for 5 min at 2,000 x g. Thirty microlitres of the supernatant were removed to concentrate the cells, and 3 µl of 10% neutral, buffered formalin were added. After fixation, samples were immediately viewed using a fluorescence microscope equipped with a Nikon B-2A filter (excitation 450 nm /emission 520 nm).

1.3.12 Confocal Microscopy

The use of the transmission electron microscope to investigate vacuole contents of ciliates co-incubated with phage was attempted, however the results did not provide clear support for or against the internalization of bacteriophage within food-vacuoles. Laser scanning confocal microscopy was used along with SYBR Gold labeling to attempt visualization of phage with ciliates. *T. thermophila* (35,000 cells/ml) were mixed with SYBR Gold-labeled T4 phage (10^8 pfu/ml final concentration) and maintained at 22^{o}C for 15 min. At this time, *Tetrahymena* were isolated using centrifugation and viewed using confocal microscopic analysis in a Zeiss LSM 510 META confocal microscope using an argon laser as a light source, at an excitation wavelength of 488 nm line and band-pass filter of 505 nm.

1.3.13 The Effect of Cytochalasin B on Decrease of Phage Infectivity

Food-vacuole formation in *T. thermophila* involves the specialized region at the base of the buccal cavity, which contains mainly microtubules arranged in such a way to allow the pinching off of vacuoles (Nilsson, 1976). Cytochalasin B (Sigma-Aldrich) is a drug that blocks the polymerization of actin, interfering with the functioning of microtubules, and inhibiting the formation of food vacuoles in *T. thermophila* (Brown and Spudich, 1979).

Nilsson, et al. (1973) observed this effect to be reversible at lower concentrations after a few hours, and cells regained proper functioning of the microtubules. To assess the effect of cytochalasin B (CB) on phage-ciliate interactions, CB was dissolved in DMSO, and added to suspensions of *T. thermophila.* These samples were maintained in Osterhout's (final concentration of 35,000 cells/ml) at CB concentrations of 0.1, 10, and 50 μg/ml; a 0 μg/ml control was made using DMSO only. The final volume of DMSO did not exceed 0.5% (v/v) in any sample, as it has been recorded to be detrimental to *T. thermophila* above this concentration (Nilsson, 1974). T4 phage stock was added to produce a final titre of 10^5 pfu/ml. At 0, 1, 2, and 24 h, 1 ml was moved to a 15 ml plastic, capped test tube and using the SGS, unassociated phage were isolated, and titre determined. Additionally, SYBR Gold-labeled T4 (10^8 pfu/ml) were added to *Tetrahymena* (35,000 cells/ml) suspended in Osterhout's solution containing 50 μg/ml CB. After two hours, cells were fixed with 10% neutral, buffered formalin and viewed using a fluorescence microscope to provide a visual assessment of cytochalasin B's effect on *Tetrahymena* and its interaction with T4 phage.

1.3.14 The Effect of Temperature on Phage Inactivation

At low temperature (4 ^{o}C) food-vacuole formation is greatly reduced in *T. thermophila* (Nilsson, 1972). Optimal growth temperature is 28^{o}C and in comparison to the rate of food-vacuole formation at this point, rates at 4^{o}C, 22^{o}C, and 37^{o}C are approximately 5%, 90%, and 80% respectively. Thus, through the manipulation of incubation temperature, the influence of food-vacuole formation on removal of phage from the mixture, was assessed. Four samples of viable *T. thermophila* (35,000 cells/ml) / T4 phage (10^5 pfu/ml) mixtures in Osterhout's solution were prepared for incubation at 4^{o}C, 22^{o}C, 28^{o}C and 37^{o}C. Protozoan-free controls

were also prepared for each temperature. Samples were assayed at 0, 4, and 24 h using the SGS and double-agar overlay. After overnight incubation at 37^{o}C, plaques were counted to assess viral titre. Additionally, SYBR Gold-labeled T4 (10^{8} pfu/ml) were added to *T. thermophila* culture (35,000 cells/ml) suspended in Osterhout's solution that had been previously chilled to 4 ^{o}C for 10 min. Following inoculation, the mixture was returned to 4^{o}C for 2 h, after which the cells were fixed with 10% neutral, buffered formalin and viewed using the fluorescence microscope, to provide a visual assessment of low temperature's effect on phage-*Tetrahymena* interactions.

1.3.15 Statistical Analysis

Throughout this project a wide variety of statistical examinations were performed on various types of data. In all cases, the GraphPad InStat® program was used for statistical analysis.

1.4 Results

1.4.1 T4 Phage Infectivity in Media Commonly Used With Ciliates

As background for the investigation of any direct effects of *T. thermophila* on T4 phage when the two were co-incubated, the effect of various media on T4 was assessed by monitoring the viral titre, or *infectivity*, in solutions used to grow and manipulate *T. thermophila*. T4 continued to be infective with little drop in titre for at least 4 days in PPYE medium or Osterhout's solution (Appendix). T4 infectivity also was unchanged by incubation

in Histopaque-1119, which was to be used in step-gradient centrifugation to separate T4 from *T. thermophila* (Appendix).

1.4.2 The Influence of Tetrahymena-conditioned Media on Phage Infectivity

The release of antiviral compounds by *Tetrahymena* into the medium was assessed through the inoculation of ciliate-conditioned medium with T4 phage. These solutions are described as being conditioned because potentially they could contain inhibitory factors released by ciliates. T4 maintained in either conditioned-PPYE or Osterhout's continued to be infectious, with no significant drop in titre over 48 hours (Appendix).

1.4.3 Separation of T4 From Tetrahymena By Centrifugation

The novel use of Histopaque-1119 for the separation of T4 from *T. thermophila* by step-gradient centrifugation was performed. The dense Histopaque phase was added in an attempt to impede the movement of *Tetrahymena* throughout the Osterhout's phase, which was intended to contain only unassociated phage. The efficiency of isolating unassociated phage using this method of viral isolation was assayed (Figure 1.2). When T4 alone in Osterhout's was layered on top of Histopaque-1119 and centrifuged, nearly all the phage were found in the top layer and only a small fraction had penetrated into the Histopaque layer. When *Tetrahymena* alone in Osterhout's was layered on top of Histopaque-1119 and centrifuged, all the ciliates as judged by light microscopy were found in the bottom Histopaque layer. In this solution, the *Tetrahymena* were slightly rounded and less motile, but they recovered their shape and motility upon being returned to Osterhout's and PPYE.

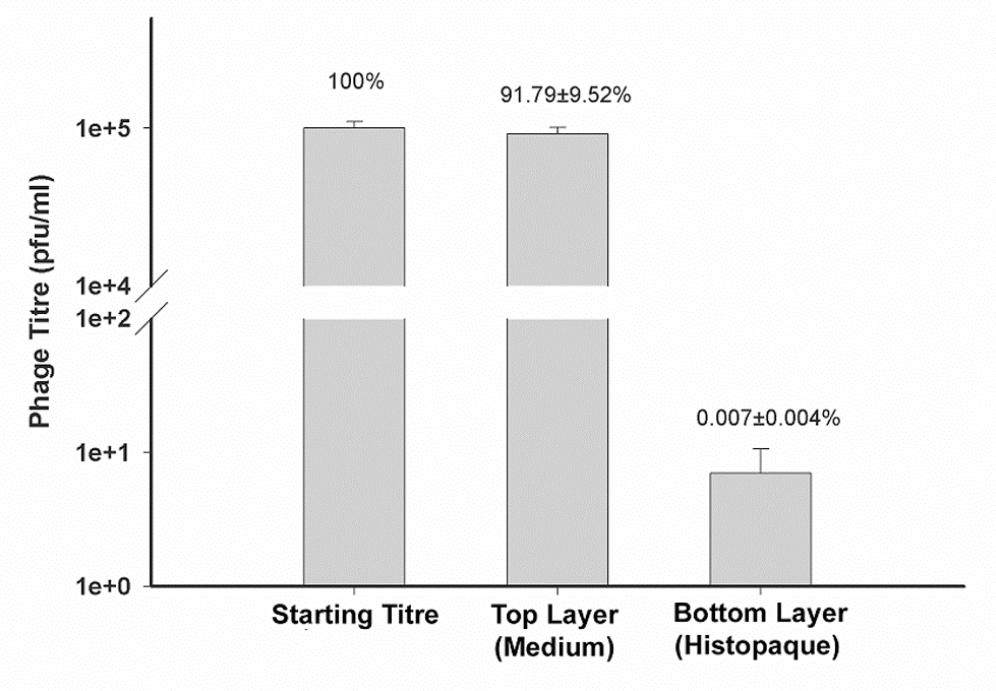

Figure 1.2 Distribution of T4 titre after mixing the phage with *Tetrahymena* and immediately subjecting the medium to step-gradient centrifugation

Within a minute of 10^5 pfu of T4 being added to a *Tetrahymena* culture, *Tetrahymena* were pelleted by centrifugation (see Figure 1.1) and the T4 titre as measured in the top (medium) and bottom (Histopaque) layer. The percent recovery of phage, in comparison to starting titre, is presented above each bar; error bars represent standard deviation. The data represent the mean of three separate trials. Means of the starting titre and titre in the top layer were compared using the paired t test and variation was not considered to be significantly different than expected by chance ($p>0.05$).

1.4.4 T4 Titre After Incubation With Tetrahymena

T4 infectivity was significantly reduced as time of incubation with viable *T. thermophila* increased (Figure 1.3). When the mixture of phage and viable *T. thermophila* was separated, the Osterhout's layer was assayed for infective T4 and phage titre declined steadily with increasing incubation time. With living *T. thermophila*, a significant decline in phage titre was seen at the first time point (4 h) examined and by 24 h the phage had been decreased by 95.3±2.1%, or 1.33±0.22 log (n=10). Little or no phage was detected in the bottom Histopaque layer. When the *Tetrahymena* pellet was lysed, very few infective phage were detected within the ciliate and as the co-incubation time increased, the titre in the pellet did not increase (Table 1). When the pelleted ciliates were isolated from a 24 h co-incubation of phage and viable *Tetrahymena,* resuspended in Osterhout's without T4 and incubated for a further 24 h, the lysate titre became even lower, with no significant level of phage released to the medium (Table 1). In contrast, when phage were incubated with *T. thermophila* that had been fixed by formalin, the phage remained unassociated with ciliates in the top layer. The Osterhout's layer titre did not show significant decline with time, judged by paired t test ($P>0.05$). Overall, the results suggested that *Tetrahymena* are actively removing and inactivated T4 from the co-incubation medium. When the titre at the start of the co-incubation was increased and the number of *Tetrahymena* kept constant, the inactivation rate increased, without saturating (Figure 1.4).

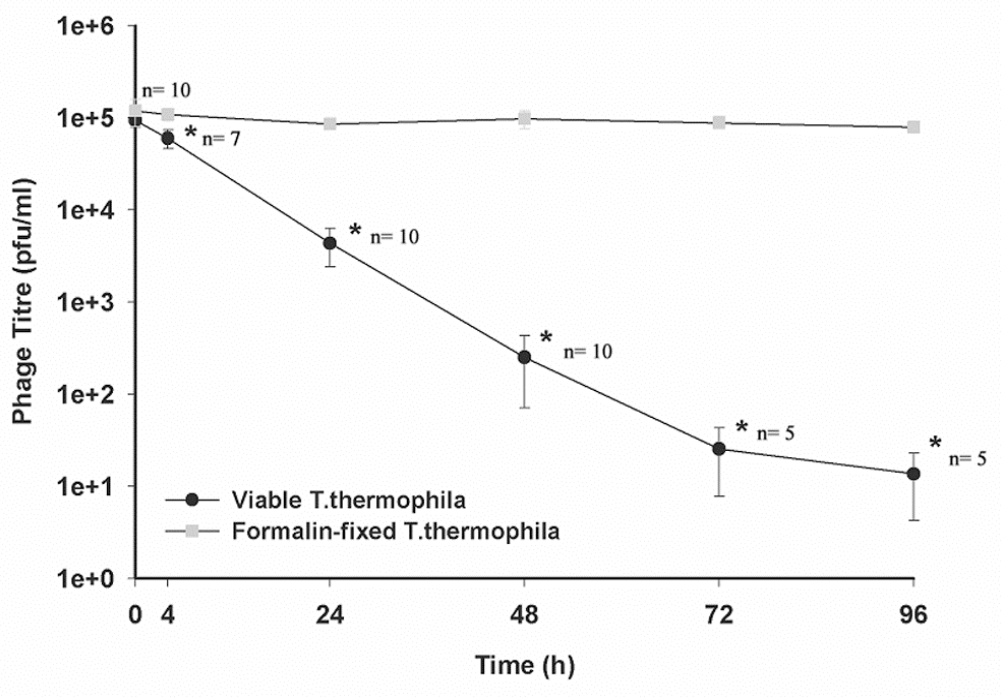

Figure 1.3 Removal of T4 from medium by live *Tetrahymena* as the co-incubation time with the phage increased

Co-incubations of T4 phage (10^5 pfu/ml) were begun in Osterhout's solution with either living or formalin-fixed *Tetrahymena* (35,000 cells/ml). At various times the *Tetrahymena* were removed by centrifugation and the remaining virus population in the medium was measured as pfu. The mean pfu with standard deviation bars (n=3 to 10) are plotted. The data were subjected to unpaired t tests and asterisks identify values where phage titre in the presence of viable ciliates was significantly different from the phage titre in the presence of fixed ciliates ($p<0.05$).

