Isolation, Characterisation and Preservation of Bacteriophages for Use in Treatment of *Aeromonas hydrophila* Infection in Finfish

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A thesis submitted in fulfilment of the requirements of
the degree of Doctor of Philosophy

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Australia

March 2022

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Abstract

Problems caused by multidrug resistant bacteria and the limited number of control strategies, highlight the need for alternative control approaches against bacterial infections in the aquaculture sector. Bacteriophages (phages) demonstrated promising results as therapeutic tools, for the effective control of bacterial diseases in aquaculture, as well as in other production animals, companion animals and humans. In the present study, a range of investigations were undertaken to assess suitability of using lytic phages in controlling *Aeromonas hydrophila* infection in fish aquaculture. *A. hydrophila* is a fish pathogen and responsible for economic damages to the aquaculture industry. The main focus of this study was to develop and use a suitable model to test the efficacy of a phage therapy by addressing the concerns associated with the potential limitations of the therapy, such as required doses and delivery methods, characterisation at the genomic level, storage methods, and the phage therapy effect, potential immune response, and effects on fish in response to multiple phage treatments.

For the isolation of suitable *A. hydrophila* phages, wastewater was used as a source and ten different *A. hydrophila* strains were used as hosts to culture and screen potential phages with lytic ability. Before use in isolation, the bacterial strains were confirmed for any prophage induction using Mitomycin C assay. The water samples were filtered to retain only virus particles and enriched with *A. hydrophila* strains, filtered again to remove the bacterial hosts and then subjected to a series of plaque assays to screen potential lytic phages. The morphology of the isolated phages was characterised by Transmission Electron Microscopy (TEM); genetic organisation was determined by enzymatic digestions of their nucleic acids and lytic ability was determined by growth pattern while growing with their respective host bacteria.

Four selected phages were subjected to whole genome sequencing using Illumina technologies with a view to determining their phylogeny, adaptive evolution, biodiversity, potential virulence and presence of any toxin genes.

The isolated and selected phages were then subjected to a study to determine their shelf lives under long-term storage conditions as a solution and in a freeze-dried form. To store as a solution, effect of different concentrations of sucrose and trehalose on the bacteriophage titre were compared with no sugar treatments under 4 °C, -20 °C and -80

°C for 56 weeks. To store in a lyophilised form, bacteriophage solutions were prepared with and without sugars (sucrose and trehalose) and then freeze dried and stored at 4 °C and room temperature for 44 weeks. For both trials, bacteriophage titre was monitored weekly for first 8 weeks and then monthly until the end of the experiments. Any changes in phage morphology after lyophilisation was determined by TEM.

The therapeutic potential of isolated phages was assessed in fish trials with a native Australian fish, silver perch (*Bidyanus bidyanus*) against one strain of *A. hydrophila*. A cocktail of four selected phages was prepared. Different phage doses were determined based on different phage titres to be tested on live fish models infected with *A. hydrophila* to understand the effects of environmental conditions and dose response of the therapeutic mode. The fish infection trial was conducted in two different phases – preliminary pilot trial and actual infection challenge trial. The preliminary pilot trial utilised 15 fish intraperitoneal injected with different quantities of *A. hydrophila* and a control group with phosphate buffered saline (PBS). After 6 days, the infected fish were euthanized and organs were collected for bacterial load determination. In the actual infection challenge trial, 144 fish were employed in three groups – control (no treatment, only PBS); *A. hydrophila* infected fish treated with different doses of bacteriophage cocktails; and uninfected fish treated with different doses of bacteriophage cocktails. At the end of the trial, bacterial load was counted from spleen and kidney and histopathology conducted on various fish organs. Fish blood was analysed for presence/absence of *A. hydrophila* and to determine lysozyme activity and IgG levels as indicators of immunological response to bacteria or the bacteriophage.

Screening for potential *A. hydrophila* phages with lytic ability resulted in the selection of four phages, coded as Ah6_G, Ah9_PP, Ah10_Se and Ah10_S. The isolated phages demonstrated complete lysis against their respective hosts and contained non-contractile tails with icosahedral heads belonging to *Siphoviridae* family (Ah6_G, Ah10_Se and Ah10_S), and icosahedral heads with short tail belonging to *Podoviridae* family (Ah9_PP). All the isolated phages contained dsDNA of > 10 Kb as their genetic materials. The whole genome analysis revealed that all these four phages are closely related to Ahp1 podovirus in terms of their genetic similarity.

The whole genome sequencing of the selected phages confirmed that all the isolates contained typical phage protein coding genes, ds DNA and smaller sizes of genomes of

Ah6_G (circular), Ah10_Se (linear) and Ah10_S (linear) (47-61 Kb) than other *Siphoviridae* phages, However, size of the genome of Ah9_PP (41 Kb, circular) was similar to other *Podoviridae* phages. Two of the phages (Ah10_Se and Ah10_S) were identified as novel linear dsDNA phages with protein coding sequences having no similarities found in the existing phage databases.

The phage shelf life study revealed that solutions of the four isolated phages could be preserved without any sugar stabilizers for 56 weeks at both 4 ℃ and -80 ℃ with no significant reduction in their titre. However, -20 ℃ was found to be less effective temperature in maintaining shelf lives of phages Ah6_G and Ah9_PP. Interestingly, the shelf life of these two phages at -20 ℃ were significantly improved when sucrose or trehalose added, sucrose being the better protectant. Nevertheless, the sugars did not have any adverse effects on their shelf lives over the experimental periods. When lyophilised, a small reduction in titre of Ah10_Se and Ah10_S was observed which was improved by adding sucrose and trehalose. Exclusion of sugars during lyophilisation caused disruption of heads and tails of the viruses. In the presence of sucrose or trehalose, the lyophilised phages were more stable when stored at 4 ℃ compared to room temperature. Nontheless, the presence of sucrose or trehalose provided better stability to the lyophilised phages at either temperatures. However, storing the phages in solution was proved to be better than lyophilisation over the experimental periods.

In the fish trial to assess therapeutic potential of isolated phages, a phage cocktail was used to treat *A. hydrophila* infections in silver perch fish model. Unfortunately, when purchased the fish had already been exposed to *A. hydrophila*. A medium dose of phage cocktail significantly reduced the background *A. hydrophila* count in the kidney and spleen of the fish group that did not receive intraperitoneal bacterial inoculation. When the fish were deliberately infected with exogenous *A. hydrophila* on top of the existing colonisation with *Aeromonas* sp., there were no significant differences in bacterial counts when challenged with any of the doses of phages. No significant immune response was observed during the challenge trial. The comparative histopathology study of different tissues of the experimental fish revealed very few significant changes that are consistently associated with *A. hydrophila* infection in teleost fish.

This thesis provides new information on the properties of phages able to kill *A. hydrophila* and that have potential for use as treatments in controlling *A. hydrophila*

infection in fish. Further studies are required to conduct larger scale fish trials involving different species of fish to expand, validate and implement the knowledge and approaches generated in this study.

Thesis declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Farida Mazumder

March 2022

Adelaide, Australia.

Acknowledgements

First and foremost I thank the Almighty for His showers of blessings throughout the immense journey of my PhD candidature.

I would like to express my deep and sincere gratitude to my principal supervisor Professor Mary Barton for her conscientious support through this long journey. Her mentoring power kept me motivated in all the ups and downs, especially when I came back to write the thesis after a long break which I took after birth of my daughter Sareena. Mary never stopped keep her faith on me and always encouraged me with her positive words to keep me going. She was my shelter and guardian with whom I could share my concerns in those times when I felt dull and demotivated. Without Professor Barton's support, I wouldn't have completed this thesis.

I am forever grateful to Dr James Munro for trusting me to start this challenging project before he left The University of Adelaide. Thanks James for giving me an opportunity to start my PhD and help design the entire project.

All the fish trials and pathology studies were supported by my co-supervisor Associate Professor Stephen Pyecroft. His positive vibes and laughter made it easy to do the laborious task of fish experiments.

My co-supervisor Associate Professor Farhid Hemmatzadeh trained me with virology and all the molecular biology methods. Despite of his hectic teaching and research involvement, he always welcomed me in his office with a wide smile and gave time as much I needed to learn bioinformatics. When I first started my experiments he allowed me to work in his lab space. His strong passion for research always inspired me.

I am thankful to Dr Timothy Barnes at the University of South Australia for supporting my storage studies and giving me the opportunity to carry out part of my experiments in his lab. He also helped me with his valuable critical comments while writing my storage chapter. I will always be grateful to Dr Susan Semple for letting me use her freeze drying machine and to Amanda Kenyon for supporting and training me to get used to the UNISA PC2 lab environment. I am grateful to Dr Lisa Elliott for providing me with bacterial strains. I was trained with basic microbiology techniques by Ken Lee from the veterinary diagnostic lab. This fun loving guy was always there for me whenever I needed. I would also like to mention Dr Rick Tearle for his outstanding support in the whole genome

analysis despite of his busy schedule. Thanks goes to Dr Michelle Hebart for assisting me with the statistical analysis for the fish trial data. I am grateful to Adelaide Microscopy for helping me take the images of my viruses.

My fish trial studies would not have been completed without hands on support from Ben Jones and Joe.

My PhD bestie Dr Liza Hasan – I want to write pages after pages about you….about your mentorship in my early PhD stages, your friendship since I first met you at Roseworthy… our never ending chats with coffee at feedlot…your yummy Malaysian Rendang! I will cherish our memories forever…

My PhD friends Sri, Dr Janet, Dr Yanhui, Entesar, Dr Jessica, Dr Vanessa, Dr Wei Yee, Dr Hue, Hanh, Hang, Indro, Dr Saad, Dr Sugi, Dr Vivek, Dr Abid, Dr Razi were a pivotal part of this journey and thanks guys for being around me in Tassie, feedlot, PC2 lab, fish lab and in the lunch parties.

I am privileged to have guidance and affection from my family friends Prof Stuart and Meredith who welcomed my family into their family and treated us with delicious foods every now and then! Prof Stuart has read the chapters critically which has helped to enrich the thesis and improve my writing skills.

Last but not the least, my dearest husband Rayhan (Dr Mahbub), whom I will be indebted to for my entire life. He was the only witness of all my PhD hurdles and my sleepless nights. He always would sit beside me and watch me like a hawk while I was writing my thesis even if it was 3 o'clock in the morning! He was the harshest critic of my writing which helped me to build up my knowledge of scientific writing.

My thesis writing took way longer than anticipated, as my sweet lovely baby girl Sareena arrived in this world and made my life even more complete and meaningful. Her every little footstep also inspired me to finish my thesis writing and keep going.

My Journey won't be worth it without them. I dedicate this thesis to my parents – my mother Nelly Mazumder and my father Dr Quamrul Hasan Mazumder who dreamt and kept faith on me that I will pursue my PhD one day. I would like to thank my one and only sister Ainura Mazumder and my brother-in-law Rashidul Hasan for being there always. I thank God for all of your existence without whom I won't be who I am today.

