BACTERIOPHAGES IN ACTIVATED SLUDGE BIOREACTOR-ROLE OF LYTIC AND LYSOGENIC PHAGE CYCLES

by

Jeongdong Choi

A dissertation submitted to the faculty of
The University of Utah
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Civil and Environmental Engineering

The University of Utah

December 2012

THE UNIVERSITY OF UTAH GRADUATE SCHOOL

STATEMENT OF DISSERTATION APPROVAL

The dissertation of **Jeongdong Choi**

has been approved by the following supervisory committee members:

Ramesh Goel	, Chair	**Aug 13th 2012** Date Approved
Steve Burian	, Member	**Aug 13th 2012** Date Approved
Markus Babst	, Member	**Aug 13th 2012** Date Approved
Otakuye Conroy-Ben	, Member	**Aug 13th 2012** Date Approved
Edward Trujillo	, Member	**Aug 13th 2012** Date Approved

and by **Chris Pantelides** , Chair of

the Department of **Civil and Environmental Engineering**

and by Charles A. Wight, Dean of The Graduate School.

ABSTRACT

Bacteriophages are viruses that infect bacteria and are known to be a very important component of microbial ecology in aquatic environments. Bacteria are involved in removing organics, ammonia and phosphorus in activated sludge processes. Frequent upsets, however, were found in the activated sludge process such as nitrification failure and filamentous bulking, causing the deteriorated effluent quality. Those upsets are caused by either unwanted bacterial growth or the death of key bacteria in an activated sludge bioreactor.

The prophage on the bacterial genome remains dormant, but can cause cell lysis under certain environmental conditions. The effect of various environmental stress factors was examined on the ammonia oxidation and prophage induction in a model ammonia oxidizing bacterium, *Nitrosospira multiformis*. The factors included in the study were pH, temperature, organic carbon, the presence of heavy metal and toxicity. The selected environmental factors are commonly encountered in wastewater treatment processes, where ammonia oxidizing bacteria play a pivotal role of converting ammonia into nitrite. All of the factors could induce prophage from *N. multiformis*, demonstrating that cell lysis due to prophage induction could be an important mechanism contributing to the frequent upset in ammonia oxidation efficiency in full-scale treatment plants.

The lytic phage could be applied to control biomass bulking in the activated sludge process using model filamentous bacteria. The lytic phage especially infecting model

filamentous bacteria was isolated from the mixed liquor of a wastewater treatment plant. Significant reduction of sludge volume index (SVI) was observed after the isolated phage addition. The phages were considerably stable after exposure to high temperature and pHs, emphasizing that phage can withstand the seasonal/operational fluctuations. The isolated phage showed no cross infectivity with other bacteria most commonly found in activated sludge systems, thus validating its suitability for biocontrol of filamentous bulking caused by filamentous bacteria. Following the application of bacteriophage-based biocontrol, successful reduction in SVI was achieved, indicating improved biomass settling. The phage application did not affect the nutrient removal efficiency of the biomass. The phage-based biocontrol, therefore, holds a great potentiality for large-scale applications as an economic agent in the mitigation of several water, wastewater and environmental problems.

I would like to dedicate this dissertation to my dear wife, Song, and my sweet daughter, Kaylee (Soyeon) and son, Kaden (Jihoon). I would not have been able to do this without your love, understanding and support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

I would like to thank my PhD advisor, Dr. Ramesh Goel, for his academic advice and drive to help me reach my research skill and goal. Without his support, this research would not have been possible. I want to thank him for giving me a lot of opportunities to professionally develop myself and advising me through all the ups and downs.

I would like to acknowledge all my committee members, Dr. Steve Burian, Dr. Otakuye Conroy-Ben, Dr. Markus Babst and Dr. Edward Trujillo, for their time, efforts and support.

I had a wonderful journey during my graduate study. I had a very good time and made many memories in our lab. I would like to acknowledge all my labmates with whom I worked in our lab for their help and support. I would like to thank Meher for his valuable research comments.

I would like to thank my parents and my brother for their constant support and love during my entire educational experience.

Finally I want to thank my family, for their help and support, and for putting a smile on my face everyday.

CHAPTER 1

INTRODUCTION

1.1 Bacteriophages and their life cycles

Bacteriophages are viruses that infect bacteria and are known to be very important components of freshwater and marine bacterial communities (Wommack and Colwell, 2000; Suttle, 2005). Bacteriophages are killers of bacteria and contribute significantly to the ecology and evolution of prokaryotes (Casjens, 2005). Up to 50 % of the daily bacterial production (heterotrophic and autotrophic) in the world's ocean is killed by bacterial predation (Wommack and Colwell, 2000). Bacteriophages have enormous influence on microbial diversity and population dynamics of host bacteria in natural as well as engineered systems (Balding et al., 2005). Bacteriophages are known to be very abundant in aquatic systems (Bergh et al., 1989) with counts of about $1x10^7$ viruses per ml. The world's oceans are estimated to contain greater than $1x10^{29}$ viruses in total (Breitbart and Rohwer, 2005). Bacteriophages in aquatic systems have been well studied over the past decade and different research efforts have investigated their influence on the abundance and composition of the bacterial community through bacterial cell lysis (Brussow, 2007).

Two categories of bacteriophages are common: virulent (lytic) and temperate (also called lysogenic). Virulent phages infect and kill the host immediately and replicate often 100s of progeny (daughter phages) in a short time (generally in minutes), called "lytic infection" (Figure 1.1). On the other hand, temperate phages recombine with their host cell DNA during "lysogenic infection", forming a dormant prophage (phage DNA integrated into the bacterial genome) where viral genes that are detrimental to bacterial cells are not expressed (Casjens, 2003). A schematic of phage lytic (Fig. 1.1a) and lysogenic cycles (Fig. 1b) is shown in Fig. 1.1.

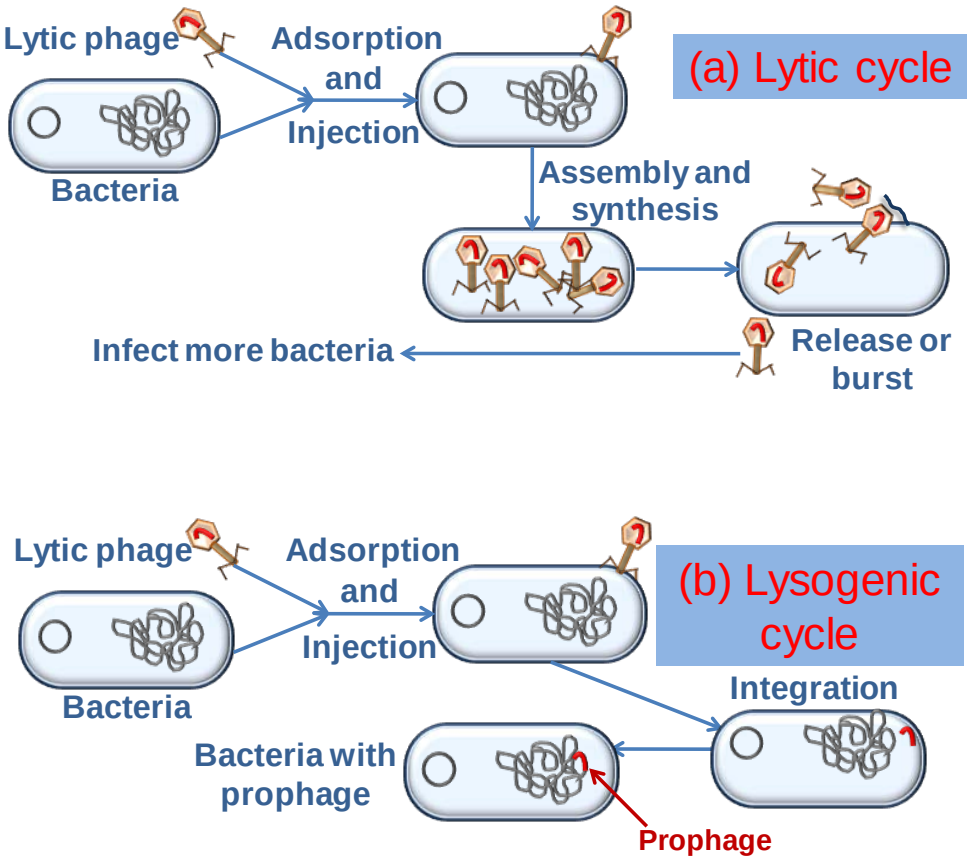

Fig. 1.1 Phage cycle. (a) Lytic cycle of virulent (lytic) phages and (b) lysogenic cycle of phages

Bacterial viruses were discovered twice, by Twort in 1915 and by d'Herelle in 1917 (Delbruck, M., 1942; Duckworth, 1976). D'Herelle coined the name "bacteriophages" for these infectious agents lysing bacteria, which literally means "eaters of bacteria". Right after their discovery, phages were also used in early forms of biotechnology to fight bacterial pathogens (Levin and Bull, 1996). In Western Europe and the United States, this phage therapy was abandoned due to ambiguous results and the discovery of antibiotics. In some Eastern European countries and the former Soviet Union, phage therapy was continued and a review was published on this topic (Sulakvelidze et al., 2001).

Bacteriophages exist in many varieties (Fig. 1.2). They may have single or double stranded RNA or DNA genomes that range in size from a few thousand to half a million base pairs (bp) in length (Casjens, 2005). Bacteriophages are tailed, polyhedral, filamentous and pleomorphic (Ackermann, 2001). Bacteriophages are classified into 1 order, 13 families and 31 genera and there are no universal criteria for genera and species (Kutter and Sulakvelidze, 2004). Among these, tailed phages are the largest and most widespread group of bacterial viruses (Ackermann, 2001). Out of approximately 5000 bacteriophages studied so far using electron microscopy, at least 4950 belonged to tailed phages (Ackermann, 2001).

1.2 Activated sludge bioreactor

Bacteriophages are widespread and it can be concluded about these tiny creatures that they exist wherever bacteria exist. Bacteria are widely used in environmental engineering to remove different contaminants from soil, hazardous waste and liquid waste.

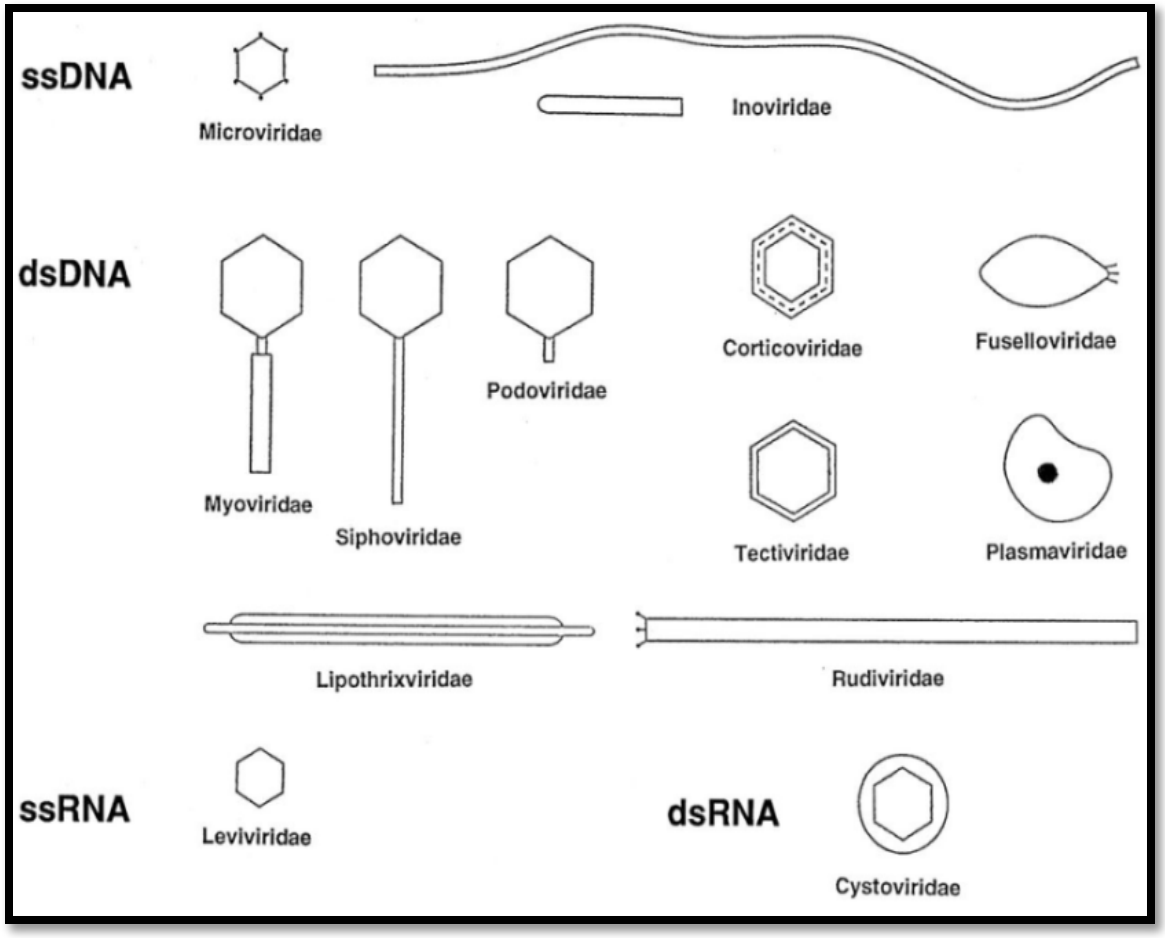

Fig. 1.2 Morphology of bacteriophages

The most common use of bacteria is in engineered bioreactors to treat liquid waste, primarily municipal wastewater. The process is commonly known as the activated sludge process.

The activated sludge treatment process (Fig. 1.3) is the widely used method for municipal wastewater treatment and is employed worldwide. A complex community of prokaryotes and protozoa is involved in the activated sludge process to degrade/remove various contaminants of concern. Various contaminants present in the influent emanating from the municipal sources enter the bioreactor in activated sludge processes.

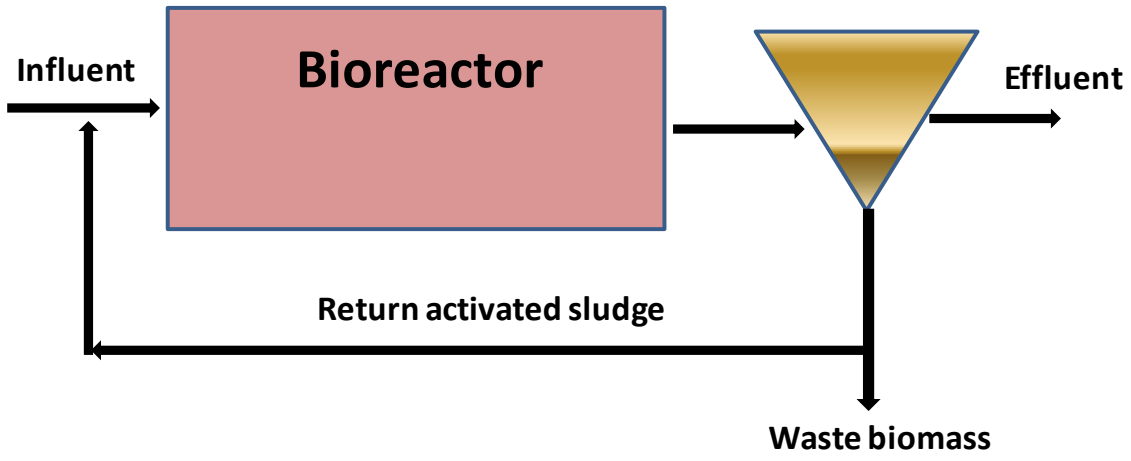

Fig. 1.3 The schematic shows a conventional activated sludge process configuration.

A variety of bacteria present in the bioreactor consume these contaminants following the specific redox chemistry in the presence and/or absence of oxygen (Grady et al., 1999). Despite being a matured engineered system with well-studied microbial ecology, operational challenges and issues related to process upsets (Love and Bott, 2000) still exist without any universally accepted engineering solution.

1.3 Bacteriophages in activated sludge bioreactor

Activated sludge has been shown to contain 10^8~10^9 viruses per ml (Ewert and Paynter, 1980; Otawa et al., 2007; Wu and Liu, 2009), a number comparable to or greater than the number of viruses which are found in most of the aquatic systems. Poor chemical oxygen demand (COD) removal, disrupted nitrogen and phosphorus removal and poor biomass settling are often observed in activated sludge processes. In addition, factors such as stress due to the presence of certain chemicals on the microbial population have been suggested and researched in the past (Love and Bott, 2002). However,

bacteriophage-mediated community changes and consequently the effect on the treatment efficiency of activated sludge processes have not been studied in detail except for those that infect influx enterobacteria (Bitton, 1987). Coliphages, viruses that infect coliform bacteria, have been studied but in the context of microbial source tracking (Cole et al., 2003). Thus, our understanding of viral abundance, distribution, rates of production, diversity and viral effects on microbial community structure and function lags significantly behind the progress made in activated sludge microbiology. Phages can impact many biogeochemical processes through changes in bacterial community composition by affecting their mortality, diversity and species distribution (Bergh et al., 1989; Proctor, 1997; Jiang and Paul, 1998; Fuhrman, 1999).

Despite significant advances in activated sludge microbiology, little is known about bacteriophages in activated sludge bioreactors. Most importantly, there is almost no information available on the effect of lytic phages on activated sludge process microbiology, especially on key bacteria such as ammonia oxidizers, polyphosphate accumulating organisms and organic oxidizers. There has not been any research performed so far on the contribution of lysogeny (schematic b in Fig. 1.1) to activated sludge microbiology. Despite process maturity and technological advances in the process design, activated sludge processes continue to suffer from upsets and operational problems such as filamentous bulking. Bacterial communites in activated sludge bioreactors often face different stresses such as the presence of toxic loading, heavy metals, pH changes and organic load variations. These stress factors cause process upsets, the reasons for which have been solely attributed to either process failure or to bacterial community inhibition. Preliminary research on genomes of the bacteria relevant in

activated sludge bioreactors has shown that many of these bacteria have a prophage element (Fig. 1.1b) on their genome. This makes these bacteria susceptible to undergo cell lysis due to the induction of a prophage element from their genome following the scheme presented in Fig. 1.4. Prophage-bacterial interactions have received tremendous attention due to the significant role that they play in the microbial ecology of various bio-systems. Researchers have also tried to study the effect of environmental factors on phage induction in natural bacterial populations (McDaniel and Paul, 2005). Environmental conditions affected the switch between the lytic and lysogenic life styles of the well-studied temperate *Escherichia* coliphage (Echols, 1972; Herskowitz and Hagen, 1980). It was reported that environmental pollutants to bacteria can be more efficient inducing agents than a universal inducer such as mitomycin-C (Cochran et al., 1998).

Municipal wastewater treatment plants frequently receive heavy metals and other toxic substances due to rapid industrialization. Negative effects of pH shift, presence of heavy metals and toxicity of substances on the performance of the ammonia oxidizing community are well established (Park and Ely, 2008; You et al., 2009). Prophage induction in lysogenic bacteria is common in the environment (Ackerman and Dubow, 1987). Lysogeny due to the prophage induction can be caused by several environmental stress factors. Lysogeny, therefore, can adversely affect the bacterial population and, in turn, the process efficiency.

In this research, one of my primary objectives was to investigate the role of stress-mediated bacteria killing in an activated sludge system due to the induction of a prophage element using the scheme presented in Fig. 1.4 .

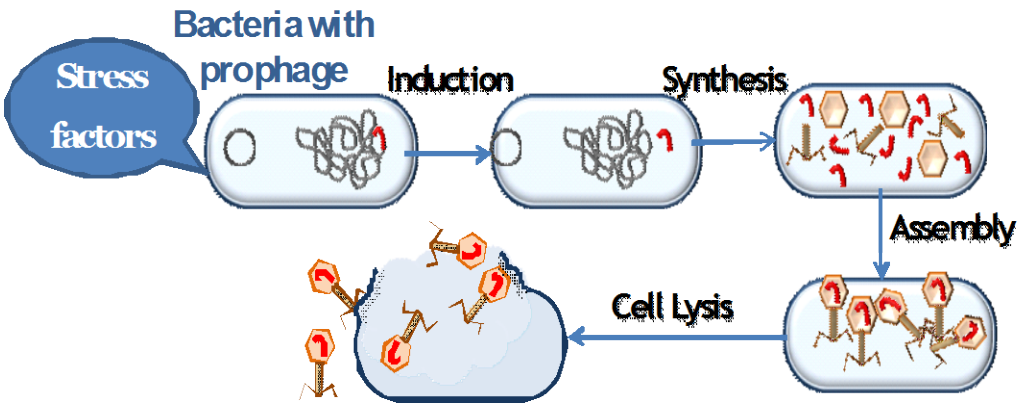

Fig. 1.4 Prophage induction leading to cell lysis in bacteria under environmental stress factors.

Apart from stress factors, other in-situ conditions such as proliferation of filamentous bacteria can cause operational problems in activated sludge systems. Filamentous bulking due to overgrowth of filamentous bacteria is the most important operational problem with no definitive solution in activated sludge processes because it causes poor biomass settling in secondary clarifiers and deteriorated effluent quality.

Current control strategies for this problem consume much labor, cost and energy, and have several side effects. According to Jenkins et al. (1993), mitigation of filamentous bulking using unspecific methods increases a plant's operational cost tremendously. According to Daigger et al. 1985, if the methods to control and avoid bulking could be identified and incorporated in a plant's expansion plan, the cost will reduce in the range of millions of dollars. The claims by Drs. Jenkins and Daigger may have been made long ago, but the truth behind these still holds true. A study by Madoni et al. (2000) in Italy observed 84 full-scale treatment plants suffering from poor settling due to filamentous bacterial growth. Likewise, Levantesi et al. (2004) found 86 full-scale treatment plants

suffering from sludge bulking due to the presence of novel filamentous bacteria belonging to *Alpha-proteobacteria*.

From wastewater treatment plants, several filamentous bacteria have been identified and isolated responsible for biomass bulking, including *Microthrix parvicella*, *Sphaerotilus natans*, Eikelboom type 1702, *Haliscomenobacter hydrossis*, *Nocardia*, *Thiothrix spp*. etc. (van Veen et al., 1973; Eikelboom, 1975; Williams and Unz, 1985; Kampfer, 1995; da Motta et al., 2003). Table 1.1 shows filamentous bacteria causing sludge bulking and foaming in the United States.

Decades of research could not fully clarify the key processes behind this phenomenon and there is still no reliable control engineering strategy. Specific and unspecific strategies are suggested for the control of bulking. Specific methods rely on finding suitable operational and environmental conditions to promote the growth of floc formers**.** Finding the right combination of operational and environmental conditions is crucial and is challenging (Martins et al., 2004). Nonspecific control strategies employ the application of external stresses to filamentous bacteria but there are several concerns associated with this approach. For example, the application of chlorine to kill filamentous bacteria is questionable due to the formation of chlorinated byproducts.

In summary, filamentous bulking in conventional activated sludge bioreactors is everlasting, and a worldwide problem in the world's most widely used wastewater treatment processes. Its root cause is bacteria. It is an adage that every single bacteria has a phage in nature which can infect (kill) it. The same concept has been used in the medical field and meat industry to kill the pathogenic bacteria using lytic phages, and the strategy is known as "phage therapy".

Table 1.1. Filamentous bacteria dominant in the United States.