Table 1. T4 titre in pellet of *Tetrahymena* after centrifugation through Histopaque[a]

Co-incubation time at 22°C before centrifugation (h)	Treatment of *Tetrahymena* pellet[b]	Subsequent centrifugations[c]	Titre (mean PFU ± SD)[d]
0[e]	Lysed[f]	none	2,867 ±702
4	Lysed	none	3,550 ±900
24	Lysed	none	3,060 ±198
24	Resuspended in buffer for 24 h[g]	Supernatant	8 ±6
		Pellet[f]	28 ±24

[a] The T4 titre at the start was 100,000 PFU for each of the coincubation conditions before centrifugation.

[b] Pellet formed after centrifugation through step gradient of Histopaque

[c] After 24 h, *Tetrahymena* were collected by centrifugation but not through Histopaque and the supernatant and pellet assayed for PFU.

[d] Titre expressed as mean of two experiments ± standard deviations (SD) .

[e] Although listed as 0 h, including the time required for Histopaque separation and wash at this time point should be considered ~20 minutes.

[f] Pellet was lysed with Triton X-100.

[g] Pellet was resuspended in Osterhout's buffer .

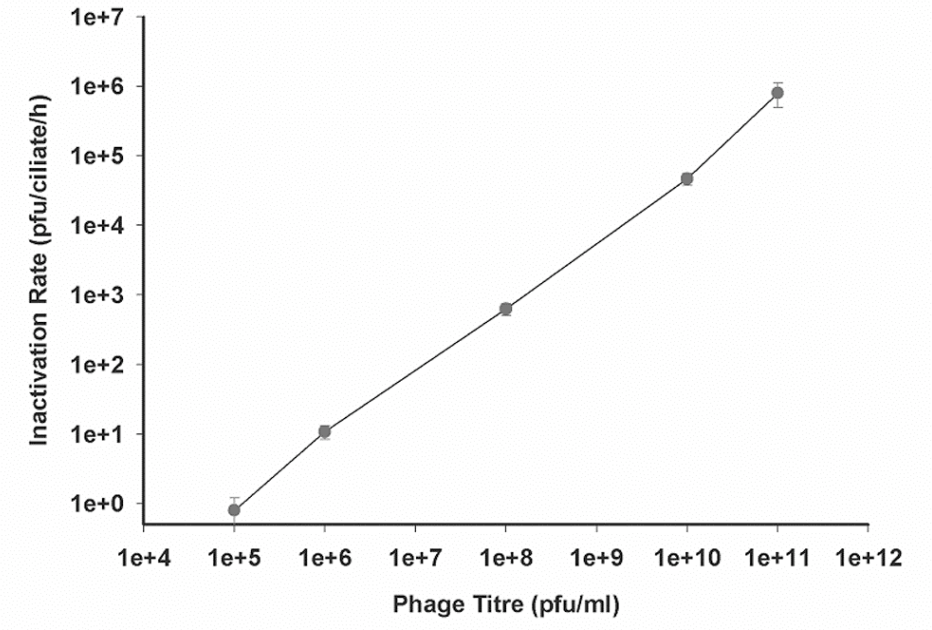

Figure 1.4 Removal of T4 by *Tetrahymena* from medium in which the starting phage titre varied

Co-incubations of T4 phage and *Tetrahymena* (35,000 cells/ml) were begun in Osterhout's solution at differing T4/*Tetrahymena* ratios, from a low of 10 to 1 to a high of 5.0×10^6 to 1. After 4 h, the *Tetrahymena* were removed by centrifugation and the remaining phage in buffer was measured as pfu. The removal of pfu was termed the inactivation rate and is expressed as pfu/ciliate/h (n=4).

1.4.5 Visualizing T4/Tetrahymena Interactions

Tetrahymena and T4 that had been fluorescently labeled with SYBR Gold were incubated together and when examined by fluorescence microscopy 2 h after the initiation of the co-incubation, *Tetrahymena* contained pockets of fluorescence (Figure 1.5D, F, H). When overlaid with corresponding phase-contrast images, in which food vacuoles can be clearly seen, these fluorescence pockets match the size and location of these food vacuoles. When examined by confocal microscopy after 15 min of co-incubation, some fluorescent particles were seen to be enclosed by a membrane at the base of the buccal cavity; the origin of food vacuole formation (Figure 1.6). In both cases, fluorescence was exclusive to the food vacuoles and the presence of fluorescent particles was lost with longer incubation.

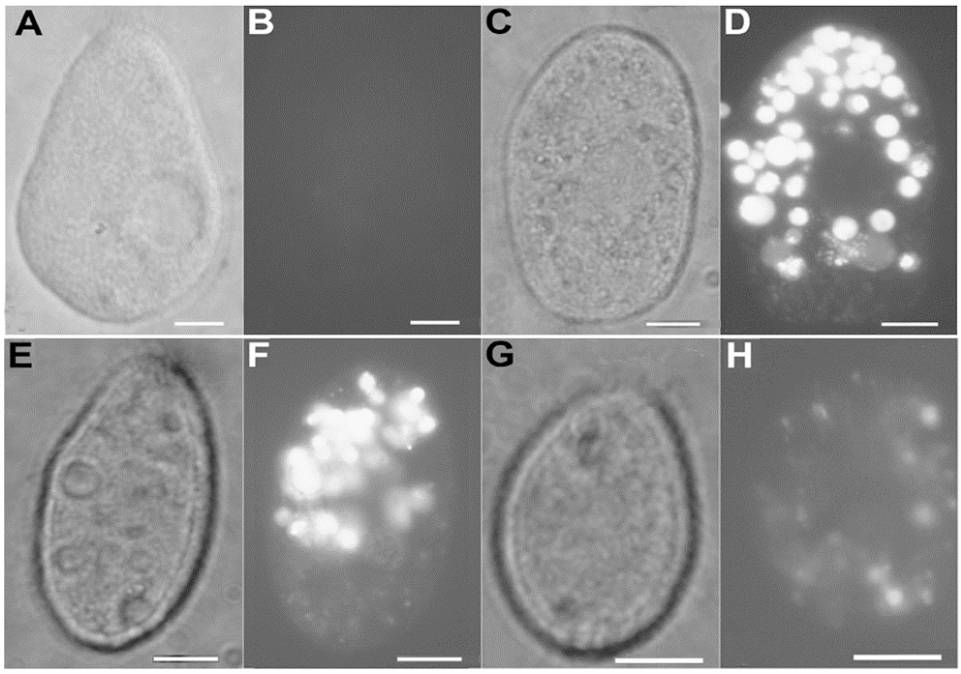

Figure 1.5 Visualizing T4/*Tetrahymena* interactions by fluorescence microscopy *Tetrahymena* (35,000 cells/ml) were exposed to SYBR Gold nucleic acid stain solution (**A, B**) or to SYBR Gold-labelled T4 phage at 10^{10} pfu/ml (**C**, **D**), 10^{9} pfu/ml (**E**, **F**), and 10^{8} pfu/ml (**G**, **H**) for 2 h at room temperature. Cultures were fixed in formalin immediately prior to microscopic analysis. Note that phase contrast photomicrographs are shown in **A**, **C**, **E** and **G**; fluorescence photomicrographs, in **B**, **D**, **F**, and **H**. Bar = 10 μm in all cases.

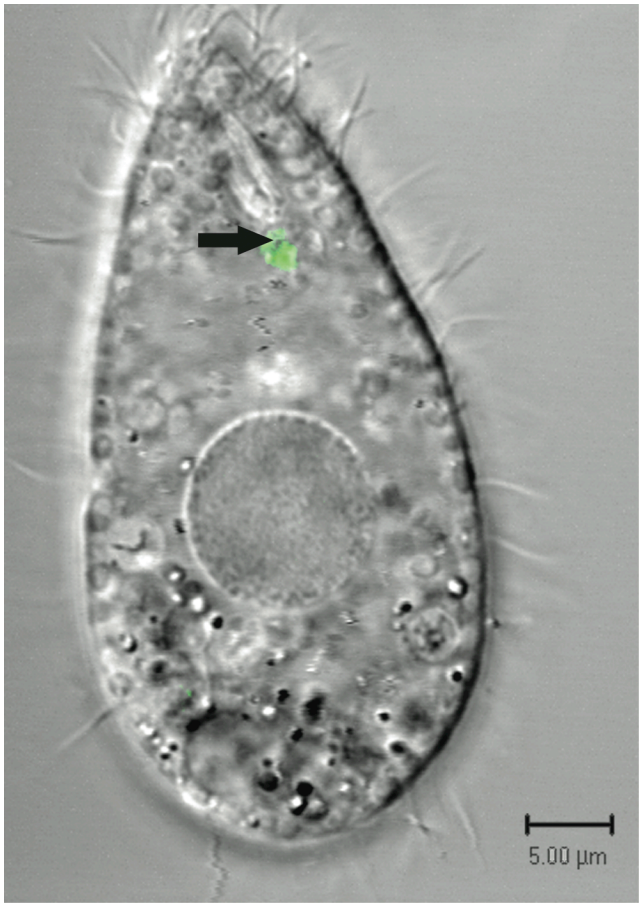

Figure 1.6 Visualizing T4/*Tetrahymena* interactions by confocal microscopy

SYBR Gold-labelled T4 phage (10^8 pfu/ml final concentration) were added to *Tetrahymena* (35,000 cells/ml). After 15 min, ciliates were separated from unassociated phage by centrifugation, isolated and viewed using confocal microscopy. Arrow indicates phage in food vacuole. The micrograph is a two-channel image taken in fluorescence and differential interference contrast (DIC) modes. Bar = 5 µm.

1.4.6 Effect of Inhibition of Food-Vacuole Formation on T4/Tetrahymena Interactions

Cytochalasin B inhibited the inactivation of T4 by *Tetrahymena* in the co-incubation and the degree of this inhibition was directly dependent on the concentration of cytochalasin B (Figure 1.7A). There was no significant difference between the titre in each sample until 24 hours, at which point the reduction of viral infectivity by ciliates at 10 and 50 μg/ml were significantly less compared to reduction in 0.1 μg/ml and the control. By 24 hours, samples with higher concentration of cytochalasin B contained a higher number of phage persisting in the co-incubation medium, resulting from a lower rate of ciliate-mediated phage inactivation. The titre in the top layer after 24 h with 50 μg/ml of cytochalasin B was not significantly different from the starting titre in this layer ($P>0.05$), while the rate of inactivation at 10 μg/ml was considered only slightly significant when compared to the initial titre of the sample, by paired t test. The presence of fluorescent particles within *Tetrahymena* after incubation with SYBR Gold-labelled T4 was notably decreased in the presence of cytochalasin B (Figure 1-.8E, F).

Low temperature, 4°C, also inhibited the removal of T4 by *Tetrahymena* (Figure 1.8B). When *Tetrahymena* and T4 were co-incubated at 4°C for 24 h, the titre in the media remained unchanged, whereas when the incubations were done at 22, 28 or 37 °C, the titre was reduced by over 90% (Figure 1-8B). It should also be noted that removal of infective T4 from the media increased at 37 ^{o}C, in comparison to removal at 22 ^{o}C, determined by an unpaired t test ($P<0.05$). In co-incubations at 4°C with T4 that had been stained with SYBR gold, no fluorescence was seen associated with *Tetrahymena* (Figure 1.8G, H).