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Abbreviations

%	percent
g	relative centrifugal force
°C	degree Celsius
µg	microgram
µl	microliter
µm	micrometre
aa	amino acid
ABR	antibiotic resistance
AH	*Aeromonas hydrophila*
AMB	*Aeromonas* medium base
ARGs	antibiotic resistance genes
Bac	bacteria
BLAST	Basic Local Alignment Search Tool
bp	base pair
CDS	coding region
cfu	colony forming unit
cm	centimetre
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
ds	double stranded
EDTA	ethylene diamine tetra acetic acid
FD	freeze dry
G	Glenelg
g	gram
h	hour
H&E	haematoxylin and eosin
HGT	horizontal gene transfer
HP	high dose phage
Ig	immunoglobulin
IP	intraperitoneal
kb	kilo base

kg	kilogram
kV	kilo volt
l	litre
LP	low dose phage
M	molar
MAS	motile Aeromonas septicaemia
mbar	millibar
MDR	multidrug resistant
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mM	millimolar
MMC	melanomacrophage centres
MMC	mitomycin C
MP	medium dose phage
NCBI	National Centre for Biotechnology Information
ng	nanogram
nm	nanometre
O/F test	oxidative/fermentation glucose test
OD	optical density
PBS	phosphate buffered saline
PEG	polyethylene glycol
Per os	by way of the mouth/oral
pfu	plaque forming unit
PP	primary sedimentation tank
q	query
RNA	ribonucleic acid
rpm	rotation per minute
RT	room temperature
S	screening tank
s	subject
Se	sewage

SE	standard error
sp.	species
ss	single stranded
TBE	tris borate EDTA
TEM	transmission electron microscopy
TMV	tobacco mosaic virus
TSA	tryptic soy agar
TSB	tryptic soy broth
V	volt

Chapter 1. Introduction

1.1. Background

Aquaculture is the fastest growing industry within the agriculture sector. With the increasing demand for commercial aquaculture production, which is an important source of nutrition and livelihood of millions of people around the developed and developing countries, there is a need to develop sustainable technology for the control of infectious diseases in the industry. Bacterial infections are considered a significant constraint to aquaculture production as they represent one of the major causes of heavy financial loss and lack of sustainability in this sector. The common method of controlling bacterial infections is the use of antimicrobial therapy. However, the widespread use of feeds supplemented with antibiotics in the aquaculture industry has resulted in the emergence of drug-resistant bacterial strains. The emergence of multidrug resistant bacterial strains limit the use of antibiotics as well as exerting a deleterious effect on the entire ecosystem. Due to international and local legislation in controlling the use of antibiotics and their residual presence in human food, antibiotics are becoming less popular with the producers. Vaccination has been found to be effective in controlling several bacterial diseases. However, there are some limiting factors such as, bacterial heterogeneity and the culture conditions of aquatic organisms, which hinders the widespread use of vaccines as a bacterial control strategy. Therefore, development of alternative, sustainable and eco-friendly infection control strategies is necessary to minimize the mortality in aquaculture, as well as to reduce the emergence of drug-resistant pathogens. The use of bacteriophages to control bacterial infections has been recognised as one of the alternative control approaches.

Based on the published examples of therapeutic and prophylactic applications of bacteriophages, phage therapy seems very promising in the aquaculture sector. However, research is still needed to develop sustainable therapeutic models for the aquaculture industries. It has been reported that bacteriophages can survive for a long period of time in both fresh and marine environments (12 to 91 days) with a moderate impact on the overall bacterial community, thus they have the potential to be used as to control infectious diseases. The majority of phage-based research has focused on *in vitro* phage use to reduce bacterial concentration. As some bacteriophages already gained regulatory acceptance, phage therapy has already become a reality but an effective therapeutic model for aquaculture is yet to be developed. Therefore, more studies need to be undertaken to

fully understand phage-bacterium interactions; and fully prove advantages of phage therapy as a biocontrol agent against bacterial infection in aquaculture.

The present study was therefore undertaken to develop a sustainable bacteriophage therapeutic or prophylactic model to test phage therapy efficacy with a view to controlling bacterial infection in aquaculture, which will enable a better understanding of phage therapy to be used within the aquaculture sector.

1.2. Aims of the current research

Finfish production is one of the major contributors in the aquaculture sector. There is a wealth of information available in the literature on the use of bacteriophages to control bacterial infection in finfish aquaculture. However, research is still underway to know how phages interact with bacteria in the aquatic systems as there are some constraints associated with the use of bacteriophages in organisms living in aquatic systems. Therefore, the aim of the present study was to develop a therapeutic tool using bacteriophages specific for *Aeromonas hydrophila*, a fish pathogen. Silver perch (*Bidyanus bidyanus*), a native Australian finfish was used in the present research as a model animal. The research presented in this thesis has four important aspects –

a) **Isolation, identification and characterisation of *A. hydrophila* bacteriophages.**
    - Isolation and purification of *A. hydrophila* bacteriophages from different wastewater samples;
    - Assessment of their lytic ability against specific *A. hydrophila* strains;
    - Characterisation of phages based on their nucleic acid types (restriction enzyme digestion) and morphological features (TEM).
b) **Whole genome sequencing of four experimental bacteriophages.**
    - Analysis of four isolated bacteriophage samples at a genomic level;
    - Assembly and comparison of each of the four phage genomes by *de novo* assembly;
    - Comparison of the sequences obtained with all known phage genomes present in the database.
c) **Bacteriophage storage method development.**

- Evaluation of the effect of different temperatures (4 °C, -20 °C and -80 °C) on phage storage in the presence or absence of cryoprotectants (sucrose and trehalose) over time;
- Assessment of the impact of lyophilisation (freeze drying) on phages in the presence or absence of cryoprotectants (sucrose and trehalose) kept in two different storage conditions (4 °C and room temperature, RT: 25-27 °C) over time;
- Observation of the changes in phage morphology resulting from lyophilisation.

d) Bacteriophage efficacy study on silver perch fish model against *A. hydrophila* infection.

- Evaluation of the potential of an experimental bacteriophage cocktail in controlling *A. hydrophila* counts in kidney and spleen samples of the fish;
- Comparison of the responses of silver perch to different doses of the phage cocktail;
- Estimation of the effect of bacteria and bacteriophage on fish (silver perch) lysozyme activity and total immunoglobulin level;
- Assessment of the histopathological changes of the tissues of infected and treated fish.

1.3. Structure of the thesis

The thesis is composed of eight chapters including introduction, review of literature, general materials and methods, experimental chapters presenting results and discussions, conclusion and future direction.

Chapter 2 presents a critical review of the existing research on bacteriophages, their structure and classification, different modes of lifecycle and application on different farmed animals including aquatic species. This chapter also critically analyses the need for establishing an alternative therapeutic tool using bacteriophages to control bacterial communities in the aquaculture sector. The research gaps are addressed and rationale of the research stated.

Chapter 3 describes general materials and methods used and applied to carry out the experiments. This section is sub divided into three sections to outline the methods used

for – a) bacterial culture investigations; b) bacteriophage isolation and purification and c) fish trial based general investigations.

Chapter 4 reports the preliminary studies conducted for the selection, isolation and identification of appropriate bacteriophages specific for different strains of *A. hydrophila* Classification of four bacteriophages based on their morphology (as observed under TEM) and restriction enzyme digestion patterns was also described here. This chapter further illustrates the *in vitro* lytic ability of the four bacteriophages against their respective host strains.

Chapter 5 describes the whole genome sequencing of the four bacteriophage samples. A comparative analysis of the whole genome of the phages was performed against other known and published phage genomes present in the database.

Chapter 6 describes the experiments conducted to monitor bacteriophage shelf life under different temperature conditions. Two sugar cryoprotectants (sucrose and trehalose) were used and bacteriophage titres were determined after storage at different temperatures over a one year period of time. Part of this chapter also describes bacteriophage lyophilisation and compares their titres before and after lyophilisation and storage at two different temperatures (4 °C and room temperature, RT: 25-27 °C).

Chapter 7 reports the results of an *in vivo* application of four phages in a live fish model. The efficacy of the phage cocktail on the test fish was determined. The appropriate bacterial and bacteriophage dose to be applied were determined through a pilot study. Then the actual fish trial was conducted following the pre-determined doses. The effect of bacteria and bacteriophage on fish lysozyme activity and total immunoglobulin level were also determined. Histopathology was used to evaluate damage to tissues resulting from the infection and bacteriophage application and the results discussed.

Chapter 8 concludes the thesis and summarises the major findings with suggestions for future research in the field.

Chapter 2. Review of literature

2.1. Introduction

Aquaculture is the cultivation of aquatic plants and animals in fresh, brackish or marine environments (Almeida et al., 2009, Bostock et al., 2010). The yield from wild fisheries has plateaued, with production from the wild fisheries appearing to have reached their maximum (Akinbowale et al., 2006). Due to this, the demand for commercial aquaculture has become of prime importance to supply the growing demand for aquatic food products (King, 2001). To secure sustainable development in the food production sector, aquaculture production has to grow to meet the increasing demand for seafood supplies (Bostock et al., 2010, Ozbay et al., 2014). Approximately 43% of the world's seafood supplies comes from the aquaculture industries, which is considered to be one of the world's fastest growing industries within the agricultural sector (Verschuere et al., 2000, Cabello, 2006, Akinbowale et al., 2006, Almeida et al., 2009, Bostock et al., 2010, Silva et al., 2014a). According to the Food and agriculture Organization (FAO) (2018), the average growth rate of the world's aquaculture production was approximately 5.2% in 2011-2016. To meet the growing demand for protein for human food consumption, a total of around 598 different aquatic species items are being cultured worldwide as of 2016 (FAO, 2018). Furthermore, the income and livelihood of approximately 59.6 million people living in developed or developing countries depends on aquaculture. As of 2016, approximately 85% of world's total population involved in aquaculture and fisheries sectors were from Asia (FAO, 2018).

One of the major impeding factors for modern aquaculture farming operations has been the increasing frequency of outbreaks of infectious diseases. This causes substantial loss in the economy as well adversely impacting on aquatic animal health in many different countries (FAO, 2015, Kiron, 2012), with losses estimated globally to be several billion US dollars per annum (Defoirdt et al., 2011). Regardless of worldwide development of the aquaculture sector, diseases caused by microbial pathogens, have led to major financial losses for the aquaculture industries (Almeida et al., 2009, Oliveira et al., 2012).

Many of the infections in farmed fish and shellfish are similar to those that occur in humans and terrestrial animals (Nakai and Park, 2002, Oliveira et al., 2012). Farmed fish tend to have higher incidences of infection compared to the equivalent wild conspecifics because of their high stocking density and lack of adequate hygienic barriers. This contributes to deterioration of their immune system and thus increases the rate of disease

transmission (Almeida et al., 2009, Cabello, 2006, Defoirdt et al., 2011, Milla et al., 2010). Diseases in aquaculture develop because of interactions between pathogen, host and environment (Snieszko, 1974, Toranzo et al., 2005, Austin and Austin, 2007).

The most common pathogens responsible for water-borne diseases in aquaculture are bacteria, viruses, protists, helminths, oomycetes and sometimes fungi (Anderson, 1992, Shao, 2001, Almeida et al., 2009, Oliveira et al., 2012). Of these organisms, diseases of bacterial origin including multidrug resistant ones are the most significant (Meyer, 1991, Oliveira et al., 2012). Almost 34% of all infections are caused by bacteria and more than 150 species of bacteria have already been recognised to infect both wild and farmed fishery (Richards, 2014, Lafferty et al., 2015). These bacteria may be of indigenous or non-indigenous origin and some are multidrug resistant (Almeida et al., 2009, Oliveira et al., 2012, Mateus et al., 2014). The most common bacterial diseases includes vibriosis, furunculosis, enteric redmouth disease, columnaris disease, bacterial gill disease syndrome, bacterial kidney disease, Edwardsiellosis, bacillary necrosis. Clinical manifestations include death, septicaemia, cutaneous lesions and, destruction of the shell (Meyer, 1991). Major bacterial pathogens responsible for these diseases are *Vibrio* spp., *Streptococcus* spp., *Aeromonas* spp., *Photobacterium* spp., *Salmonella* spp., and *Escherichia coli* (Almeida et al., 2009). Some virulent bacteria such as some *Vibrio* spp. may cause up to 100% mortality in infected animals (Silva et al., 2014b). Bacterial diseases in aquatic environment are relatively common as it is easy for the pathogens to infect contact animals particularly under adverse environmental conditions such as overfeeding, high temperature, infrequent water exchange and irregular removal of sick and dead fish from farming sites (Almeida et al., 2009, Mateus et al., 2014).