Rank	**Filamentous organism**	**Percentage of treatment plants with bulking sludge where filament was observed to be dominant (a)**
1	*Nocardia* spp.	31
2	Type 1701	29
3	Type 021 N	19
4	Type 0041	16
5	*Thiothrix* spp.	12
6	*Sphaerotilus natans*	12
7	*Microthrix parvicella*	10
8	Type 0092	9
9	*Haliscomenobacter hydrossis*	9
10	Type 0675	7
11	Type 0803	6
12	*Nostocoida limicola*	6
13	Type 1851	6
14	Type 0961	4
15	Type 0581	3

Source: Bitton, 2005

(a) Percentage of 525 samples from 270 treatment plants with bulking problems.

The available literature on the application of phage therapy in these applications is enormous and has been reviewed previously (Alisky et al., 1998; Bull et al., 2002; Nakai and Park, 2002; Dixon, 2004). In this study, I conducted research (my second primary objective) to apply the concept of phage therapy (hereafter called the phage-mediated biocontrol) in activated sludge systems with filamentous bulking as the model operational challenge.

CHAPTER 2

RESEARCH HYPOTHESES

The following hypotheses have been formulated based on the aforementioned discussion about bacteriophage and activated sludge process upsets.

2.1 Hypothesis 1 (H1)

The hypothesis for phage-bacteria interactions in activated sludge processes leading to lysogeny of bacteria is: lysogeny is common in activated sludge bacteria and is controlled by various operational and environmental parameters that are relevant in activated sludge systems.

Task 1 - Maintain healthy population of key model bacteria relevant in activated sludge processes.

Task 2 - Develop methodology of virus quantification.

Task 3 - Study lysogenic cycle phage under variation of environmental stress factors.

The tasks for hypothesis 1 were shown in Chapter 3.

2.2 Hypothesis 2 (H2)

The underlying hypothesis for the phage-mediated biocontrol of bulking is: the lytic phages do exist in activated sludge bioreactors, but reduction in the specific bacteria population (filamentous organisms in this case) due to the phage predation must take place at a higher phage-to-host ratio to affect the community.

Task 4 - Maintain healthy population of filamentous bacteria.

Task 5 – Isolate and characterize the isolated bacteriophages infecting filamentous bacteria.

Task 6 – Demonstrate the bacteriophage-based bicontrol of filamentous bacteria.

The tasks for hypothesis 2 were shown in Chapter 4 and 5.

CHAPTER 3

VARIOUS PHYSICO-CHEMICAL STRESS FACTORS CAUSE PROPHAGE INDUCTION IN *NITROSOSPIRA MULTIFORMIS* 25196- AN AMMONIA OXIDIZING BACTERIA (H1-TASK 1, 2 AND 3)

3.1 Introduction

Bacteriophages are virsues that infect bacteria. Bacteriophages are known to be very important components of freshwater and marine bacterial communities (Wommack and Colwell, 2000; Suttle, 2006). The world's oceans are estimated to contain greater than 1×10^{29} phages in total (Breitbart and Rohwer, 2005). On the other hand, activated sludge systems have been shown to contain 10^{8}-10^{9} phages per ml (Ewert and Paynter, 1980; Otawa et al., 2007), a number comparable to or greater than the number of phages found in most of the aquatic systems. Even though the role of bacteriophages in changing bacterial communities in marine environments is well understood, the role they play in maintaining any bacterial population in activated sludge systems is not properly understood. Several research attempts (Ewert and Paynter, 1980; Havelaar et al., 1991; Hantula et al., 1991; Hertwig et al., 1999; Khan et al., 2002a,b; Thomas et al., 2002;

Farahbakhsh and Smith, 2004) have been made on the diversity and physiological identity of phages in activated sludge processes, but these studies limited their scope to the hetrotrophic population.

Two categories of bacteriophages are common: virulent and temperate. Virulent phages infect and kill the host and replicate often 100s of progeny (daughter phages) in a short time, called “lytic infection”. On the other hand, temperate phages can recombine with host cell DNA during “lysogenic infection”, forming a dormant prophage (phage DNA integrated into the bacterial genome) where viral genes that are detrimental to bacterial cells are not expressed (Casjens, 2003) and are incorporated into the bacterial genome. Temperate phages can provide necessary virulence and fitness factors affecting cell metabolism, bacterial adhesion, colonization, immunity, destruction of competing bacteria, antibiotic resistance and serum resistance (Wagner and Waldor, 2002). It is speculated that more than 80% of bacterial strains contain prophages and phage DNA can contribute to as much as 10-20% of a bacterium’s genome (Canchaya et al., 2003).

Ammonia nitrogen (NH_3-N) removal in wastewater treatment plants is accomplished through biological oxidation of ammonia by ammonia oxidizing bacteria (AOBs). AOBs are chemolithoautotrophic organisms which catalyze the biochemical oxidation of NH_3-N to nitrite-nitrogen (NO_2-N).

AOBs are extremely slow growers and their growth is sensitive to many environmental factors such as pH, dissolved oxygen, heavy metals and toxic chemicals (Shammas, 1986; Blum and Speece, 1991; Dangcong et al., 2000). Effective ammonia oxidation requires a high solid retention time (SRT) in order to ensure a healthy population of AOBs in the system and to prevent biomass washout (Rittmann and

McCarthy, 2001). Incomplete ammonia oxidation has been attributed to low specific activities of nitrifying bacteria as a result of toxic inhibition by chemicals or ammonia concentration in the influent wastewater (Burgess et al., 2002). The failure of ammonia oxidation has also been attributed to a low population or the absence of AOBs (Wanner et al., 2004). One of the possibilities often overlooked about the poor ammonia oxidation in treatment plants is the phage infection to AOBs, either by lysogenic or lytic viral attack.

Lysogeny has been extensively studied in heterotrophic bacteria but not for ammonia oxidizing communities. Recently, prophage-bacterial interactions have received tremendous attention due to the significant role that they play in the microbial ecology of various bio-systems. Researchers have also tried to study the effect of environmental factors on phage induction in natural bacterial populations (McDaniel and Paul, 2005). Environmental conditions affect the switch between the lytic and lysogenic life styles of the well-studied temperate Escherichia coliphage (Echols, 1972; Herskowitz and Hagen, 1980). It has been shown for bacterial communities that environmental pollutants can be more efficient inducing agents than a universal inducer such as mitomycin-C (Cochran et al., 1998).

Due to rapid industrialization, municipal wastewater treatment plants frequently receive heavy metals and other toxic substances. Negative effects of pH shift from the suggested optimum values, presence of heavy metals and substances causing toxicity on the performance of ammonia oxidizing communities are well established (Park and Ely, 2008; You et al., 2009). Lysogeny and induction of prophages into the lytic cycle in lysogenic bacteria are common in the environment (Ackerman and Dubow, 1987). Lysogeny due to the induction of prophages can be caused by several environmental

factors. Lysogeny can adversely affect the bacterial population and, in turn, the process efficiency. However, the significance of lysogeny in ammonia oxidizing bacteria due to pH shift, presence of heavy metals and toxicity has not been studied.

There has been very little research to date on lysogeny in autotrophic AOBs. In this research, we studied the effect of selected physical and chemical factors on the lysogeny of AOBs with *Nitrosospira multiformis* (ATCC # 25196) as the model AOB. Several studies have shown that genus *Nitrosospira* is the dominant terrestrial population among the three AOB genera: *Nitrosomonas, Nitrosospira* and *Nitrosococcus* (Avrahami and Bohannan, 2007). The strain *N. multiformis* ATCC 25196 has been extensively studied and its genomes have been completely sequenced (Norton et al., 2008). From the genomic data, the strain was established to contain prophage. Taking into consideration the importance of the strain and the pre-established presence of prophage in the *N. multiformis* genome, this AOB was suitable for studying phage induction and its effect on ammonia oxidation under the applied environmental stresses. The performance of *N. multiformis* for ammonia oxidation and phage induction (viral abundance) under the effect of stresses caused due to changes in pH, the presence of heavy metals and toxic substances was investigated in this research and is reported here. To the best of our knowledge, no such study has been conducted in the past which investigated the AOB performance affected by phage induction due to environmental stresses. The present study was an endeavor in this direction to understand the influence of various stress factors on phage induction in a model AOB, *N. multiformis* ATCC 25196.

3.2 Materials and methods

3.2.1 Bacterial strain, culture conditions and experimental setup

N. multiformis ATCC 25196T was grown and maintained lithoautotrophically in ATCC medium 929 at 28 ºC in the dark as described previously (Norton et al., 2002). ATCC medium 929 contained NH_3-N. Hence, the batch reactors were not supplemented with NH_3-N. The NH_3-N concentration in the bulk liquid during all batch tests was 351 ±19 mg/L. Cultures grown for 48 h (4e+10 cells/mL) were used for all experiments. The experimental setup used for stress experiments is depicted in Fig. 3.1. Batch reactors containing 200 mL of *N. multiformis* culture and supplied with a regulated amount of filter sterilized air were agitated using a magnetic stirrer. pH was maintained using sterile 1 N HCl and 1 N NaOH solutions. Samples were aseptically collected at 0 and 5 h for monitoring the performance of the batch reactor in terms of ammonia oxidation and prophage induction. Collected samples were immediately processed for subsequent analysis for NH_3-N, NO_2-N, viral and bacterial enumeration. AOBs are shown to be very sensitive to pH changes, the presence of heavy metals and toxic substances (Stasinakis et al., 2003; You et al., 2009). Hence, three stress factors viz., pH, Cr (VI) as heavy metal and potassium cyanide (KCN) as toxic substance were considered in the study. Furthermore, since temperature and the presence of organics (represented by COD) can also affect the performance of AOBs, these factors were also considered in this study.

The pH values of 5, 7 and 8 were considered to evaluate the effect of pH on phage induction. Since Cr and KCN were toxic, subvalues of the corresponding minimum inhibitory concentration (MIC) reported in the literature were considered.

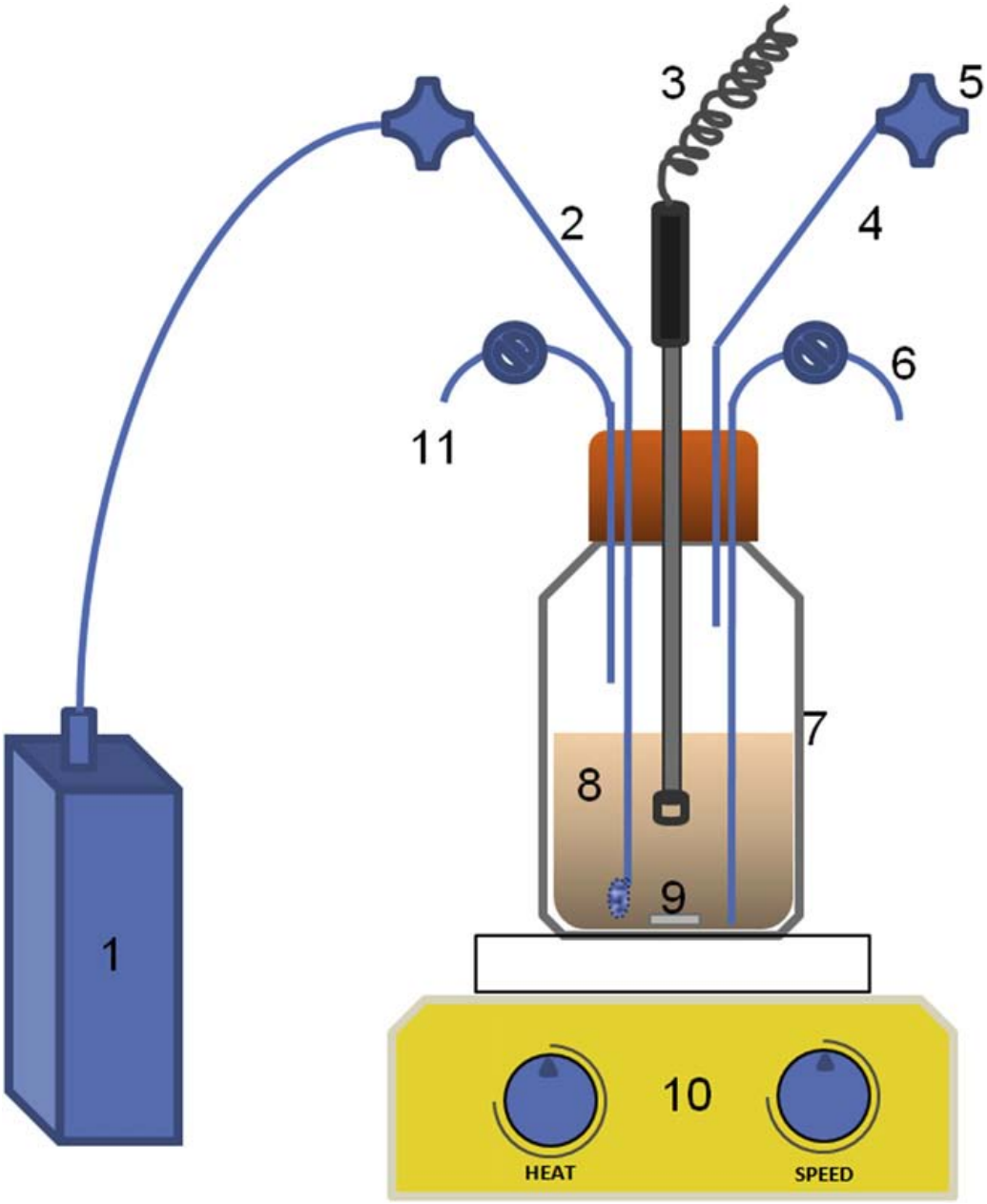

Fig. 3.1 - Schematic of batch reactor used for stress tests and ammonia oxidation by *N. multiformis*. In the schematic, (1) aquarium pump to supply air, (2) pH probe, (3) cable to the pH controller, (4) pressure release tube, (5) 0.22 micron filter, (6) acid or base tube, (7) glass bottle, (8) growth media, (9) magnetic stir bar, (10), magnetic stir plate, (11) feed tube.

To study the effect of fluctuations in chemical oxygen demand (COD) on prophage induction in the model AOB, glucose concentrations corresponding to COD values of 100-400 mg/L were supplemented to the *N. multiformis* liquid cultures. Similarly, an experimental temperature range of 4 ºC - 45 ºC was considered to study the effect of temperature fluctuations on bacteriophages induction in *N. multiformis*.

3.2.2 Mitomycin-C induction and phage extraction

To induce prophage from *N. multiformis*, 15 mL of 1 mg/mL mitomycin was added to 15 mL of fresh culture of *N. multiformis*. The mixture was incubated overnight on a gyratory shaker. Afterwards, the mixture was processed for phage extraction and enumeration. The sample was filtered through 0.22 mm pore size filter paper (Millipore, Co., Bedford, MA) to remove the bacterial debris. The filtered sample was transferred to a fresh and pre-autoclaved glass tube containing 100 mL of the reaction buffer and 2 mL of RNase free DNase I (Invitrogen, CA). After DNase treatment, samples were incubated at room temperature for 20 min and 35 mL of 0.5 M EDTA solution was added thereafter to stop the DNase activity.

3.2.3 Epifluorescent microscopy (EFM) and viral enumeration

Aliquots of DNase treated sample (100 mL) were suspended in 900 mL of sterile deionized water and were vacuum filtered through a stack of 25 mm filters consisting of 0.02 μm Anodisc (Whatman Int'l Ltd., Maidstone, England), a 0.22 μm Durapore membrane filter (Millipore, Ireland), and a glass fiber prefilter (Millipore, Ireland). Anodisc containing captured virus-like particles (VLPs) were stained by adding 10 ×

SYBR Gold dye (Invitrogen Co.). Anodisc was incubated for 20 min in the dark and analyzed by epifluorescence microscope (EFM) with BX 51 microscopy (Olympus, Japan) using a Cy3 filter. Pictures were digitally captured at a magnification of 1000X with a DPI-71 camera. VLPs were enumerated manually from the micrographs using a glass slide with grid.

3.2.4 Transmission electron microscopy

Mitomycin-C induced 500 mL culture of *N. multiformis* was centrifuged at 4500 × g for 20 min to pallet down the cell debris. The supernatant containing the induced prophages was further centrifuged at 8300 × g overnight at 4 ºC. The phage particles were purified by isopycnic centrifugation at 115,000 × g for 3 h through cesium chloride gradient. 5 mL of the purified phage extract was loaded on 400 grid formvar coated copper grids (Fisher Scientific) and allowed to settle for 1 min. Excess liquid was soaked by holding bibulous paper (Fisherbrand) at 90º to the grid. The grids were stained for 1 min using 2 mL freshly prepared and filtered 1% uranyl acetate and excess stain was soaked by holding bibulous paper at 90º to the grid. Dried grids were subsequently examined under Tecnai T12 Transition Electron Microscope (FEI, Japan). The accelerating voltage used for imaging was 80 kV and images of negatively stained phage particles were recorded.

3.2.5 Bacterial enumeration using live/dead assay

The samples (1 mL) for bacterial enumeration were filtered through 0.22 μm (PCTE black, GE Water & Process Technologies) using a vacuum manifold. The bacteria

captured on the membrane filters were stained with Baclight^{TM} bacterial viability kit (Molecular Probes Inc.), incubated in the dark for 20 min and analyzed under BX 51 microscope (Olympus, Japan) using Cy3 and FITC filters to capture live and dead cells, respectively. At each parameter tested, multiple pictures were captured and each picture was divided into four compartments using a grid system. The number of live and dead cells in each compartment was manually counted and was averaged. The percentage increase or decrease in the number of cells was calculated based on the average values.

3.2.6 Other analytical methods

Ammonia (NH_3) concentration was analyzed spectrophotometerically using HACH reagent kit (DR 5000, HACH), 10020 (Chromotropic Acid method).

3.3 Results and discussions

A blast search at the GenBank database using terminase gene revealed that *N. multiformis* contained prophage with greater than 25 kb sequence length. Terminase gene synthesizes terminase protein, which helps bacteriophages in DNA packaging (Casjens, 2008). Because of the presence of prophage element on the genome of *N. multiformis*, this AOB was considered for stress experiments as a model AOB. The performance of the bacteria for ammonia oxidation and phage induction under pH, temperature and COD changes, due to the presence of Cr (VI) and toxic potassium cyanide, was investigated. It is highlighted here that the purpose of this research work was not to evaluate the inhibitory effects of these selected stress factors on AOB kinetics. The effects of toxic substances, heavy metals and other operational factors have been thoroughly researched

in the past (Neufeld et al., 1986; You et al., 2009). This manuscript details one of the mechanisms (phage induction) that may be responsible for inhibitory effects of the studied stress factors. In other words, in this manuscript, we have tried to answer the question of "why AOB activity is inhibited in the presence of certain stress factors which have been shown to be important by the past researchers".

3.3.1 Prophage induction of *N. multiformis* using mitomycin-C

Recent studies have confirmed that *N. multiformis* (ATCC 25196T) has two prophages integrated within its chromosome (Norton et al., 2008). It is also an established fact that under the influence of environmental stress factors, prophages can be induced (coming out of the chromosome), causing cell lysis of the host organism. The process of induction releases phages in the surrounding environment. Mitomycin-C is an universal prophage-inducing agent used widely to induce prophages. Mitomycin-C is not naturally found in the wastewater treatment systems and therefore was a suitable surrogate for an environmental stress factor on *N. multiformis*.

Mitomycin-C has been employed in previous studies as a positive control for prophage induction (Cochran et al., 1998). Mitomycin-C was added to a batch containing freshly grown *N. multiformis* culture. The effect of mitomycin-C on *N. multiformis* with time is shown in Fig. 3.2. The bright big dots shown by yellow arrows in all micrographs are *multiformis* cells and the tiny small dots shown by red arrows are VLPs. An increase in viral population and a decrease in *N. multiformis* population were observed over the time (Fig. 3.2a-d). Lysis is clearly evident comparing the micrographs 2a (at 0 time) and 2d (after 24 h) in Fig. 3.2.

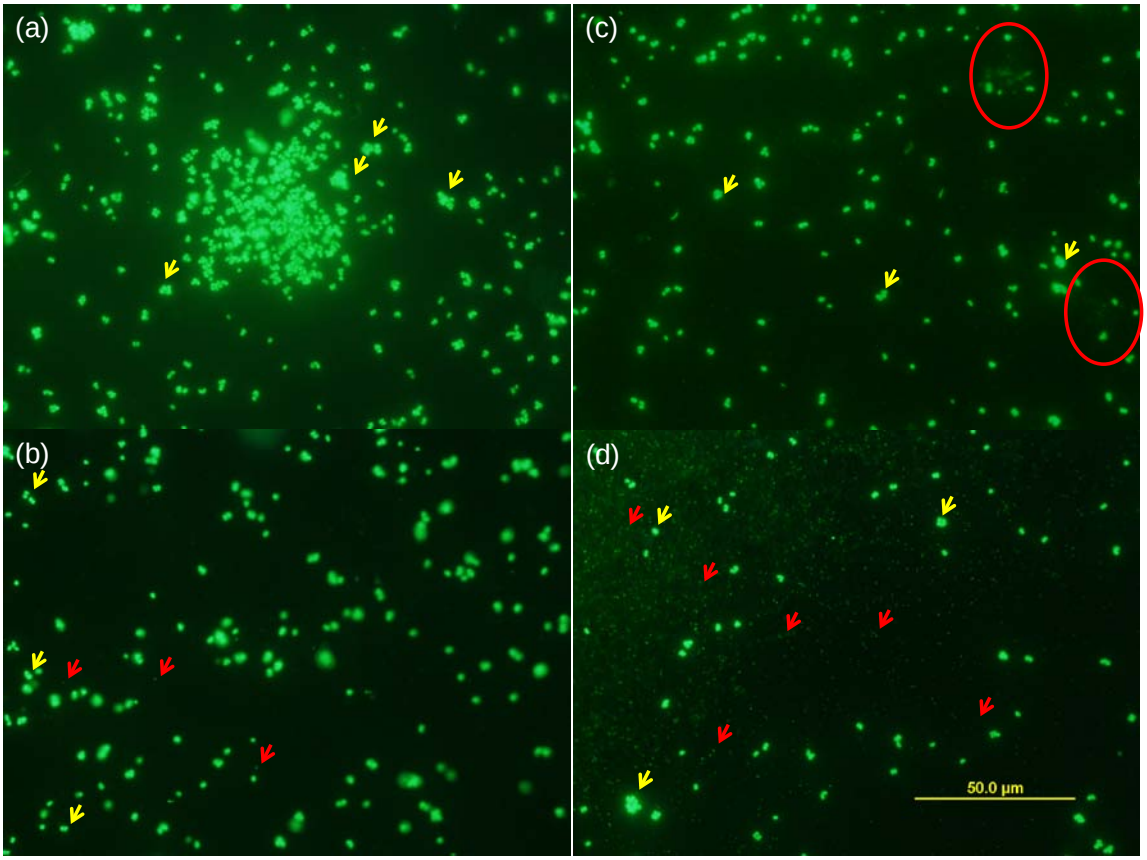

Fig. 3.2. Epifluorescent VLPs micrographs on *N. multiformis* induced by mitomycin-C. The micrographs show *N. multiformis* (yellow arrows) and VLPs (red arrows) in the mitomycin-C induced sample (a) at the beginning, (b) after 3 h, (c) after 12 h and (d) after 24 h. The red circles represent VLPs released after cell lysis due to prophage induction with mitomycin-C. The scale bar represents 50 µm.