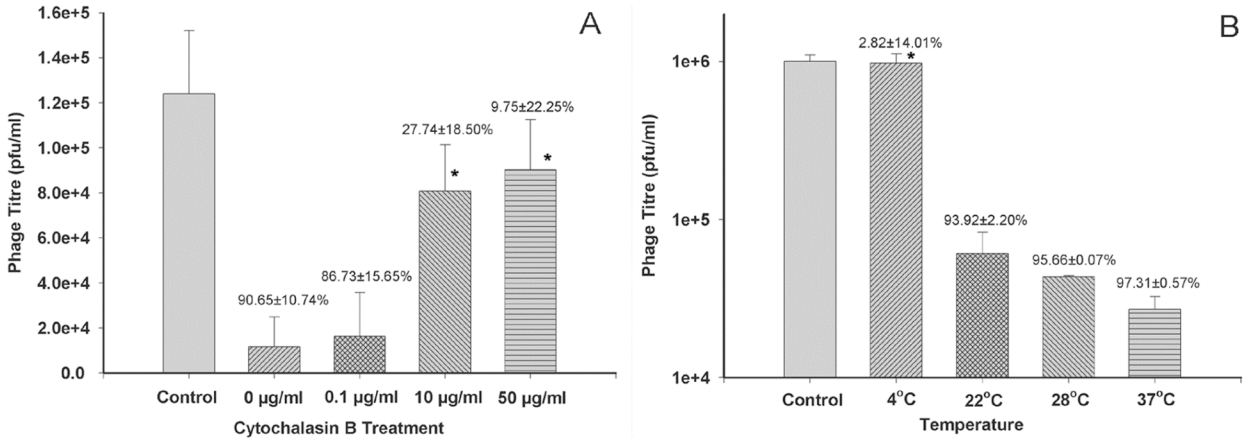

Figure 1.7 Effect of cytochalasin B and temperature on removal of T4 by *Tetrahymena*

Co-incubations of *Tetrahymena* and T4 phage were begun in Osterhout's solution with a known T4 phage titre and were allowed to proceed under different conditions for 24 h at which time cultures were centrifuged to remove the *Tetrahymena* and to permit measurement of the remaining virus as pfu. The mean pfu with standard deviation (n=2 to 4) are plotted, with the percent decrease in pfu from time zero listed above the bars. The data were subjected to a paired t test. **A.** The co-incubations were begun at 10^5 pfu and proceeded with cytochalasin B (CB) at 0.1, 10, or 50 µg/ml or without (0 µg/ml). The control bar represents cell-free, phage-inoculated medium. Compared to 0 µg/ml, significant inhibition of phage inactivation occurred at 10 and 50 µg/ml of cytochalasin B, determined by unpaired t test ($p<0.01$). **B.** The co-incubations were begun at 10^6 pfu and proceeded at 4°C, 22°C, 28°C and 37°C. The control bar represents cell-free, phage-inoculated medium maintained at 4°C. Compared to higher temperatures, low temperature (4^oC) was found to significantly inhibit phage inactivation, determined by unpaired t test ($p<0.05$). At 4^oC, there was no significant decrease in phage titre from 0 to 24 hours, determined by paired t test ($P>0.05$).

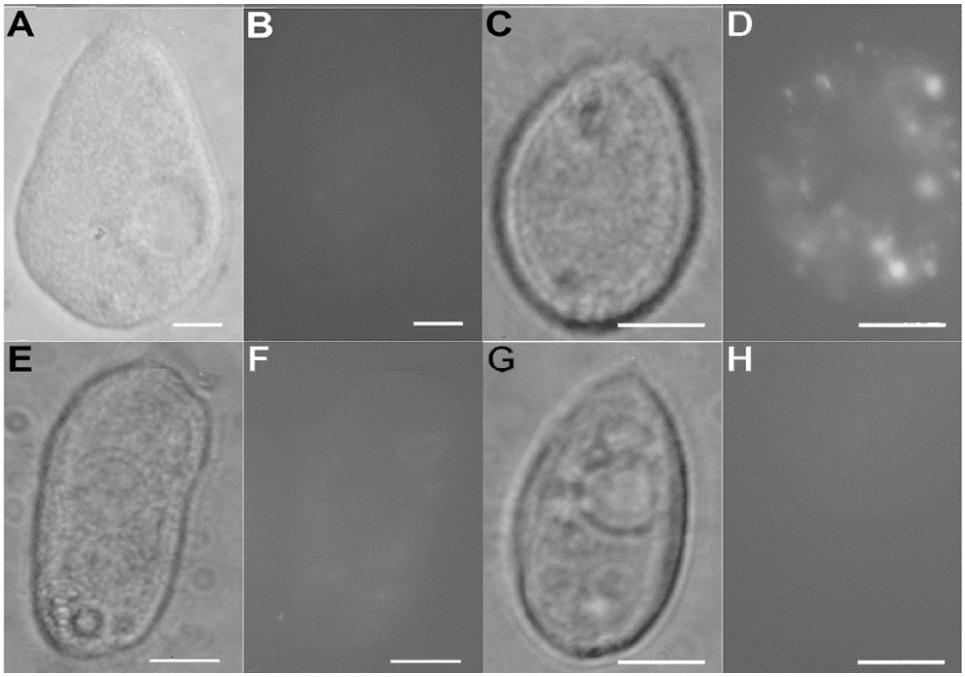

Figure 1.8 Effect of cytochalasin B and temperature on T4/*Tetrahymena* interactions as visualized by phase contrast and fluorescence microscopy

Tetrahymena (35,000 cells/ml) were exposed to SYBR Gold nucleic acid stain solution (**A, B**) or to SYBR Gold-labelled T4 phage at 10^8 pfu/ml for 2 h at room temperature (**C, D**) for 2 h at room temperature in the presence of cytochalasin B (**E, F**) or at 4 °C for 2 h (**G, H**). Cultures were fixed in formalin immediately prior to microscopic analysis. Phase contrast photomicrographs are shown in **A**, **C**, **E**, and **G**; fluorescence photomicrographs, in **B**, **D**, **F**, and **H**. Note, bar = 10 μm in all cases.

1.5 Discussion

In the studying of phage-ciliate interactions, a major problem was the isolation of these highly motile protozoa from unassociated phage, long enough to assay phage titre. The use of Histopaque in establishing a step gradient for the separation of T4 phage and *Tetrahymena* from co-culture will pellet the *Tetrahymena* with associated T4 in the dense sucrose solution, which will limit ciliate dispersal while not causing significant long-term effects to the cells. This process will leave only free or unassociated T4 in the supernatant. A similar method has been used to separate ciliates from bacteria (Berk *et al.,* 1976), but appears to have only been used with viruses for their separation from spermatozoa (Hanabusa *et al.,* 2000). In the case of T4 and *Tetrahymena* co-incubations, a simple differential centrifugation, or one not involving a gradient, would likely have accomplished the same separation, but because *Tetrahymena* is very motile it is likely they would rapidly disperse from the pellet into the supernatant, making precise separation difficult. This would be especially relevant when multiple cultures are being handled at the same time. By contrast, with the step gradient, the *Tetrahymena* remained in the dense Histopaque phase for many minutes and the supernatant is clearly defined. This facilitates removal of the supernatant, and viral titre assay to follow the disappearance or removal of T4 from the co-incubation medium.

T4 phage were removed from co-incubation medium only if living *Tetrahymena* were present, while when phage were mixed with formalin fixed ciliates, the T4 titre was unchanged and the phage remained in the supernatant. Further, T4 phage were not inactivated by the *Tetrahymena* conditioned growth medium, suggesting that T4 removal did not result from the release of antiviral components. A lack of any significant reduction in T4 titre was also

present when co-incubations were maintained at 4 ^{o}C. This is supported by epifluorescence microscopy, which confirms a lack of fluorescent particles either on or in *Tetrahymena* cells when phagocytosis was inhibited by temperature. These observations suggest the adsorption of phage to the *Tetrahymena* was not the mechanism behind T4 removal. The rate of removal of T4 by living *Tetrahymena* suspended in Osterhout's solution was exponential for at least 48 h.

The cellular process carried out by *Tetrahymena,* resulting in T4 removal, appeared to depend on proper food-vacuole formation. Phage removal was inhibited by the action of cytochalasin B; the disruption of actin polymerization, a necessary procedure in the formation of food vacuoles by *Tetrahymena* (Nilsson *et al.*, 1973). Secondly, T4 removal did not occur at 4°C, a temperature shown to inhibit formation of food vacuoles in *Tetrahymena* (Nilsson, 1972). Finally, the time course of T4 removal was consistent with the development of food vacuoles. *Tetrahymena's* digestive cycle, from food-vacuole formation to egestion takes about 2 h, with new vacuoles being formed approximately every 20 minutes (Nilsson, 1976). The earliest co-incubation time point to be examined was 4 h, thus the ciliate will have carried out several cycles of food-vacuole formation and digestion of their contents (Nilsson, 1979). The decline in plaque-forming units (pfu) at this point was significant, thus the disappearance of T4 from the co-incubation medium by *Tetrahymena* was most likely due to an active engulfment and digestion of T4 by the ciliates.

As well as forming food vacuoles *Tetrahymena* also forms receptor dependent, smaller vesicles with an apparent clathrin coat at the parasomal sacs of *Tetrahymena* (Nilsson and van Deurs, 1983; Elde *et al.*, 2005). These appear not to be involved in T4 removal by *Tetrahymena* because removal could not be saturated through modification of the

phage/protozoan ratio and fluorescence was never observed on the cell surface onto which the parasomal sacs open.

In their natural habitat, *Tetrahymena* would typically feed on bacteria-sized food particles, with an optimum clearance rate on particles of about 0.5 - 1.0 μm in diameter (Frankel, 2000). These food particles are ingested in food vacuoles, or phagosomes and their formation is phagocytosis (Nilsson, 1979). In fluid axenic medium, *Tetrahymena* can form food vacuoles filled with culture fluid and these fluid-filled food vacuoles are called pinosomes and are essential in internalizing fluid; their formation is termed pinocytosis (Nilsson, 1979). Growth rate and food vacuole production of *Tetrahymena* in fluid culture medium is maximized if there is particulate material present (Nilsson and van Deurs, 1983). Thus, the particulate nature of T4 may stimulate the ingestatory functions of *Tetrahymena*. Phagocytosis is generally defined as involving particles greater than 500 nm in diameter (Nilsson and van Deurs, 1983). T4 viruses are approximately 250 nm long with a diameter of 85 nm (Mesyanzhinov, 2004). Therefore, the removal of single T4 particles by *Tetrahymena* would not fit the definition of phagocytosis. Phage will commonly adsorb to larger solids, or to each other however, in which case these "clumps" may fall within the known size range for phagocytosis. Barring this, this phage inactivation by *Tetrahymena* could be a consequence of at least one form of pinocytosis, which is the process of internalizing fluid. One form of pinocytosis is macropinocytosis, which creates vesicles larger than 1 μm in diameter by an actin-dependent process (Rupper and Cardeli, 2001; Swanson and Watts, 1995). *Tetrahymena* does create vesicles with these characteristics in the cytopharynx of the oral apparatus. These are fluid-filled food vacuoles (Nilsson, 1972), which by current terminology can be considered

macropinosomes. Therefore, macropinocytosis in the cytopharynx is the likely mechanism behind the removal of T4 phage by *Tetrahymena*.

Co-incubation of *Tetrahymena* and T4 appears to result in T4 inactivation resulting from an active process by the ciliate. Firstly, pfu declined continuously with time and when removed from phage-inoculated medium, no phage were released to the environment. This suggests that *Tetrahymena* offered no transitory refuge to this bacteriophage. Previously, a mammalian rotavirus was found to adsorb to *Tetrahymena*, but with time the virus was released again (Benyahya *et al.*, 1998). Secondly, although pfu were detected from the pellet of *Tetrahymena*, there was a significant decrease in pellet pfu at 24 h following the transfer of *Tetrahymena* from phage-inoculated solution to phage-free solution. Finally, transmission electron microscopy (TEM) failed to provide unequivocal evidence of T4 being associated with *Tetrahymena* (data not shown). This is likely due to the rapid loss of their characteristic morphology by digestion in secondary lysosomes (Nilsson, 1979). At least one ciliate, *Euplotes*, has been reported to digest viral particles or phage under natural conditions. When *Euplotes* from a lake were examined over a year by TEM, some had food vacuoles with bacteria that were lysing and releasing phage particles, which were then digested, in the food vacuoles (Clark, 1998). Overall the removal of T4 by *Tetrahymena* likely involves T4 engulfment by macropinocytosis and subsequent T4 digestion in secondary lysosomes.