2.2. Control measures for bacterial infections in aquaculture settings

2.2.1. Antibiotics

Since their discovery in 1940s, antibiotics have played a significant role in controlling bacterial infections in the aquaculture industry (Defoirdt et al., 2011, Oliveira et al., 2012). The “golden age” of antibiotics lasted for more than four decades until concerns related to resistance started to arise (Altamirano and Barr, 2019, Hassan et al., 2021). Antibiotics are normally used to control disease outbreaks and infections as a supplement in the food used in both intensive and semi-intensive environments (Mateus et al., 2014).

There are a number of reasons for the widespread use of antibiotics in aquaculture industry including the following:

- As a therapeutic tool or medication for sick and diseased animals
- Prophylactic use during the time of infection risk
- For the improvement of feed utilization
- Production enhancement and growth promotion (Mathew et al., 2007, Pratt et al., 2003)

However, concerns have arisen over the use of antimicrobial drugs in aquaculture because of the development of the antibiotic resistant strains, resulting in failure of the antibiotic treatment (Verschuere et al., 2000, Oliveira et al., 2012, Martínez-Díaz and Hipólito-Morales, 2013, Mateus et al., 2014, Chen et al., 2020). It is already well proven that approximately 75% of antibiotic drugs applied to animal body cannot be digested properly, thus remaining within the animal body and then passes through the environment (Chee-Sanford et al., 2009, Jassim and Limoges, 2014). Resistance to antibiotics can be transferred during bacterial replication and by horizontal gene transfer from resistant to sensitive bacteria which may then spread in the aquaculture system and possibly ultimately to humans (Verschuere et al., 2000).

The worldwide risk of antibiotic resistance is now very common because of the increasing rate of international trade of food products. As a result, there is an increased opportunity for antibiotic resistant bacteria to emerge in any environment around the world, hence antibiotic use in aquaculture also affects other sectors (Ishida et al., 2010). Moreover, rules and controls on the use of the amount of antibiotics vary widely in different countries and may lead to barriers in trade (Alderman and Hastings, 1998, Defoirdt et al., 2011). As a result of this greater awareness of antibiotic resistant bacteria and antibiotic residues, industry sectors are becoming more aware of the regulation of antibiotic use and the pressure to reduce antibiotic use (Defoirdt et al., 2011).

2.2.2. Vaccines

The use of vaccines to control diseases in aquaculture began in the 1970s (Shao, 2001). Some DNA vaccines have been found to show protective effect against some aquaculture bacterial pathogens such as *Vibrio* spp., *Streptococcus iniae*, *Edwardsiella tarda*, *Flavobacterium psychrophilum*, *Cryptocaryon irritans*, *Cryptobia salmocitica* and

Ichthyophthrius multifiliis (Hølvold et al., 2014). Vaccination has been found to be an effective and successful preventive measure against some bacterial infections but their availability is still very limited (Hølvold et al., 2014, Adams, 2019, Matsuura et al., 2019, Mohd-Aris et al., 2019, Mzula et al., 2019, Sommerset et al., 2005). Moreover, the costs associated with vaccine production and undesirable side-effects hinders the use of vaccine in aquaculture sector (Cipriano and Austin, 2006). Emergence of different types of infectious diseases over a variety of aquaculture organisms makes the use of vaccine more difficult (Nakai and Park, 2002). Moreover, some bacteria such as *Aeromonas hydrophila* exhibits biochemical and serological heterogeneity which hinders the production of a vaccine for the control of this pathogen (Le et al., 2018). Besides, the effectiveness of vaccination is influenced by factors like water temperature, the size of the fish, and the maintenance of sterility during handling for anaesthesia or intraperitoneal injection (Håstein et al., 2005). Vaccinated fish when exposed to disease were found to have a lower mortality than unvaccinated ones, but the exact mode of action of their immune system is not clear yet (Almeida et al., 2009).

2.2.3. Probiotics

The use of probiotics to combat bacterial diseases has been known since 1974 (Parker, 1974). Probiotics are a microbial means of improving the balance of the microbiota of the host organism (Gatesoupe, 1999, Wang et al., 2008, Tan et al., 2016). Intestinal tract, gills, skin mucus and the environment where the animal resides are all potential sources of probiotics in the aquaculture environment (Hai, 2015). Probiotics not only work to improve growth and health of animals, but also they were reported to improve reproduction and stress tolerance or act as an immunostimulant (Martínez Cruz et al., 2012). Different studies have already demonstrated the successful use of probiotics in controlling bacterial infections. However, despite the beneficiary outcomes, probiotics may cause difficulties if they are used excessively in the aquaculture sector due to the costs associated with their storage and application (Wang et al., 2008). In addition, the route of probiotics administration and adequate guidelines for proper dosage of probiotics requires further investigation (Jahangiri and Esteban, 2018, Hai, 2015). Probiotics can lead to increased microbial virulence through horizontal gene transfer or they can increase pathogenicity to human and other hosts (Pérez-Sánchez et al., 2014).

2.3. Use of bacteriophages as an alternative to control bacterial diseases

Although management practices as control measures have improved in recent times, bacterial infections still cause major losses in hatcheries and grow-out facilities, leading to between 70 to 90% mortality. Hence the use of bacteriophages as an alternative to chemotherapeutics can offer a more effective and sustainable technology to meet this need (Fernández et al., 2019, Sieiro et al., 2020, Kowalska et al., 2020, Ramos-Vivas et al., 2021).

2.4. What are bacteriophages?

The name bacteriophage came from the fusion of two words- “bacteria” and “phagein” (Greek origin) which implies that phages are capable of invading or eating bacteria (Sulakvelidze et al., 2001). Bacteriophages (or phages) are obligate bacterial parasites and have the ability to invade, multiply within the host bacteria and destroy them specifically by disrupting the cell wall, thus killing the bacteria within a few minutes (Hanlon, 2007, Huff et al., 2010, Wittebole et al., 2014, Rao and Lalitha, 2015, Penadés et al., 2015). Because of their bacteriolytic nature with no side effects to the host animal, bacteriophages have gained a great deal of interest to replace the use of antibiotics (Jun et al., 2014). Recently more than 6000 bacteriophages have been discovered and described from natural environments, among them 6196 are bacterial and 88 are archeal viruses (Wittebole et al., 2014). These bacteriophages can infect one or more different types of bacteria (Drulis-Kawa et al., 2012). Their bacteriolytic capacity makes them unique and a natural means of suppressing bacterial infections and also shaping the natural bacterial communities (Huff et al., 2010).

Bacteriophages are highly diverse and abundant in the environment and capable of controlling bacterial infections in both humans and animals (Penadés et al., 2015, Podlacha et al., 2021, Secor and Dandekar, 2020, Fernández et al., 2019). There is almost a limitless source of phages in the environment (Altamirano and Barr, 2019). They are extremely diverse and occupy a great variety of natural habitats in the biosphere where their specific hosts are available, but they mainly reside in topsoils, vegetation and aquatic systems (fresh and marine). Bacteriophages also occur within the microbial community of an animal or human body (Skurnik and Strauch, 2006, Ackermann, 2011, Drulis-Kawa et al., 2012, Wittebole et al., 2014). The number of phages is in direct proportion to the number of host bacteria present in their environment, usually higher

than the bacteria by a factor of ten. More than 10^{32} (10^4 to 10^8 virions per ml aquatic systems and 10^9 virions per g in soil) of bacteriophages are present in the environment at any given time, which is almost equivalent to a trillion per gram of sand grains (Furfaro et al., 2018). This estimation makes them the most abundant organism on earth (Weinbauer, 2004, Skurnik and Strauch, 2006, Hanlon, 2007, Ackermann, 2011, Orlova, 2012, Drulis-Kawa et al., 2012, Adesanya et al., 2020, Clokie et al., 2011). Thus bacteriophages play an important role in maintaining the microbial balance in the environment (Wittebole et al., 2014). Since their discovery, the fatal effect of bacteriophages on their host bacteria has been very much well-known (Haq et al., 2012b, Merril et al., 1996). They are useful for the treatment and prevention of bacterial diseases as well as destroying pathogens on food (Huff et al., 2010).

2.4.1. Historical context

The antibacterial properties of bacteriophages were first reported in 1896 by a British bacteriologist, Ernest Hanbury Hankin while working with cholera-inducing bacterial cultures in the waters of the Ganges and Jamuna rivers in India and subsequently it was found that this material can pass through Millipore filters (Sulakvelidze et al., 2001, Wittebole et al., 2014). Within two years of Ernest Hankin's observations, a similar fact was observed by a Russian bacteriologist, Gamaleya, while working with *Bacillus subtilis* (Samsygina and Boni, 1984, Sulakvelidze et al., 2001). Then in 1915, a British microbiologist Frederick Twort noticed some filterable isolates during his experiments on these viruses in cell-free agar media (Carlton, 1999, Sulakvelidze et al., 2001).

Two years after Twort in 1917, a similar finding was reported by a French-Canadian microbiologist Félix d'Herelle while working on the outbreak of severe haemorrhagic bacillary dysentery. He found some antibacterial factors after filtering stool samples from some of his shigellosis patients. This active filtrate was able to lyse Shiga bacillus cultures (Carlton, 1999, Sulakvelidze et al., 2001, Summers, 2001, Wittebole et al., 2014, Rao and Lalitha, 2015).

The findings of Twort and d'Herelle independently are considered to be the official discovery of bacteriophages (Barrow and Soothill, 1997, Carlton, 1999, Sulakvelidze et al., 2001, Huff et al., 2010, Rao and Lalitha, 2015). The name bacteriophage was given by d'Herelle who confirmed that the activities of bacteriophages are due to a live virus (Sulakvelidze et al., 2001, Kutter et al., 2005).

The potential action of bacteriophages against different bacterial infections was widely studied until the 1930s, but after World War II, the research and clinical application of bacteriophages for therapeutic purpose was abandoned by the Western world and became confined only within the Eastern Europe and former Soviet Union.

2.4.2. Properties of bacteriophages

Bacteriophages are very small infectious particles and have a wide range of shapes and forms (Orlova, 2012). The viral nature and structure of phages was confirmed in 1940 by a German doctor, Helmut Ruska, after the invention of the electron microscopy (Ackermann, 2003, Wittebole et al., 2014). Phages are only able to infect and replicate in prokaryotes, that is, bacteria and archaea. For the most part, there are three basic structural forms of phages. Some phages have a head or capsid with a tail (tailed phages) (Figure 2-1); while others consist of a head but no tail (polyhedral or pleomorphic) and some phages have a filamentous form (filamentous phages) (Rao and Lalitha, 2015). The head or capsid is made of proteins and long nucleic acid polymer (either DNA or RNA) which is packaged within this protein shell. The phage nucleic acid carries all the genetic information required and regulates phage properties. Capsids are icosahedral in shape except for filamentous phages and are connected to a tail through a collar or neck (Haq et al., 2012a, Orlova, 2012, Wittebole et al., 2014, Rao and Lalitha, 2015). The tails can be contractile, non-contractile or short in structure (Ackermann, 2003). The distal end of some tails consist of tail fibres, which allows bacteriophages to attach or bind to the receptors which consist of distinctive carbohydrate, protein and lipopolysaccharide molecules present profusely on the bacterial cell surface (Kutter et al., 2005, Haq et al., 2012a, Wittebole et al., 2014). The viral nucleic acid passes or is injected through the tail into the bacterial host body to generate new phage progenies within the bacterial cell. Often phages are able to produce 100 progenies from each host cell body within just half an hour of time (Kutter et al., 2005).