Over 90% of the *N. multiformis* population was dead after 24 h following the induction with mitomycin-C. Transmission electron micrography (TEM) was performed on the induced phages and the TEM micrographs are shown in Fig. 3.3. The induced phages belong to the family podoviridae. Brightly stained capsids of diameter 70-85 nm without any tails were observed enclosing darkly stained DNA within them.

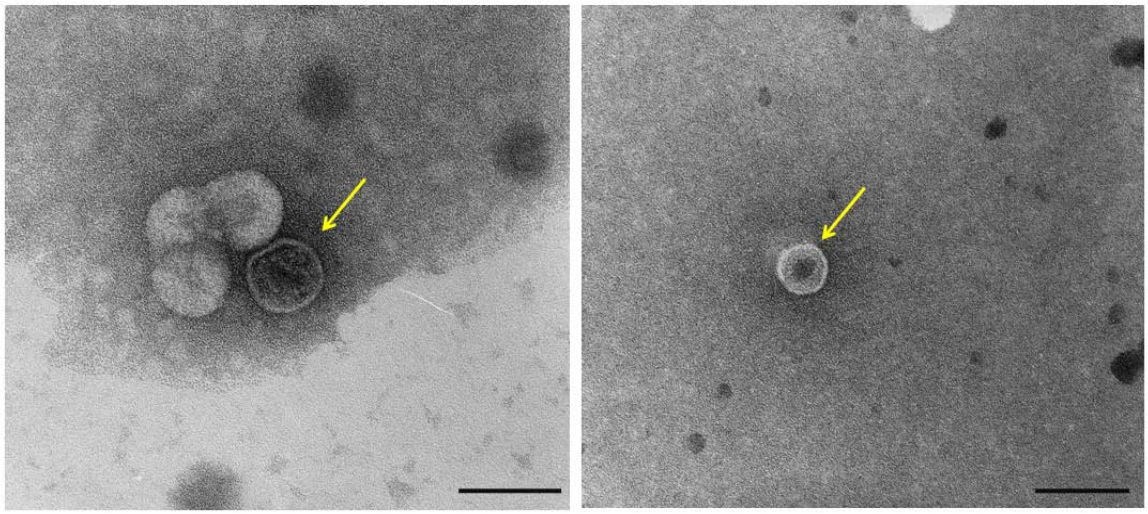

Fig. 3.3. Transmission electron micrograph. Yellow shows the morphology of bacteriophage from *N. multiformis* induced by mitomycin- C. Scale bar: 100 nm

3.3.2 Effect of pH

The optimum pH condition for the ammonia oxidation by *N. multiformis* ranges from 6.0 to 7.3 with an optimum value of 7 (Norton et al., 2002). Performance of *N. multiformis* for the ammonia oxidation and phage induction was tested at lower and higher pH values with respect to the neutral pH. *N. multiformis* cell viability, number of VLPs, ammonia disappearance and nitrite production were monitored and the data are shown in Fig. 3.4. The number of VLPs increased at pH 5 and 8, indicating prophage induction, and the induction was more prominent at pH 5. The number of VLPs increased by 2.3E+10 in 5 h as compared to the VLP number at 0 time when the pH was 5. Similarly, the VLPs increased by 9.67E+9 and 1.57E+10 in 5 h at pH 7 and 8, respectively.

Although the increase in VLPs at pH 5 and 8 was almost 2 and 1.5 fold more, respectively, than the number of VLPs at pH 7 (control), it was surprising to see phage

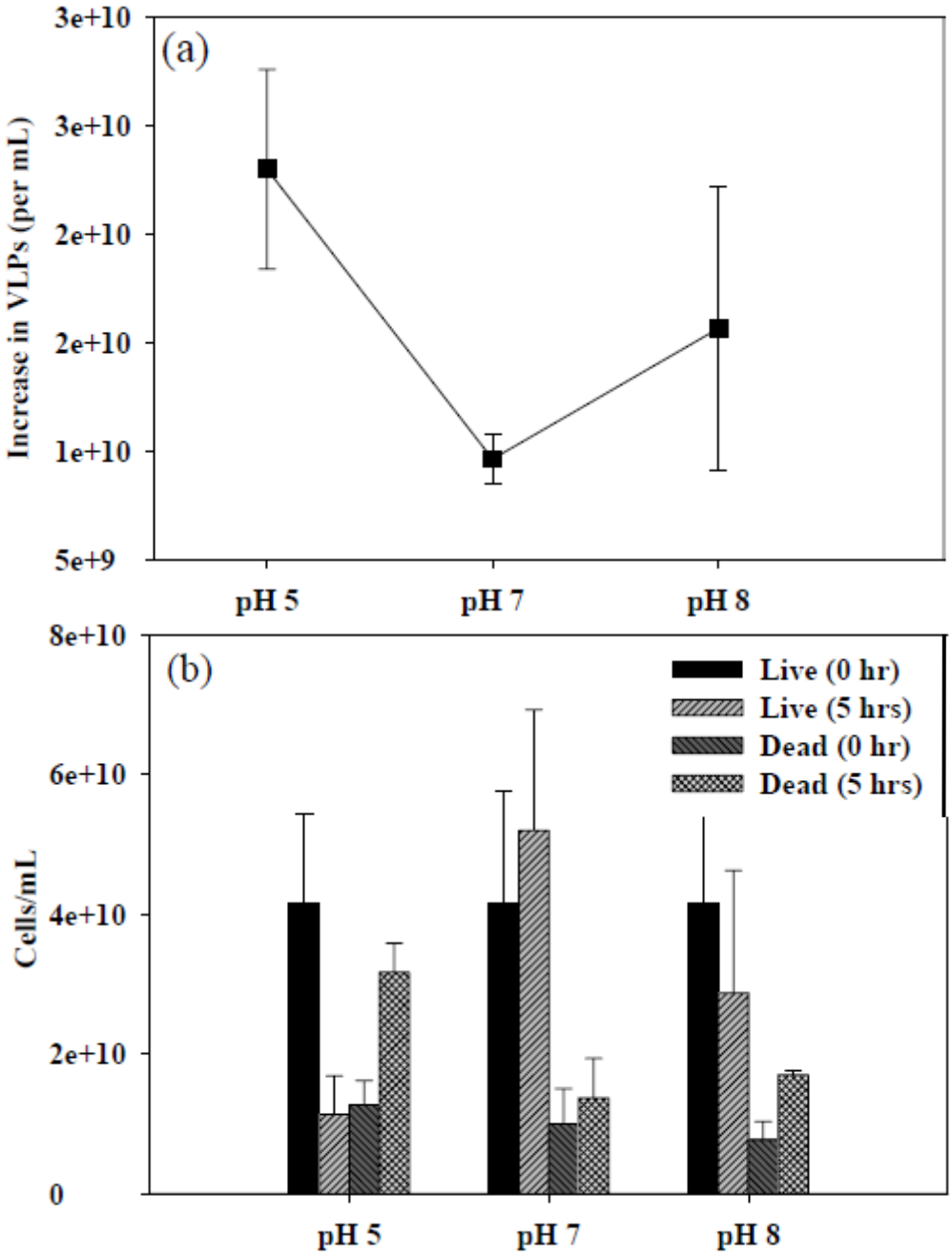

Fig. 3.4. Changes in (a) number of virus-like particles and (b) number of live and dead cells at different pH values.

induction at pH 7. VLPs were monitored at the beginning and at the end without any intermediate monitoring. It is also noticeable from Fig. 3.4b that dead cells were present in all batch reactors at different pH values at the beginning. Although the increase in the number of dead cells at pH 7 from time zero to time 5 h was 37% and considerably less than the corresponding numbers at pH values of 5 (150%) and 8 (121%), this small decrease must have contributed to the increase in VLPs in the batch at pH 7. Furthermore, a net increase of almost 29% in the number of live cells was recorded at pH 7 because of the growth of *N. multiformis*. On the other hand, 64% and 36% decreases in the number of live cells were recorded at pH values of 5 and 8, respectively. Assuming first order degradation, the rate of NH_3-N oxidation was 3.62 mgN $L^{-1}h^{-1}$ at pH 7. A considerable decrease in ammonia oxidation rates was observed at pH values of 5 and 8. The NH_3-N oxidation rates were 1.9 mgN $L^{-1}h^{-1}$ and 1.5 mgN $L^{-1}h^{-1}$ at pH values of 5 and 8, respectively. All batch experiments at different pHs were originally started from the same source. Hence, the number of live cells was nearly the same in all batches. The increase in VLPs and the decrease in viable *N. multiformis* cells at pH5 and 8 are concomitant with the corresponding decrease in ammonia oxidation rates at these pH values with respect to *N. multiformis* performance at pH 7.

The process of ammonia oxidation leads to a net acidification of the environment. Where ammonia deposition and nitrification rates are high, this may contribute to the lowering of the environmental pH (Beiderbeck et al., 1996; Kowalchuk and Stephen, 2001). Chemolithoautotrophic nitrifying bacteria like *N. multiformis*, which catalyze the first step of ammonia to nitrite, are known to be sensitive to low pH values. Bacterial growth of ammonia oxidizing bacteria (AOB) is usually limited at pH of 5.8 (Watson et

al., 1989) and their activity ceases typically below pH of 5.5 (Hayatsu and Kosuge, 1993) in liquid pure culture. Even though the failure of AOB to cope with acidic condition is thought to be caused by the unavailability of the substrate with decreasing pH values, lysogenic-lytic conversion of phage life cycle can be the reason for failure of AOB growth and ammonia oxidation, as evident by results from this research. High pH values above 8 are also shown to be detrimental for AOBs (Painter, 1986). Adverse effects of either low or higher pH than the optimal pH were observed in this research where more VLPs, slow ammonia oxidation by *N. multiformis* and greater number of dead cells were observed at pH values of 5 and 8. These results suggest that fluctuations in pH may act as a potential stress factor for AOBs, resulting in prophage induction. The results also project that prophage induction is more imminent in acidic pH than in basic pH.

3.3.3 Effect of Cr (VI) (heavy metal)

In the current experimental scenario, five different concentrations of Cr (VI) ranging from 0.002 mM to 1 mM were used to evaluate their effect on *N. multiformis* performance and its phage induction. The results for number of VLPs and cell viability are shown in Fig. 3.5. At Cr (VI) concentrations of 0.002 mM, 0.01mM and 0.1 mM, the number of VLPs increased by 5.00E+09, 6.33E+09 and 10.30E+09, respectively, as depicted in Fig. 3.5a. The corresponding NH_3-N oxidation rates were 2.7, 2.1 and 1.3 N $L^{-1}h^{-1}$ at Cr (VI) concentrations of 0.002, 0.01 and 0.1 mM, respectively. It can be seen that NH_3-N oxidation rates decreased with increasing concentrations of Cr (VI). These concentrations also caused significant decrease in live *N. multiformis* cells, as shown in Fig. 3.5b.

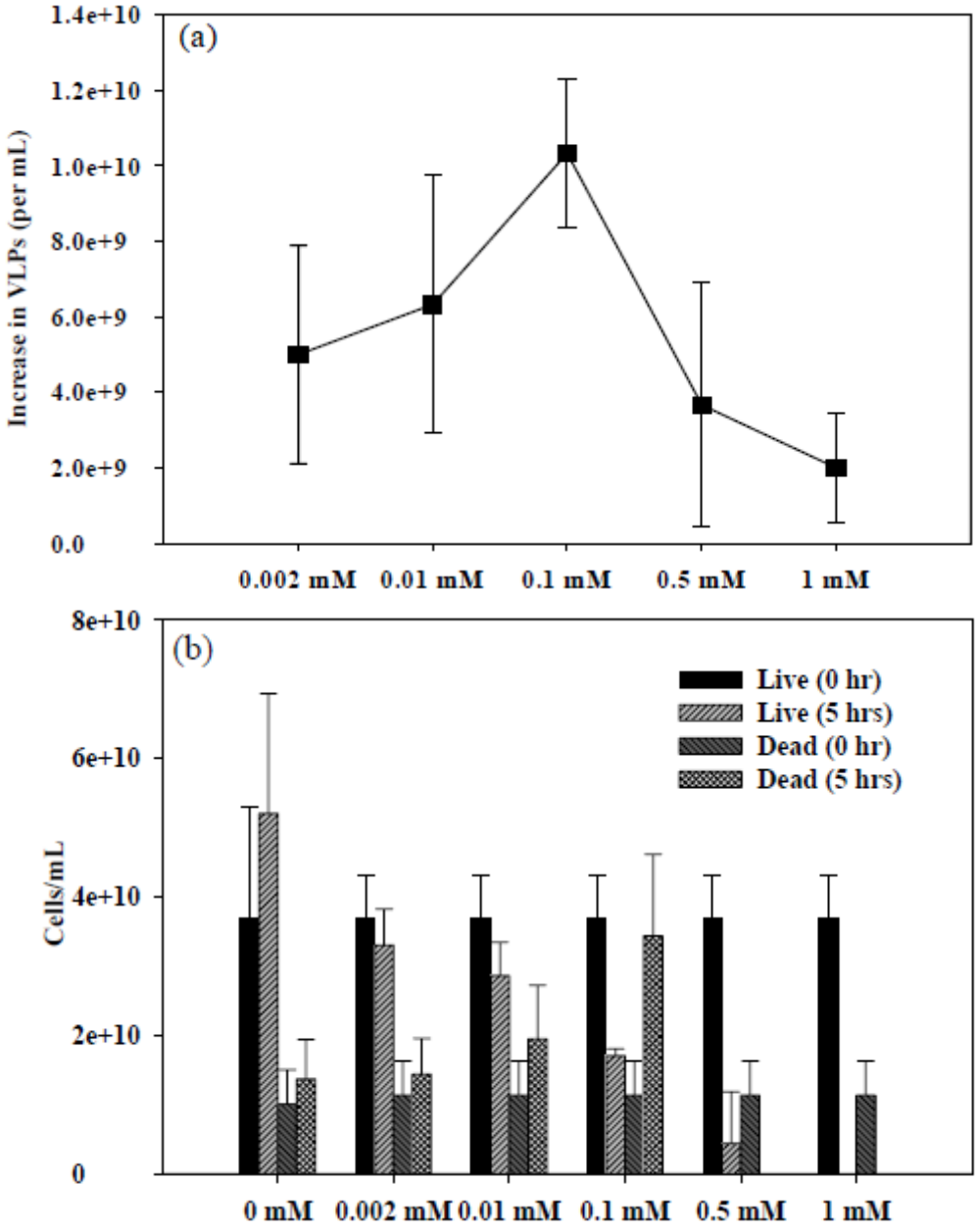

Fig. 3.5. Changes in (a) number of virus-like particles and (b) number of live and dead cells at different Cr (VI) concentrations.

At Cr (VI) concentrations of 0.002 mM, 0.01mM and 0.1 mM, decreases in the number of live cells were 11, 33 and 54%, respectively. The corresponding increases in the number of dead cells were 26, 73 and 203% at Cr (VI) concentrations of 0.002 mM, 0.01 mM and 0.1 mM, respectively.

The results suggest that the number of live cells decreased and the number of dead cells increased with the corresponding increase in Cr(IV) concentrations. These results are in agreement with those obtained by Stasinakis et al. (2003). These researchers demonstrated that nitrification efficiency decreased with increasing concentration of Cr (VI) in laboratory-scale reactors containing heterotrophs and ammonia oxidizers. Increase in the number of induced prophages (in terms of VLPs) also suggests that heavy metals can trigger prophage induction in AOBs and, in general, all bacteria, which, in turn, may initiate the lysogenic cycle of host-phage resulting in death of bacteria.

At higher concentrations of Cr (VI) beyond 0.1 mM, the increase in the number of VLPs dropped with a significant decrease in the number of live cells. This contradicts the fact that a decrease in live cells should cause a corresponding increase in the number of VLPs as it was seen at other lower concentrations of Cr (IV). This could be explained in light of the mechanisms of cell lysis caused at higher concentrations of Cr (VI). There are two primary mechanisms for cell lysis: (1) lysis due to the direct toxic effect of Cr (VI) on the cell wall where this heavy metal binds onto functional groups of proteins and inactivates them, rapidly bringing metabolism to a standstill (Talaro, 2005) and, (2) lysis due to the induction of intracellular prophage because of the stress generated by the presence of heavy metal. At concentrations above 0.1 mM, the Cr(VI) was too toxic for the cells, resulting in leakage of cell contents, and cell lysis was dominated by the first

mechanism. However, at lower concentrations of Cr (VI), cell lysis was primarily caused by induction of prophages and, as a result, an increase in the number of VLPs was observed at lower Cr (VI) concentrations. At 1.0 mM, complete inhibition of *N. multiformis* metabolism and consequently no ammonia oxidation were observed.

3.3.4 Effect of KCN (toxicity effect)

The results of exposure of *N. multiformis* to various concentrations of potassium cyanide, a known toxic substance to the bacterial cell, are shown in Fig. 3.6. Four different concentrations ranging from 0.015 µM to 0.3 µM were used. Exposure to various concentrations of KCN also resulted in the induction of prophage in *N. multiformis*. A concentration of 0.15 µM resulted in the highest induction of prophages with nearly a 2-fold increase when compared with results using 0.015 µM KCN (Fig. 3.6a). At 0.3 µM concentration, both live and dead bacterial populations dropped to zero after 5 h exposure and a low number of VLPs were recorded (Fig. 3.6a). Consequently, the rates of ammonia oxidation were affected as KCN concentrations increased and were almost completely inhibited at 0.3 µM KCN concentration.

3.3.5 Effect of COD loading and temperature

3.3.5.1 Effect of COD

Under the influence of COD fluctuations, there was no increase in the number of VLPs observed, suggesting no prophage induction. Furthermore, ammonia oxidation rates showed 2.4 mg $NL^{-1}hr^{-1}$ and 3.6 mg $NL^{-1}hr^{-1}$ at COD concentrations of 300 mg/L and 400 mg/L, respectively, implying no negative effect of high COD concentrations on AOBs.

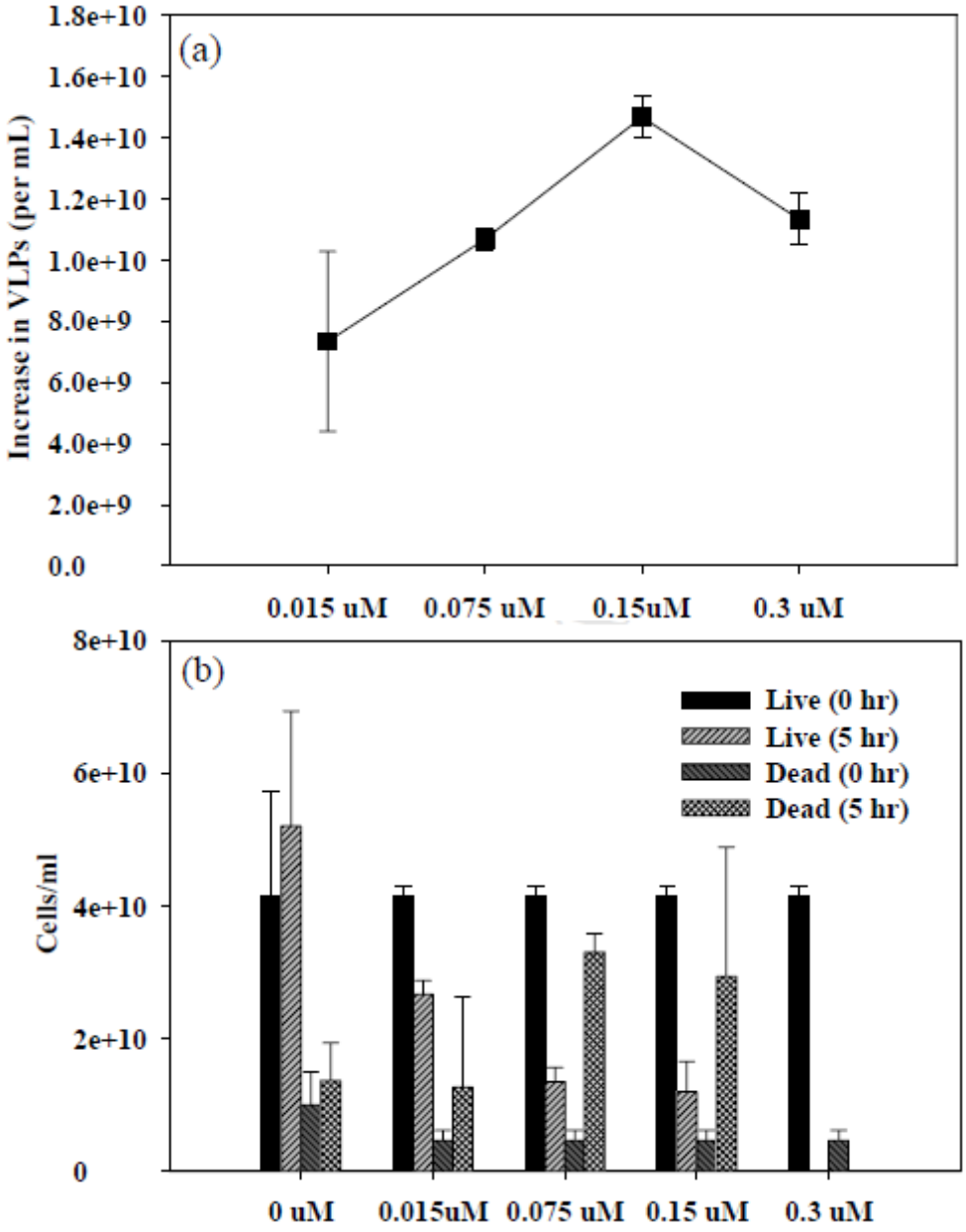

Fig. 3.6. Changes in (a) number of virus-like particles and (b) number of live and dead cells at different cyanide concentrations.

However, in actual wastewater treatment plants, fluctuations in COD are known to effect AOB population and nitrification in general. It may therefore be speculated that higher biodegradable organic COD directly promote growth of heterotrophic bacterial populations but, doesn't directly influence AOBs. The subsequent increase in heterotrophic population may indirectly influence AOB population by competing for space, oxygen and other nutrients (Rittmann and McCarty, 2001).

3.3.5.2 Effect of temperature

The growth of *N. multiformis* was inhibited at 4 ºC. However, there was no significant change in VLPs, suggesting that no prophage induction or lysogeny occurred at this low temperature. However, at 35 ºC significant decrease in live *N. multiformis* cells and significant increase in VLPs was observed. The total number of *N. multiformis* cells decreased by 2.30E+10 when the temperature was raised from 4 ºC to 35 ºC. The corresponding increase in number of VLPs was 6.50E+10. At much high temperature of 45 ºC, the growth of bacterial cells was inhibited by heat shock or heat inactivation of cellular enzymes and proteins. As a result, although decrease in the number of live *N. multiformis* cells was recorded at 45ºC, increase in the number of VLPs was minimal. This suggests that the death of *N. multiformis* cells at 45 ºC was not due to lysogeny as a result of prophage induction. Even though, there is evidence that high temperature (42º C) treatment of mutants lysogenic bacteria leads to prophage induction and release of bacteriophage (Schuster et al., 1972), it was more likely that there is no prophage induction at 45 ºC occurred in the present study as no significant increase in VLPs was

detected. Ammonia oxidation did not occur at 4 ºC and 45 ºC as it was apparent from a no increase in nitrite-nitrogen.