1.6 General Conclusions

Tetrahymena were able to remove T4 phage from solutions in which the ciliate and virus had been co-incubated. Metabolically active ciliates were required for removal and the

cellular process responsible had the characteristics of macropinocytosis. Removal appeared to coincide with inactivation. Ciliates are known to continuously undergo macropinocytosis, which could mean that they are continuously inactivating phage. Phages, in turn, kill their bacterial hosts. By influencing both participants in phage-host interactions - by inactivating phage as well as grazing on bacteria - the ciliates might have a more important and intricate role in determining the microbial ecology of water and in turn water quality than suggested from their relatively low numbers in the environment.

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Chapter 2

Investigating the impact of virophagy by the grazing ciliate, *Tetrahymena thermophila,* on bacteriophage MS2 when mixed in culture

2.1 Abstract

As the ciliate, *Tetrahymena thermophila*, recently has been shown to inactivate the bacteriophage T4, a study has been done of the interaction between *Tetrahymena* and another phage, MS2, which is often a surrogate for poliovirus in studies of viruses in aquatic environments. MS2 and either living or fixed ciliates were co-incubated for varying times prior to centrifugation through Histopaque to form a pellet of ciliates and a supernatant of MS2, whose numbers were quantified as plaque forming units (pfus). Living but not fixed *Tetrahymena* removed MS2 from the supernatant. Approximately 9 h was required to reduce the starting titre by 50%. MS2 removal did not occur when co-incubation was done at 4°C or when actin polymerization was blocked by cytochalasin B. Removal of the phage was attributed to inactivation because few pfus were found in the pellet of ciliates. When labeled with SYBR gold prior to co-incubation, MS2 were seen within vesicles inside *Tetrahymena*. All these features were similar to the inactivation of T4, but one difference was found. Over 48 h of co-incubation, T4 was reduced to less than 1 % of the starting pfu, whereas MS2 reached a plateau of approximately 10 %. Therefore, viruses could vary in their susceptibility to inactivation by ciliates.

2.2 Introduction

The inactivation of viruses in natural and man-made aquatic ecosystems is important to study in order to understand aquatic ecology and to protect human health. The most abundant category of viruses in water is the bacteriophages (Weinbauer, 2004). These viruses infect bacteria and regulate bacterial populations. Experimentally, bacteriophages are often used as models to determine the stability and movement of human viruses in the environment (Grabow, 2001). Yet even with model viruses, inactivation in ecosystems is poorly understood, but both abiotic and biotic mechanisms likely contribute. In several environments, abiotic mechanisms are thought to be especially important. For surface waters, UV is thought to be a significant inactivation mechanism (Weinbauer, 2004). In the sewage treatment process, adsorption is critical: viruses adsorb to floc, which ultimately settles to become sludge (Curds, 1992). By contrast, biotic mechanisms of viral inactivation and their importance are poorly understood. A few studies have briefly investigated phage inactivation by bacteria, single-cell protozoa, and invertebrates (Benyahya et al., 1998; Clark, 1998; Kim and Unno, 1996; Pinheiro *et al*., 2007). Ciliates are protozoa of interest because they are ubiquitous in aquatic environments and in sewage treatment plants (Curds, 1973)

The susceptibility of a virus to inactivation by a particular abiotic mechanism often depends on the properties of the virus, making the selection of an ideal model virus difficult. Two commonly selected bacteriophages are PRD-1 and MS2 (Dowd *et al.*, 1998). Less frequently studied is T4. The genomes for PRD-1 and T4 are linear double-stranded DNA, whereas the MS2 genome consists of a single, positive sense strand of RNA. Commonly MS2 is used as a surrogate for poliovirus, which also has a single stranded RNA genome. However MS2 was less sensitive than poliovirus to UV inactivation (Shin et al., 2005). The inactivation

of viruses by adsorption depends on structural properties, particularly the isoelectric point (surface charge). MS2 phage has a lower isoelectric point (pH of 3.9) than that of PRD-1 and T4 (pH of 4.2), and thus, has a different potential to be inactivated by adsorption to surfaces (Sakoda et al., 1997).

Less is known about the influence of general viral properties on biotic viral inactivation. Recently, we developed a method to study the interaction between bacteriophages and the ciliated protozoan, *Tetrahymena thermophila.* This method involves co-incubation of phage and ciliates, followed by centrifugation through Histopaque to yield a pellet of ciliates and ciliate-associated phage and a supernatant of free phage, which could be easily monitored for plaque forming units (pfus). With this method, *Tetrahymena* was shown to remove T4 from the co-incubation medium by internalizing the phage through macropinocytosis (Pinheiro et al., 2007). Internalization resulted in T4 inactivation (Pinheiro et al., 2007). How representative this interaction with *Tetrahymena* is for other viruses is unknown. In this paper the same method has been used to study the interaction between the ciliate and MS2. Structurally, MS2 is very different from T4. T4 is one of the largest phages; MS2, one of the smallest. T4 has a tail; MS2 has an icosahedral shape (Grabow, 2001). We find that MS2 is also engulfed by *T. thermophila* but the level of MS2 removal over 2 days was much less than with T4.

2.3 Materials and Methods

2.3.1 MS2 Bacteriophage and Bacterial Host

MS2 bacteriophage (ATCC®: 15597-B1™) is a common model phage in studies involving downstream contamination by sewage treatment plants, and was used in this study. Available from the American Tissue Culture Collection, the phage stock solution used for these experiments was graciously provided, along with the bacterial host, by Dr. Maria Mesquita and Dr. Monica Emelko of the University of Waterloo Department of Civil Engineering. Phage stock was maintained in tryptone, yeast extract (TYB) medium, a nutrient-rich broth consisting of 1% (w/v) tryptone (Becton-Dickinson, Sparks, MD), 0.1% (w/v) yeast extract (Becton-Dickinson), 0.8% (w/v) NaCl (BioShop, Burlington, Ont), 0.03% (w/v) $CaCl_2 \bullet 2H_2O$ (Sigma, St. Louis, MO) dissolved in MilliQ water. For storage, phage stock was kept at -20 ^oC and would only be thawed prior to use. Although not required by the phage, the nutrient-rich TYB medium is used to promote bacterial growth of the host strain. Bacteriophage require the presence of their host strain in order to replicate. This prevents any increase in phage number while a co-incubation proceeds, but necessitates the use of viable host strain for the proposed method of enumeration, the production of plaques on Petri plates. The MS2 host strain is *Escherichia coli* C-3000 (ATCC®: 15597™). This strain of *E. coli* was found to be prone to the development of phage resistance, and best results for the formation of plaques was achieved by inoculating host culture for 8 hours in TYB medium at 37^oC prior to use in experimentation, rather than overnight incubation.

2.3.2 Enumerating bacteriophage in a sample

The double-agar overlay was the chosen method of enumeration due to its relative accuracy, and its ability to measure only infective phage, and for this study a modified process of Adams' original method was used (1959). This involved mixture of 1 ml of phage dilution, 100 μl of host bacteria, and 3 ml of "top" agar, which was then poured over "bottom" agar-coated Petri plates. Top agar consisted of 1% (w/v) tryptone, 0.1% (w/v) yeast extract, 0.8% (w/v) NaCl, 0.7% (w/v) agar (Fisher Scientific, Fair Lawn, NJ), and 0.03% (w/v) $CaCl_2 \bullet 2H_2O$, while bottom agar differed only in agar content [1.5% (w/v) agar]. Plates were incubated overnight at 37 ^{o}C, to allow for the formation of plaques, or zones of clearing in the bacterial lawn. A single plaque represents the theoretical presence of one infective phage particle, and the number of plaques present on a plate is the number of phage present in a sample. Dilutions in TYB were made of samples, with plates containing between 20 and 200 plaques being counted. For every assessment of titre, all samples were plated in duplicate.

2.3.3 Tetrahymena thermophila

Tetrahymena thermophila starter cultures are available from the American Type Culture Collection (ATCC), however the lab culture holds its origins at the University of Guelph, and was originally donated by Dr. Denis H. Lynn (Department of Zoology, University of Guelph, Guelph, ON, Canada, N1G 2W1). The method used for culturing *Tetrahymena thermophila* was adapted from one originally designed by Gilron *et al* (1999). A batch culture was maintained at 22^{o}C in 10 ml of proteose peptone, yeast extract-enriched medium (PPYE), composed of 0.125% (w/v) dextrose (Sigma), 0.5% (w/v) proteose peptone (Becton-Dickinson) and 0.5% (w/v) yeast extract (Becton-Dickinson) dissolved in MilliQ

water. These batch cultures were started, maintained, and sub-cultured every 7-14 days. In preparation for experimentation, 1 ml of the *T. thermophila* batch culture was added to 10 ml of fresh PPYE in order to maintain a constant supply of healthy ciliate stock. The remaining 9 ml were added to 50 ml of fresh PPYE in a 75cm^2 non-tissue culture treated flask and kept at 22^{o}C, on an orbital shaker (50 RPM) overnight. Enumeration of ciliate cultures was performed using a Z2 Coulter Particle Counter and Size Analyzer (Coulter, Luton, UK), using an upper particle limit diameter of 50 μm and a lower limit diameter of 20 μm, the average size of the ciliate (Nilsson, 1976). In order to count *Tetrahymena* using the particle counter, set-up involved combining 19 ml of the isotonic IsoFlow Sheath Fluid solution (Coulter), 500 μl of *T. thermophila* culture, and 500 μl of 10% neutral, buffered Formalin (Sigma). Four counts were done per sample and these values were added then multiplied by 20 to convert to cells/ml.

2.3.4 General Protocol for Bacteriophage/Protozoan Co-incubation

The preferred medium in the investigation of *Tetrahymena's* effect on MS2 is the simple salt solution originally designed by Osterhout (1906), as it will maintain an isotonic environment for the ciliates. Prior to mixture with phage, the ciliates required transfer to this simple salt medium and enumeration. Following enumeration, *T. thermophila* culture was centrifuged for 10 min at 400 x g, and the supernatant was removed. Pelleted ciliates were washed twice, then resuspended, using Osterhout's medium, in each case. MS2 bacteriophage stock was added as required to produce the desired final titre, which varied with the proposed experiment. Mixtures were maintained in 15ml capped, plastic test tubes at 22^{o}C (unless otherwise stated) on an orbital shaker (50 RPM). Such mixtures were then subsampled for

analysis as indicated in each experimental method and *Tetrahymena* titre was determined as described above.

2.3.5 Step-gradient Separation (SGS) of the Bacteriophage/Protozoan Mixture

The separation of phage from ciliates used step-gradient separation as a means of impeding the movement of motile ciliates through to the supernatant following centrifugation (Figure 1.1). Efficient separation of ciliates from unassociated phage is important, to ensure that any phage that may remain infective on or in the ciliate will not produce a plaque in the agar overlay. This procedure involved adding an aliquot of phage-ciliate mixture to sterile, capped test tubes. To this, a sterile Pasteur pipette was used to add 1 ml of Histopaque-1119 (Sigma-Aldrich, St. Louis, MO) for every 1 ml of sample, beneath the protozoan-virus mixture, creating a second denser liquid phase. This was centrifuged at 400 x g for 10 min, isolating ciliates and any associated phage to the pellet. The top Osterhout's phase was removed and diluted with TYB as required for use in the double-agar assay. After overnight incubation at 37^{o}C, plaques were counted to assess titre.