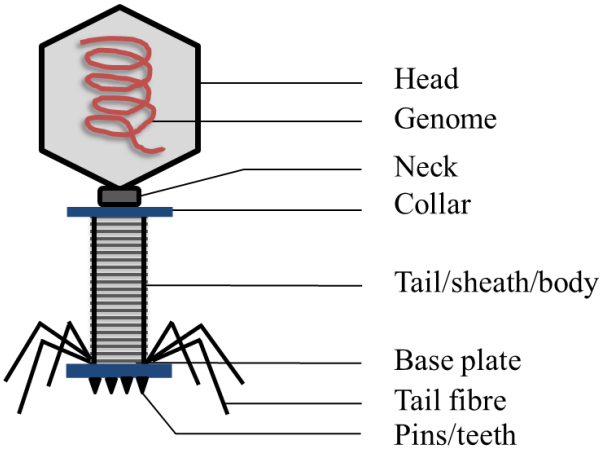

Figure 2-1. A typical bacteriophage. Redrawn from Hanlon (2007), Doss et al. (2017).

2.4.3. Classification

Bacteriophages can be classified according to their genetic material, morphology, their host specificity, habitats or their lifecycle (Ackermann, 2003, Skurnik and Strauch, 2006, Ackermann, 2011, Orlova, 2012, Wittebole et al., 2014, Matsuzaki et al., 2005). The classification of bacteriophages has undergone a number of modifications since phages were first discovered. But the key factors for bacteriophage classification are their nucleic acid or genomic properties and virion morphology (Orlova, 2012, Adriaenssens and Brister, 2017). However, there are generally more than 40 considerable bases to classify phages up to genera and species level (Chandrarathna et al., 2020). There are 24 bacteriophage families have been classified according to the Bacterial and Archaeal Viruses Subcommittee (BAVS) of the International Committee on the Taxonomy of Viruses (ICTV) (Table 2-1) (Adriaenssens et al., 2020, Sieiro et al., 2020).

The genomic constituent of bacteriophages is either DNA or RNA and either double-stranded or single-stranded (Ackermann, 2011). The vast majority of bacteriophages have double stranded DNA (dsDNA). However single stranded DNA (ssDNA), double stranded RNA (dsRNA), or single stranded RNA (ssRNA) can also be found in some small groups of phages (Ackermann, 2004, Skurnik and Strauch, 2006, Orlova, 2012).

In regard to their structural morphology, bacteriophages can be tailed, polyhedral (icosahedral or quasi-icosahedral), filamentous or pleomorphic. Out of all bacteriophages observed under transmission electron microscopy, tailed phages were found to constitute about 96.2% of all phages and are considered the most diverse and widespread group of bacterial viruses (Ackermann, 2003, Ackermann, 2011, Hassan et al., 2021). There are 23 genera of tailed phages described by the 9th ICTV. Depending on their tail structure bacteriophages can be assigned to three different families: *Myoviridae* (helical contractile tail), *Siphoviridae* (non-contractile and long tail) and *Podoviridae* (short tail) (Hanlon, 2007, Ackermann, 2011).

The filamentous or helical phages (*Inoviridae, Lipothrixviridae, Rudiviridae, Plectroviridae*) consist of long, rigid or flexible filaments of different lengths (Ackermann, 2003, Orlova, 2012, Hanlon, 2007, Adriaenssens et al., 2020). There are pleomorphic phages belonging to the *Fuselloviridae*, *Plasmaviridae, Halspiviridae, Ovaliviridae* and *Thaspiviridae* families – all possesses ds DNA (Adriaenssens et al., 2020). Around 3.7%

of total known phages are polyhedral, filamentous and pleomorphic (Ackermann, 2003, Orlova, 2012). Polyhedral, filamentous and pleomorphic phages consist of a total of ten families on the basis of nucleic acid content and morphology difference

Table 0-1. Classification of bacteriophages (Ackermann, 2003, Hanlon, 2007, Adriaenssens et al., 2020, Mäntynen et al., 2020, Thanh et al., 2020)

Order	**Family**	**Shape**	**Feature**	**Genetic material**	**Example**
Caudovirales	*Myoviridae*	Tailed	Contractile tail	ds DNA	T4
Caudovirales	*Siphoviridae*	Tailed	Long, non-contractile tail	ds DNA	Λ
Caudovirales	*Podoviridae*	Tailed	Short, non-contractile tail	ds DNA	T7
Caudovirales	*Demerecviridae*	Tailed	Icosahedral, non-enveloped capsid		T5
Caudovirales	*Drexlerviridae*	Tailed	Non-enveloped capsid	DNA	T1
Caudovirales	*Herelleviridae*	Tailed	Icosahedral capsid with long contractile tail	ds DNA	SPO1
Caudovirales	*Autographiviridae*	Tailed	Non-enveloped, icosahedral capsid	ds DNA	SP6, K1-5
Caudovirales	*Chaseviridae*	Tailed	Isometric heads, long contractile tails	ds DNA	φEcoM-GJ1
Caudovirales	*Ackermannviridae*	Tailed	Icosahedral capsid with contractile tail	ds DNA	EspM4VN
Ligamenvirales	*Lipothrixviridae*	Filamentous	Enveloped filaments, lipids	ds DNA	TTV1
Ligamenvirales	*Rudiviridae*	Filamentous	Helical rods, resembles TMV	ds DNA	SIRV1
Tubulavirales	*Inoviridae*	Filamentous	Non-enveloped, long and flexible filaments	ss DNA	M13
Tubulavirales	*Plectroviridae*	Filamentous	Non-enveloped, rigid and asymmetric rods	ss DNA	MV-L1
Petitvirales	*Microviridae*	Polyhedral	Icosahedral capsid, 12 knoblike capsomeres	ss DNA	φX174
Vinavirales	*Corticoviridae*	Polyhedral	Complex icosahedral capsid with lipid layer	ds DNA	PM2

Kalamavirales	*Tectiviridae*	Polyhedral	Icosahedral capsid with inner lipoprotein vesicle, pseudo-tail	ds DNA	PRD1
Norzivirales	*Leviviridae*	Polyhedral	Poliovirus-like, quasi-icosahedral capsid	ss RNA	MS2
Mindivirales	*Cystoviridae*	Polyhedral	Icosahedral capsid, segmented, lipidic envelope	ds RNA	φ6
Unclassified	*Finnlakeviridae*	Polyhedral	Icosahedral capsid	ss DNA	FLiP
Unclassified	*Fuselloviridae*	Pleomorphic	Spindle-shaped, enveloped, lipids, no capsid	ds DNA	SSV1
Unclassified	*Halspiviridae*	Pleomorphic	Spindle-shaped	ds DNA	His1
Unclassified	*Ovaliviridae*		Ellipsoid, an envelope encases a striated capsid	ds DNA	SEV1
Unclassified	*Plasmaviridae*	Pleomorphic	Quasi-spherical, No capsid, lipidic envelope	ds DNA	L2
Unclassified	*Thaspiviridae*	Pleomorphic	Spindle-shaped with short fibres at one pole	ds DNA	NSV1

*ds – double stranded; *ss – single stranded

2.4.4. Lifecycle

Bacteriophages are obligatory intracellular parasites in bacterial cells and to maintain their sustainability in the environment they display different mode of lifecycles described as chronic infection, pseudo-lysogenic, lysogenic and lytic development (Figure 2-2) (Weinbauer, 2004, Drulis-Kawa et al., 2012, Wittebole et al., 2014). Of these the lytic development is the virulent one and therefore, lytic phages are the ones most effective for phage therapy (Almeida et al., 2009, Oliveira et al., 2012, Rao and Lalitha, 2015, Wittebole et al., 2014). The four modes of infection are:

a. Chronic infection

No bacterial lysis occurs in this type of infection. After infecting the bacterial host body, the phage progeny starts to release themselves outside the host body by budding or an

extrusion method without causing any lethality. This type of cycle is not so commonly found in aquatic environment (Weinbauer, 2004, Sime-Ngando, 2014).

b. Pseudo-lysogenic cycle

This state of the lifecycle is also known as the phage-carrier state where a constant state of phage production occurs in the presence of high host cell abundance (Weinbauer, 2004, Wommack and Colwell, 2000). This type of cycle does not result in the total host cell lysis as in lytic cycle, but rather in a state whereby a high number of phages coexist with exponential bacterial host growth (Almeida et al., 2009). Here the phage genome (DNA) usually does not integrate into the bacterial genome (DNA) as in lysogenic cycle, but rather remains in a dormant state inside the host cell and thus no replication and segregation of phage occurs along with bacterial replication into all new daughter cells (Wommack and Colwell, 2000, Almeida et al., 2009, Oliveira et al., 2012). Pseudolysogeny in viruses or phages normally occurs when the viral surrounding is adverse and there is a scarcity of nutrients in the environment and consequently the starved host cell cannot find enough energy to induce viral gene expression (Sime-Ngando, 2014). When the surrounding growth condition improves, phages can either continue with lytic division or may undergo lysogenization following the termination of pseudo-lysogeny. This viral state of division represents an important characteristic of phage-host interaction in the environment (Łoś and Węgrzyn, 2012).

c. Lysogenic cycle

Compared to pseudo-lysogeny, the lysogenic cycle is a more stable association between the phage genome and the bacterial host cell (Wommack and Colwell, 2000). Phages which establish a lysogenic cycle are known as temperate phages (Weinbauer, 2004, Hanlon, 2007, Penadés et al., 2015). Temperate phages are capable of replicating themselves inside the host body without causing death to the bacterial cell. After attaching to a suitable bacterial cell surface receptor, lysogenic bacteriophages create a pore in the bacterial cell wall and integrate their nucleic acid material (DNA or RNA) into the bacterial chromosome and initiate the lysogenic cycle (Oliveira et al., 2012, Rao and Lalitha, 2015, Wittebole et al., 2014, Secor and Dandekar, 2020). The phage capsid remains outside the host body (Wittebole et al., 2014). This creates the prophage state and the viral nucleic acid starts to replicate along with multiplication of their host bacterium (Penadés et al., 2015, Secor and Dandekar, 2020). This technique is known as

lysogenization (Richards, 2014). In some cases, prophages may also be found as a plasmid inside the host body without inserting genetic material into the host genome (Hanlon, 2007, Rao and Lalitha, 2015). The prophages may stay in a dormant stage for long periods of time, even for years and continue to replicate within the bacterial cell up to several generations without creating any major metabolic changes in the cell (Rao and Lalitha, 2015, Almeida et al., 2009). Both the prophage and the bacterial host cell gets benefit from the lysogenic cycle. The lysogenic cycle allows the phage to keep going even at the times when there is a scarcity of host cells in the environment (Sime-Ngando, 2014, Paul, 2008). Temperate phages are considered as K-selected viruses which have longer maturity period and survives longer because of their small burst size and longer coexistence with their host body (Suttle, 2007).

The lysogenic state is maintained by a phage repressor gene, but under specific environmental conditions or stressors the repressor and its production are inhibited. This leads the temperate phages to induce the virion productive cycle or lytic cycle (Skurnik and Strauch, 2006, Hanlon, 2007, Rao and Lalitha, 2015, Penadés et al., 2015, Howard-Varona et al., 2017). Several environmental (physical and chemical) and biological stressors can induce the lytic cycle including the host resistance mechanisms, UV irradiation, different pollutants and carcinogens, changes in salinity, differences in temperature or nutrient concentration and use of pesticides, Mitomycin C, and PCBs (Cochran et al., 1998, Hanlon, 2007, Almeida et al., 2009, Richards, 2014, Rao and Lalitha, 2015). Once the lytic cycle is initiated, replication of the phage genome and the manufacture of head and tails leads to the release of new phage progenies from the host bacteria (Rao and Lalitha, 2015).