3.4 Summary

The overall effect, as observed from the experiments was that prophage was induced under all tested environmental factors from *N. multiformis* indicating lysogeny could be an important pathway for the occasional deterioration in ammonia oxidation efficiency in full scale treatment plants. *N. multiformis* cells responded to stress caused by pH changes, heavy metal and toxic substance through prophage infection. More VLPs were recorded under acidic pH conditions than under basic pH values when compared to the VLPs number at the neutral pH. During the process of ammonia oxidation to nitrite, alkalinity in form of carbonated or bicarbonates is consumed which causes the system pH to go down and the ammonia oxidation to slow down. Evidence that low pH induces prophage in ammonia oxidizers is novel.

Although the effect of Cr (VI) and cyanide (Neufeld et al., 1986; Kelly et al., 2004; Vazquez et al., 2006) has been fully studied on the process of nitrification, their effect on phage induction has not been evaluated. Both Cr (VI) and KCN caused significant induction in *N. multiformis* even at concentrations recommended as minimum inhibitory values. These results suggest that these toxic compounds can not only inhibit the activity of AOBs through their toxic effect but also put some sort of environmental stress that ultimately leads to prophage (if present) induction in these organisms. Higher concentrations of Cr (VI) and KCN completely inhibited ammonia oxidation by *N. multiformis*. Kim et al. (2008) observed significant reduction in nitrification efficiency by

free cyanide in an anoxic-aerobic activated sludge system. Kim et al. (2008) also demonstrated that ammonia nitrogen as high as 350 mg/L was not inhibitory for ammonia oxidizers. Hence, there was no possibility of phage induction due to inhibitory effect of ammonia.

It was interesting to note that, among the factors tested pH has stronger inducing ability than Cr (VI) and KCN. This can be further advocated by the fact that disturbance in performance of nitrification in wastewater treatment plants due to the fluctuations in pH is more common than attributed for presence of heavy metals like Cr (VI) or chemicals like KCN. Nevertheless, further studies are essential to establish the precise mechanism behind induction of prophages under the influence of stress factors. Also it is necessary to investigate how the released phages can affect other microbial communities in wastewater systems.

CHAPTER 4

BIOCONTROL OF BIOMASS BULKING CAUSED BY HALISCOMENOBACTER HYDROSSIS USING A NEWLY ISOLATED LYTIC BACTERIOPHAGE (H2-TASK 4, 5 AND 6)

4. 1 Introduction

Bacteriophages are viruses that infect bacteria and are known to be very important components of freshwater and marine water ecosystems. Phages have the ability to drive and control bacterial community structure (Breitbart and Rohwer, 2005). Metagenomic analyses have shown that viral communities in the environment are incredibly diverse (Weinbauer, 2004). There are an estimated 5000 viral genotypes in 200 L of seawater and possibly a million different viral genotypes in 1 kg of marine sediment (Breitbart and Rohwer, 2005). An abundance and diversity of phages have been reported in natural, marine ecosystems, oceanic ice, sediments, soil (Wommack and Colwell, 2000; Weinbauer, 2004) and also in engineered systems such as drinking water distribution systems, wastewaters and activated sludge bioreactors (Weinbauer, 2004; Otawa et al.,

2007; Wu and Liu, 2009). The published information on bacteriophages in aforementioned natural and engineered systems provides sufficient evidence that bacteriophages play a pivotal role in all nutrient cycles and engineered bioprocesses.

The activated sludge process is one of the widely used methods for municipal wastewater treatment. The performance of the activated sludge process is driven by a complex community of prokaryotes. The community composition in activated sludge systems is as complex and as diverse as that in many other aquatic systems, including the oceans. Activated sludge systems have been shown to contain 10^8-10^9 phages per ml (Ewert and Paynter, 1980; Otawa et al., 2007), a number comparable to or greater than the number of phages found in most of the aquatic systems. It would not be an overstatement to say that the "viruses rule the world". However, phage-mediated changes in the bacterial community in activated sludge systems and the effect of phages on the process performance have not been investigated hitherto. Albeit, the activated sludge process is an established treatment technique that is being employed worldwide; frequent process upsets, biomass bulking and foaming are a few undisputed problems that still haunt these processes.

Biomass bulking in activated sludge processes is caused due to the over-development of filamentous bacteria (Eikelboom, 1977; Eikelboom and van Buijsen, 1981; Ziegler et al., 1990) and is one of the main operational problems in activated sludge systems. From wastewater treatment plants, several filamentous bacteria have been identified, isolated and found responsible for biomass bulking viz., *Microthrix parvicella*, *Sphaerotilus natans*, Eikelboom type 1702, *Haliscomenobacter hydrossis*, *Nocardia*, *Thiothrix* spp. etc. (van Veen et al., 1973; Eikelboom, 1975; Williams and Unz, 1985; Kampfer, 1995;

da Motta et al., 2003). Biomass bulking causes poor settling in the secondary clarifier and allows the unsettled biomass to escape with the effluent. Engineering manipulations are conventionally employed to solve the problem of biomass bulking and these manipulations are primarily based on the past engineering observations without sufficient microbiological insight. Thus, a cause and an effect relationship between the specific microorganisms and their role in filamentous bulking is unclear. In efforts towards controlling the biomass bulking, physico-chemical methods like manipulation of flow rates of the return activated sludge, increased aeration, the addition of flocculants/coagulants and oxidants have been tried in the past (Xie et al., 2007). Surfactants and chlorine are also used to control the filamentous bulking in different attempts (Se´ka et al., 2003; Caravelli et al., 2007; Xie et al., 2007). For various reasons, most of the above-mentioned attempts at mitigating filamentous bulking are not acknowledged as sustainable and/or cost-effective. For example, the addition of chlorine may trigger the formation of halogenated organics, which could pose potential threat to the receiving waters. Lou and de los Reyes (2008) proposed a conceptual framework where they attribute kinetics of substrate (nutrients) concentration and substrate diffusion rates as primary factors driving the growth of filamentous bacteria. An engineering parameter often used to distinguish between good and bad biomass settling is sludge volume index (SVI). SVI value of between 50 ml/g and 100 ml/g is considered to be good, that between 100 ml/g and 150 ml/g is filamentous growth and above 150 ml/g is bulking (Lee et al., 1983).

It is an established concept that bacteriophages can infect a single host or multiple hosts (Jensen et al., 1998; Weinbauer, 2004). Based on the host specificity of

bacteriophages, phage therapy has been used in medical applications and in meat industry where the objectives have been to infect the target bacteria to cure the disease and to disinfect the meat, respectively (Withey et al., 2005; Kropinski, 2006). Likewise, biomass bulking is most often caused due to the overgrowth of filamentous bacteria (called filaments) and therefore could be controlled using phage therapy or biocontrol using phages to reduce the filamentous bacterial populations. There are no published reports on the application of phage-based biocontrol to regulate the population of filamentous bacteria and to improve the biomass settleability. Furthermore, the information on the types of phages and their genetic diversity in activated sludge systems is scarce (Breitbart and Rohwer, 2005). Nevertheless, there is evidence that lytic phages infecting these filamentous bacteria exist in nature (Thompson, 1979; Kampfer, 1995; Jensen et al., 1998; Winston and Wommack and Colwell, 2000).

Withey et al. (2005) provided an excellent review on the possibility of using bacteriophages to mitigate several problems related to the bacterial ecology in activated sludge systems. With this driving force, we approached the idea of using bacteriophage-based biocontrol of biomass bulking. *H. hydrossis* is a sheathed filamentous bacterium that has been detected worldwide in activated sludge samples because of its easily recognizable morphological appearance: rigid straight filament, length between 10 and 200 mm, diameter between 0.3 and 0.5 mm extending from the floc surface (thin needle shape appearance) and gram negative staining (Eikelboom, 2006). This study is limited to *H. hydrossis* as the model filamentous bacterium and bulking caused by this bacterium. The objectives of this study were to (i) isolate, purify and characterize a lytic phage specifically infecting *H. hydrossis*, a model filamentous bacterium; (ii) demonstrate the

application of biocontrol using the isolated phage in laboratory-scale set-ups; and (iii) investigate whether the addition of the newly isolated phage will have any effect on the activated sludge process performance for organic and nutrient removals. After the proof of concept of the biocontrol of the bulking caused by *H. hydrossis*, the application will be transformative to be applied to several other filamentous bacteria as well as to foam forming bacteria that are also found predominantly in activated sludge systems. Furthermore, since phages are host-specific and are not pathogenic to higher organisms (Weinbauer, 2004; Withey et al., 2005), use of phages to biocontrol the population of filamentous bacteria and other unwanted organisms does not pose any health-related threats.

4.2 Materials and methods

4.2.1 Bacterial strains

H. hydrossis (ATCC # 27776) was obtained from the American Type Culture Collection (ATCC) and was grown as per ATCC's instructions in 733 SCY medium (Tripticase soy broth without dextrose 0.25 g, casitone 0.75 g in 1 L deionized water) at 37 ºC. *Escherichia coli* K12, *Pseudomonas aeruginosa*, *Nitrosospira multiformis* (ATCC # 25196), *Nitrosomonas europea* (ATCC # 19718) and *Desulfovibrio desulfuricans* ND132 were used for cross infectivity studies.

4.2.2 Isolation of bacteriophage with *H. hydrossis* as the host

Biomass sample (called the mixed liquor) from a full-scale wastewater treatment plant was used as a source of virulent phages. Mixed liquor sample was sequentially filtered

through 0.45 mm and 0.2 mm filters (Millipore, CA) to remove the bacteria and other suspended impurities. The filtrate, which mostly contained dissolved substances and phages, was collected in a pre-autoclaved glass flask. The filtrate was concentrated for phages using Amicon Ultra-4 (Millipore, CA) Uitracel-30k with MWCO 30,000. The concentrated phage extract was stored at 4º C until further use. To isolate the lytic phage specific to the filamentous bacterium, an established top-agar plating technique was used. The model filamentous bacterium, *H. hydrossis*, was grown overnight and 10 mL of this overnight grown culture was mixed with 1 mL of the concentrated phage extract and 3 mL of 0.75% SCY agar (SCY medium þ 0.75% w/v bacto-agar). This mixture was vortexed thoroughly and overlaid on a premade 2% SCY base agar plate. The top layer of 0.75% agar does not solidify as quickly as the base 2% agar and therefore, it would allow uniform distribution of host bacterium and phages within the agar. This results in formation of isolated plaques following incubation. The plate was allowed to solidify and was incubated at 37 ºC overnight. A negative control plate was also prepared following the same protocol. The control plate contained everything mentioned above except the concentrated phage extract. One of the capillary stubs of the plaques that appeared on the plate following the incubation was carefully picked and resuspended in the SMG buffer (5.8 g/L NaCl, 2.0 g/ L $MgSO_4$-$7H_2O$, 5.0 mL/L of a 5% solution of gelatin, 50 mL/L of 1 M Tris-HCl pH 7.5). The plaque was further purified by repetitive infections on the fresh *H. hydrossis* plates. The purified isolated phage was stored at 4 ºC in the SMG buffer until further use.

4.2.3 Viral enumeration using epifluorescence microscopy (EFM)

From the bacteria-phage mixture, 1 mL triplicate samples were taken for the viral enumeration. The samples were first filtered through a 0.22 mm pore size filter (Millipore Co. Bedford, MA) to remove the dead unlysed bacteria and other suspended solids. The filtrate was collected in a fresh pre-autoclaved flask. In order to exclude the free bacterial DNA present in the phage extract, 900 mL of phage solution was transferred into a fresh polypropylene tube containing 100 mL of RQ1 reaction buffer and 2 mL of RNase free DNase I (Invitrogen). After the addition of DNase, samples were incubated at 37 ºC for 20 min and 35 mL of 0.5 M EDTA was added to stop the DNase activity. Aliquots of DNase treated sample (100 mL) were suspended in 900 mL of sterile deionized water and were vacuum filtered through a stack of 25 mm filters consisting of 0.02 µm Anodisc (Whatman Int'l Ltd., Maidstone, England), a 0.22 µm Durapore membrane filter (Millipore, Ireland) and a glass fiber prefilter (Millipore, Ireland). The anodisc containing captured virus-like particles was stained by adding 10× SYBR Gold dye (Invitrogen Co.). The anodisc was incubated for 20 min in the dark. The anodisc was viewed under an Olympus BX 51 epifluorescence microscope (Olympus, Japan) using a Cy3 filter. At least 10 fields were captured from each anodisc digitally at a magnification of ×1000 with an Olympus DP-71 camera. Virus-like particles (VLPs) were enumerated manually from the micrographs and the average VLPs were calculated for all micrographs. Uninfected *H. hydrossis* culture was considered as a control for the VLPs enumeration.

4.2.4 Live and dead bacterial enumeration

Live and dead bacterial cell analysis was done using a Baclight^{TM} bacterial viability kit (Molecular Probes Inc.). The samples (1 mL) for bacterial enumeration were filtered through 0.22 mm (PCTE black, GE Water & Process Technologies) using a vacuum manifold. The bacteria captured on the membrane filters were stained with a mixture of two dyes supplied with the Baclight^{TM} bacterial viability kit (Molecular Probes Inc.). The bacterial cells with the dye mixture were incubated in the dark for 20 min and were analyzed using a BX 51 microscope (Olympus, Japan) using Cy3 and a FITC filters to capture live and dead cells, respectively. At each parameter tested, multiple pictures were captured and each picture was divided into four compartments using a grid system. Number of live and dead cells in each compartment was manually counted and was averaged.

4.2.5 Preparation of lysate stock

The newly isolated bacteriophage (>10^8 plaque forming units (PFU)/mL) was mixed with 0.1 mL of the *H. hydrossis* suspension in 1:1 ratio and the mixture was incubated for 20 min at 37 ºC. To the incubated culture, 3 mL of the molten SCY agar (0.7% w/v) was added and the mixture was immediately poured into petri dishes containing solidified SCY agar (1.5% w/v). After the overnight incubation at 37 ºC, the plates were removed from the incubator and 5 ml SMG buffer was added to these plates. The plates were stored at 4 ºC for 12 h with intermittent gentle shaking. A Pasteur pipette was used to collect the overlying SMG buffer from the plate after the overnight incubation. The collected buffer was transferred into a fresh and pre-autoclaved polypropylene tube (13 ×

100 mm). Fresh SMG buffer (1 mL) was again added on the top of each incubated plate slowly. The plates were allowed to sit for 15 min. Thereafter, the overlying buffer was collected and was added to the previously collected buffer. Chloroform (0.1 mL) was added to the collected SMG buffer solution followed by gentle vortexing for 1 min. The tube containing the SMG buffer and the chloroform was centrifuged for 10 min at 4000×g and 4 ºC. The supernatant containing phages was carefully transferred to a fresh tube and stored at 4 ºC.

4.2.6 Phage titer

In order to estimate the phage titer for the isolated bacteriophage, 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} dilutions of the original phage extract in triplicate were prepared using SMG buffer. Subsequently, 200 mL of the fresh *H. hydrossis* culture (O.D.600 ≥1) was added to 10 mL of each set of the diluted phage extract. The mixture was vortexed thoroughly and incubated for 30 min at room temp. To each tube containing this mixture, 3.0 mL of 0.75% SCY agar (45 ºC) was added, vortexed thoroughly and poured onto premade 2% SCY base agar plates. Triplicate plates for each dilution were incubated overnight at 37 ºC for 16-24 h. Plaques formed on each plate were counted and the plaque numbers were averaged from three plates to calculate the phage titer. Phage titer was calculated by multiplying number of plaques with the corresponding dilution factor (Madigan et al., 2000).

4.2.7 One-step growth curve of bacteriophage

In order to construct the one-step growth curve of the isolated phage, 1 mL of freshly grown *H. hydrossis* culture (ca. 1×10^9) was taken into sterile polypropylene tubes (13 mm× 100 mm) in triplicate. Subsequently, 1×10^{10} plaque forming units (PFU) of the isolated phage were added to each tube and the tubes were incubated at 37 ºC for 1 min. The bacterial culture containing the isolated phage particles was immediately centrifuged at 3000 × g for 1 min at 4 ºC. The supernatant was discarded and the pallet was re-suspended in fresh SCY growth medium. The resuspended pallet was added to freshly grown 1 L cell suspension of *H. hydrossis* in log growth phase. The mixture was incubated at 37 ºC on a shaker and triplicate samples at every 10 min were taken from the mixture. Each subsample from the set of triplicate samples at each time step was divided into two equal halves, of which one half was used to calculate the total PFU (free phage plus any infectious intracellular phage particles) after adding the chloroform and the other half was used to calculate the free PFU (extracellular/nonadsorbed). The counts obtained from triplicate values were averaged and were plotted to obtain the one-step growth curve (Ellis and Delbrück, 1939). The burst size was calculated by dividing the number of virus-like particles released from the cell with the number of virus particles initially added.

4.2.8 Transmission electron microscopy (TEM)

Phages were allowed to grow for 12 h in 1 L fresh cultures of *H. hydrossis*. Chloroform (10 mL) was added to the culture and the mixture was kept on a gyratory shaker for 2 h. Once the cell lysis was visible, the mixture was centrifuged at 5500 × g for

20 min to pellet down the lysed bacteria. The supernatant containing the phage particles was carefully transferred to a fresh tube and was centrifuged at 8890 × g overnight at 4 ºC. The clear pellet was resuspended in the SMG buffer. The phage particles were further purified by isopycnic centrifugation at 115000 × g for 3 h in sucrose cesium chloride gradient. Subsequently, 5 mL of the purified phage was loaded on 400 grid formvar coated copper grids (Fisher Scientific) and was allowed to settle for 1 min. Excess liquid was soaked by holding bibulous paper (Fisher brand) at 90º to the grid. The grids were stained for 1 min using 2 mL freshly prepared filtered 1% uranyl acetate and excess stain was soaked by holding bibulous paper at 90º to the grid. Dried grids were subsequently examined under Tecnai T12 Transition Electron Microscope (FEI, Japan). The accelerating voltage used for imaging was 80 kV and images of negatively stained phage particles were recorded subsequently.

4.2.9 Phage-based biocontrol of biomass bulking caused by *H. hydrossis*

Fresh biomass without any settling problem was obtained from an ongoing laboratory-scale sequencing batch reactor. Subsequently, 50 mL of this sludge in triplicate was mixed with an equal volume of overnight grown culture of *H. hydrossis* in 250 mL in an Erlenmeyer flask. The resulting mixture was grown under limited dissolved oxygen (DO) and nutrient conditions for 48 h to acclimatize the spiked *H. hydrossis* with the biomass. Afterwards, the biomass was divided into two halves and one of those was spiked with the isolated phage extract at 1000:1 host to phage ratio and was further allowed to sit for 3 h. The second half without the phage addition was considered as the control. The samples were poured in separate measuring cylinders. For each case, sludge volume

index for 30 min settling (SVI_{30}), settling velocity and turbidity were determined at 0 and 3 h time intervals (Lee et al., 1983). Turbidity of the supernatant was measured using a turbidometer (2100N Turbidimeter, HACH, CO, USA) and reported in terms of nephalometric turbidity units (NTU). The supernatant was also examined qualitatively for microbial population by staining with 4,6-diamidino-2- phenylindole (DAPI) (Sigma) and observing under the 100× objective of the epifluorescent microscope (Olympus, Japan) using a DAPI filter. DAPI is a fluorescent stain (excitation maximum 358 nm/emission maximum 461 nm) that binds strongly to DNA. Samples in triplicate at each location were collected from the supernatant at different depths during settling tests using a Pasteur pipette and were examined using DAPI staining technique. During sample collection, care was taken so as not to disturb the settled biomass.

4.2.10 Effect of pure oxygen in phage-based biocontrol of biomass bulking

The effect of pure oxygen on phage-based biocontrol was evaluated using batch tests. Four batch tests were performed over a period of 12 h. The first batch contained the biomass with *H. hydrossis* and served as a control. The second batch contained biomass, *H. hydrossis* and phage. The third batch contained biomass, *H. hydrossis* and purged pure oxygen. The last batch contained the biomass, filamentous bacteria, the isolated lytic phage and purged pure oxygen. Air was purged in the first and second batches. Care was taken that all four batch tests contained same concentrations of biomass and had identical volumes. These tests were done in triplicate to eliminate manual errors. For each case, SVI_{30}, settling velocity and turbidity were determined at 0 h, 3 h and 12 h. Samples in

triplicate were taken at the beginning of the experiment (0h), middle (3 h) and at the end of 12 h and analyzed for COD and ammonia.

4.2.11 Stability of isolated phage, cross infectivity tests and its effect on biomass performance

Stability of the isolated phage was tested under the storage medium, temperature and pH changes. Storage media considered were water and SMG buffer over a storage period of 9 months. The temperature range of -20 ºC to 45 ºC and the pH range of 5-8 were considered for stability studies. Stability in terms of infectivity titer was determined for each parameter in triplicates. Cross infectivity tests were performed to evaluate the possibility of infection by the isolated phage to selected model bacteria found in activated sludge systems. For cross infectivity studies, bacterial strains that are relevant in the wastewater treatment were considered. Not all of the considered strains (viz., *Nitrosomonas*) could grow easily as lawn on agar plates, so they were tested for infectivity in liquid cultures. To conduct the cross infectivity tests, 100 mL of each bacterial culture was grown overnight in their respective nutrient media and subsequently spiked with 1 mL SMG buffer containing the isolated phage (resulting in a phage to host ratio of ~1:1000). The resulting mixture was incubated for 3-6 h at 37 ºC and was tested for the plaque formation using standard plating technique or for the presence of VLPs using the epiflourescence microscopy. The effect of the addition of the isolated phage on the biomass performance in terms of chemical oxygen demand (COD) and nutrient removal was evaluated using batch tests. Three batch tests were performed over a period of 12 h simultaneously. The first batch contained the biomass without any other addition

and served as a control. The second batch contained the biomass and *H. hydrossis*. The last batch contained the biomass, *H. hydrossis* and the isolated phage. Care was taken that all three batch tests contained the same concentrations of biomass, had identical final volume and were incubated under the same temperature and agitation. Samples for inorganic species estimation were taken at the beginning of the experiment and at the end of 12 h.

4.2.12 Cost analysis between phage-based biocontrol and chlorine addition

The cost analysis for the control of filamentous bacteria was performed between chlorine disinfection and phage-based biocontrol. Data for chlorine price and dose were obtained from Central Valley Water Reclamation Facility (CVWRF).

4.2.13 Other analytical methods

COD was quantified using HACH's low range COD method (Hach, Loveland, CO). Dissolved P, NH_3-N, NO_3-N and NO_2-N were quantified using HACH methods 8048 (Ascorbic Acid method), 10020 (Chromotropic Acid method), 10031 (Salicylate method) and 8153 (Ferrous Sulfate method), respectively.

4.3 Results and discussion

The purpose of this research was to demonstrate bacteriophage-mediated biocontrol of filamentous bulking using lytic bacteriophages. For this purpose, *H. hydrossis* was

chosen as the model filamentous bacterium and the biocontrol of biomass bulking caused by it was investigated.

4.3.1 Isolation and characterization of *H. hydrossis* phage

Lytic phage specific to *H. hydrossis* (referred to as HHY-phage onwards) was isolated from the mixed liquor sample from a full-scale wastewater treatment plant. The purification of the HHY-phage was performed using repeated plaque assay technique.