2.3.6 MS2 Phage Infectivity in Media Commonly Used With Ciliates

Although other bacteriophage have been found to remain stable in ciliate media, MS2 has been found to rapidly decay naturally, and this susceptibility to degradation may make the phage more sensitive to these media (Pinheiro et al., 2007). Simply being maintained in different medium could potentially alter the titre of phage however, as it has been found that MS2 decay quickly at room temperature (Allwood *et al.,* 2003). To insure that viral decay was not going to impede this study, infectivity was assessed in the various media that would be

used. The stability of MS2 was measured by assaying titre in sterile PPYE and Osterhout's. For this assay, 10 ml of the tested medium was inoculated with MS2 stock to produce a final titre of 10^6 pfu/ml, in the absence of protozoa. Samples were kept in capped, plastic test tubes at 22°C. At 0, 4, 24, and 48 h, samples were serially diluted using TYB and plated using the double-agar overlay. After overnight incubation at 37°C, plates with 20-200 plaques were counted to assess viral titre

2.3.7 The Influence of Tetrahymena-conditioned Media on Phage Infectivity

Again, the high natural rate of MS2 decay may make the phage more susceptible to the presence of *Tetrahymena*-produced antiviral products released to the environment. To assess this susceptibility, media that *T. thermophila* had previously conditioned, was inoculated with MS2 (10^6 pfu/ml). This conditioned medium, either PPYE or Osterhout's, had sustained a ciliate culture for two weeks and was then centrifuged at 400 x g for 10 minutes, and was then filtered using a 0.2 μm syringe filter. At 0, 4, 24 and 48 h, samples were serially diluted with TYB and plated using double-agar overlay. Plates were incubated at 37 °C overnight and plates containing 20-200 plaques were counted to determine titre.

2.3.8 Assessment of MS2 Infectivity in the Presence of T. thermophila

Due to the affinity MS2 phage show toward adsorbing to suspended surfaces, to assess the role of this potential passive interaction between the two organisms, along with a sample of viable ciliates, formalin-fixed ciliates were assessed for their effects on phage infectivity. Both viable and fixed *T. thermophila* samples began with an overnight culture (35,000 cells/ml final density), which had been transferred to Osterhout's medium. These were transferred to a 15ml

test tube and for the fixed sample, 1 ml of 10% neutral, buffered formalin was added for every 3 ml of sample and allowed to sit for 10 min; viable samples were inoculated with Osterhout's solution in place of formalin. To each of these samples, MS2 phage stock was added to produce a final titre of 10^6 pfu/ml. Sterile Osterhout's was also assayed to express the degree of MS2 decay that naturally occurred. At 0, 4, 24, and 48 h after mixture, the SGS was performed and phage infectivity was assessed by the double-agar overlay. A 1,000 fold dilution of the SGS supernatant was assayed at 0 and 4 h, with decreasing dilutions being plated as phage infectivity decreased to allow a countable (20-200) number of plaques per plate.

2.3.9 Pellet Assay

To further build the case supporting phagocytic or pinocytic uptake, over surface adsorption, the phage titre of ciliate lysate was measured. An overnight culture of *T. thermophila* (35,000 cells/ml) was suspended in Osterhout's buffer and inoculated with MS2 phage (10^5 pfu/ml). At 0, 4, and 24 h, 2 ml of sample were chilled on ice. An SGS was performed to isolate the ciliates and they were resuspended in 1 ml 20 mM Tris-HCl, then returned to ice to chill. To this, was added 2 x lysis buffer (1.4 M sucrose, 4 mM EDTA, 2% Triton X-100) in a 1:1 ratio to the Tris-HCl volume, and the sample was vortexed for one minute. The lysed sample was then plated and any phage, which may have recently been internalized, should be detectable using the double-agar overlay.

Existing evidence highlights that infective viral particles may survive the digestive process and ultimately be returned to the environment as infective agents (Clarke, 1998). To assess this possibility with MS2, a method was devised to test whether *T. thermophila* were

releasing infective phage. Ciliates, which were maintained in the presence of MS2 phage for 24 hours, were removed through centrifugation (400 x g for 10 min), washed twice with fresh Osterhout's solution, and resuspended in fresh Osterhout's. Twenty-four hours following this transfer to fresh buffer, ciliates were pelleted using the SGS method. The viral titre of the supernatant was assayed to determine if any new phage were released to the medium. Additionally, pelleted ciliates were isolated and were resuspended in 1 ml 20 mM Tris-HCl and placed on ice to chill. Lysis of the cells was carried out through the addition of 2 x lysis buffer in a 1:1 ratio to the Tris-HCl volume, and the sample was vortexed for one minute. Plating the internal contents of these cells would detect the presence of any infective phage.

2.3.10 Epifluorescence of MS2 Phage

The use of SYBR Gold nucleic acid stain, to label the MS2 single-stranded RNA genome will allow the location of phage on or in the ciliate to be visualized using a fluorescent microscope (Mosier-Boss *et al.,* 2003). The production of phage stock solutions requires the lysis of host bacteria, expelling pieces of the host genome into the medium. If these artifacts of lysis remain in the media at the time of SYBR Gold addition, it is possible that this genetic material will become labeled and produce inaccurate results. To limit remnants of the phage replication process, prior to SYBR Gold labeling, MS2 stock was treated with RNase-free, DNase I (100 U/ml) for 10 minutes. SYBR Gold stock dye solution (provided as a 10,000 x concentrate) was diluted and added to the phage stock, resulting in a 2.5 x dye solution and allowed to sit in the dark for 10 min (Mosier-Boss *et al.*, 2003). The labeled-phage solution was then passed through a 100,000 MW Centricon® concentrator (Fisher) and centrifuged at 2000 x g for 5 min to remove unbound dye. The labeled-phage solution was rinsed twice by

passing 1 ml of Osterhout's solution through the concentrator at 2000 x g for 5 min. As a negative control, a solution (consisting of Osterhout's solution, SYBR Gold dye solution, and DNase-free RNase I) was also passed through a fresh concentrator and rinsed twice with Osterhout's solution. In both cases, the concentrate was returned to its original volume using Osterhout's solution. The labeled-phage solution was diluted as required and added to *T. thermophila* (35,000 cells/ml), which had previously been suspended in Osterhout's solution, to produce samples of 10^{10}, 10^{9}, and 10^{8} pfu/ml. A control sample was also produced, consisting of *T. thermophila* suspended with the T4-free dye solution. Samples were kept in the dark for 2 h, after which 60 µl were moved to a microcentrifuge tube and centrifuged for 5 min at 2,000 x g. Thirty microlitres of the supernatant were removed to concentrate the cells, and 3 µl of 10% neutral, buffered formalin was added. After fixation, samples were immediately viewed using a fluorescence microscope equipped with a Nikon B-2A filter (excitation 450 nm /emission 520 nm).

2.3.11 The Effect of Cytochalasin B on Decrease of Phage Infectivity

Cytochalasin B's (CB) effect on phage-ciliate interactions could build significant support for either an active (phagocytosis) or passive (adsorption) origin to these intra-species relationships, as it inhibits the process of actin-polymerization necessary for phagocytosis (Nilsson, 1976). CB was dissolved in the carrier, DMSO, and added to suspensions of *T. thermophila.* These samples were maintained in Osterhout's (final concentration of *Tetrahymena* 35,000 cells/ml) at CB concentrations of 0.1, 10, and 50 µg/ml. A 0 µg/ml control was made by adding DMSO to the ciliate culture. DMSO has been shown to be cytotoxic to *Tetrahymena* at even low concentrations, thus the final volume of DMSO did not

exceed 0.5% (v/v) in any sample; a concentration not seen to illicit a significant negative effect on the cells (Nilsson, 1974). MS2 phage stock was added to produce a final titre of 10^6 pfu/ml. At 0, 2, 4, 24, and 48 h, 1 ml was moved to a 15ml plastic, capped test tube and using the SGS, unassociated phage were isolated, and the titre determined.

2.3.12 The Effect of Low Temperature on Phage Inactivation

Ciliates at low temperature (i.e. 4 ^{o}C) will experience various decreases in cellular functions, including reduced motility and a near-complete reduction in food-vacuole formation (Nilsson, 1972). The resulting effects on phage reduction were investigated by combining viable *T. thermophila* (35,000 cells/ml) and MS2 phage (10^6 pfu/ml, final titre) in Osterhout's solution at 4^{o}C. A protozoan-free control was prepared to monitor the level of viral decay experienced during the experiment time period. Samples were assayed at 0, 4, 24 and 48 h using the SGS and double-agar overlay, and after overnight incubation at 37^{o}C plaques were counted to assess viral titre.

2.3.13 Statistical Analysis

Throughout this project a wide variety of statistical examinations were performed on various types of data, primarily paired and unpaired t tests. The GraphPad InStat® and Prism® program was used for statistical analysis of raw data.

2.4 Results

2.4.1 MS2 Phage Infectivity in Media Commonly Used With Ciliates

Since MS2 are not commonly used in either ciliate growth medium (PPYE) nor Osterhout's simple salt buffer, any effects to the viral titre were assayed by inoculating these media with phage and performing plaque assays at various times over 48 h. Titre of MS2 in both media decreased to a degree corresponding with data presented in Allwood *et al.* (2003). Comparison of titre between PPYE and Osterhout's at each time point (Figure 2.1) did not differ significantly, as determined by an paired t test ($P>0.05$).

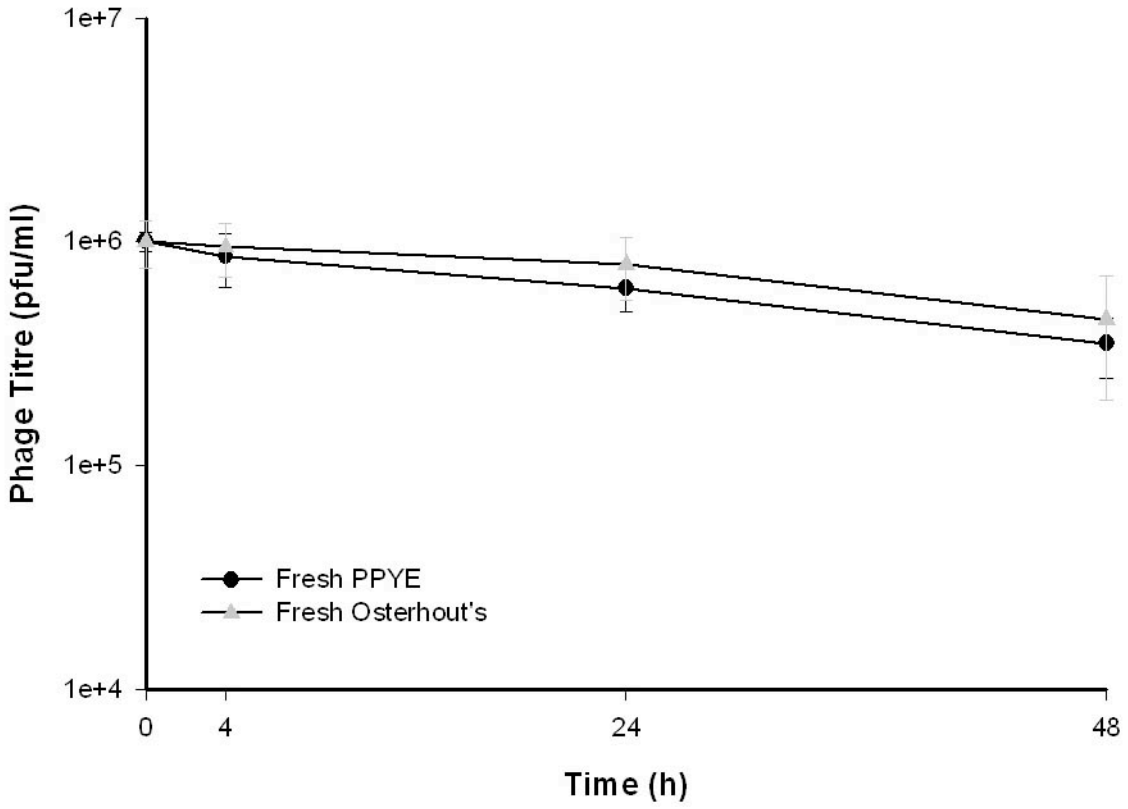

Figure 2.1 MS2 Phage Infectivity in Media Commonly Used With Ciliates

Ciliate growth medium (PPYE) and isotonic simple salt solution (Osterhout's) were assessed for any detrimental effects to inoculated phage titre. Phage were added to sterile media (10^6 pfu/ml) and at each time point viral titre was assessed using the double-agar overlay. The mean pfu with standard deviation bars (n=4) is plotted. At each time point, the titres of the two samples were compared to one-another by unpaired t test and were not found to differ significantly ($p>0.05$). Titre at 48 h was compared to the initial titre for each sample, and in both cases there was a significant decrease in MS2 titre, determined by paired t tests ($P<0.05$).