After the initiation of lytic cycle from the lysogenic state, prophages may occasionally carry host genetic material along with their genome (Rao and Lalitha, 2015, Wittebole et al., 2014, Penadés et al., 2015). This may result in the transference of host cell genes which may confer virulence, antimicrobial resistance, antigenic changes, or changes to metabolism in a newly infected host (Hanlon, 2007, Wittebole et al., 2014). Temperate phages can be used for phage typing, phage display or phage vaccines (Almeida et al., 2009, Haq et al., 2012a). But these phages are not suitable for phage therapy since they do not directly destroy bacterial pathogens and they may transfer undesirable host derived genes into the new host body (Hanlon, 2007, Almeida et al., 2009).

d. Lytic cycle

The lytic cycle of bacteriophages represents a virulent mode of infection (Rao and Lalitha, 2015). Bacteriophages going through the lytic cycle are known as virulent phages as they have the ability to kill the bacterial host by lysis (Sulakvelidze et al., 2001). Lytic bacteriophages follows r-selection strategy since they are highly virulent, have large burst size and grow and replicates rapidly (Suttle, 2007).

The steps of a typical lytic cycle are: adsorption, penetration or injection, latent period, maturation and lysis (Brovko et al., 2012). The lytic phage attaches to a bacterial receptor site and then injects the phage genome into bacterial body (Sime-Ngando, 2014). The phage nucleic acid takes over all the bacterial synthetic machinery and starts to replicate their genetic material to produce a number of daughter phages inside bacterial cell. Finally, upon the lysis of cell wall, a large number of new phage progenies are released into the environment where they are ready to start another cycle by attacking new neighbouring bacterial cells (Almeida et al., 2009, Oliveira et al., 2012, Wittebole et al., 2014, Rao and Lalitha, 2015, Penadés et al., 2015). The total number of new released phage particles per bacteria (the burst size) depends on factors such as, the type of phage, state of the host bacteria, and availability of nutrients for the host bacteria (Wittebole et al., 2014). It may take 20 to 60 minutes to complete the whole lytic cycle and amplification will be continued as long as they can find a suitable host body to infect (Rao and Lalitha, 2015). The rate of replication is higher when the bacterial population is greatest and the rate will decrease gradually as the bacterial population is killed (Almeida et al., 2009). This feature of lytic bacteriophages makes them an attractive option to be used in biological control as well as in phage therapy (Jassim and Limoges, 2014, Kowalska et al., 2020).

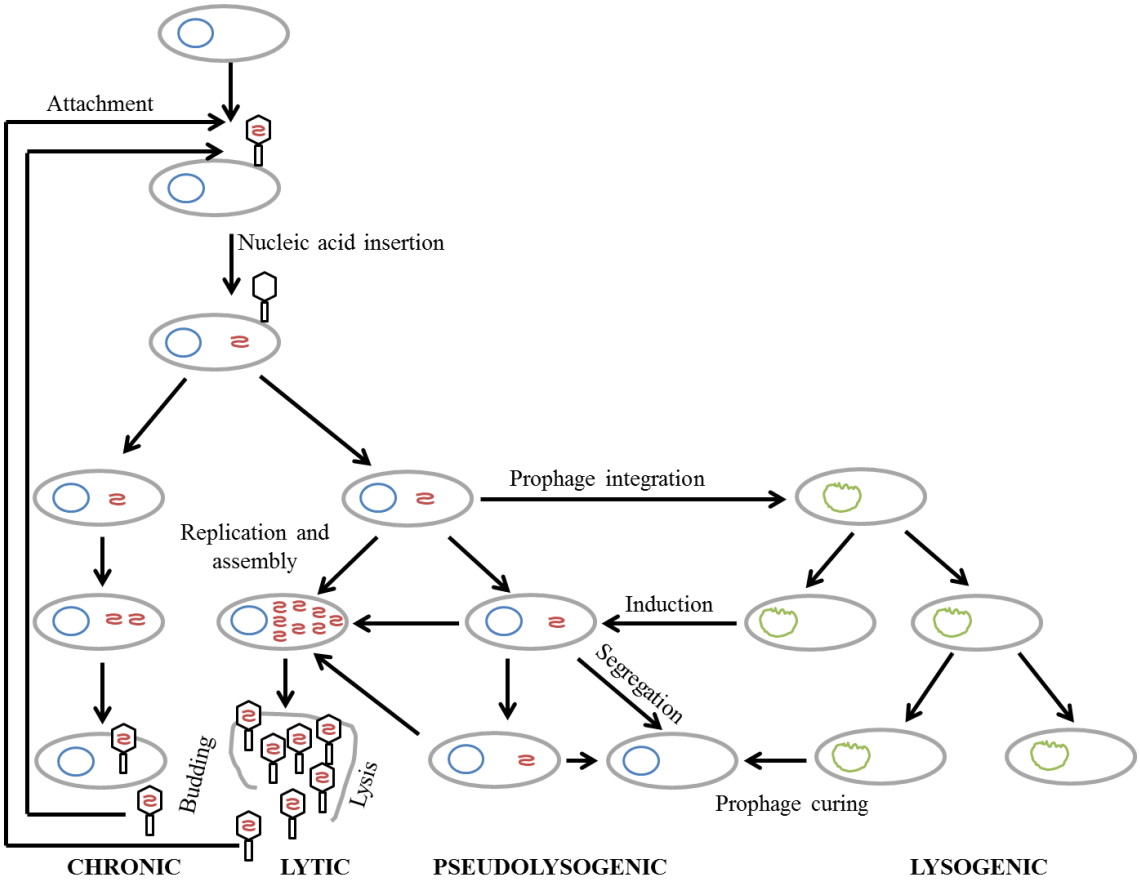

Figure 2-2. Diagrammatic representation of different modes of bacteriophage life cycle. Adapted and re-drawn from Weinbauer (2004).

2.5. Bacteriophage therapy

After the initial discovery in 1920s and their identification, bacteriophages were subject to extensive research and were considered a potential therapeutic tool for controlling bacterial infections (Caflisch et al., 2019, Kortright et al., 2019). Several commercial companies and laboratories within the United States of America and parts of Europe started to use different phage products for the treatment and prophylaxis of bacterial infections. But after the Second World War, research and clinical applications related to phages were abandoned in the Western world but continued in Georgia, Russia, Poland and former Soviet Union region for almost 80 years (O'Flynn et al., 2004, Kropinski, 2006, Kutateladze and Adamia, 2010, Matsuzaki et al., 2005). Due to the controversies associated with mixed result of therapeutic success, lack of adequate experimental data and poor knowledge in understanding phage biology and with the advent of antibiotic era in 1940s, the potential of a phage therapeutic approach was not properly realized and eventually phased out of research (O'Flynn et al., 2004, Kropinski, 2006, Kutateladze and

Adamia, 2010, Haq et al., 2012b). Although there was some knowledge of their mode of action, very little data were published showing the use of phages to treat and prevent bacterial infections, until the last four decades (Hermoso et al., 2007, Almeida et al., 2009, Klumpp et al., 2012, Nikolich and Filippov, 2020). Phage therapy has been re-evaluated due to the emergence of multidrug resistant (MDR) bacteria (Hermoso et al., 2007, Vincent et al., 2017, Matsuzaki et al., 2005). Advances in the understanding of biology, genetics, immunology and pharmacology of phages stimulated their use as a potential complement or alternative to antibiotics for treating bacterial infections (Altamirano and Barr, 2019). In the 1980s, Smith and Huggins published evidence of using *E. coli* infections in farm animals and mice as models to demonstrate the possible uses of phages as a therapy, and this was considered to be the rediscovery of the principle of phage therapy (Smith and Huggins, 1983, Smith and Huggins, 1982, Smith et al., 1987, Barrow and Soothill, 1997, Park et al., 2000). In recent times, some countries in the West such as Belgium and France started to accept the therapeutic use of phages. The rediscovery of phage therapy combined with the decline in the efficacy of antibiotics against MDR infections has revitalised the use of phages as a potential bactericidal approach.

2.6. Advantages of bacteriophage therapy to control bacterial pathogens

Bacteriophages possess some distinct characteristics which makes them more suitable for use as a therapeutic tool compared to other biocontrol strategies (Sulakvelidze et al., 2001, Inal, 2003, Haq et al., 2012b, Oliveira et al., 2012, Martínez-Díaz and Hipólito-Morales, 2013, Matsuzaki et al., 2005).

1. Abundance – There is an abundant supply of bacteriophages in the environment and they can be found wherever their host organism is present (Inal, 2003, Garcia et al., 2008, Abedon, 2009, Kutateladze and Adamia, 2010, Haq et al., 2012a, Haq et al., 2012b, Jun et al., 2014, Wittebole et al., 2014). The prevalence of phages is normally ten times higher than that of bacteria (Ackermann, 2011, Hanlon, 2007, Orlova, 2012, Skurnik and Strauch, 2006)
2. Range of activity – Bacteriophages are active against all types of Gram-negative and Gram-positive bacteria, including multidrug resistant bacterial pathogens present in the environment (Wittebole et al., 2014, Matsuzaki et al., 2005).

3. Host specificity – Bacteriophages are host specific having a relatively narrow antibacterial range. This allows them to infect only a single targeted species and sometimes only a strain or serotype within a species. Because of this specificity no beneficial bacteria will be affected (Sulakvelidze et al., 2001, Inal, 2003, Garcia et al., 2008, Oliveira et al., 2012, Haq et al., 2012b, Martínez-Díaz and Hipólito-Morales, 2013, Wittebole et al., 2014, Matsuzaki et al., 2005). This specificity also helps to minimize secondary infection development as well as resistance development in normal microflora (Oliveira et al., 2012, Sulakvelidze et al., 2001).
4. Multiplication rate – Bacteriophage populations increase and decline rapidly in the presence of bacterial host. The rate is the highest when the host is in the mid-exponential growth phase and is reduced as the host multiplication rate approaches stationary phase (Inal, 2003, Almeida et al., 2009, Kutateladze and Adamia, 2010, Oliveira et al., 2012, Haq et al., 2012b). Therefore, phage therapy is a flexible and relatively fast approach to controlling bacterial growth.
5. No side effects – Because of their restricted host range, bacteriophages will not affect the non-targeted bacteria nor do they affect normal eukaryotic or mammalian cells (Sulakvelidze et al., 2001, Garcia et al., 2008, Kutateladze and Adamia, 2010, Wittebole et al., 2014, Matsuzaki et al., 2005). Moreover phages do not have any adverse effects on plants, animals or environment because they only consists of nucleic acid encapsulated by a protein coat (Martínez-Díaz and Hipólito-Morales, 2013).
6. Cost effective – Considering the efficiency of phage therapy for the control of bacterial infection, the cost is lower compared to that of conventional antibiotic treatment (Wittebole et al., 2014, Matsuzaki et al., 2005).
7. Administration route – There are many options for the route of administration. Bacteriophages may be administered orally, in impregnated feed, by aerosols, or immersion, or by injection (Oliveira et al., 2012). They can penetrate through the blood brain barrier, hence they are effective against central nervous system infections. Some phages also have been found to be able to disrupt bacterial biofilms (Kutter et al., 2010, Garcia et al., 2008, Wittebole et al., 2014).