Fig. 4.1a shows an agar plate containing several plaques obtained after serial infection of *H. hydrossis*. From the figure, it is also evident that the plaques were distinct, clear and round shaped with 1-2 mm diameter. Distinct bacteriolysis was visible when *H. hydrossis* in suspension was infected with the HHY-phage. A significant number of virus-like particles (VLPs) were also observed when the phage extract was viewed under epifluorescent microscope (Fig. 4.1b). The titer of the HHY-phage with *H. hydrossis* was calculated to be $5.2 \pm 0.3 \times 10^5$ PFU/mL. Compared to the reported values (Synnott et al., 2009; Uchiyama et al., 2009; Verma et al., 2009), the HHY-phage was found to have lower titer, which may be attributed to the slow growth rate of *H. hydrossis*. Furthermore, no turbid or bull-eyed plaques, which are characteristics of lysogenic phages (Ellis, and Delbru¨ ck, 1939; Uchiyama et al., 2009), were observed (Fig. 4.1b).

TEM analysis (Fig. 4.2) revealed that the isolated phage belongs to the Myoviridae family with hexagonal head, collar, contractile tail, tail fibers and icosahedral symmetry. The HHY-phage was around 203 nm long and depicted an icosahedral head (diameter ~81 nm) and contractile tail (length ~126 ± 10 nm; diameter ~18 nm).

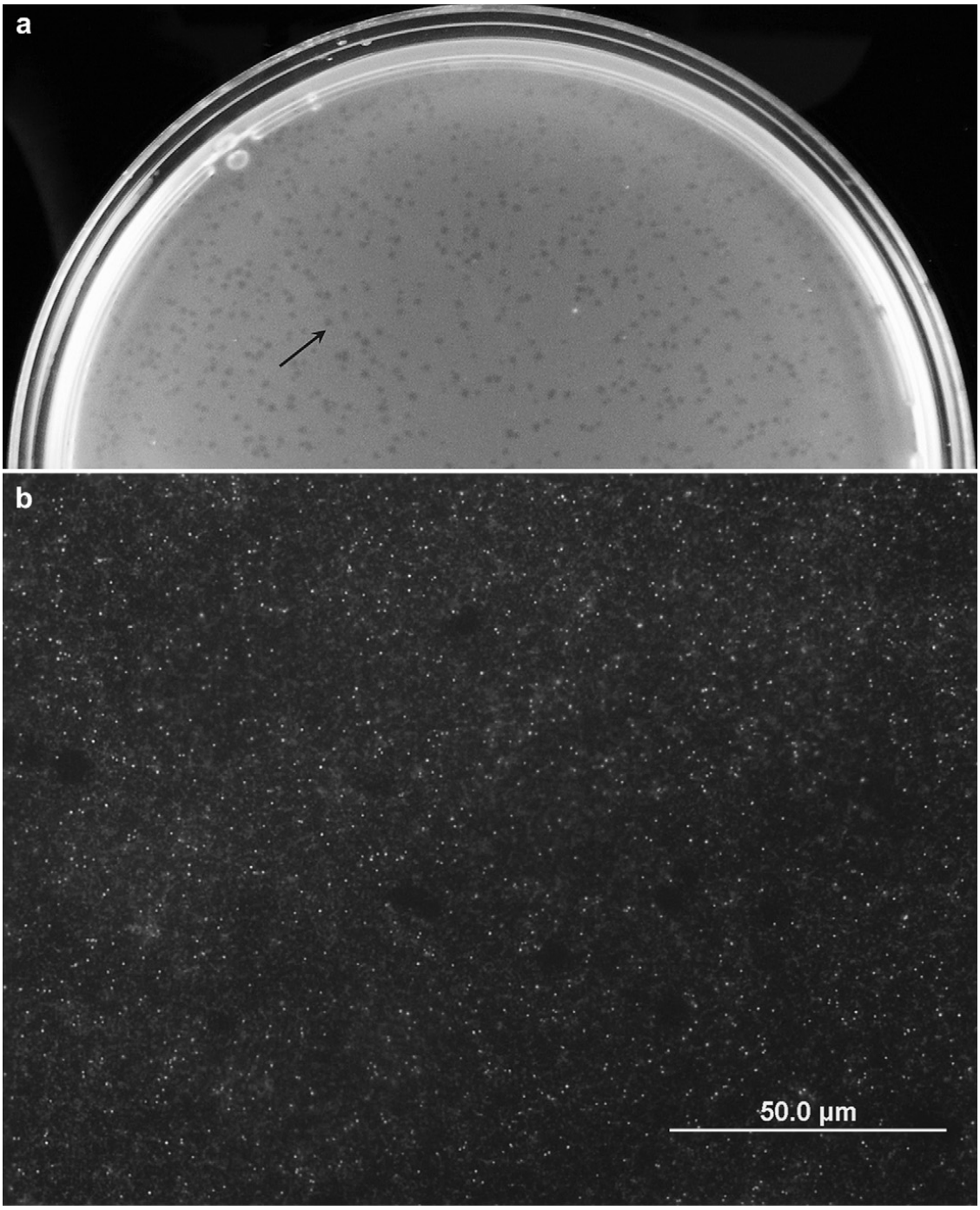

Fig. 4.1 –Plaque formation and virus-like particles: a) Plaque obtained from phages infecting *H. hydrossis* (arrow pointing at the plaque originating from isolated phages) b) Micrographs obtained from EFM of phage extract stained with SYBR Gold-VLPs can be seen as tiny dots.

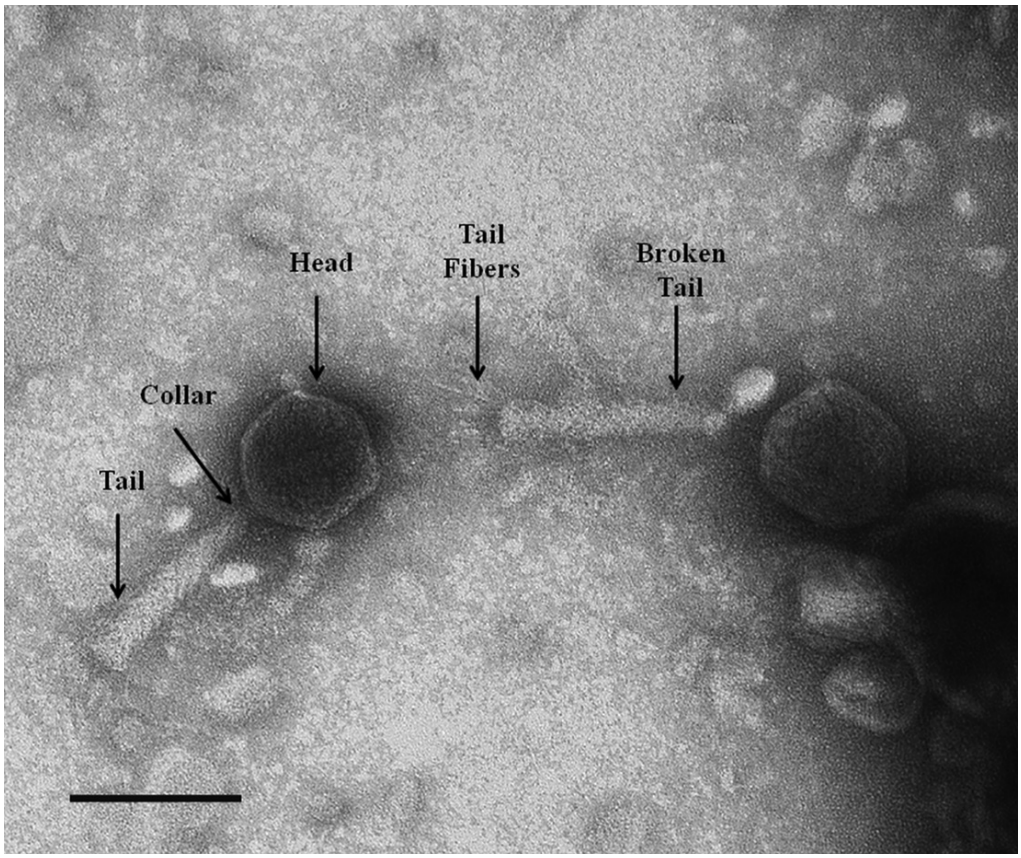

Fig. 4.2 Micrographs obtained from TEM of phage extract infecting *H. hydrossis* (bar represents 100 nm).

Myoviruses are typically virulent phages, meaning they do not integrate their genetic material with their host cells (lysogenise), and they usually kill their host cell (Suttle, 2005). This trait of HHY-phage (myoviruses) would be most beneficial for the biocontrol of *H. hydrossis* in wastewaters, because it negates the risk of lytic phage turning into lysogenic phage under low host populations.

One-step growth curve experiment performed on the isolated phage confirmed that the phage was lytic in nature. Fig. 4.3 shows the one-step growth curve for the HHY-phage. The latent and eclipse period of the bacteriophage were found to be 30 min and 20 min, respectively, which are typically similar to that of a T4 type bacteriophage. Latent and

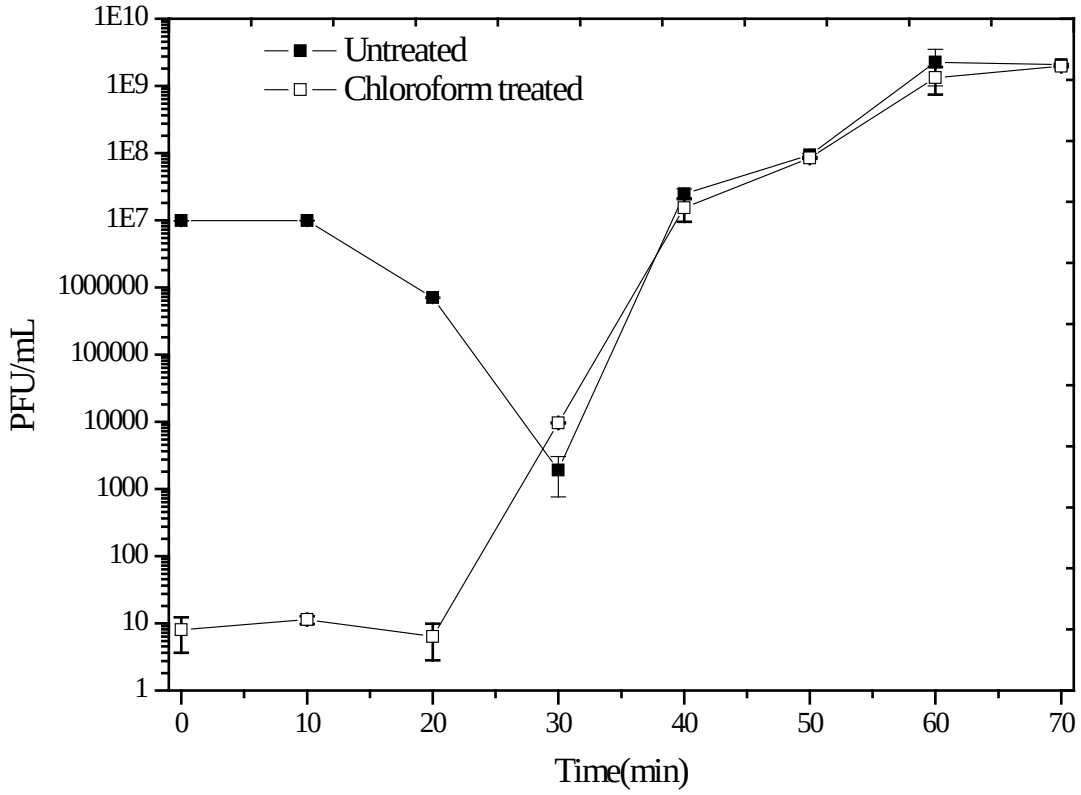

Fig. 4.3 One-step growth curve of HHY-phages.

eclipse periods are important parameters for bacteriophage growth, especially during the process of biocontrol using the bacteriophage. The latent time period spans from the point of phage adsorption to the point at which host lysis occurs and the eclipse time period spans from the point of phage adsorption to the point at which the first phage progeny have matured within an infected cell. The burst size was calculated to be 105 ± 7 PFU/infected cell.

4.3.2 Stability under different conditions and cross infectivity of HHY-phage

The biggest challenge for the sustainability of biocontrol would be the resistance of the host towards phage infection over time (Holmfeldt et al., 2007). Therefore, virulence studies were conducted before the HHY-phage could be used as remedial biocontrol for the biomass bulking. In this direction, the infectivity titer of the HHY-phage was monitored across a 9-month time period. HHY-phage was stable in sterile SMG buffer at 4 ºC during the storage time period. No appreciable decrease in infectious titer was observed following the storage of the HHY-phage in the SMG buffer. However, the storage of the HHY-phage in water reduced the infectious titer to a considerable extent (~80%). Negligible change in the infectivity titer after 9 months suggested the stability of the phage and the absence of any developed resistance of the host towards the phage infection. An alternative solution to the problem of host resistance against the lytic phage would be to isolate more than one lytic phage for the host filamentous bacterium. That will ensure long-term suitability of the demonstrated biocontrol strategy for biomass bulking caused due to a specific filamentous bacterium. Exposure to temperatures ranging from -20 ºC to 35 ºC had no effect on the infectious titer of the HHY-phage. The phage was relatively stable upon exposure to the high temperature for about 2 h. The decrease in infectious titer after incubation at 42 ºC for 2 h was negligible. However, exposure to temperatures higher than 42 ºC resulted in negative effect on the phage infectivity, suggesting possible denaturation of capsid proteins and nucleic acids at higher temperatures (Caldeira and Peabody, 2007). The HHY-phage remained equally infectious after overnight incubation (8-10 h) at pH 5 and 8. The average value of the HHY-phage

titer after 9 months at different pH values was 5.0 ± 0.1 × 10^5 PFU/mL, which was very close to the original titer value of 5.2 (±0.3) × 10^5 PFU/mL. The isolated phage was sufficiently stable under varying pH and temperature conditions that are commonly encountered in activated sludge processes. The observed stability characteristics of the HHY-phage endorse the fact that this newly isolated phage is a potential candidate for an intended biocontrol application in activated sludge processes, which typically encounters pH and temperature fluctuations.

For the biocontrol using the bacteriophage in activated sludge processes, it is very important that the applied phage is host-specific and it does not have any cross infectivity with other important indigenous bacterial communities in activated sludge processes. In cross infectivity studies using pure cultures of several bacteria, it was observed that HHY-phage did not produce any plaques when attempted to infect *Escherichia coli*, *Pseudomonas aeruginosa, N. multiformis*, *N. europea* and *D. desulfuricans* cultures. These bacteria represented communities responsible for COD removal, ammonia oxidation and anaerobic sulfate reduction. Live-dead assay for each of these bacteria after addition of HHY-phage showed no death caused due to phage infection. No signs of bacteriolysis in suspended growth experiments or plaques on agar plates were observed for these pure strains of bacteria. This demonstrated the suitability of the isolated phage for the biocontrol of filamentous bacteria in activated sludge processes. The phages leaving with the effluent after the biocontrol may pose a threat to the receiving waters. However, given the host specificity of the isolated phages, the phages leaving with the effluent will not multiply further and will die when the phages will not find their host in receiving waters.

Phage to host ratio (PHR) was another critical parameter that was evaluated in this research. Suitable PHR for infection was found to be 1:1000 where the bacterial death of ~54% was recorded and final viral count was $1.89 \pm 0.3 \times 10^{12}$ per mL (Table 4.1). Higher PHR caused more than 90% mortality of *H. hydrossis* and PHR lower than 1:1000 resulted in mortality less than 10%. It is worth mentioning that just the presence of filamentous bacteria is not sufficient to cause the biomass bulking.

In fact, at lower concentrations, filamentous bacteria form an integral part of the floc and help in floc integrity. Hence, the overdeath of filamentous bacteria could cause negative effects on the biomass settleability. The activated sludge bioreactor consists of a complex community of a variety of organisms. Hence, a higher ratio may be needed to achieve optimum lytic effect of the bacteriophage on the targeted filamentous bacteria.

Furthermore, more than one filamentous bacterium may also exist in activated sludge mixed liquor. Under such a case, it may be necessary to be isolated lytic phage for each filamentous bacterium and then use the isolated phages for the biocontrol of bulking caused by multiple filamentous bacteria.

Table 4.1 Effect of initial HHY-phage/host ratio on bacteriolysis and VLPs count after 12 h.

Initial phage/host ratio	**Final O. D. $_{600}$**	**Final Bacteria Live/Dead ratio**	**Final VLP count (/ mL)**
Blank	0.08±0.03	97.8±0.5	-
0.01:100	0.100±0.01	89.9±0.4	$7.03 \pm 0.4 \times 10^{10}$
0.1:100	0.072±0.001	54.5±0.5	$1.89 \pm 0.3 \times 10^{12}$
1:100	0.012±0.004	6.1±0.3	$9.12 \pm 0.5 \times 10^{12}$
10:100	0.003±0	0.5±0.3	$2.99 \pm 0.6 \times 10^{13}$

4.3.3 Effect of biocontrol on sludge settling

Upon visual examination, difference in height of settled sludge of batches was recorded following the application of the HHY-phage (Fig. 4.4a and b). Relatively clear supernatant (Fig. 4.4b) observed after application of the HHY-phage also suggested better sludge settling. Distinct difference in sludge settling characteristics was also encountered when the two batches were compared at 0 h and 3 h of infection with the HHY-phage (Fig. 4.4c and d, respectively).

As it may be seen from Fig. 4.4d, in the batch spiked with the HHY-phage, the settling velocity of the sludge was higher and the height of final settled sludge was lower. The biomass acclimatized with *H. hydrossis* showed a SVI >156 ± 3 ml/g, (Fig. 4.5a) resulting in poor settling. On the other hand, the biomass sample containing *H. hydrossis* and spiked with the HHY-phage at 1:1000 PHR showed signs of bacteriolysis.

A SVI value 105 ± 2 ml/g for the biomass sample containing *H. hydrossis* and the HHY-phage was recorded after 12 h. This is a significant finding and demonstrates that phage therapy indeed can be applied for a complex system such as an activated sludge bioreactor. At the time of dividing the *H. hydrossis* culture into two halves for the purpose of the control and the phage spiked biomass settling tests, care was taken to divide the biomass sample such that the resulting subsamples will have nearly identical cell concentrations.

Hence, the possibility of different *H. hydrossis* numbers in the control and the phage spiked biomass can be ignored. One possibility to monitor the numbers of *H. hydrossis* in the control and the phage spiked biomasses was to count *H. hydrossis* cells.

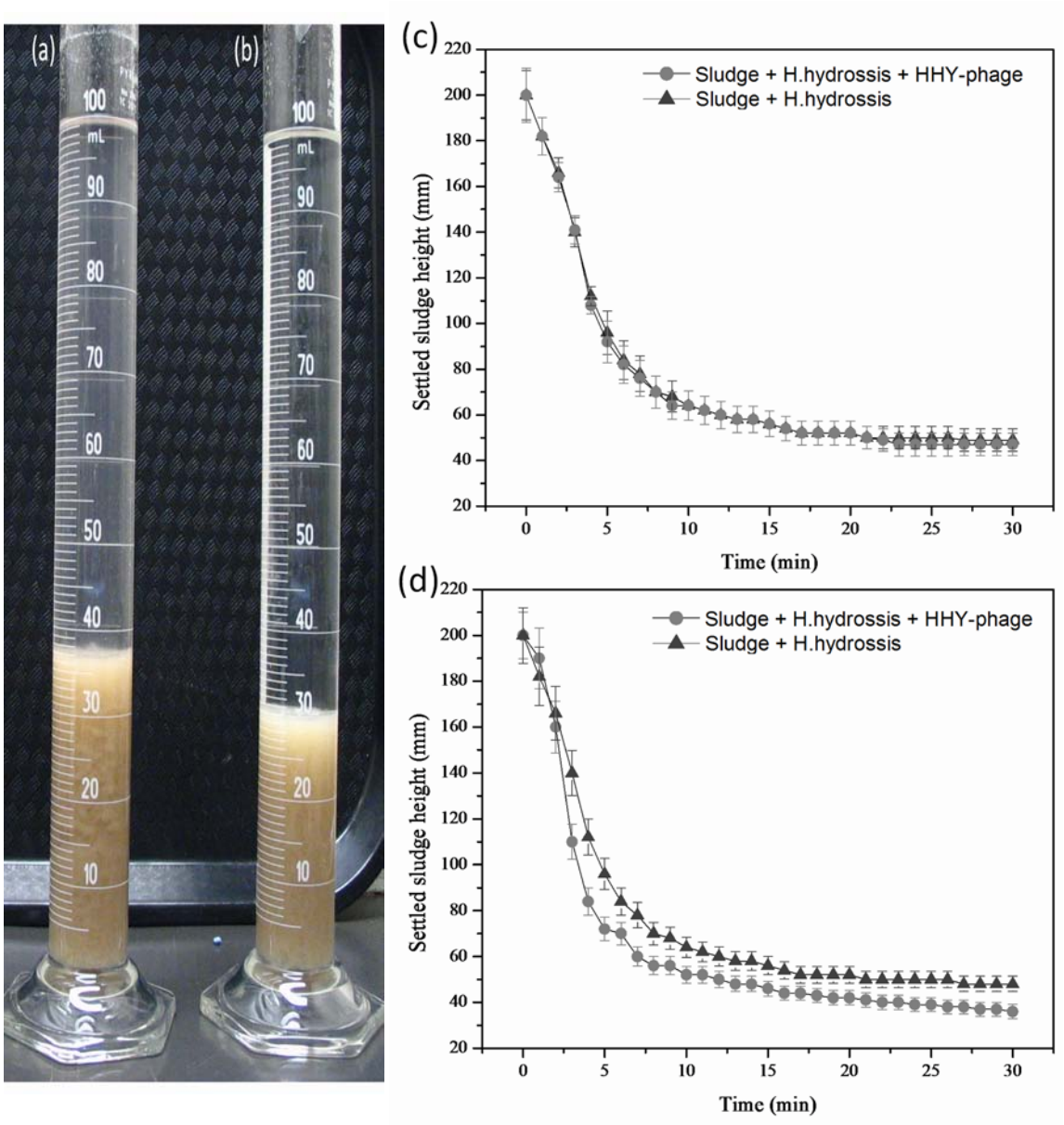

Fig. 4.4 Settled biomass after 30 min a) biomass acclimatized with *H. hydrossis* b) biomass acclimatized with *H. hydrossis* and spiked with HHY-phage. Settling curves of mixed liquor of two batches at c) 0 h, d) 3 h after phage addition.