2.4.2 The Influence of *Tetrahymena*-conditioned Media on Phage Activity

Ciliate-conditioned medium was assayed for its effect on viral titre. Pinheiro *et al. (2007)* have shown that ciliate conditioned medium did not have a significant effect on the titre of bacteriophage T4 over an extended period of time, however MS2's genome is much different than T4's and is much less stable. The presence of antiviral components in this media was determined by adding MS2 to conditioned PPYE and Osterhout's, and assessing the titre at various times over 48 hours (Figure 2.2). The means for each time point were compared between PPYE and Osterhout's data and were not found to be significantly different by unpaired t test ($P>0.05$). Additionally, the mean for each time point was compared to its corresponding time point from the sterile media data by unpaired t test. At each time point, neither the conditioned PPYE titre nor the conditioned Osterhout's titre was found to vary significantly from sterile PPYE or sterile Osterhout's, respectively ($P>0.05$).

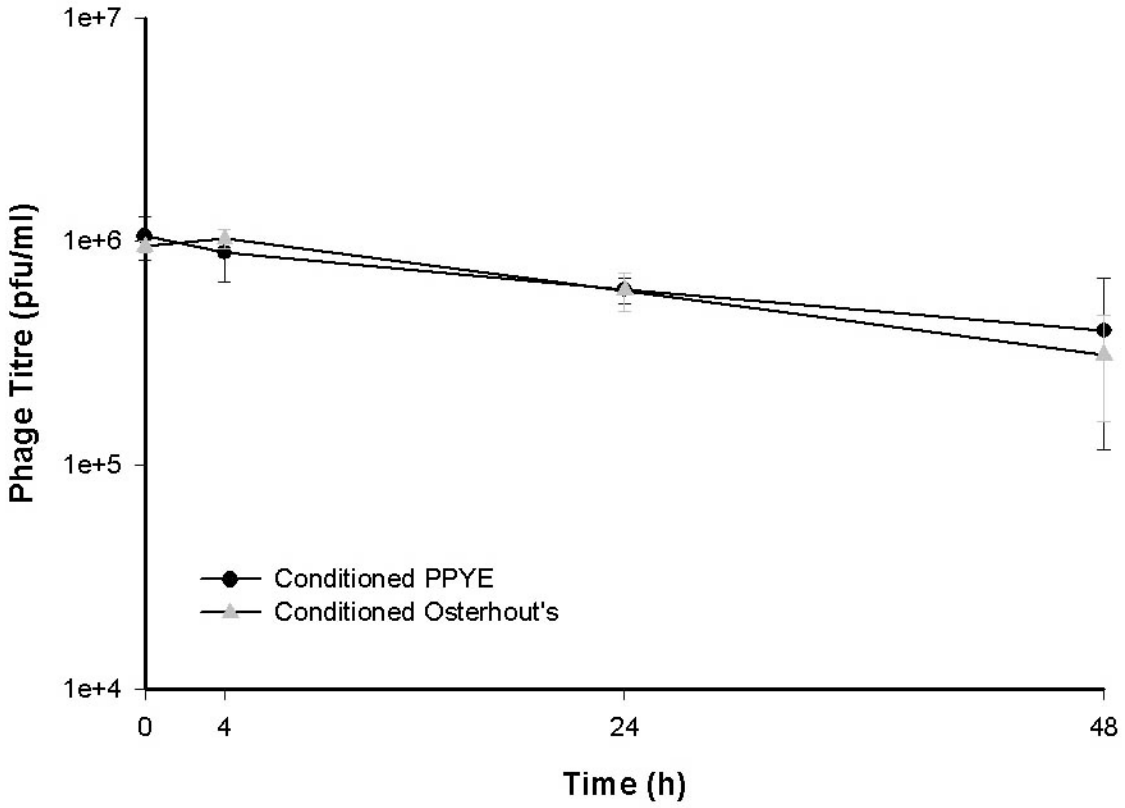

Figure 2.2 The Influence of *Tetrahymena*-conditioned Media on Phage Activity *Tetrahymena* cultures were grown in PPYE or Osterhout's starvation buffer for two weeks. The ciliates were removed using centrifugation, syringe-filtered, and this "conditioned" media was then inoculated with MS2 phage and pfu assayed over time at 22^{o}C. The mean pfu with standard deviation (n=4) was plotted. The data for conditioned media samples were compared to the corresponding data from the assay of titre in phage-inoculated fresh media. Unpaired t tests showed that the titre from conditioned media samples was not significantly reduced in comparison to fresh media data points ($P>0.05$).

2.4.3 Co-incubation of MS2 Phage With Viable or Formalin-Fixed *Tetrahymena*

When MS2 phage were co-incubated with formalin-fixed *Tetrahymena*, the titre decreased only slightly over time (Figure 2.3). When these titres were compared to corresponding data in the cell-free control, this decrease did not vary significantly between the treatments, by unpaired t tests ($P>0.05$). When in the presence of viable ciliates, reduction of MS2 titre occurred and decreased by 39±7% (SD) by 4 h, and 47±6% by 24 h in comparison to the titre in the cell-free control at these respective times. This reduction in phage titre in the presence of viable ciliates, compared to the cell-free control was significant at 4 h after initiation of co-incubation and remained so for the remainder of the investigation, judged by unpaired t tests ($P<0.05$). When compared to the fixed-ciliate data, the viable ciliate-dependant reduction was similarly significant ($P<0.05$). Thus, the presence of viable *Tetrahymena* results in a decrease in MS2 titre over time, and this viable ciliate-dependant rate of reduction was greater than the natural rate of phage decay. This increase in titre reduction is not seen in the presence of fixed ciliates, where the rate of MS2 decay was similar to its natural rate of decay.

When lysates of pelleted *Tetrahymena* were assayed for infective phage, a consistent but low number of phage was found at 0, 4, and 24 hours following co-incubation, and this number of pfus corresponded to ~9% of the initial titre (Table 2). Isolated ciliates, which were previously incubated in the presence of MS2 for 24 hours, were washed and resuspended in sterile Osterhout's. After 24 hours in this buffer, the ciliates were lysed and assayed. The consistent number of phage, previously found in the lysate of ciliates in the presence of MS2, had decreased to nearly zero, and no infective phage had been released to the medium (Table 2). This suggests that although infective phage may be found inside *Tetrahymena* at a single

moment in time, these phage are quickly inactivated and no MS2 phage particles are able to evade the digestive mechanisms of the ciliate, to be expelled to the medium intact.

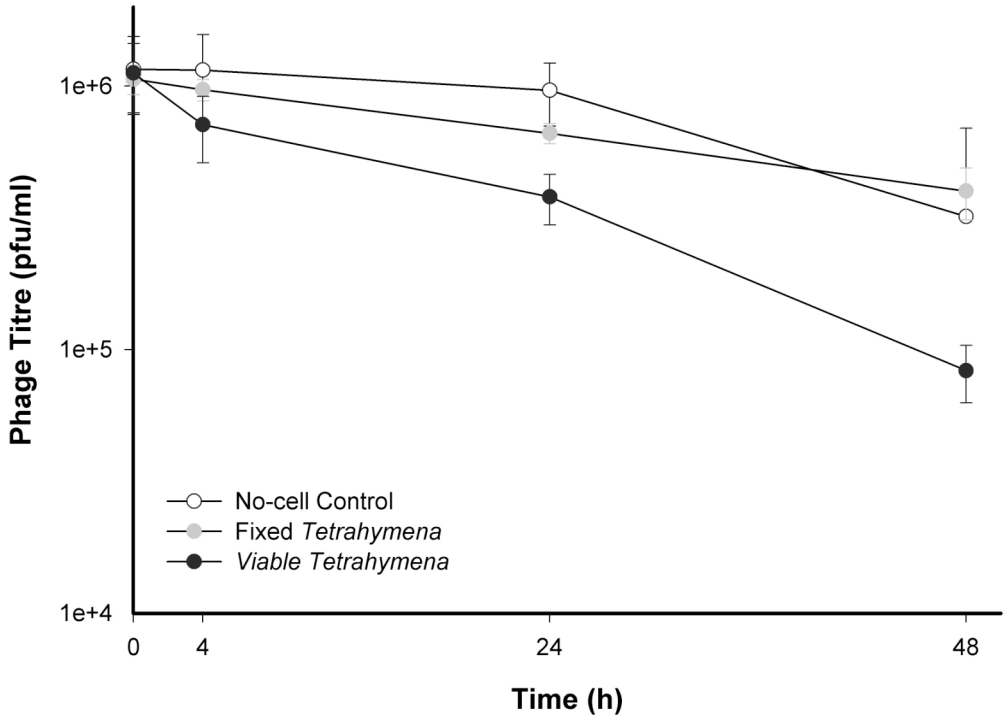

Figure 2.3 Co-incubation of MS2 Phage With Viable or Formalin-Fixed *Tetrahymena*

Co-incubations of T4 phage (10^6 pfu/ml) were begun in Osterhout's solution with either living or formalin-fixed *Tetrahymena* (35,000 cells/ml). At various times the *Tetrahymena* were removed by step-gradient centrifugation and the titre of MS2 remaining in the medium was assayed. MS2 phage's high natural rate of decay made the inclusion of a no-cell control necessary to compare any reduction in either ciliate-containing sample to the decrease in titre from decay. The mean pfu with standard deviation (n=4 for formalin-fixed sample; n=6 for the no-cell control and viable trials) are plotted. Fixed-ciliate data were compared to the no-cell control values by unpaired t tests, and were not found to vary significantly ($P>0.05$). As early as 4 h, viable *Tetrahymena* were found to reduce MS2 titre significantly, compared to both fixed-*Tetrahymena* samples and the no-cell control ($P<0.05$). The starting pfu was determined for cultures with and without *Tetrahymena* and for living and fixed *Tetrahymena*. As these starting values should be the same, these values were pooled to give the zero time (0 h) value.

Table 2. MS2 titre in pellet of *Tetrahymena* after centrifugation through Histopaque[a]

Co-incubation time at 22°C before centrifugation (h)	Treatment of *Tetrahymena* pellet[b]	Subsequent centrifugations[c]	Titre (mean PFU ± SD)[d]
0[e]	Lysed[f]	none	8,780 ±2,133
4	Lysed	none	9,140 ±1,549
24	Lysed	none	8,715 ±3,728
24	Resuspended in buffer for 24 h[g]	Supernatant	0 ±1
		Pellet[f]	3 ±4

[a] The MS2 titre at the start was 100,000 PFU for each of the coincubation conditions before centrifugation.

[b] Pellet formed after centrifugation through step gradient of Histopaque

[c] After 24 h, *Tetrahymena* were collected by centrifugation but not through Histopaque and the supernatant and pellet assayed for PFU.

[d] Titre expressed as mean of two experiments ± standard deviations (SD) .

[e] Although listed as 0 h, including the time required for Histopaque separation and wash this time point should be considered ~20 minutes.

[f] Pellet was lysed with Triton X-100.

[g] Pellet was resuspended in Osterhout's buffer .

2.4.4 Visualizing MS2/*Tetrahymena* Interactions

The use of SYBR Gold-labeled T4 was successful in visualizing phage sequestered by viable ciliates within food-vacuoles (Pinheiro *et al.,* 2007). Although MS2 is much smaller than T4, visualization of MS2 interacting with *Tetrahymena* was conducted (Figure 2.4). At both 10^9 and 10^{10}, fluorescent particles were seen within the ciliate after 2 h of co-incubation. When compared to phase-contrast images of the same *Tetrahymena*, in which food-vacuoles are easily identifiable, fluorescent pockets are seen to correlate to the location of food-vacuoles, and share a size similar to recorded food-vacuole diameters from the literature (Frankel, 2000). This fluorescence was not found in the case of *Tetrahymena* exposed to dye-only treatments.