2.7. Experimental data available on phage therapy

Since their discovery, there has been active research into bacteriophages in human medicine, the food industry, waste water treatment, and agriculture (D'Herelle, 1929, Skurnik and Strauch, 2006, Almeida et al., 2009, Ormala and Jalasvuori, 2013, Wittebole et al., 2014, Pereira et al., 2016, Fernández et al., 2018, Hassan et al., 2021). Several phage-based bio-engineering methods, such as recombinant phages and phage display have been demonstrated their effectiveness as therapeutic and biological control agents and large scale of application of phage therapy has showed promising results (Oliveira et al., 2012, Chandrarathna et al., 2020). Initially use of phages for therapeutic purposes was confined to the clinical sectors. With the advent of better knowledge on phage biology, phage advantages over antibiotics and their mode of action, phage use has become more popular and started to be applied in many other areas of biotechnology and medical sciences including phage typing (Clark and March, 2004, Clark and March, 2006, Haq et al., 2012a). So far, a number of phage-based therapeutic products have also been accepted by different regulators such as, FDA (Food and Drug Administration), FSANZ (Food Standards Australia and New Zealand) and EPA (US Environmental Protection Agency) to control infections caused by *Listeria monocytogenes, E. coli*, *Pseudomonas putida* and *Salmonella*. In addition, different bacteriophage ecological studies such as their abundance in nature, role in maintaining microbial communities and balancing different environmental mechanisms has gained attention over the last several years (Talledo et al., 2003, El-Araby et al., 2016). Moreover phage particles or their components have also been used as a pathogen detection tool (Pierce et al., 2011, Klumpp et al., 2012).

The therapeutic potential of phages was first tried against *Vibrio cholerae*, where the *in vitro* trial was found to be more effective than the *in vivo* trial (Adams, 1959, Rao and Lalitha, 2015). This was followed by a number of studies of prophylactic treatment of human diseases caused by different bacterial pathogens (Sulakvelidze et al., 2001, Kutter et al., 2010). Most of the investigations of phage producution, formulation and therapy for human treatment were conducted in Eastern Europe and the former Soviet Union (Sulakvelidze et al., 2001, Kutter et al., 2010). Moreover, in the early 1980s phage-based experiments were carried out by Smith and Huggins to control *E. coli* infections in mice, piglets, lambs and calves (Smith and Huggins, 1983, Barrow and Soothill, 1997).

As well as the experiments decribed above, work in chickens carried out by Huff and his colleagues showed that respiratory infections caused by *E. coli* were successfully treated by phage therapy (Huff et al., 2002, Huff et al., 2003a). Several studies have also showed the decrease in *Campylobacter* and *Salmonella* concentrations in chicken using bacteriophage biocontrol strategy (Atterbury et al., 2007, Kittler et al., 2013). In addition to mice and chickens, control of infections by phage therapy has been shown in rats, rabbits, calves, lambs as experimental models (Oliveira et al., 2012, Wittebole et al., 2014). Bacteria used in these studies included *E. coli*, *Chronobacter turicensis*, *Staphylococcus aureus* and some MDR bacteria (Wittebole et al., 2014). Most of these studies showed reductuion in mortality using phage therapy, often better than the results of antibiotic treatment (Wittebole et al., 2014).

2.8. Bacteriophage therapy application in aquaculture

In addition to the attempts to control human and other livestock pathogens, the control of aquaculture diseases as well as to maintain biogeochemical cycles in marine ecosystems, bacteriophage therapy has gained increasing interest over the last few decades (Stenholm et al., 2008, Abedon, 2009, Oliveira et al., 2012, Doss et al., 2017, Kowalska et al., 2020, Żaczek et al., 2020). The therapeutic potential of phages in controlling fish-borne pathogens was first described by Wu et al. (1981) using the Japanese eel *Anguilla japonica*. Other studies on the identification and characterization of phages to use as a therapeutic agent against fish-borne pathogens have also been described (Wu et al., 1981, Stevenson and Airdrie, 1984, Merino et al., 1990a, Munro et al., 2003, Almeida et al., 2009).

The therapeutic use of phage in controlling *Lactococcus garviae* and *Pseudomonas plecoglossicida* infection in cultured ayu fish (*Plecoglossus altivelis*) *in vivo* has been reported by Nakai and Park (2002), Park and Nakai (2003), Skurnik and Strauch (2006), Park et al. (2000). The research demonstrated that a) fish fed with phage-impregnated-food were protected from infection; b) to avoid pathogen transmission in water, phage treatment could be used; c) there was evidence of phage resistance as well as phage-neutralizing antibodies; and d) phage therapy was effective in minimizing infection and reducing the fish mortality rate (Park et al., 2000, Park and Nakai, 2003, Oliveira et al., 2012).

The use of phage combinations to treat furunculosis (caused by *Aeromonas salmonicida* infection) in brook trout (*Oncorhynchus fontinalis*) in a fish farm showed promising results (Imbeault et al., 2006, Almeida et al., 2009, Oliveira et al., 2012, Kim et al., 2015). An *in vitro* study by Kim et al. (2010) also demonstrated the efficacy of bacteriophages in reducing the impact of *Flavobacterium psychrophilum* infection on ayu fish. Another study by Higuera et al. (2013) observed 100% survival rate in Atlantic salmon when phage suspension was added to the *Vibrio anguillarum* infected fish. A pilot study by Khairnar et al. (2013) also showed promising results of phage therapy for the control of *Pseudomonas aeruginosa* in freshwater catfish *Clarias gariepinus*.

In addition to fish, a number of bacteriophage therapy studies have been conducted on shrimps, lobsters and bivalve molluscs (Oliveira et al., 2012). The use of bacteriophages isolated from the environment were effective in the control of luminous vibriosis caused by *V. harveyi in vitro* as well as in the shrimp hatchery (Karunasagar et al., 2005, Vinod et al., 2006, Karunasagar et al., 2007). Other studies have described the effectiveness of phage therapy in controlling *Vibrio* spp. pathogens from larval cultures of tropical rock lobster, pacific oysters and pearl oysters (Pass et al., 1987, Sugumar et al., 1998, Park and Nakai, 2003, Berthe, 2005, Vinod et al., 2006, Crothers-Stomps et al., 2010).

Most of the bacteriophage therapeutic studies done for aquatic organisms are still in their research stage and require to be more investigated and described. However, the experimental work summarised here provides an optimistic outlook on the fact that phages can be used successfully for treating diseases in aquatic organisms.

2.9. *Aeromonas hydrophila* infection in aquaculture and their bacteriophages

Aeromonas hydrophila is one of the major causative agents of disease outbreaks and mortality in the aquaculture environment (Janda and Abbott, 2010). This ubiquitous, Gram negative rod is an opportunistic pathogen and responsible for a number of fish diseases including haemorrhagic septicaemia, ulcerative infections and red sore disease resulting in elevated mortality in aquatic organisms (Beaz-Hidalgo and Figueras, 2013). *A. hydrophila* is also capable of producing siderophores, biologically active extracellular products (ECPs) and toxins or enzymes (such as, cytotoxins, haemolysins, and enterotoxins) and also of biofilm formation in various biotic and abiotic environments (Anand et al., 2018, Chenia and Duma, 2017, Casabianca et al., 2015, Igbinosa et al.,

2013). Until now the most commonly accepted method of controlling *A. hydrophila* infection in aquatic organisms is the use of antibiotics. However, as a consequence of emergence of MDR bacteria in different aquatic organisms and aquaculture systems, there is an ongoing need for studies targeting the isolation and characterisation of new phages that are specifically pathogenic to *A. hydrophila* and develop a bacteriophage therapeutic tool which will aid in minimising the use of antibiotics in the fish aquaculture operations.

In the past few years, some studies on *A. hydrophila* phages have shown promising results. Hsu et al. (2000) conducted an *in vitro* study where they isolated two *A. hydrophila* phages along with some other phages targeting infections in eels (*Anguilla japonica*). Another successful study by Le et al. (2018) showed promising results where they successfully isolated two phages active against *A. hydrophila* and the phage cocktail was able to treat Motile *Aeromonas* Septicemia (MAS) in striped catfish (*Pangasianodon hypophthalmus*) using intraperitoneal injection. In addition to that, a study conducted by El-Araby et al. (2016) demonstrated the potential of two phages ZH1 and ZH2 to treat infection caused by *A. hydrophila* in Nile tilapia with a survival rate up to 82%. Other studies described by Jun et al. (2013) and Akmal et al. (2020) showed the potential of phages, pAh1-C, pAh6-C and Akh-2 respectively as therapeutic agents against *A. hydrophila* infection in cyprinid loach (*Misgurnus anguillicaudatus*). Furthermore, Easwaran et al. (2016) isolated and characterised an *A. hydrophila* phage, pAh-1 and described its protective effects in experimentally infected zebrafish (*Danio rerio*).

Other than the studies stated here, a handful of studies have been conducted by a number of scientists which primarily focused on isolation of *A. hydrophila* phages from different aquatic environments and their characterisation up to genomic level to enrich phage related data within scientific community.

The present study focused on the isolation and characterisation of phages specific to *A. hydrophila* and the evaluation of the safety and effectiveness of these phages with the aim of developing a therapeutic tool as a replacement to antibiotics with a long term view of controlling this pathogen in aquaculture.

2.10. Conclusion

Based on the examples described on therapeutic and prophylactic applications of bacteriophages, phage therapy seems very promising in the aquaculture sector. However,

phage based therapeutic models in aquaculture have not been fully explored yet (Oliveira et al., 2012, Almeida et al., 2009). Moreover there is still a lack of information about phage and bacteria diversity and ecology (Jassim and Limoges, 2014).

Some factors should be considered prior to the selection of phages for effective therapeutic purposes. The most important factors for the selection of phages are the burst size and the host range of bacteriophages. Phages with large burst size have been found to be more effective in lysing host bacteria and their use also reduce the chance of selecting phage- resistant bacteria (Hyman and Abedon, 2010, Mirzaei and Nilsson, 2015). Moreover there are some virulent phages with a wide host range that are active against a wide variety of bacteria, and so more cost effective for therapeutic use (Mirzaei and Nilsson, 2015).

It has been reported that bacteriophages can survive between 12-91 days in marine environments with a moderate impact on bacterial communities leading to a reduction in bacterial diseases (Pereira et al., 2011). The majority of publications have focused on phage isolation and characterisation to reduce bacterial biomass *in vitro* for different aquaculture species. There need to be more *in vivo* studies to validate the use of phage therapy in aquaculture.

Some bacteriophages have already gained regulatory acceptance for phage therapy (Oliveira et al., 2012). However additional studies are required to fully understand phage-bacterium interactions, and demonstrate advantages of phage therapy as a biocontrol agent against bacterial infection in aquaculture.

Chapter 3. General Materials and Methods

3.1. Bacterial investigations

3.1.1. Bacterial strains

Ten different strains of *Aeromonas hydrophila* were used for the isolation of *Aeromonas* phage. Bacterial strains (named as Ah1, Ah2, Ah3, Ah4,) were obtained from the bacterial culture collection (fish-pathogenic clinical isolates) held within the School of Animal and Veterinary Sciences, Roseworthy Campus, The University of Adelaide. The other six bacterial strains (isolated from crayfish and named as Ah5, Ah6, Ah7, Ah8, Ah9, and Ah10) were obtained from AusPhage Pty Ltd, Townsville, Queensland (Hayakijkosol et al., 2017).

3.1.2. Bacterial culture media

All bacterial strains were streaked from the frozen culture stock onto Tryptic Soy Agar (TSA, Edwards Group Pty Ltd, NSW, Australia) (17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean, 2.5 g dextrose, 5.0 g NaCl, 2.5 g K_2HPO_4, 1.5% Bacto-agar, 1000 ml deionized water) plates and their identity was confirmed following the standard biochemical procedures (Markey, 2013) and by growth in ampicillin supplemented *Aeromonas* selective media (*Aeromonas* Medium Base, Thermo Fisher Scientific, Australia). After identification, the bacterial strains were kept in glycerol broth (20% glycerol in TSB) at -80 °C for long term storage. Frozen cultures were reactivated onto TSA plates at 28 °C for routine use.