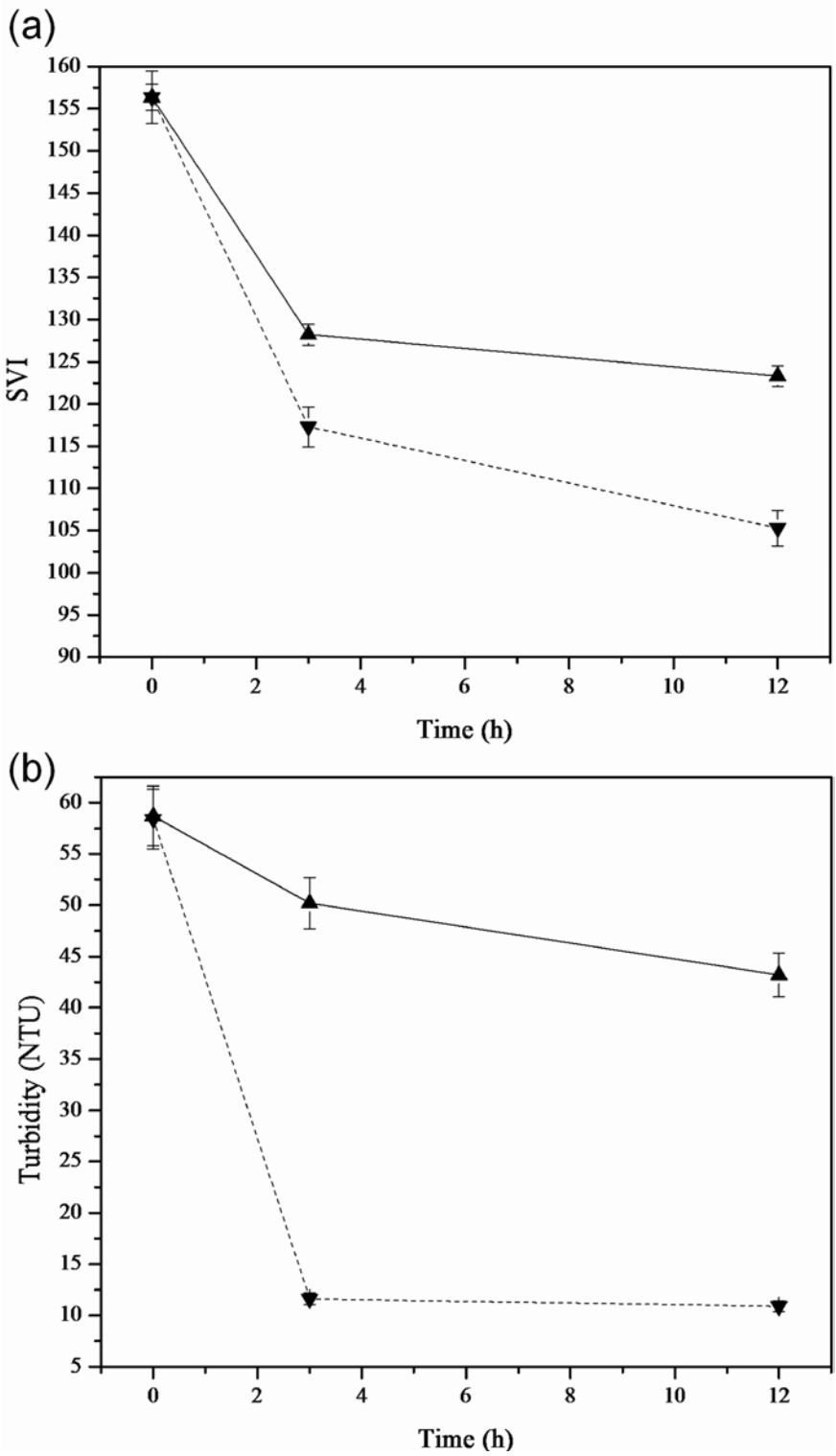

Fig. 4.5 Sludge volume index and turbidity: a) SVI and b) Turbidity profiles of supernatant, of the two batches: biomass acclimatized with *H. hydrossis* (▲) and biomass acclimatized with *H. hydrossis* and spiked with HHY-phage (▼).

However, it was not easy in practice because *H. hydrossis* is filamentous in morphology and integrates itself within biomass flocs, thus making it difficult to count the number.

The turbidity profiles of the supernatant in both batches are shown in Fig. 4.5b. It is evident from this figure that the turbidity of the supernatant after 3 h in the batch containing the biomass and the *H. hydrossis* spiked with HHY-phage decreased (from 58.4 NTU to 11.6 NTU) with time. This reduction in turbidity may be attributed to the bacteriolysis of *H. hydrossis* as a result of infection by HHY-phage. Turbidity of the supernatant provided another strong evidence of bacteriolysis of *H. hydrossis*. Turbidity of the supernatant in the batch test containing the biomass and *H. hydrossis* was greater than that in the batch containing the biomass and *H. hydrossis* spiked with the HHY-phage. The higher turbidity of the supernatant in the batch containing the biomass and the HHY-phage may be due to the presence of unsettled smaller flocs containing *H. hydrossis* which were not sufficiently heavy to settle to the bottom. The turbidity of the supernatant in the batch containing the biomass and *H. hydrossis* spiked with HHY-phage further dropped after 12 h of settling (data not shown).

It is noteworthy that the settling tests were done with a much greater population of *H. hydrossis* to demonstrate the concept of biocontrol. However, in a real-time scenario, the number of filamentous bacteria will be present in much smaller number as compared to other bacteria in the activated sludge process mixed liquor and the turbidity of the supernatant should not pose a problem.

Microscopic examination of the supernatant was performed to investigate the presence of *H. hydrossis* in the supernatant. Since the purpose of DAPI staining was only

qualitative, cells were not counted but the floc size and the morphology were recorded. The representative micrographs of DAPI stained supernatant samples from both the batches are presented in Fig. 4.6. In the supernatant from the settling cylinder with *H. hydrossis* but no phage extract, pin flocs (<10 mm diameter) were observed which were typical for filamentous bulking caused by *H. hydrossis* (van Veen et al., 1973; Eikelboom and van Buijsen, 1981). However, no pin flocs were present in the supernatant in the settling cylinder containing biomass with *H. hydrossis* and spiked with the phage extract. Bigger floc sizes around 50 mm diameter were observed in settled biomass with *H. hydrossis* and spiked with the phage extract. This finding further strengthens the earlier speculation that following application of the HHY-phage, the decrease in the population of *H. hydrossis* occurred resulting in the increase of the floc size, leading to clearer supernatant and better sludge settling.

The reduction in the SVI that led to a better settling was due to the increase in settling velocity, which was possibly a result of the increase in floc sizes due to the killing of *H. hydrossis* by the HHY-phage. It has been reported earlier that sludge settling velocity is dependent on filamentous population and overpopulation can result in decreased settling velocity (da Motta et al., 2002). It is also an established fact that the overpopulation of filamentous bacteria like *H. hydrossis* can cause smaller floc sizes and poor settling (Eikelboom, 1975). Good settling in the batch spiked with *H. hydrossis* and HHY phage was noticed only after 30 min of settling tests using batch experiments (Fig. 4.4c).

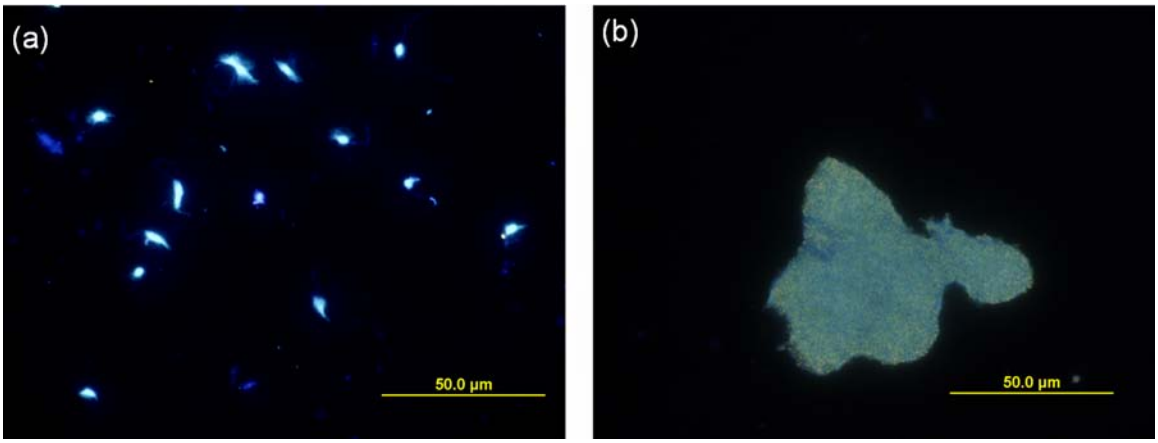

Fig. 4.6 Epifluorescent micrographs of DAPI stained samples of (a) supernatant of batch containing biomass acclimatized with *H. hydrossis* and (b) settled biomass acclimatized with *H. hydrossis* and spiked with HHY-phage.

This may be due to the reason that the latent period for the HHY-phage was around 30 min. During this period, HHY-phage may have only adsorbed onto the *H. hydrossis* cells, but no lysis and reduction in the population had taken place. Furthermore, a higher phage to bacteria ratio may be needed in full wastewater treatment plants experiencing filamentous bulking problems because of the mixed liquor complexity and the transport limitations that the added phage/phages will face to come in contact with the filamentous bacteria.

4.3.4 Phage-based biocontrol of biomass bulking under pure oxygen effect

Pure oxygen effect was performed on the phage-based biocontrol of biomass bulking. Sludge settling height was recorded following the application of the isolated phage under pure oxygen (Fig. 4.7 a and b). Clear supernatant was observed in both batches containing pure oxygen and pure oxygen with the phage.

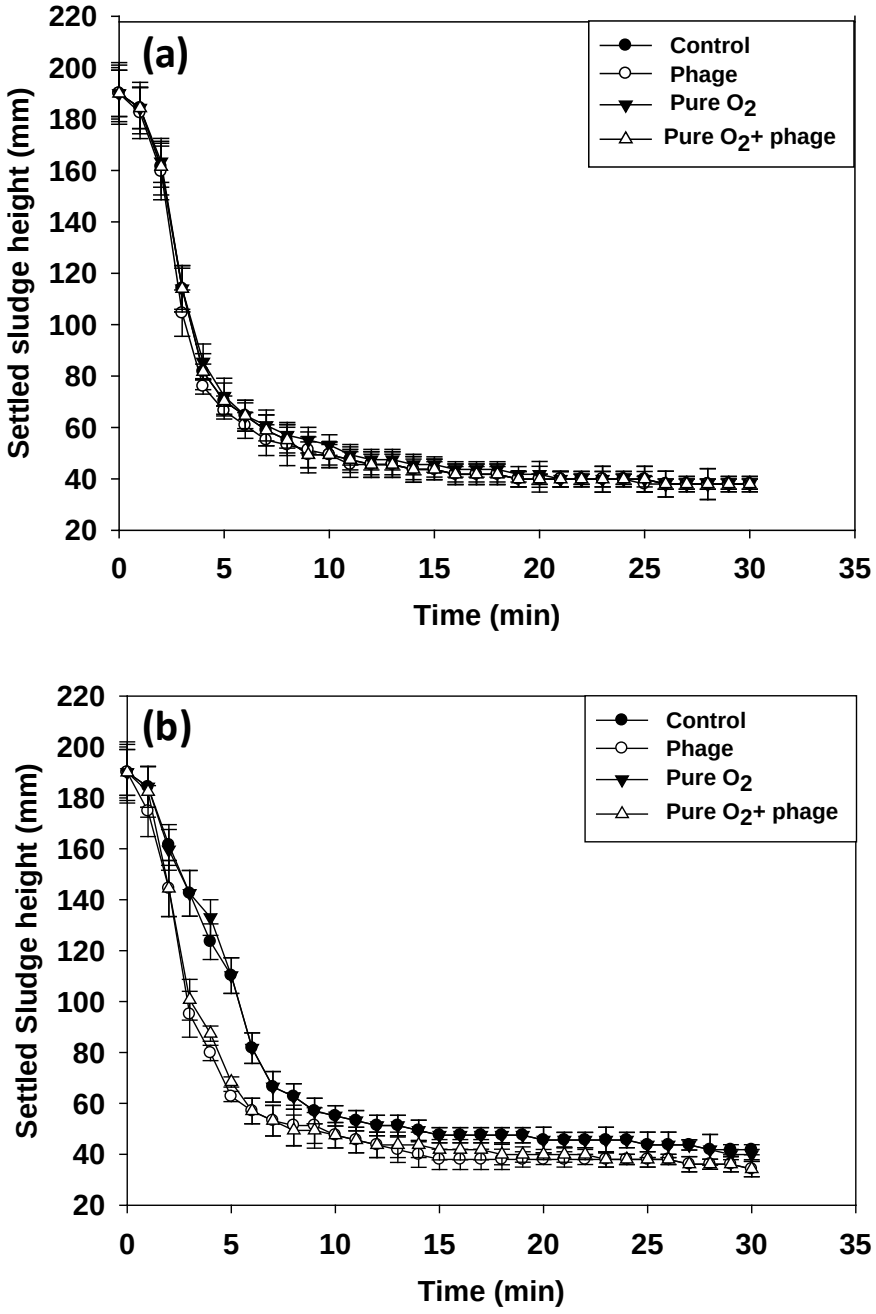

Fig. 4.7 Settling curves of mixed liquor of four batches at (a) 0 h and (b) 3 h after lytic phage infection (control: biomass+*H.hydrossis*, phage: biomass+*H.hydrossis*+HHY phage, pure O_2: biomass+*H.hydrossis* + pure O_2, pure O_2+phage: biomass+*H.hydrossis* + pure O_2+HHY phage).

No distinct difference in sludge settling was encountered when the batches were compared at 0 h and 3 h of infection and pure oxygen exposure.

SVI values decreased in batches containing the phage and the phage with pure oxygen. Less reduction of SVI in the batch containing the phage with pure oxygen was recorded compared to the batch containing only the phage. The turbidity profile of the supernatant in batches is shown in Fig. 4.8 b. Based on the turbidity profile, pure oxygen did not affect turbidity in phage-based biocontrol in 3 h infection. However, the turbidity under pure oxygen increased at 12 h, suggesting that the pure oxygen may affect bacterial growth or particle disruption in a batch test.

As described earlier, bacteriophage-based biocontrol did not affect COD and nutrient removal in the batch test for 12 h (Table 4.2). The COD and ammonia removals in four batches were 79~85 % and 77~96%, respectively. More COD and ammonia removals were observed in batches purged with pure oxygen. Pure oxygen enhanced biological oxidation of ammonia in constructed treatment wetlands due to the high transfer efficiency (Beutel and Horne, 1999).

4.3.5. Effect of phage application on COD and nutrient removal efficiency

Cross infectivity studies of the HHY-phage with selected pure strains of bacteria showed no infectivity of HHY-phage on these strains. However, it was necessary to determine whether the COD and nutrient removing efficiencies of the original biomass were affected due to the addition of HHY-phage.

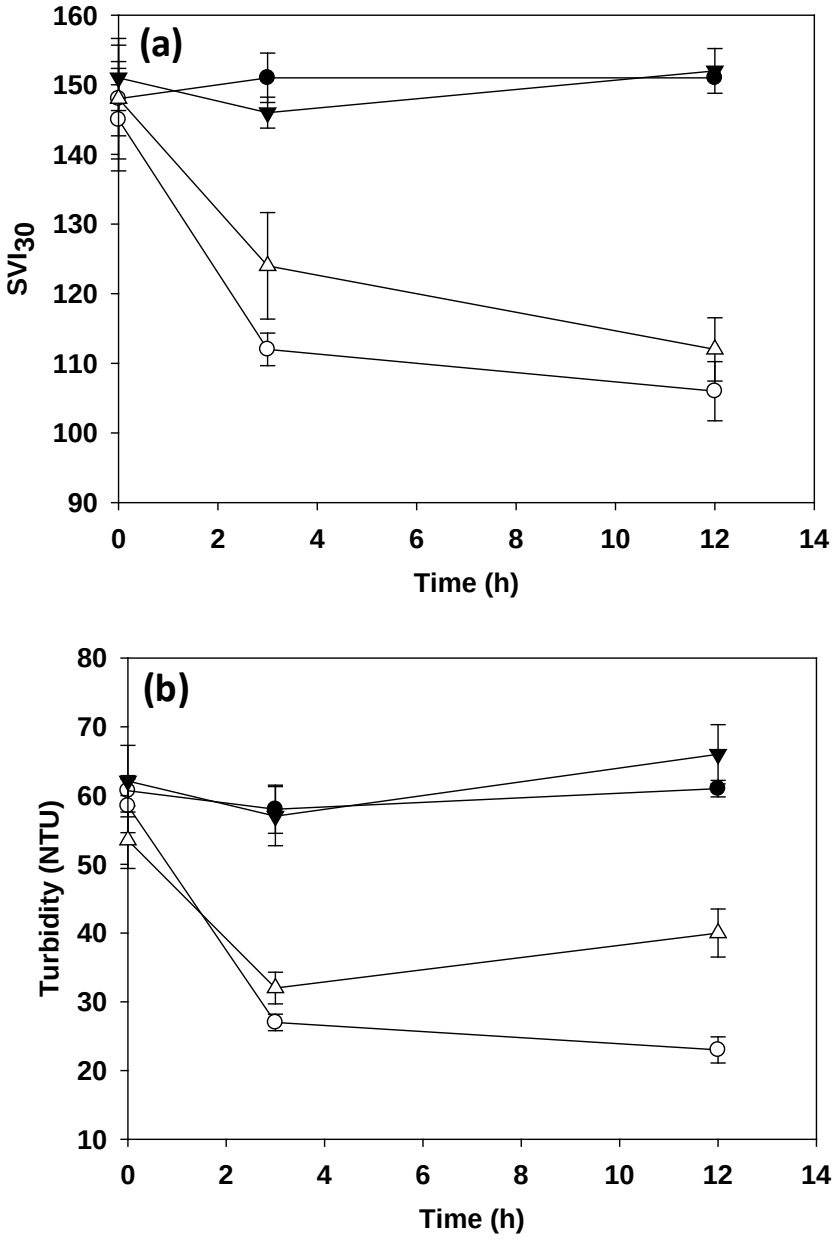

Fig. 4.8 Sludge volume index and turbidity: (a) SVI_{30} and (b) Turbidity profiles of supernatant of four batches (● biomass+ *H.hydrossis*, ○: biomass+ *H.hydrossis*+HHY phage, ▼: biomass+*H.hydrossis* + pure O_2, △: biomass+*H.hydrossis* + pure O_2+HHY phage).

Table 4.2 Nutrient profile of the biocontrol of *H. hydrossis* under pure oxygen effect

	Time	Biomass+*H. hydrossis*	Biomass+*H. hydrossis*+phage	Biomass+*H. hydrossis*+pure O_2	Biomass+*H. hydrossis*+pure O_2+ phage
COD (mg/L)	**0**	150	144	152	148
	3	120.5	116.5	121.2	98.7
	12	30.2	25.5	20.6	21.6
NH_3-N (mg/L)	**0**	25.6	23.2	24.8	26.2
	3	19.2	20.4	18.4	19.4
	12	4.35	5.25	1.42	0.99
COD removal (%)		79.87	82.29	86.45	85.41
Ammonia removal (%)		83.01	77.37	94.27	96.22

The sequencing batch reactor from which the biomass was sampled was efficiently removing COD, nitrogen and phosphorus. It was observed that the phage application did not affect the COD and the nutrient removal efficiencies for 24 h of treatment (Table 4.3). The COD and ammonia nitrogen removals in the control batch (no *H. hydrossis* and HHY-phage) test were 74% and 100%, respectively.

The corresponding removal efficiencies over 24 h of these species in the batch containing the biomass and *H. hydrossis* were 63 and 96%, respectively, which indicates that the COD and ammonia nitrogen removal efficiencies in this batch slightly went down as compared to the control. The marginal difference in COD and nutrient removal efficiencies between the control batch and the batch containing *H. hydrossis* could possibly be due to the interference of *H. hydrossis* with other bacteria, putting substrate diffusion limitations to both organics and ammonia nitrogen.

Table 4.3 pH and nutrient profile (at 0, 12, 24 h) on the biocontrol of *H. hydrossis*.

	Time	Sludge	Sludge + virus	Sludge + *H. hydrossis*	Sludge + *H. hydrossis* + virus
pH	**0**	7.6	7.5	7.5	7.3
	12	7.4	7.5	6.8	6.9
	24	7.1	7.4	6.9	7.1
COD (mg/L)	**0**	31.4	31.1	30.9	30.9
	12	9.0	7.9	12.3	7.5
	24	8.3	7.5	11.4	7.3
Turbidity (NTU)	**0**	31.5	30.9	58.7	50.4
	12	28.2	27.9	50.4	11.6
	24	21.5	18.0	50.2	5.9
NH_3-N (mg/L)	**0**	27.8	27.8	27.8	27.8
	12	0.5	0.6	1.1	0.6
	24	0	0	0.2	0

The removal efficiencies of COD and ammonia nitrogen in the batch containing the biomass with *H. hydrossis* and spiked with HHY-phage was nearly close to the control batch test, indicating that the HHY-phage did not infect other key organisms responsible for COD and ammonia nitrogen removals. This finding further demonstrates that the addition of phage in fact reduced *H. hydrossis* population to overcome the substrate diffusion limitations caused by the presence of *H. hydrossis*.

4.3.6 Cost comparison of bacteriophage-based biocontrol and chlorine addition

Filamentous bacteria can be controlled by adding chlorine to the return activated sludge to kill filamentous microorganisms. Sludge bulking control by chlorination was used over 50 years ago and this practice is probably a cost-effective and short-term control method for filamentous bacteria (Bitton, 2005). Chlorine concentration adding to

the return activated sludge should be 10-20 mg/L. If the concentrations are more than 20 mg/L, It may cause deflocculation and formation of pin-point flocs. In addition, chlorine-resistant filamentous bacteria (e.g., chlorine-resistant 021N) have been reported (Se´ ka et al., 2001), indicating that chlorination is sometimes unsuccessful in bulking control.

Based on the calculation for cost analysis, chlorine addition was estimated to be 943 dollars/day (see Appendix). For phage application, the cost was calculated to be 171 dollars/day. This indicates that phage application would be beneficial compared to oxidants treatment.

4.4 Summary

So far, the phage-based biocontrol (phage therapy) using lytic phages has been researched and practiced for medical and sanitation applications only (Kropinski, 2006). Although some review papers (Withey et al., 2005) have discussed the possibility of using bacteriophages as biocontrol agents for problems such as sludge dewatering and anaerobic digestion in wastewater treatment application in activated sludge processes, the actual research efforts in this direction are wanting. Biomass bulking resulting in poor biomass settling in the secondary clarifier due to the presence of filamentous bacteria is one of the most common operational problems, which is still encountered at many full-scale biological wastewater treatment plants. Although several engineering parameters have been linked to the biomass bulking, a definite engineering solution to this is yet to be evolved. This research demonstrated the biocontrol of the biomass bulking using a lytic bacteriophage as an alternative solution to this problem. The proposed idea is innovative and falls outside the existing paradigm of current engineering practices in

municipal wastewater treatment for biomass bulking control. Furthermore, this is the first report on the isolation, characterization and application of a lytic bacteriophage for the biocontrol of filamentous bacterium *H. hydrossis* responsible for sludge bulking. Hence, the research has both microbiological as well as environmental engineering significance.

Currently, characterization of HHY-phage at the genetic and proteomic level is underway which would provide further insight into this phage. Further work to obtain the complete genome sequence of the isolated phage is also in progress. This would not only help understand the newly isolated bacteriophage but also may provide ways to genetically engineer the phage for better performance and extended applications. Unlike bacterial genomes, there are limited phage genomes available in the common public databases, which poses a challenge to researchers in conducing blast searches for genes specific to and conserved in bacteriophages. Such a database would also be useful to compare phage genomes among themselves and also with bacterial genomes. In the least, it would promote the development of phage-based biocontrol applications to a new level. Parallel research work is also in progress for the isolation of lytic phages infecting other important filamentous and foam-forming bacteria. This is aimed at constructing a pool of bacteriophages that could together form a consortium of lytic phages that can infect any filamentous bacteria that is responsible for biomass bulking or foaming. An ideal solution, however, would be to have a single (natural/engineered) bacteriophage which has broad host range to infect all filamentous bacteria without any collateral damage to nutrient/COD removal efficiency.