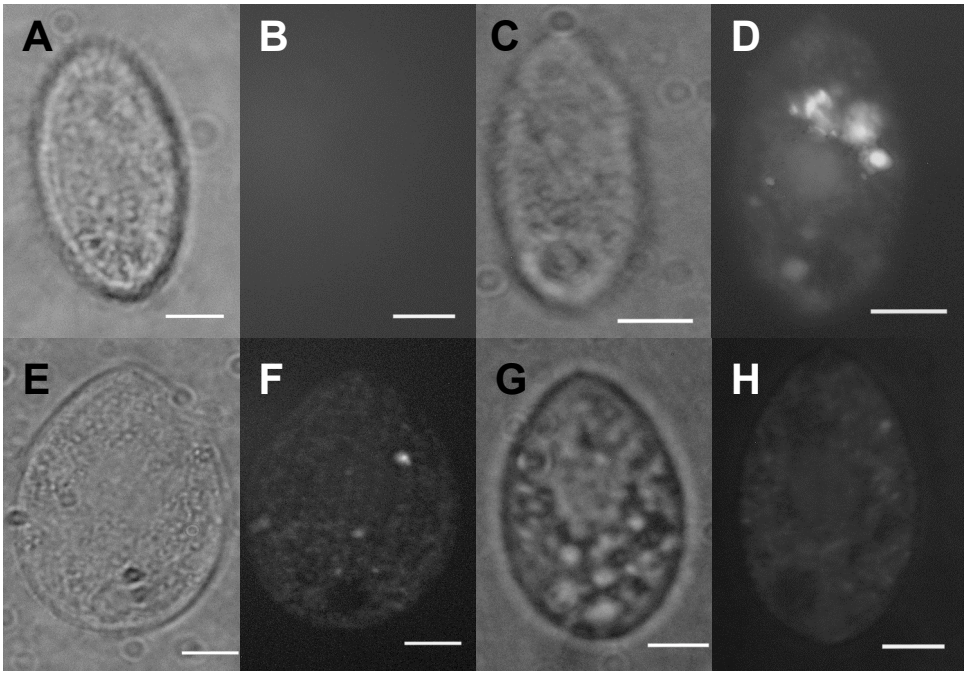

Figure 2.4 Visualizing MS2/*Tetrahymena* Interactions

Tetrahymena (35,000 cells/ml) were exposed to SYBR Gold nucleic acid stain solution (**A, B**) or to SYBR Gold-labeled MS2 phage at 10^{10} pfu/ml (**C**, **D**), 10^{9} pfu/ml (**E**, **F**), and 10^{8} pfu/ml (**G**, **H**) for 2 h at room temperature. Immediately prior to viewing, cultures were fixed in 10% neutral-buffered formalin. Note that phase contrast photomicrographs are shown in **A**, **C**, **E** and **G**; fluorescence photomicrographs, in **B**, **D**, **F**, and **H**. Bar = 10 µm in all cases.

2.4.5 Effect of Inhibition of Food-Vacuole Formation on MS2/*Tetrahymena* Interactions

The fungal-produced compound, Cytochalasin B, is a chemical inhibitor of actin polymerization; an essential process to the formation of food vacuoles by *Tetrahymena*. Ciliates were exposed to cytochalasin B prior to co-incubation with MS2 to assess the importance of food-vacuole formation on the reduction of phage titre. Variance between the rates of inactivation across the various cytochalasin B concentrations first became significant at the 24 h time point (Figure 2.5). This was determined by performing unpaired t tests, comparing the titres of differing cytochalasin B concentrations against one-another ($P<0.05$). The recorded MS2 titres at 24 h, for 0 and 0.1 μg/ml samples, and 10 and 50 μg/ml samples were compared using unpaired t tests and were not seen to significantly differ from the other ($P>0.05$). However, when data from 0 or 0.1 μg/ml samples were compared to 10 or 50 μg/ml data, titre differed significantly ($P<0.05$). Contrary to 0 and 0.1 μg/ml samples, neither 10 nor 50 μg/ml titres were found to vary significantly from the no-cell control, by unpaired t test ($P>0.05$).

When *Tetrahymena* are maintained at 4 ^{o}C they will cease food-vacuole production (Nilsson, 1976). Low temperature was exploited as an environmental method of inhibiting vacuole formation. *Tetrahymena* was placed at low temperature prior to the addition of MS2, and the titre was plotted after 24 h of co-incubation at this temperature, along with a sample maintained at 22 ^{o}C (Figure 2.6). At low temperature, *Tetrahymena* was not found to significantly reduce the MS2 titre, in comparison to the no-ciliate control ($P>0.05$), while titre in the *Tetrahymena*-containing sample dropped significantly compared to its no-ciliate control ($P<0.05$).

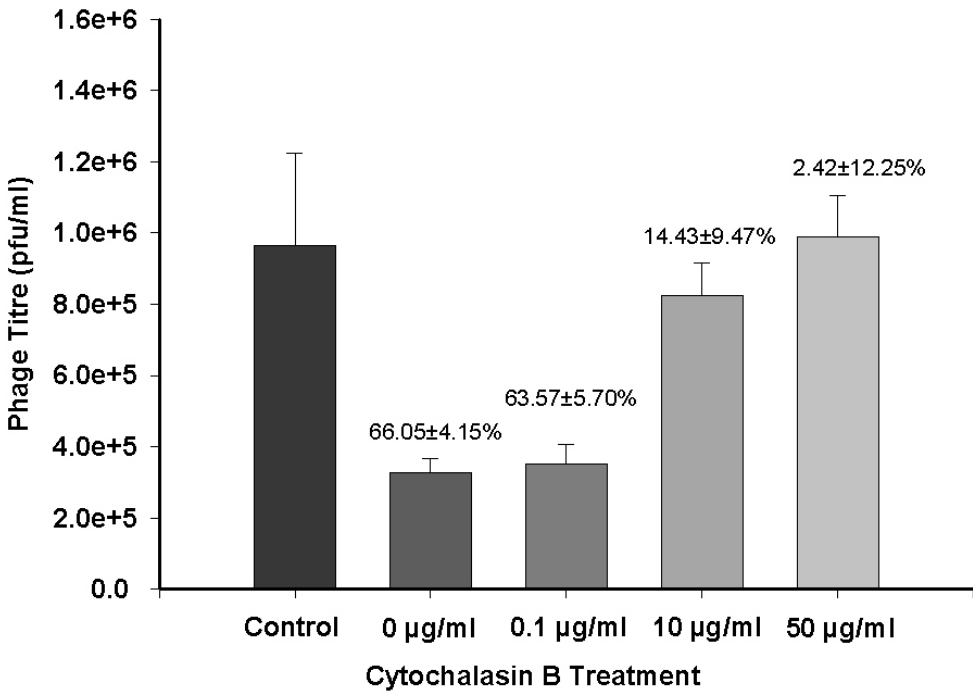

Figure 2.5 Effect of Cytochalasin B on MS2/*Tetrahymena* Interactions

Prior to MS2 phage (10^6 pfu/ml) and *Tetrahymena* (35,000 cells/ml) being co-incubated in Osterhout's simple salt solution, *Tetrahymena* were treated with Cytochalasin B, which was added to the samples dissolved in DMSO. Twenty-four hours following the addition of MS2, cultures were centrifuged to remove the *Tetrahymena* and to permit measurement of the remaining virus as pfu. The mean pfu with standard deviation (n=4) are plotted, with the percent decrease of titre from the no-cell control listed above the bars. The data were subjected to unpaired t test, comparing titre for each concentration to the no-cell control. Of all the samples, 10 and 50 µg/ml titres were not significantly different from the control titre ($P>0.05$), while 0 and 0.1 µg/ml sample both show significantly lower titres ($P<0.05$). Comparisons amongst the samples showed no significant difference between 0 and 0.1 µg/ml, nor 10 and 50 µg/ml, by unpaired t tests ($P>0.05$). Conversely, unpaired t tests comparing either 0 or 0.1 µg/ml to 10 or 50 µg/ml show titre were significantly different ($P<0.05$).

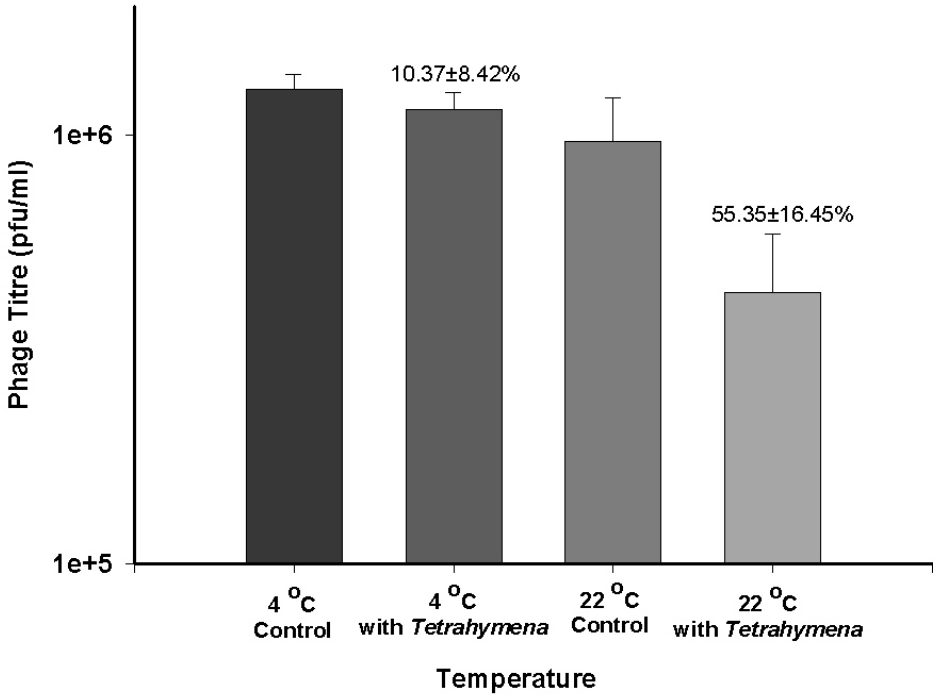

Figure 2.6 Effect of Low Temperature on MS2/*Tetrahymena* Interactions

Tetrahymena (35,000 cells/ml), prior to being co-incubated with MS2 (10^6 pfu/ml), was maintained at low temperature (4 ^{o}C) in Osterhout's simple salt solution for 15 minutes. Twenty-four hours following the addition of MS2, cultures were centrifuged to remove the *Tetrahymena* and to permit measurement of the remaining virus as pfu. The mean pfu with standard deviation bars (n=4) are plotted, with the percent decrease of titre from the no-cell control listed above the bars of the *Tetrahymena-containing* samples. The data were subjected to unpaired t test, comparing titre for each temperature to its no-cell control. At 4 ^{o}C, the *Tetrahymena*-containing sample did not show a significant reduction in titre by 24 h, compared to the no-cell control at this temperature by unpaired t test ($P>0.05$). The 22 ^{o}C *Tetrahymena*-containing sample, by contrast, shows significant reduction in titre from its control, and both 4 ^{o}C samples, by unpaired t test ($P>0.05$).

2.5 Discussion

Previously, we have shown the potential for the grazing ciliate, *Tetrahymena thermophila* to engulf and inactivate bacteriophage T4, a model phage that is relatively large and stable (Pinheiro *et al.,* 2007). These results suggested that the grazing ciliates may play a much greater role in both pathogen inactivation during sewage treatment, and also a regulatory role in the environment, by influencing the bacterial-phage loop and nutrient cycling. However, in order to broaden the scope of that research, the ciliate's ability to interact with a variety of viruses needed to be shown. In contrast to T4, MS2 is much smaller, has a RNA rather than DNA genome, and shows much higher potential for adsorption (Sakoda *et al.,* 1997). By applying the same methods that were used to demonstrate *Tetrahymena's* removal of T4 phage, we have shown that these ciliates will remove and inactivate MS2 phage from a co-incubation through a process that is broadly similar to that for T4 removal.

Tetrahymena and MS2 were co-incubated for varying times prior to centrifugation through Histopaque to form a pellet of ciliates and a supernatant with free MS2, whose numbers could be quantified as plaque forming units (pfus). As the time in which MS2 was incubated with and without the ciliates was increased, MS2 pfus in the supernatant steadily declined and the decline was much greater for incubation with *Tetrahymena*. If the *Tetrahymena* had been fixed, MS2 removal by the ciliate was stopped. These results suggest that MS2 is not being removed by passive adsorption to the ciliates. If incubated in medium in which *Tetrahymena* had been grown and then removed, MS2 pfus declined at the same slow rate as in medium in which *Tetrahymena* had not been grown. This result suggests that the removal of MS2 by the ciliates is not due to the release of virucidal agents. If MS2 and the

ciliates were co-incubated at 4 °C, the removal of MS2 by the ciliate was inhibited. Together, these results suggest that like T4, MS2 is removed by *Tetrahymena* through an active process, likely involving the engulfment of phage by the ciliates.