3.2. Bacteriophages

3.2.1. Isolation and enrichment of bacteriophages

A total of five sewage/waste water samples were collected from three sampling locations (Table 4-1) for the isolation of bacteriophages. Published methods with minor modifications were used for the isolation, enrichment and purification of bacteriophages from the collected sewage and waste water samples (Merabishvili et al., 2009, Akhtar et al., 2014, Silva et al., 2014b). Following the transportation, water samples were centrifuged at 3000 ×*g* at 4 °C for 10 min (Centrifuge MPW-351R) and the supernatants filtered through 0.45 µm polycarbonate membrane syringe filters (Millex-HP 33 mm, Adelab Scientific, South Australia). Then 50 ml of filtered water samples were mixed with 50 ml of double strength (2×) TSB and 1 ml of exponentially grown *A. hydrophila* culture which was prepared on the same day. The mixtures of the filtered water samples

and *A. hydrophila* hosts were incubated at 28 °C, shaken at 60 rpm for 18-20 h. Following incubation, the mixed samples were centrifuged at 3000 ×*g* at 4 °C for 15 min and filtered with 0.45 µm polycarbonate membrane syringe filters. These filtrates potentially contained bacteriophages which were used for further analyses.

3.2.2. Bacteriophage plaque assay (spot test)

Following the filtration, the phage preparations were used to conduct spot tests to determine the growth of the cultured bacteriophages. *Aeromonas hydrophila* bacterial strains were initially grown in TSB media at 28 °C overnight. The next day, bacterial lawns were prepared by spreading 100 µl of log phase *A. hydrophila* aliquots on to the TSA plates and allowed to dry for 20 min in a biological safety cabinet Class II. Then 10 µl of each phage preparation was pipetted onto each bacterial lawn. The plates were then incubated overnight in an inverted position at 28 °C for plaque formation.

3.2.3. Bacteriophage amplification in liquid medium

The standard protocol for liquid medium amplification of bacteriophages from was followed from the methods described by (Kutter, 2005, Goodridge et al., 2003), briefly 10 ml of overnight culture of *A. hydrophila* was inoculated in 500 ml of fresh sterile PBS (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1000 ml deionized water) and then same volume (500 ml) of respective bacteriophage (~10^9 pfu/ml) solution was added to the bacterium-PBS solution. The culture was then incubated overnight with very mild shaking (65 rpm) at 28 °C to infect bacterial cells with bacteriophages. The next day when bacterial lysis occurred, few drops of chloroform was added to the bacterium-phage suspension and mixed gently to induce and complete lysis of the host cell and release the phage progeny which may still left inside the host. This amplified phage solution was then transferred to chloroform-free Eppendorf tubes and centrifuged at 4000 ×*g* for 20 min and the supernatant was then filtered with 0.45 µm polycarbonate membrane syringe filters to get rid of the remaining bacterial cell debris. The final phage titre was checked and the phage lysate was stored at 4 °C for further analyses. The amplification was carried out in order to make larger volumes of bacteriophages to facilitate further experiments.

3.2.4. Transmission Electron Microscopy (TEM)

Transmission Electron Microscopy (TEM) (Adelaide Microscopy) was used to determine the morphology of the isolated and purified bacteriophages. Bacteriophage solutions were negatively stained using 2% solutions of phosphotungstic acid (PTA) or uranyl acetate (UA) and fixed on a carbon and hydrophilic pioloform-coated 200-mesh copper grid. One drop (10 µl) of bacteriophage solution was first placed on the copper grid and allowed to settle for 2 min and the excess phage solution fluid was then blotted carefully using a filter paper. Then one drop (10 µl) of negative stain PTA or UA was placed on the copper grid containing the sample and left for 2 min. Excess stain was then blotted. These copper grids with stained bacteriophage sample were then placed in a FEI Tecnai G2 Spirit Transmission Electron Microscope (TEM) operating at 100 kV to observe their specific morphological features and measure their sizes.

3.3. Fish infection trials

3.3.1. Bacterial stock solution preparation for fish infection trials

A single strain of *Aeromonas hydrophila* (Ah10) was selected for the infection trial. The day before the infection of the fish, bacteria were grown in TSB (Tryptic Soy Broth, Edwards Group Pty Ltd, NSW, Australia) (17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean, 2.5 g dextrose, 5.0 g NaCl, 2.5 g K_2HPO_4, 1000 ml deionized water) and incubated overnight in a shaker incubator for 12 h with 120 rpm at 28 °C. The next day growth was observed and the bacterial concentration in the broth was adjusted to an approximate final optical density of suspension (OD) of 0.180 at a wavelength of 600 nm. The bacterial broth culture was then centrifuged at 3000 $\times g$ for 10 min. The supernatant was discarded and the bacterial pellet was resuspended with the same volume of sterile PBS solution. This reconstituted bacterial suspension became the stock solution and was then serially diluted in PBS solution 1 in 1000 (10^{-3}) to give a viable count of approximately 6.7×10^7cfu/ml. This was the bacterial inoculum used for the fish infection trials.

3.3.2. Preparation of fish (sedation and recovery) for the preliminary pilot trial and actual infection challenge trial

Silver perch (*Bidyanus bidyanus*), a native fish of Australia, was used in this study as a model to test the therapeutic effectiveness of bacteriophages against an *A. hydrophila*

pathogen. Prior to dose inoculation, each fish was anaesthetised by emersion using Aqui-S (active ingredient: Isoeugenol) solution at a dose rate of 20-30 mg/l water in a separate tank and fish were kept there until they showed signs of sedation. Then each individual fish was weighed and the body length was measured to determine the dose/volume for bacteria and PBS (control) for inoculation. Following the inoculation, fish were kept in a separate recovery tank with proper aeration and then returned to their designated tanks. Fish were not fed on the day of their infection.

The dose/volume to be injected (control, bacteria and bacteriophage dose) was determined using a body mass-volume calculation (0.1 ml dose per 10 kg fish) as described by Treves-Brown (2013) in an attempt to deliver a consistent number of bacteria per gram body weight for each of the fish in the separate treatment groups.

3.3.3. Fish organ collection for bacterial counts

A sample of kidney and spleen were dissected out from each fish of every treatment and then suspended in individual sterile tubes containing PBS for homogenisation. Each PBS containing tube was weighed before and after adding the kidney and spleen samples to get actual sample weight. Aseptic conditions were maintained throughout the procedure. The samples were then homogenised and kept in PBS solution for 10-20 min before undertaking serial dilutions of the supernatant to aid in bacterial plate counts (see below).

3.3.4. Bacterial counts in kidneys and spleens

The homogenised samples were serially diluted (10-fold) in PBS and then plated onto TSA and AMB agar plates. All agar plates were incubated overnight at 28 °C. The next day plates were observed for bacterial growth, colonies counted and bacterial cfu/g were calculated to determine bacterial carriage rate in the treated fish.

Chapter 4. Isolation, partial characterization and *in vitro* lytic ability of bacteriophages targeting *Aeromonas hydrophila*

4.1. Introduction

Aeromonas hydrophila is one of the many fish pathogens that is recorded as causing severe economic losses in global aquaculture (Ackermann et al., 1985, Fang et al., 2004, Poobalane et al., 2010). *A. hydrophila* is a Gram negative, oxidase and catalase positive, rod-shaped, motile and facultative anaerobe (Flint, 1996, Saavedra et al., 2004, Citterio and Biavasco, 2015, Fernández-Bravo and Figueras, 2020). Their occurrence is ubiquitous in the environment and they are known to cause diseases in different animals including fish living in freshwater, marine, or estuarine habitats (Merino et al., 1990a, Flint, 1996, Dong et al., 2018). Disease outbreaks caused by *A. hydrophila* in freshwater fish has been reported to be more common in the aquaculture industry (Noga, 2011, Yu et al., 2005, El-Araby et al., 2016, Dhanapala et al., 2021). Most common diseases caused by this pathogen includes motile Aeromonas septicaemia (MAS)/haemorrhagic septicaemia, epizootic ulcerative syndrome, ulcerous dermatitis, infectious abdominal dropsy, fin and tail rot in various fish species (Zhang et al., 2016, Noga, 2011, Swift et al., 1999, Ventura and Grizzle, 1988, Harikrishnan and Balasundaram, 2005, Saavedra et al., 2004, Harikrishnan et al., 2010, Zhang et al., 2015). A number of fresh water fish including carp (*Labeo rohita*, *Catla catla*, *Cyprinus carpio*, *Cirrhinus cirrhosis*, etc), loach (*Misgurnus anguillicaudatus*) eel (*Anguilla japonica*), channel catfish (*Ictalurus punctatus*), striped catfish (*Pangasianodon hypophthalmus*), tilapia (*Tilapia nilotica*), ayu (*Plecoglossus altivelis*), goldfish (*Carassius auratus*) have been reported to be infected with *A. hydrophila* and leading to high mortality levels in various aquaculture farming systems worldwide (Harikrishnan and Balasundaram, 2005, Le et al., 2018, Jun et al., 2010). This species exhibits variable pathogenicity and is generally considered to be a secondary or opportunistic pathogen which is able to cause disease in already infected or immunologically compromised fish (Haq et al., 2012b, Harikrishnan et al., 2003). However *A. hydrophila* can also act as a primary pathogen infecting healthy fish (Lehane and Rawlin, 2000). This bacterium is also responsible for diseases in higher vertebrates, even in humans causing meningitis, septicaemia, or wound infections. Ingestion of infected fish or infection through damaged skin while fish handling can cause diseases associated with *A. hydrophila* (Noga, 2011, Saavedra et al., 2004, Handfield et al., 1996).

Antibiotics are extensively used in aquaculture industries for the treatment of bacterial infections as well as a growth supplement. Overuse and unregulated administration of these drugs has contributed to the emergence of antibiotic resistance (ABR) in the wider

environment through exchange of antibiotic resistance genes (ARGs) (Defoirdt et al., 2011, Lee and Wendy, 2017). ARGs are commonly found on bacterial mobile genetic elements such as plasmids, transposons and integrons which can interchange between bacterial species through a process called horizontal gene transfer (HGT) (Dzidic and Bedeković, 2003). Selection and co-selection of these ARGs are facilitated by the presence of antibiotics in the polluted environment where aquatic animal and human pathogens can take up new genes that may confer resistance to the respective antibiotics resulting in the failure of antimicrobial therapy (Akinbowale et al., 2006, Mathew et al., 2007, Tenover, 2001, Chang et al., 2015). Aquatic industries serve as reservoirs of ARGs. Therefore, keeping the food safety concerns in mind, research projects are being undertaken to develop alternative control approaches for bacterial infections in aquaculture (Defoirdt et al., 2011, Matsuzaki et al., 2005). Since the discovery of bacteriophages, their ability to kill the respective host bacteria (lytic ability) has been tested to control both human and animal pathogens (Carlton, 1999, Kutateladze and Adamia, 2010, Vinod et al., 2006). Lysogenic viruses undergo a latent stage when they integrate their genome in the bacterial DNA (prophage) and therefore are not useful in phage therapy. Lytic bacteriophages are preferred for controlling bacterial infections as they are capable of destroying bacterial host cell within a short period of time (Breitbart, 2012, Rao and Lalitha, 2015).