CHAPTER 5

BACTERIOPHAGE-BASED BIOCONTROL OF BIOLOGICAL SLUDGE BULKING IN WASTEWATER (H2-TASK 4, 5 AND 6)

5.1 Introduction

The main objective of this work is to check the extendibility of bacteriophage-based biocontrol of filamentous bulking beyond the model microbe tested in the previous work (Kotay et al., 2011). It had been previously established that lytic bacteriophage can be used to mitigate the overgrowth of filamentous bacteria (Kotay et al., 2011). The application, therefore, is a feasible, cost-effective and efficient method to improve settling in activated sludge processes with filamentous bulking. However, beyond the model organism (*Halicomenobacter hydrossis*) tested, filamentous bulking can be caused by several other bacteria, such as *Sphaerotilus natans*, *Nocardia* spp., *Microthrix parvicella*, etc., which have been identified and isolated from wastewater treatment plants (Kampfer, 1995; Eikelboom, 1975; da Motta et al., 2003). Since bacteriophages have host specificity, filamentous sludge bulking can be controlled only by applying lytic

bacteriophage specific to that bacterium which is causing bulking (Kropinski, 2006). An ideal solution would be to have a pool of lytic bacteriophages which can control the growth of all known filamentous bacteria that can cause bulking.

5.2 Isolation and characterization of lytic bacteriophage infecting *S. natans*

Lytic phage specific to *S. natans* (referred to as SN-phage) was isolated from the mixed liquor of local municipal wastewater treatment plant. Purification of SN-phage was performed following the protocol described by Kotay et al. Figure 5.1A shows plaque formation on an agar plate containing several plaques obtained after serial infections of *S. natans*. Plaques were distinct, clear and round shaped with 0.5-1 mm diameter. A significant amount of virus-like particles (VLPs) were also observed under epifluorescent microscope (Fig. 5.1 B). Based on transmission electron micrographs, SN-phage belonged to the Myoviridae family, which is typically a virulent phage, inducing cell lysis without a lysogenic cycle (Suttle, 2005). The SN-phage was around 166 nm long and depicted an icosahedral head with contractile tail (Fig. 5.1C). Myoviridae phages may be beneficial for the biocontrol because it negates the risk of the lytic phage turning lysogenic following the decline of the host population. However, some phages found in wastewater treatment plants were of the *Siphoviridae* family (Thomas et al., 2002). *Siphoviridae* morphology may have an evolutionary benefit in activated sludge processes since their flexible tail seems to have the highest specific enzymatic activity and they are capable of remaining viable under adverse environments, such as exposure to toxic materials. Figure 5.1D shows the one-step growth curve for the SN-phage.

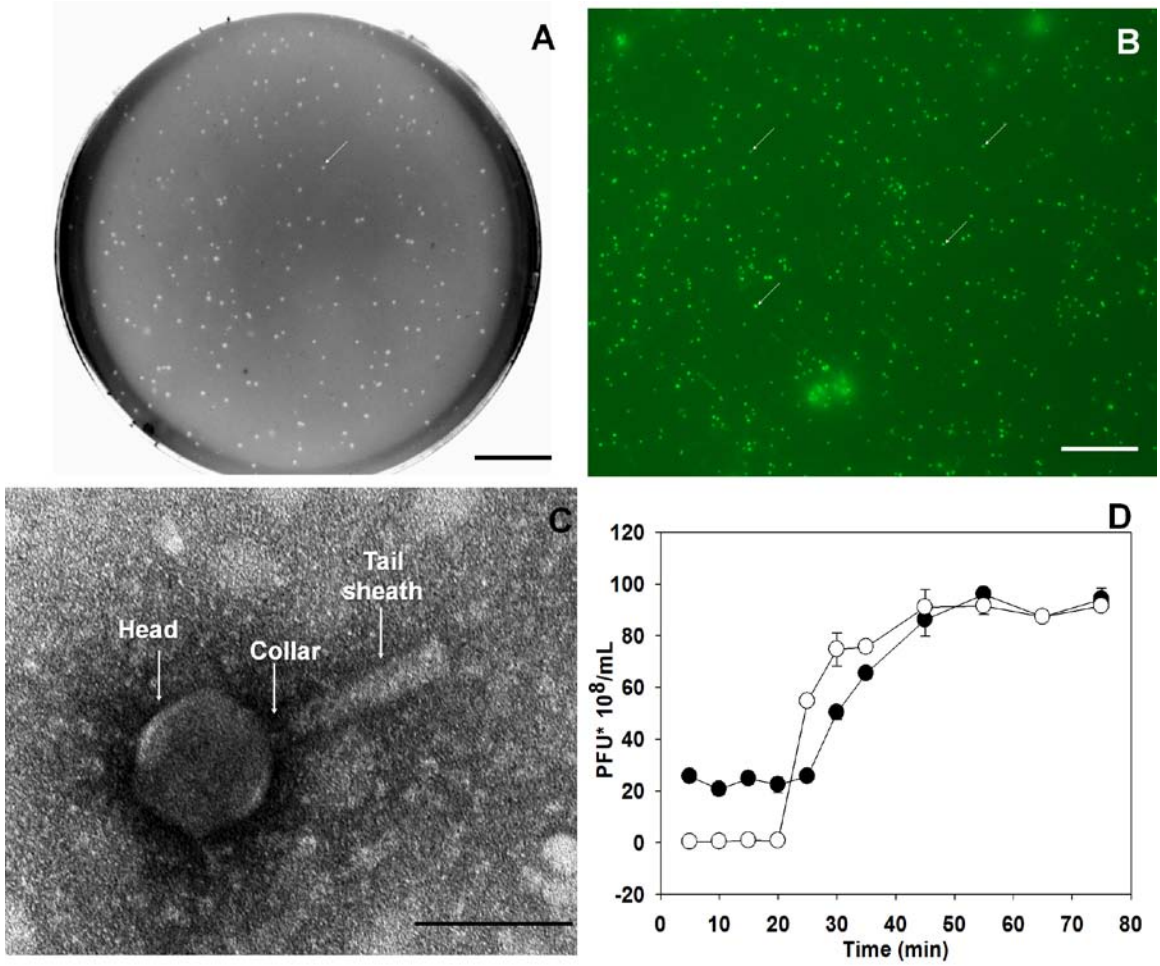

Fig. 5.1. Characteristics of isolate phage-(A) Plaque formation on agar plate (bar represents 2 cm), (B) epifluorescent micrograph of phage extract stained with SYBR Gold (arrows show virus-like particles), (C) transmission electron micrograph revealing morphology of SN-phage (bar=100 nm) and (D) one-step growth curve of SN-phage.

The one-step growth curve obtained from the isolated phage indicated that the phage was lytic in nature. The latent and eclipse period of the bacteriophage were observed to be 25 and 20 min, respectively, which are similar to that of a HHY-phage (Kotay et al., 2011).

In the latent period, the titer of active virions inside the cells rises dramatically, but newly synthesized virions have not yet appeared outside the cell. In the eclipse period, the virus nucleic acid becomes separated from its protein coat, and the virion no longer exists as an infectious entity. The burst size was calculated to be 89±6 PFU/ infected cell.

Phage stability for biocontrol is important to the bacteriophage infection over time (Holmfeldt et al., 2007). The isolated phage was stable for over 9 months and negligible change in the infectivity was observed, suggesting the sustainability of biocontrol in filamentous organisms. Efficiency of the phage infection did not vary upon storage at temperatures between -20º C and 35º C. However, exposure to temperatures higher than 42 º C resulted in negative effect on the biocontrol, suggesting possible denaturation of the protein coat and nucleic acid at high temperature (Caldeira and Peabody, 2007). The isolated phage was also quite stable under pH fluctuation that is commonly encountered in activated sludge processes. No cross infection was observed for the other bacteria tested viz., *E. coli, P. aeruginosa, N. multiformis* and *N. europaea*, indicating the host specificity of SN-phage.

5.3 Biocontrol of filamentous bulking

Remediation of sludge bulking in wastewater treatment plants is associated with high operational costs (Jenkins et al., 1993). If remedial methods to control or avoid sludge

bulking could be identified, cost would be reduced significantly. Physico-chemical methods such as manipulation of sludge recycle ratio and the addition of flocculants and oxidants have been tried in the past (Xie et al., 2007). Chlorination controls filamentous extension from the floc surface and merely reduces the symptom of sludge bulking. Thus, filamentous bacteria will regrow with vengeance after the termination of chlorine addition.

The biomass acclimatized with *S. natans* showed a SVI of 168±2 mL/g (Fig. 5.2A), indicating poor sludge settling. After 12 h of the infection with SN-phage, SVI descended to 102 ± 2 mL/g. Significant reduction of the turbidity was observed in the batch containing SN-phage after 12 h (Fig. 5.2B). The reduction of the turbidity provided the evidence of bacteriolysis of *S. natans*.

Microscopic examination of the supernatant from the batches was performed to investigate the effect of SN-phage infection after 0 h and 8 h (Fig. 5.2C and D, respectively). Live cells (green fluorescent) with distinct filamentous morphology of *S. natans* were found to be dominant at 0 h in the supernatant, suggesting filamentous bulking. After 8 h of phage infection, dead (red fluorescent) and lysed cells of *S. natans* were observed in multiple fields examined, suggesting successful infection and lysis caused by phage.

The sludge settling characteristics of the two batches, control and infected with SN-phage, were significantly different (Fig. 5.2 E and F, respectively). As can be seen in Figure 5.2 F, the settling velocity in the batch spiked with SN-phage and the height of sludge volume was lower due to the reduction of *S. natans* population.

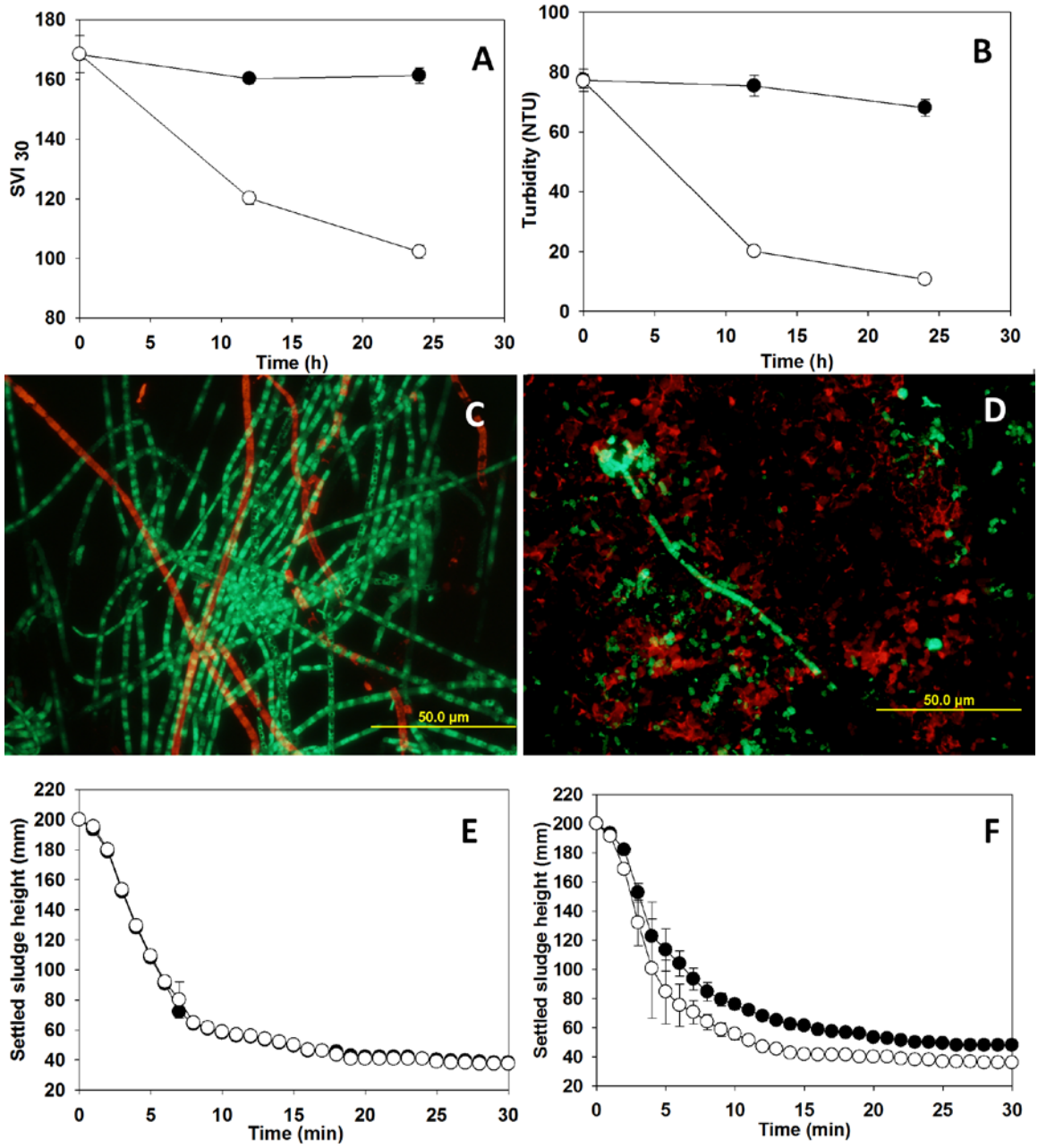

Fig. 5.2 Profiles of (A) SVI, (B) turbidity of supernatant; epifluorescent micrographs from Live and Dead cell analysis of supernatant samples at (C) 0 h, (D) 8 h of SN-phage infection; and settling curves of biomass at (E) 0 h, (F) 8 h after infection.

In chemical oxygen demand (COD) and nutrient removal efficiency, the marginal difference was observed, compared with control without the isolated phage. This finding strengthens the fact that the SN-phage did not infect other bacteria that were involved in COD and nutrient removal.

5.4 Summary

Very little research has been devoted on sustainable solutions towards mitigation of biomass bulking in activated sludge processes. The majority of research regarding the control of sludge bulking has been focused on engineering manipulations, which are temporary solutions. The biocontrol method we investigated in this work is a microbiological approach, which used lytic bacteriophage and brings a sustainable solution to repress the over-proliferation of filamentous bacteria in wastewater treatment plants. The biocontrol method now has been tested successfully against two model filamentous bacteria, *H. hydrossis* and *S. natans*. However, further studies are necessary to fully understand the mechanisms underlying how host bacterium could attain resistance to phage infection. Despite these challenges, the integrated approach of wastewater engineering and bacteriophage application promise to be an excellent strategy to solve biomass bulking in activated sludge processes.

CHAPTER 6

CONCLUSIONS

The objectives of this dissertation research were to investigate the role of stress-mediated bacteria killing in activated sludge bioreactors due to the prophage induction and apply the concept of bacteriophage-based biocontrol in activated sludge systems with filamentous bulking as the model operational challenge.

In the first objective, the selected environmental factors could induce prophage from *N. multiformis*, demonstrating that cell lysis due to prophage induction could be an important mechanism contributing to the frequent upset in ammonia oxidation efficiency in full-scale wastewater treatment plants. Among the stress factors considered, pH in the acidic range was the most detrimental to the nitrification efficiency by *N. multiformis*. Cell lysis due to stress resulting in phage induction seemed the primary reason for deteriorated ammonia oxidation by *N. multiformis* at lower concentration of Cr (VI) and potassium cyanide. However, direct killing of *N. multiformis* due to the binding of chromium and potassium cyanide with cell protein, as demonstrated in the literature at higher concentrations of toxic compounds, was the primary mechanism of cell lysis of *N. multiformis*. Organics represented by the COD did not have any effect on the phage induction in *N. multiformis*. This AOB remained dormant at low temperature without any

phage induction. Significant decrease in the number of live cells with a corresponding increase in the number of VLPs was recorded. Death of *N. multiformis* at 45 ºC was attributed to the destruction of cell wall rather than to the phage induction. Therefore, the overall effect that prophage was induced under all tested environmental factors from *N. multiformis* could be an important pathway for the occasional deterioration in ammonia oxidation efficiency in full-scale wastewater treatment plants.

The second objective was to demonstrate the application of lytic bactriophage-based biocontrol of biomass bulking in the activated sludge process using *H. hydrossis* and *S. natans* as model filamentous bacteria. The lytic phage specifically infecting *H. hydrossis* and *S. natans* was isolated from the mixed liquor of a local wastewater treatment plant. SN-phage belonged to the Myoviridae family which is typically a virulent phage, indicating cell lysis without lysogenic cycle. Significant reduction in sludge volume index and turbidity of the supernatant was observed in batches containing filamentous biomass following addition of lytic phages. Phage to host ratio for the optimal infection was found to be 1:1000 with ~52 % host death. The phage was stable after exposure to high temperature (42 ºC) and pH between 5 and 8, emphasizing that it can withstand the seasonal/operational fluctuations under real-time applications. Microscopic examination confirmed that the isolated lytic phage can trigger the bacteriolysis of filamentous bacteria. This finding strengthens our hypothesis of bacteriophage-based biocontrol of overgrowth of filamentous bacteria and the possibility of phage application in activated sludge processes, the world's most widely used wastewater treatment processes.

CHAPTER 7

SCIENTIFIC CONTRIBUTION AND DISSEMINATION OF RESULTS

Publications

Research paper number 1

Various physico-chemical stress factors cause prophage induction in *Nitrosospira multiformis* 25196 – an ammonia oxidizing bacteria

Choi, J., Kotay, S. M., Goel, R. (2010) Water Research (44) 4550-4558

Abstract: Bacteriophages are viruses that infect bacteria and contribute to significant changes in the overall bacterial community. Prophages are formed when temperature bacteriophages integrate their DNA into the bacterial chromosome during the lysogenic cycle of the phage infection to bacteria. The prophage (phage DNA integrated into bacterial genome) on the bacterial genome remains dormant, but can cause cell lysis under certain environmental conditions. This research examined the effect of various environmental stress factors on the ammonia oxidation and prophage induction in a model ammonia oxidizing bacteria *Nitrosospira multiformis* ATCC 25196. The factors included in the study were pH, temperature, organic carbon (COD), the presence of heavy metal in the form of chromium (VI) and the toxicity as potassium cyanide (KCN). The

selected environmental factors are commonly encountered in wastewater treatment processes, where ammonia oxidizing bacteria play a pivotal role of converting ammonia into nitrite. All the factors could induce prophage from *N. multiformis*, demonstrating that cell lysis due to prophage induction could be an important mechanism contributing to the frequent upset in ammonia oxidation efficiency in full-scale treatment plants. Among the stress factor considered, pH in the acidic range was the most detrimental to the nitrification efficiency by *N. multiformis*. The number of virus-like particles (VLPs) increased by 2.3E+10 at pH 5 in 5 h under acidic pH conditions. The corresponding increases in VLPs at pH values of 7 and 8 were 9.67E+9 and 1.57E+10 in 5 h, respectively. Cell lysis due to stress resulting in phage induction seemed the primary reason for deteriorated ammonia oxidation by *N. multiformis* at lower concentrations of Cr (VI) and potassium cyanide. However, direct killing of *N. multiformis* due to the binding of Cr (VI) and potassium cyanide with cell protein, as demonstrated in the literature at higher concentration of these toxic compounds, was the primary mechanism of cell lysis of *N. multiformis*. Organics represented by the chemical oxygen demand (COD) did not have any effect on the phage induction in *N. multiformis*. This AOB remained dormant at low temperature (4ºC) without any phage induction. Significant decrease in the number of live *N. multiformis* cells with a corresponding increase in the number of VLPs was recorded when the temperature was increased to 35 º C. Death of *N. multiformis* was 45 ºC was attributed to the destruction of cell walls rather than to the phage induction.

Research paper number 2

Bacteriophage-based biocontrol of biological sludge bulking in wastewater

Choi, J., Kotay, S. M., Goel, R. (2011) Biogineered Bugs 2:4, 1-4; July/August

Abstract: Previously, we reported the first ever application of lytic bacteriophage (virus)-mediated biocontrol of biomass bulking in the activated sludge process using *Halicomenobacter hydrossis* as a model filamentous bacterium. In the current study, we extended the biocontrol application to another predominant filamentous bacterium, *Sphaerotilus natans*, known to be a notorious cause of filamentous bulking in wastewater treatment systems. Similar to the previous study, one lytic bacteriophage was isolated from wastewater that could infect *S. natans* and cause lysis. Significant reduction in sludge volume index and turbidity of the supernatant was observed in batches containing *S. natans* biomass following addition of lytic phages. Microscopic examination confirmed that the isolated lytic phage can trigger the bacteriolysis of *S. natans*. This extended finding further strengthens our hypothesis of bacteriophage-based biocontrol of overgrowth of filamentous bacteria and the possibility of phage application in activated sludge processes, the world's most widely used wastewater treatment processes.

Research paper number 3

Biocontrol of biomass bulking caused by *Haliscomenobacter hydrossis* using a newly isolated lytic bacteriophage

Kotay, S. M., Datta, T., Choi, J., Goel, R. (2011) Water Research (45) 694-704

Abstract: This research demonstrates the first ever application of lytic bacteriophage (virus)-mediated biocontrol of biomass bulking in the activated sludge process using

Haliscomenobacter hydrossis as a model filamentous bacterium. Bacteriophages are viruses that specifically infect bacteria only. The lytic phage specifically infecting *H. hydrossis* was isolated from the mixed liquor of a local wastewater treatment plant. The isolated bacteriophage belongs to the Myoviridae family with a contractile tail (length-126 nm; diameter-18 nm) and icosahedral head (diameter-81 nm). Titer of the isolated phage with *H. hydrossis* was calculated to be $5.2\pm0.3 \times 10^5$ PFU/mL and burst size was found to be 105 ± 7 PFU/infected cell. The phage was considerably stable after exposure to high temperature (42 ºC) and pH between 5 and 8, emphasizing that it can withstand the seasonal/operational fluctuations under real-time applications. Phage to host (bacteria) ratio for the optimal infection was found to be 1:1000 with ~54% host death. The isolated phage showed no cross infectivity with other bacteria most commonly found in activated sludge systems, thus validating its suitability for biocontrol of filamentous bulking caused by *H. hydrossis*. Following the phage application, successful reduction in sludge volume index (SVI) from 155 to 105 was achieved, indicating improved biomass settling. The application of phage did not affect nutrient removal efficiency of the biomass, suggesting no collateral damage. Similar to phage therapy in medical applications, phage-mediated biocontrol holds a great potentiality for large-scale applications as an economic agent in the mitigation of several water, wastewater and environmental problems. The present study in this direction is a novel effort.

Research paper number 4

Viruses to the rescue?

Choi, J., Kotay, S. M., Goel, R. (2012) Water Environment & Technology. (June), 38-41

Abstract: Widely used to treat municipal and industrial wastewater, membrane bioreactors (MBRs) frequently experience biofouling, the result of excessive growth of biofilm-forming bacteria. Biofouling can cause such operational problems as loss of filtration efficiency, pipe corrosions and impaired water quality. Although methods exist for controlling biofouling in MBRs, these methods have shown certain limitations. Seeking an improved method for addressing the problem of biofouling, researchers at the University of Utah (Salt Lake City) conducted a feasibility study to assess the possibility of using lytic bacteriophages-viruses that infect bacteria and cause lysis, meaning that the bacteria break open, to control growth of the microorganisms responsible for biofouling within MBRs. The study found that using bacteriophages, or "phages" as they are commonly known, holds promise for reducing biofilm formation in MBRs.