Several observations support the engulfment of MS2 into vesicles as the process by which the phage is removed. Firstly fluorescently labelled phages were observed within vesicles. Secondly treatments that are known to inhibit vesicle formation blocked both the appearance of fluorescent phage within vesicles and the removal of MS2 by *Tetrahymena*. These treatments were low temperature (4 °C) and treatment with cytochalasin B, which have been shown to inhibit food vacuole formation by macropinocytosis and phagocytosis in ciliates (Nilsson, 1972; Nilsson, 1979). Their actions on micropinocytosis are less studied in *Tetrahymena* but low temperature and cytochalasin B clearly inhibit this process in cells of higher organisms (Pratten and Lloyd, 1979). Based partially on the size of T4 (250 nm long with a diameter of 85 nm), macropinocytosis appeared to be responsible for the uptake of T4 by *Tetrahymena* (Pinheiro et al., 2007). However, in the case of MS2, the small size of the virus (~25 nm) means that potentially they could be taken up by micropinocytosis as well as by macropinocytosis. Micropinocytosis is usually considered to involve vesicles less than 200 nm in diameter (Rupper and Cardeli, 2001). In the case of the *Tetrahymena,* pinocytic vesicles could form in the buccal cavity and at the cell surface (Elliott and Clemmons, 1966; Nilsson, 1972; Nilsson and van Deurs, 1983).

The kinetics of MS2 removal showed differences from the kinetics of T4 removal (Table 3), however, when the time required for *Tetrahymena* to reduce the starting titre by 50% were compared, for both phages, by an unpaired t test, they were not seen to be significantly different ($P>0.5$). MS2 removal reached a plateau of approximately 25 % by 48 h, whereas T4

continued to be removed and pfus were less than 1 % of the starting values by 48 h. As had been found previously for T4 (Pinheiro et al., 2007), the removal of MS2 by *Tetrahymena* appeared to inactivate the phage. Two observations support this. Firstly, after 24 h of co-incubation with the phages, the pellet of *Tetrahymena* contained many fewer pfus than the pfus lost or removed from the co-incubation medium. This was true for both phages. With MS2, approximately 9% of the initial pfus were associated with *Tetrahymena*, while only 3% of the starting T4 pfus were associated with the ciliate. These low pfu numbers were maintained over 24 hours of incubation with the phage. These pfus are attributed to the recently internalized but yet to be inactivated phage. If this interpretation is correct, this suggests that upon engulfment by the ciliate, MS2 might be slower to be inactivated than T4. A second observation supports the contention that phage internalization can lead to inactivation, as had been found previously for T4 (Pinheiro et al., 2007). When pellets of ciliates from co-cultures with MS2 were transferred to phage-free medium, the number of pfus declined over 24 h to a very low level, with no infective phage being expelled by the ciliate into the supernatant.

Table 3. Kinetics of phage inactivation in co-culture with *Tetrahymena thermophila*

Bacterio-phage	Trial #	One-phase exponential decay parameters[a]			
		Time to 50% (h)[b]	95% interval	Plateau (% of control titre)[c]	95% interval
T4[d]	1	3.3	1.99 to 9.55	2.04	-7.5 to 11.58
	2	7.87	4.71 to 23.91	-3.36	-15.31 to 8.6
	3	7.36	6.28 to 8.90	-1.31	-4.68 to 2.07
	4	6.49	5.28 to 8.44	-0.63	-4.55 to 3.29
MS2[e]	1	6.77	2.17 to 0.0	27.76	-0.47 to 55.99
	2	18.49	4.28 to 0.0	17.60	-87.89 to 123.1
	3	2.29	1.03 to 0.0	27.39	14.17 to 40.62

[a] One-phase exponential decay determined through performing nonlinear regression on data using Prism v. 4.0c. The equation used in this analysis was Y=Span*exp(-K*X)+Plateau .

[b] Time to 50% defined as 0.69/K, where K is defined by software in the determination of one-phase exponential decay.

[c] Plateau is the theoretical final titre determined by the software, as determined by the rate of decline (K) and the preceding data. Negative values may be considered as zero.

[d] Data used in this analysis for T4 include values from 0, 4, 24 and 48 h of co-incubation with *Tetrahymena thermophila*. This data was originally presented in Pinheiro *et al.* (2007).

[e] Data used in this analysis for T4 include values from 0, 4, 24 and 48 h of co-incubation with *Tetrahymena thermophila*.

The reason for the differences in the removal and inactivation of MS2 versus T4 is unknown at this time. Differences might be considered at three interconnected steps within the ciliates: phage internalization into vesicles, phage stability within digestion vesicles, and possibly phage defecation at the cytoproct. At the level of internalization, the uptake of MS2 possibly could be receptor mediated and subject to saturation and/or regulation, whereas T4 likely is not (Pinheiro et al., 2007). For changes within vesicles, MS2 might be more stable than T4 in the acidic and enzymatic environment of secondary lysosomes. Additionally T4 and MS2 might differ in the degree to which they form aggregates within these vesicles, with the larger T4 being more likely to aggregate. In turn, aggregation could be critically important if a potential third step, phage defecation, occurs. Although viral defecation has yet to be described, observations have been made recently of *Tetrahymena* releasing viable *Salmonella enterica* within vesicles (Brandl et al., 2005). If aggregated phage were to be defecated, an aggregate might be counted as a single pfu, and greater aggregation would lead to greater apparent inactivation. Currently distinguishing between these possibilities is difficult because the number of phage particles cannot be counted other than as pfus and removal and inactivation of the phages cannot be measured independently. In the future, unraveling these complexities will be important for understanding the role of ciliates in microbial ecology and water quality.

2.6 General Conclusions

As was done previously with T4 phage, centrifugation of co-cultures of *Tetrahymena* and MS2 phage was used to separate free phage from *Tetrahymena*-associated phage in order to monitor the removal of phages by the ciliates. Only living *T. thermophlia* were able to

remove MS2, as was found previously for the T4 removal. When fluorescently labelled prior to being added to co-cultures, MS2 could be visualized inside vesicles within *Tetrahymena*, as observed previously for T4. In the case of T4 uptake, the process appeared to be by macropinocytosis, whereas micropinocytosis also might have contributed to MS2 internalization. Over the time frame of 2 days, T4 had been reduced to less than 1 % of the starting pfus, whereas approximately 25% of the initial pfus still remained in suspension for MS2. As relatively few pfus were found in the pellet of ciliates, removal of T4 and MS2 meant inactivation. The substantially higher survival of MS2 than T4 suggests that viruses could vary in their susceptibility to inactivation by ciliates.

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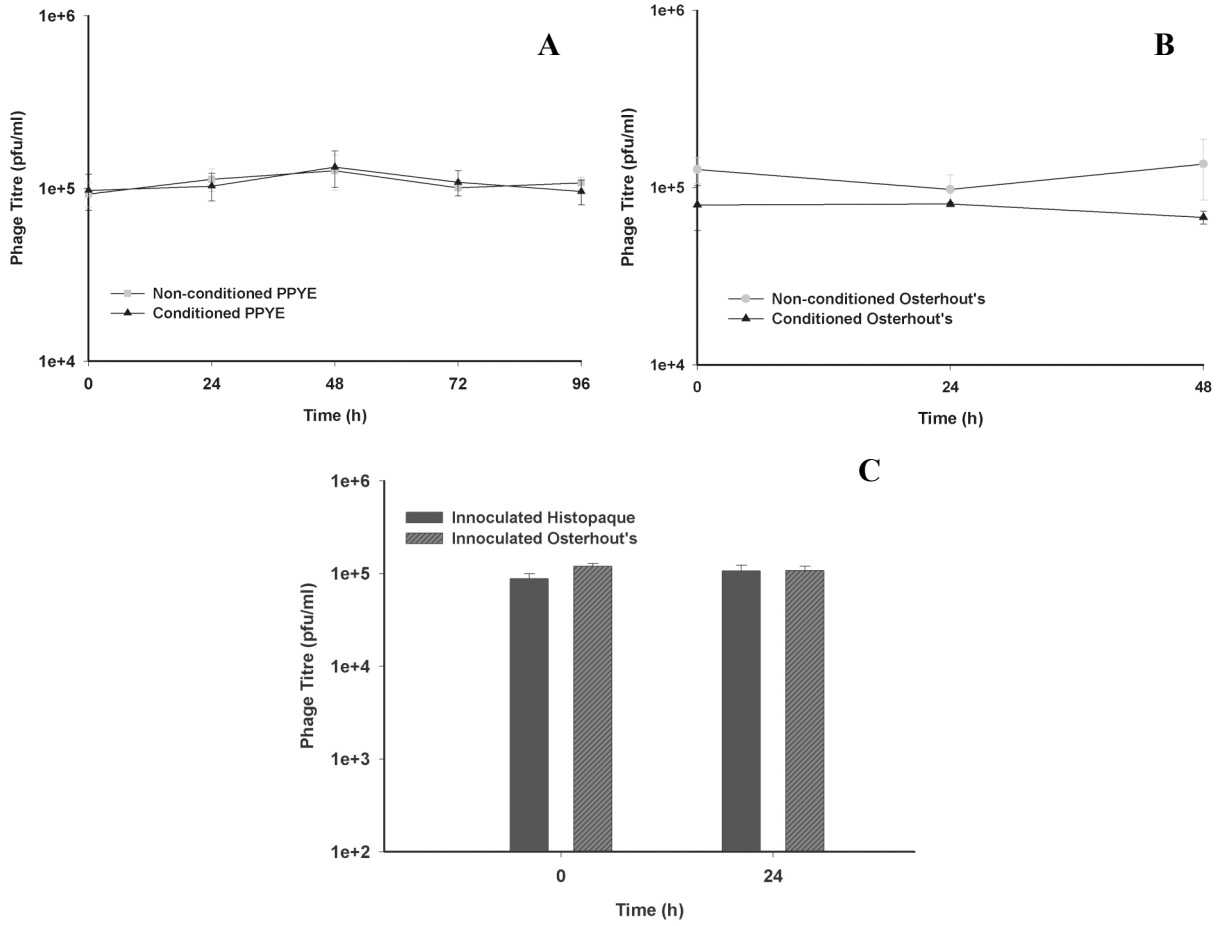
Future Research

Having monitored the effect of the grazing ciliate, *Tetrahmena thermophila* upon a population of bacteriophage, I can propose several directions that these results could lead. Firstly, by inhibiting food vacuole acidification (using chemical inhibiters) a greater understanding of the cellular mechanism responsible for inactivation could be gained. The use of different bacteriophage, to expand the range of physicochemical properties represented, with the ciliate would help to broaden the scope of *Tetrahymena's* impact on natural phage populations. Further, because of the increasing ubiquity of pharmaceuticals in the aquatic environments, and the high concentrations found in sewage, the chemicals could be added to phage-ciliate co-culture to assess any negative effects on inactivation of phage. If the ciliate digestive cycle is interrupted in some way, it will impact bacterial and phage populations, along with the ecosystem, as a whole. The influence of *Tetrahymena* could be investigated on human pathogens (such as attenuated Poliovirus), in order to assess the ability of ciliates to inactivate viruses infecting higher organisms. Alternatively, focusing on the sewage treatment environment, there are a wide variety of grazers present. By isolating different species of grazers, differences in their inactivation capacity could be studied. It is clear that this project has presented many new opportunities to study, and many new questions regarding the importance of ciliates to ecosystem diversity that can be answered.

Appendix



T4 Bacteriophage Infectivity in Various Media

Tetrahymena was allowed to grow in PPYE (**A**) or Osterhout's buffer (**B**) for two weeks, at which point ciliates were removed through centrifugation. This "conditioned" media was then inoculated with T4 phage and assayed for infectivity. This was compared to infectivity of T4 suspended in fresh media. The mean pfu with standard deviation bars (n=4) plotted. The data were subjected to one-way ANOVA, but no significant variation in the means was found ($p>0.05$). Additionally, T4 phage was added to Histopaque-1119 and Osterhout's medium (**C**) and maintained at room temperature for 24 h, at which point pfu was compared. The mean pfu

with standard deviations (n=4) are plotted. The means between zero time and 24 h for each medium were not statistically different as judged by one-way ANOVA ($p>0.05$).