Conferences

Jeongdong Choi, Shireen Meher Kotay Ramesh Goel (2011) Feasibility study on biocontrol of membrane biofouling using lytic bacteriophages. WEFTEC 2011 Oct 15-19. Los Angeles Convention Center, Los Angeles, CA, USA

Jeongdong Choi, Shireen Meher Kotay, Ramesh Goel (2011) Effect of various environmental stress factors on prophage induction in *Nitrosospira multiformis*-an ammonia oxidizer. US-KOREA conference 2011 Aug 10-14, Canyons Resort, Park City, Utah, USA

Shireen Meher Kotay, Jeongdong Choi, Ramesh Goel (2010) Phage therapy for filamentous bacteria: A novel direction to address biomass bulking in activated sludge systems. WEFTEC 2010 Oct 2-6. New Orleans Morial Convention Center, New Orleans, Louisiana, USA

Shireen Meher Kotay, Jeongdong Choi, Ramesh Goel (2010) Phage biocontrol to mitigate filamentous bulking: Application of novel bacteriophages infecting filamenous bacteria, *Spherotilus natans* and *Haliscomenobacter hydrossis*. ASM 110th General meeting May 23-27, San Diego, CA, USA

Jeongdong Choi, Shireen M Kotay, Tania Datta, Ramesh Goel (2009) Dynamics of bacteriophages (viruses) in biological nutrient removing activated sludge processes-correlating viral abundance with performance. WEFTEC 2009 Oct 10-14. Orange County convention center, Orlando, Florida.

APPENDIX

CALCULATION FOR COST ANALYSIS

Table A. 1 Cost comparison of phage-based biocontrol and chlorination

Chlorination [1)]			
Plant capacity (mgd)	**Chlorine price ($/ton)**	**Labor cost ($/hr)**	
50	491	28	
Isolation of lytic bacteriophage			
Item	**Price ($)**	**Qt in item**	**Unit price ($)**
0.4 µm filter	66.29	100	0.663
0.2 µm filter	136.91	50	2.738
Petri dish plate	231.7	300	0.772
media			0.275
Agar media			0.162
Phage stock production			
Item	**Unit price ($/gram)**	**Gram used in 1L (g)**	**Cost in 1 L ($)**
Tripticase soy broth without dextrose	0.16	0.25	0.04
Casitone	0.314	0.75	0.235
Electricity (kwh) [2)]	-	-	0.05
Total cost (isolation and phage stock, $)	-	-	**4.5**

1) Information for chlorination was obtained from Central Valley Water Reclamation Facility (CVWRF).

2) Electrical use includes centrifugation and autoclave.

Calculation for cost analysis

For chlorination dose:

$Q \times C = 1.92 \times 10^8$ L/d × 10 mg/L = 1.92×10^9 mg/d = 1.92 tons/d

(10 mg/L Chlorine is used for sludge bulking control, Bitton, 2005)

1.92 tons/d × 491 $/ton = **942.72 dollars/d for Chlorine dose.**

For phage application:

Assuming that *H. hydrossis* exists 40% in VSS. (VSS of sludge=2975 mg/L from CVWRF), phage stock concentration = 10^{12} virus/mL, and phage to host ratio (PHR)= 1:1000,

The amount of *H. hydrossis* in VSS = 1.92×10^8 L/d × 2975 mg/L × 0.4 × (1.6×10^8 Cells/mg VSS)

= 3.8×10^{19} Cells/d

(Here, 1.6×10^8 Cells/mg VSS is experimental value.)

Applying PHR, (3.8×10^{19} Cells/d)/1000 = 3.8×10^{16} Cells/d (This is the amount of virus added for phage-based biocontrol).

Determine the amount of phage stock used in wastewater treatment plant,

$Q \times C = Q \times 10^{15}$ virus/L = 3.8×10^{16} virus/d

Q = 38 L/d

38 L/d × (4.5 $/1L-phage) = **171 dollars/day for phage stock dose.**

The cost of labor was not included in this calculation.

REFERENCES

Ackerman, H.W., DuBow, M.S., 1987. Viruses of Prokaryotes: General Properties of Bacteriophages. CRC Press, Boca Raton, Florida.

Ackermann, H. W., 2001. Frequency of morphological phage descriptions in the year 2000. Brief review. Arch. Virol. 146, 843–857.

Alisky, J., Iczkowski, K., Rapoport, A., Troitsky, N., 1998. Bacteriophages show promise as antimicrobial agents. J. Infect. 36, 5-15.

Avrahami, S., Bohannan, B.J.A., 2007. Response of Nitrosospira sp. strain AF-like ammonia oxidizers to changes in temperature, soil moisture content, and fertilizer concentration. Appl. Environ. Microbiol. 73, 1166-1173.

Balding, C., Bromley, S. A., Pickup, R. W., Saunders, J. R., 2005. Diversity of phage integrases in Enterobacteriaceae: development of markers for environmental analysis of temperate phages. Environ. Microbiol. 7, 1558–1567.

Beiderbeck, V.O., Campbell, C.A., Ukrainetz, H., Curtin, D., Bouman, O.T., 1996. Soil microbial and biochemical properties after ten years of fertilization with anhydrous ammonia. Can. J. Soil Sci. 76, 7-14.

Bergh, O., Borsheim, K. Y., Bratbak, G., Heldal, M., 1989. High abundance of viruses found in aquatic environments. Nature 340, 467-468.

Beutel, M.W., Horne, A.J., 1999. A review of the effects of hypolimnetic oxygenation on lake and reservoir water quality. Lake and Reserv. Manage. 15(4), 285-297.

Bitton, G., 1987. Fate of bacteriophage in water and wastewater treatment plants, p 181-195, In: Phage Ecology, Goyal et al., Eds., Wiley, N.Y.

Bitton, G., 2005. Wastewater Microbiology, John Wiley & Sons, INC., Hoboken, New Jersey.

Blum, D.J.W., Speece, R.E., 1991. A database of chemical toxicity to environmental bacteria and its use in interspecies comparisons and correlations. J. Water Pollut. Control Fed. 63, 198-207.

Breitbart, M., Rohwer, F., 2005. Here a virus, there a virus, everywhere the same virus? Trends in Microbiol. 13, 278-284.

Brussow, H., 2007. Phage Therapy: The Western Perspective. in S. McGrath and D. van Sinderen (eds.) Bacteriophage: Genetics and Molecular Biology, Caister Academic Press, Norfolk, UK. ISBN 978-1-904455-14-1.

Bull, J.J., Levin B.R., DeRouin T., Walker N., Bloch C.A., 2002. Dynamics of success and failure in phage and antibiotic therapy in experimental infections. BMC Microbiol. 2,35.

Burgess, J. E., Stuetz, R. M., Stehpeoson, T., 2002. Dinitrogen oxide detection for process failure early warning systems. Water Sci. Technol. 45, 247-254.

Caldeira, J. C., Peabody, D. S., 2007. Stability and assembly in vitro of bacteriophage PP7 virus-like particles. J. Nanobiotechnol. 5, 10.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A., Bru¨ssow, H., 2003. Prophage genomics. Microbiol Mol. Biol. Rev. 67, 238-276.

Caravelli, A., Giannuzzi, L., Zaritzky, N., 2007. Inhibitory effect of a surfactant on pure cultures of a filamentous and a floc forming micro-organism. Environ. Technol. 28, 137-146.

Casjens, S.R., 2008. Diversity among the tailed-bacteriophages that infect the enterobacteriaceae. Research in Microbiol. 159 (5), 340-348.

Casjens, S., 2005. Comparative genomics and evolution of the tailed-bacteriophages. Curr. Opinion Microbiol. 8:1-8.

Casjens, S., 2003. Prophages and bacterial genomics: what have we learned so far? Mol. Microbiol. 49 (2), 277-300.

Cochran, P.K., Kellogg, C.A., Paul, J.H., 1998. Prophage induction of indigenous marine lysogenic bacteria by environmental pollutants. Mar. Ecol. Prog. Ser. 164, 125-133.

Cole, D., Long, S.C., Sobsey, M. D., 2003. Evaluation of F+ RNA and DNA coliphages as source specific indicators of fecal contamination in surface waters. Appl. Environ. Microbiol. 69 (11), 6507-6514.

da Motta, M., Pons, M.N., Roche, N., 2002. Study of filamentous bacteria by image analysis and relation with settleability. Water Sci. Technol. 46, 363-369.

da Motta M, Pons MN, Roche N., 2003. Monitoring filamentous bulking in activated sludge systems fed by synthetic or municipal wastewater. Bioprocess Biosyst. Eng. 25, 387-393.

Daigger, G. T., Robbins, M. H., Marshall. B. R., 1985. The design of a selector to control low F/M filamentous bulking. Water Pollution Control Federation. 57 (3), 220-226.

Dangcong, P., Bernet, N., Moletta, R., 2000. Effects of oxygen supply methods on the performance of a sequencing batch reactor for high ammonium nitrification. Water Environ. Res. 72, 195-200.

Delbruck, M., Luria, S. E., 1942 Interference between bacterial viruses. I. Interference between two bacterial viruses acting upon the same host, and the mechanism of virus growth. Arch. Biochem. 1, 111-141.

Dixon, B., 2004. New dawn for phage therapy. Lancet Infect. Dis. 4,186.

Duckworth, D. H., 1976. "Who discovered bacteriophage?" Bacteriol. Rev. 40**,** 793–802.

Echols, H., 1972. Developmental pathways for the temperate phage: lysis vs lysogeny. Annu. Rev. Genet. 6, 157-190.

Eikelboom, D.H., 1975. Filamentous organisms observed in activated sludge. Wat. Res. 9, 365-388.

Eikelboom, D.H., 1977. Identification of filamentous organisms in bulking activated sludge. Progr. Water Technol. 8, 153-161.

Eikelboom, D.H., van Buijsen, H.J.J., 1981. Microscopic Sludge Investigation Manual. TNO Res. Inst. for Env. Hygiene, Delft, The Netherlands.

Eikelboom, D.H., 2006. Identification and Control of Filamentous Microorganisms in Industrial Wastewater Treatment Plants. IWA Publishing, London, UK.

Ellis, E.L., Delbru¨ ck, M., 1939. The growth of bacteriophage. J. Gen. Physiol. 22, 365-384.

Ewert, D.L., Paynter, M.J., 1980. Enumeration of bacteriophages and host bacteria in sewage and the activated sludge treatment process. Appl. Environ. Microbiol. 39, 576-583.

Farahbakhsh, K., Smith, D.W., 2004. Removal of coliphages in secondary effluent by microfiltrationdmechanisms of removal and impact of operating parameters. Water Res. 38 (3), 585-592.

Fuhrman, J. A. 1999. Marine viruses and their biogeochemical and ecological effects. Nature 399, 541–548.

Grady, C.P.L.Jr., Daigger, G. T., Lim, H.C., 1999. Biological Wastewater Treatment 2nd edition. Marcel Dekker, Inc. Newyork.

Hantula, J., Kurki, A., Vuoriranta, P., Bamford, D., 1991. Ecology of bacteriophages infecting activated sludge bacteria. Appl. Environ. Microbiol. 57, 2147-2151.

Havelaar, A.H., Butler, M., Farrah, S.R., Jofre, J., Marques, E., Ketratanakul, A., Martins, M.T., Ohgaki, S., Sobsey, M.D., Zaiss, U., 1991. Bacteriophages as model viruses in water quality control. Water Res. 25 (5), 529-545.

Hayatsu, M., Kosuge, N., 1993. Autotrophic nitrification in acid tea soils. Soil Sci. Plant Nutr. 39, 209-217.

Herskowitz, I., Hagen, D., 1980. The lysis-lysogeny decision of phage lambda: explicit programming and responsiveness. Annu. Rev. Genet. 14, 399-455.

Hertwig, S., Popp, A., Freytag, B., Lurz, R., Appel, B., 1999. Generalised transduction of small yersinia enterocolitica plasmids. Appl. Environ. Microbiol. 65, 3862-3866.

Holmfeldt, K., Middelboe, M., Nybroe, O., Riemann, L., 2007. Large variabilities in host strain susceptibility and phage host range govern interactions between lytic marine phages and their *Flavobacterium* hosts. Appl. Environ. Microbiol. 73, 6730-6739.

Jenkins, D., Richard, M.G., Daigger, G.T., 1993 Manual on The Causes and Control of Activated Sludge Bulking and Foaming, 2nd ed. Lewis Publishers, Chelsea, MI, 193 p.

Jensen, E.C., Schrader, H.S., Rieland, B., Thompson, T.L., Lee, K.W., Nickerson, K.W., Kokjohn, T.A., 1998. Prevalence of broad-hostrange lytic bacteriophages of Sphaerotilus natans, Escherichia coli, and Pseudomonas aeruginosa. Appl. Environ. Microbiol. 64, 575-580.

Jiang, S.C., Paul, J.H., 1998. Gene transfer by transduction in the marine environment. Appl. Environ. Microbiol. 64, 2780-2787.

Kampfer, P., 1995. Physiological and chemotaxonomic characterization of filamentous bacteria belonging to the genus *Haliscomenobacter*. Syst. Appl. Microbiol. 18, 363-367.

Khan, M.A., Satoh, H., Katayama, H., Kurisu, F., Mino, T., 2002a. Bacteriophages isolated from activated sludge processes and their polyvalency. Water Res. 36, 3364-3370.

Khan, M.A., Satoh, H., Mino, T., Katayama, H., Kurisu, F., Matsuo, T., 2002b. Bacteriophageehost interaction in the enhanced biological phosphate removing activated sludge system. Water Sci. Technol. 46, 39-43.

Kelly II, R.T., Henriques, I.D.S., Love, N.G., 2004. Chemical inhibition of nitrification in activated sludge. Biotechnol. Bioeng 85, 683-694.

Kim, Y.M., Park, D., Lee, D.S., Park, J.M., 2008. Inhibitory effects of toxic compounds on nitrification process for cokes wastewater treatment. J. Hazard. Mater. 152, 915-921.

Kotay SM, Datta T, Choi J, Goel R., 2011. Biocontrol of biomass bulking caused by *Haliscomenobacter hydrossis* using a newly isolated lytic bacteriphage. Wat. Res. 45, 694-704.

Kowalchuk, G.A., Stephen, J.R., 2001. Ammonia oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol. 55, 485-529.

Kropinski, A.M., 2006. Biocontrol - everything old is new again. Can J. Infect Dis. Med. Microbiol. 17, 297-306.

Kutter and Sulakvelidze, 2004. Bacteriophages: biololgy and application CRC Press, Boca Raton, Florida.

Lee, S.E., Koopman, B., Bode, H., Jenkins, D., 1983. Evaluation of alternative sludge settleability indices. Water Res. 17, 1421-1426.

Levantesi, C., Beimfoh, C., Geurkink, B., Rossetti, S., Thelen, K., Krooneman, J., Snaidr, J., Van Der Waarde, J & Tandoi, V., 2004. Filamentous Alphaproteobacteria associated with bulking in industrial wastewater treatment plants. Syst. Appl. Microbiol. 27, 728–736.

Levin, B. R., Bull, J. J., 1996. Phage therapy revisited : the population biology of a bacterial infection and its treatment with bacteriophage and antibiotics. American Naturalist 147, 881-898.

Lou, I.C., de los Reyes III, F.L., 2008. Clarifying the roles of kinetics and diffusion in activated sludge filamentous bulking. Biotechnol. Bioeng. 101, 327-336.

Love, N.G., Bott, C.B., 2000. A Review and Needs Survey of Upset Early Warning Devices. Water Environment Research Foundation, Report No. 99-WWF-2. Alexandria, VA.

Love, N. G., Bott, C. B., 2002. Evaluating the role of microbial stress response mechanisms in causing biological treatment system upset. Water Sci. Technol. 46 (1-2), 11-18.

Madigan, M.T., Martinko, J.M., Parker, J., 2000. Brock: Biology of Microorganisms, ninth ed. Prentice-Hall International Limited, London.

Martins, A. M. P., Pagilla, K., Heijnen, J. J., van Loosdrecht, M. C. M., 2004. Filamentous bulking sludge – a critical review. Water Res. 38, 793–817.

Madoni, P., Davoli, D., Gibin G., 2000. Survey of filamentous microorganisms form bulking and foaming activated sludge plants in Italy. Water Res. 34 (6), 1767-1772.

McDaniel, L., Paul, J.H., 2005. Effect of nutrient addition and environmental factors on prophage induction in natural populations of marine Synechococcus species. Appl. Environ. Microbiol. 71, 842-850.

Meyn, M.S., Rossman, T., Troll, W., 1977. A protease inhibitor blocks SOS functions in Escherichia coli: antipain prevents lambda repressor inactivation, ultraviolet mutagenesis, and filamentous growth. Proc. Natl. Acad. Sci. U S A. 74, 1152-1156.

Nakai, T., Park, S.C., 2002. Bacteriophage therapy of infectious diseases in aquaculture. Res. Microbiol. 153,13-18.

Neufeld, R., Greenfield, J., Rieder, B., 1986. Temperature, cyanide and phenolic nitrification inhibition. Water Res. 20, 633-642.

Norton, J.M., Klotz, M.G., Stein, L.Y., Arp, D.J., Bottomley, P.J., Chain, P.S., Hauser, L.J., Land, M.L., Larimer, F.W., Shin, M.W., Starkenburg, S.R., 2008. Complete genome sequence of Nitrosospira multiformis, an ammonia-oxidizing bacterium from the soil environment. Appl. Environ. Microbiol. 74, 3559-3572.

Norton, J.M., Alzerreca, J.J., Suwa, Y., Klotz, M.G., 2002. Diversity of ammonia monooxygenase operon in autotrophic ammonia oxidizing bacteria. Arch Microbiol. 177, 133-149.

Otawa, K., Lee, S.H., Yamazoe, A., Onuki, M., Satoh, H., Mino, T., 2007. Abundance, diversity, and dynamics of viruses on microorganisms in activated sludge processes. Microb. Ecol. 53, 143-152.

Painter, H.A., 1986. Nitrification in the treatment of sewage and wastewaters. Spec. Publ. Soc. Gen. Microbiol. 20, 185-213.

Park, S., Ely, R.L., 2008. Candidate stress genes of Nitrosomonas europaea for monitoring inhibition of nitrification by heavy metals. Appl. Environ. Microbiol. 74, 5475-5482.

Proctor, L. M., 1997. Advances in the study of marine viruses. Microsc. Res. Tech. 37, 136–161.

Rittmann, B.E., McCarthy, P.L., 2001. Environmental Biotechnology: Principles and Applications. McGraw-Hill, New York.

Schuster, H., Beyersmann, D., Mikolajczyk, M., Schlicht, M., 1972. Prophage induction by high temperature in thermosensitive DNA mutants lysogenic for bacteriophage lambda. J. Virol. 11 (6), 879-885.

Se´ ka, M.A.,Y. Kalogo., F. Hammes., J. Kielemoes, Verstraete, W., 2001. Chlorine-susceptible and chlorine-resistant type 021N bacteria occurring in bulking activated sludges. Appl. Environ. Microbiol. 67, 5303-5307.

Se´ ka, M.A., Hammes, F., Verstraete, W., 2003. Predicting the effects of chlorine on the micro-organisms of filamentous bulking activated sludges. Appl. Microbiol. Biotechnol. 61, 562-568.

Shammas, N.K., 1986. Interactions temperature, ph, and biomass on the nitrification process. J. Water Pollut. Control Fed. 58, 52-58.

Stasinakis, A.S., Thomaidis, N.S., Mamais, D., Papanikolaou, E.C., Tsakon, A., Lekkas, T.D., 2003. Effect of chromium (VI) addition on the activated sludge process. Water Res. 37, 2140-2148.

Sulakvelidze, A., Alavidze, Z., Morris, J. G., Jr., 2001. Bacteriophage therapy. Antimicrob. Agents. Chemother. 45, 649–659.

Suttle, C.A., 2005.Viruses in the sea. Nature 437, 356-361.

Synnott, A.J., Kuang, Y., Kurimoto, M., Yamamichi, K., Iwano, H., Tanji, Y., 2009. Isolation from sewage influent and characterization of novel Staphylococcus aureus bacteriophages with wide host ranges and potent lytic capabilities. Appl. Environ. Microbiol. 75, 4483-4490.

Talaro, K.P., 2005. Foundations in Microbiology, fifth ed.. McGraweHill, New York, NY.

Thomas, J.A., Soddell, J.A., Kurtboke, D.I., 2002. Fighting foam with phages? Water Sci. Technol. 46, 511-518.

Uchiyama, J., Rashel, M., Matsumoto, T., Sumiyama, Y., Wakiguchi, H., Matsuzaki, S., 2009. Characteristics of a novel Pseudomonas aeruginosa bacteriophage, PAJU2, which is genetically related to bacteriophage D3. Virus Res. 139, 131-134.

van Veen, W.L., van der Kooij, D., Geuze, E.C., van der Vlies, A.W. 1973., Investigations on sheathed bacterium Haliscomenobacter hydrossis gen. nov., sp. nov., isolated from activated sludge. Anton Van Leeuwenhoek 39, 207-216.

Vazquez, I., Rodrıguez, J., Maranon, E., Castrillon, L., Fernandez, Y., 2006. Study of aerobic biodegradation of coke wastewater in a two and three-step activated sludge process. J. Hazard. Mater. 137, 1681-1688.

Verma, V., Harjai, K., Chhibber, S., 2009. Characterization of a T7- like lytic bacteriophage of Klebsiella pneumoniae B5055: a potential therapeutic agent. Curr. Microbiol. 59, 274-281.

Wagner, P.L., Waldor, M.K., 2002. Bacteriophage control of bacterial virulence. Infect. Immun. 70 (8), 3985-3993.

Wanner, J., Ruzickova, I., Krhutoka, O., Probyl, M., 2004. Activated sludge population dynamics and wastewater treatment plant design and operation. Water Sci. Technol. 41, 217-225.

Watson, S.W., Bock, E., Harms, H., Koops, H.-P., Hooper, A.B., 1989. Nitrifying bacteria. In: Staley, J.T., Bryant, M.P., Pfennig, N., Holt, J.G. (Eds.), Bergey's Manual of Systematic Bacteriology, vol. 3. The Williams and Wilkins Co., Baltimore, Md, pp. 1808-1834.

Weinbauer, M.G., 2004. Ecology of prokaryotic viruses. FEMS Microbiol. Rev. 28, 127-181.

Williams, T.M., Unz, R.F., 1985. Isolation and characterization of filamentous bacteria present in bulking activated sludge. Appl. Microbiol. Biotechnol. 22, 273-282.

Winston, V., Thompson, T.L., 1979. Isolation and characterization of a bacteriophage specific for Sphaerotilus natans which contains an unusual base in its deoxyribonucleic acid. Appl. Environ. Microbiol. 37, 1025-1030.

Withey, S., Cartmell, E., Avery, L.M., Stephenson, T., 2005. Bacteriophages e potential for application in wastewater treatment processes. Sci. Total Environ. 339, 1-18.

Wommack, K.E., Colwell, R.R., 2000. Virioplankton: viruses in aquatic ecosystems. Microbiol. Mol. Biol. Rev. 64, 69-114.

Wu, Q., Liu, W., 2009. Determination of virus abundance, diversity and distribution in a municipal wastewater treatment plant. Water Res. 43, 1101-1109.

Xie, B., Dai, XC., Xu, YT., 2007. Cause and pre-alarm control of bulking and foaming by *Microthrix parvicella* - a case study in triple oxidation ditch at a wastewater treatment plant. J. Hazard. Mat. 143, 184-191.

You, S.J., Tsai, Y.P., Huang, R.-. Y., 2009. Effect of heavy metals on nitrification performance in different activated sludge process. J. Hazard. Mater. 165, 987-994.

Ziegler, M., Lange, M., Dott, W., 1990. Isolation and morphological and cytological characterization of filamentous bacteria from bulking sludge. Water Res. 24, 1437-1451.