Some reports have demonstrated the development of antibiotic resistance in *A. hydrophila* due to their indiscriminate exposure to various antibiotics (Zdanowicz et al., 2020, Scarano et al., 2018, Dahanayake et al., 2019). Compared to other bacteria causing diseases in fish, *Aeromonas* sp. are known to be more resistant to many antibiotics used in treating and controlling infection (Harikrishnan and Balasundaram, 2005).

Bacterial viruses or phages have been found as a suitable tool in controlling antibiotic resistant bacteria as they have a completely different mechanism of killing or lysing specific bacteria than that of the antibiotics (Lin et al 2017). The self-limiting factors of bacteriophages (i.e. present in very low numbers after target hosts have been killed) makes them very attractive to use as a significant means of controlling MDR pathogens (Nakai and Park, 2002). A number of bacteriophage based therapeutic studies against various aquatic animal pathogens have already showed promising results.

Antibiotics are still considered to be the most frequently used therapeutic to treat *Aeromonas* spp. infections in aquaculture, some recent studies however isolated multidrug resistant (MDR) *A. hydrophila* from different aquatic organisms (Chandrarathna et al., 2020). Therefore isolation, characterisation and application of virulent bacteriophages targeting *A. hydrophila* is a promising strategy to reduce economic losses in aquaculture sector.

The present study describes the isolation and characterization of four novel lytic bacteriophages which are active against three different strains of *A. hydrophila* that are pathogenic to fresh water fish with the aim to use them to design an efficient biocontrol method as an alternative treatment strategy for *A. hydrophila*. Additionally, we investigated their lytic property towards the host bacteria under controlled condition in the laboratory. The *in vitro* analyses done in this study demonstrate that the isolated phages have the potential for use in treating *Aeromonas hydrophila* infection in aquaculture.

4.2. Materials and methods

4.2.1. Bacterial strains and media used

The detail of the bacterial strains and their culture condition are described in section 3.1 of chapter 3. Briefly, ten different strains of *Aeromonas hydrophila* namely Ah1, Ah2, Ah3, Ah4, Ah5, Ah6, Ah7, Ah8, Ah9, and Ah10 were used for the isolation of *Aeromonas* phage. A loop full of frozen culture of each bacterial strains were streaked onto Tryptic Soy Agar plates and incubated at 28 °C. The next day a single colony from the agar plates were transferred to 50 ml Eppendorf tubes containing 25 ml of Tryptic Soy Broth (TSB) and grown overnight with 120 rpm shaking at 28 °C.

4.2.2. Prophage detection by Mitomycin C (MMC) assay

Induction of prophage in the ten experimental *Aeromonas* bacterial strains was investigated using Mitomycin C (MMC) (Acros Organics). Bacterial inocula were prepared by growing each strain overnight to an approximate optical density (OD_{600}) of 0.2. A stock solution of 1000 µg/ml MMC was used to prepare three different final concentrations of 100 ng/ml, 200 ng/ml and 400 ng/ml MMC in TSB. The controls did not contain any MMC (TSB only). MMC solutions (from each concentration) of 190 µl were dispensed into the wells of 96-well sterile microtiter plates that were then inoculated

with 10 µl of individual bacterial strains from overnight grown cultures. Blank wells were filled with 200 µl TSB broth. The inoculated plates were incubated at 28 °C and growth was monitored for 18 h at 30 min intervals using xMark^{TM} Microplate Spectrophotometer SW MPM6 (BIO-RAD). Growth curves were generated by plotting OD_{600} vs time (h). Growth of each bacterial strains in the presence of different concentrations of MMC was compared with the controls to determine the presence and induction of prophage. All samples from each concentration were tested in triplicate for each bacterial strain.

4.2.3. Sample collection

For the isolation of *Aeromonas* phages, a total of five sewage/waste water samples were collected from three sampling locations (Table 4-1) – a) raw effluent of sewage water samples collected from the sewage tank located at the Roseworthy campus, The University of Adelaide; b) three different raw effluents of waste water collected from the Bolivar waste water treatment plant, Bolivar, Adelaide, SA; and c) raw effluent samples of waste water collected from the Glenelg waste water treatment plant, Glenelg, Adelaide, SA. One litre of each sewage and waste water sample was collected in sterile glass bottles and kept on ice during transportation to the laboratory. Samples were processed immediately and stored at 4 °C.

Table 0-1. Description of the samples collected from different places for bacteriophage isolation

Collection Location	Sample name	Sample type
Roseworthy campus	Se	Raw sewage sample
Bolivar	PP	Raw water effluent from primary sedimentation tank
Bolivar	S	Raw water from screening tank
Bolivar	C	Raw waste water effluent
Glenelg	G	Raw waste water effluent

4.2.4. Bacteriophage isolation and enrichment

Published methods with minor modifications were used for the isolation, enrichment and purification of bacteriophages from the collected sewage and waste water samples (Merabishvili et al., 2009, Akhtar et al., 2014, Silva et al., 2014b). Water samples were centrifuged at 3000 ×*g* at 4 °C for 10 min (Centrifuge MPW-351R) and the supernatants filtered through 0.45 µm polycarbonate membrane syringe filters (Millex-HP 33 mm, Adelab Scientific, South Australia). Then 50 ml of filtered water samples were mixed with 50 ml of double strength (2×) TSB and 1 ml of exponentially grown *A. hydrophila* culture which was prepared on the same day. The mixtures of the filtered water samples

and *A. hydrophila* hosts were incubated at 28 °C, shaken at 60 rpm for 18-20 h. Following incubation, the mixed samples were centrifuged at 3000 ×*g* at 4 °C for 15 min and filtered with 0.45 µm polycarbonate membrane syringe filters. These filtrates potentially contained bacteriophages which were used for further analyses.

4.2.5. Bacteriophage confirmation by plaque assay and host range determination

Following the filtration, the phage preparations were used to conduct spot assays to determine the growth of the cultured bacteriophages. The spot assay was performed following the protocol described in section 3.2.2 of chapter 3.

To determine the host range of the isolated bacteriophages, all samples were subjected to spot testing against all ten bacterial strains. Not all plates showed positive results; plates exhibiting phage plaques only were used for further investigation. The next day, individual plaques from each positive plate were picked off with a sterile pipette tip and suspended in 1 ml SM buffer (5.8 g NaCl, 1.2 g MgSO4, 50 ml 1M Tris-HCl, pH 7.5, 0.1 g gelatin, 1000 ml deionized water) and kept for 1 h at room temperature (25-27 °C) to obtain a uniform plaque solution in SM buffer. The phage-buffer solution was then filtered with 0.45 µm polycarbonate membrane syringe filters before further use.

4.2.6. Bacteriophage purification and final stock preparation

For the purification of the bacteriophages, the filtered phage-SM buffer solution was serially diluted in TSB (10-fold) and plated on to lawn plates as conducted in the spot test. The plates were incubated overnight at 28 °C. Following incubation, a single phage plaque was carefully picked from the plate with a sterile pipette tip and suspended in SM buffer and kept for 1 h at room temperature (25-27 °C) to obtain a uniform plaque solution. This solution was then filtered with 0.45 µm polycarbonate membrane syringe filters. This whole process of making Phage-SM buffer solution from a purified single plaque and then the filtration was repeated three times to confirm a pure bacteriophage solution.

To make the final phage stock, purified single phage culture was again serially diluted in TSB (10-fold) and 20 µl aliquots of each dilution were plated onto lawn plates and incubated overnight at 28 °C. The next day, a pure bacteriophage solution was serially

diluted make up a 500 µl solution using the highest dilution of phage that just caused 100% clearing. An equal volume (500 µl) of log phase culture of host bacteria was then added to the phage-SM buffer solution and the mixed solution kept in an inverted position for 5 min. Then 2-3 agar lawn plates were prepared, incubated overnight and the following day 20 ml of SM buffer poured onto the plates exhibiting nearly 100% plaque clearing and held at room temperature for 2 h to infuse the phage preparations with SM buffer. Then using a sterile pipette, the phage-SM buffer solution was removed from the agar plates and poured into sterile 50 ml conical centrifuge tubes. The solution was then centrifuged at 3000 ×*g* for 30 min to remove any cell debris and filtered through 0.45 µm polycarbonate membrane syringe filters to obtain the pure phage stock culture. The purified final bacteriophage in SM buffer stocks were stored at 4 °C for further analyses.

4.2.7. Transmission Electron Microscopy (TEM)

To determine bacteriophage morphology, Transmission Electron Microscopy (TEM) (Adelaide Microscopy) was carried out following the methods described in section 3.2.4 of chapter 3.

4.2.8. Extraction of bacteriophage nucleic acid, enzymatic digestion and gel electrophoresis

Extraction of bacteriophage nucleic acid was carried out as described by Vinod et al. (2006) with some minor modifications. For phage precipitation, 20 µl (2M) sterile zinc chloride ($ZnCl_2$) was added to 1 ml of phage solution (1:50 ratio) and incubated at 37 °C for 5 min. Then the solution was centrifuged at 4000 ×*g* for 5 min. After discarding the supernatant, the pellet was resuspended in 200 µl of TENS buffer (50 mM Tris, pH 8.0, 100 mM EDTA, 100 mM NaCl and 0.3% SDS) and 0.5 µl of 40 µg/ml Proteinase K added and incubated at 65 °C for 10 min. To remove excess proteins, the same volume (200 µl) of phenol/chloroform/isoamyl alcohol (Sigma-Aldrich) was added to the TENS buffer solution, vortexed for 1 min and then centrifuged at 10,000 ×*g* for 10-15 min. The clear supernatant was transferred to a new tube. The protein extraction step was repeated twice and then 700 µl of isopropanol was added to the phage nucleic acid for precipitation and centrifuged at 10,000 ×*g* for 10-15 min. The precipitated pellet was then resuspended and washed with 1 ml of 70% ethanol and centrifuged again at 10,000 ×*g* for 10-15 min. The pellet was then resuspended in 30-50 µl of diethyl pyrocarbonate (DEPC) treated

water. The concentration (ng/µl) of nucleic acids of each sample were measured using the NanoDrop® Spectrophotometer, ND-1000, BIOLAB.

After the extraction of nucleic acids, RNase A, S1 Nuclease, and BamHI enzyme treatments were conducted according to the manufacturer's protocols (Thermo Scientific Australia) to confirm the nature of the extracted nucleic acids (Phumkhachorn and Rattanachaikunsopon, 2010, Vinod et al., 2006). Then the digested samples were run on 0.8% agarose gel electrophoresis at 100V for 1 h and the gel was imaged by Molecular Imager® ChemiDoc^{TM} XRS+ Imaging System (BIO-RAD).

4.2.9. Determination of the lytic ability of the isolated bacteriophages

To determine if the isolated bacteriophages could produce mortality in target host bacteria, four bacteriophages (Ah6_G, Ah9_PP, Ah10_Se and Ah10_S) were grown with their respective *A. hydrophila* bacterial strains (Ah6, Ah9 and Ah10). An equal volume of exponentially grown bacteria and bacteriophage were mixed together and then triplicate phage-bacteria mixtures were observed for bacterial lysis in a sterile 96-well microtiter plate. Each well of the microplate contained 100 µl of exponential bacteria + 100 µl bacteriophage. Control wells contained 100 µl bacteria + 100 µl of pure TSB broth. Blank wells were filled with 200 µl TSB broth. The optical density (OD_{600}) of each microplate well was measured using xMark^{TM} Microplate Spectrophotometer SW MPM6 (BIO-RAD) at 8 min intervals for a total of 13 h. The growth curves for each bacterial strain (with bacteriophage and control) were generated from the spectrophotometer readings.

4.3. Results

4.3.1. Induction of prophage in bacterial strains

Mitomycin C (MMC) is known to induce prophages which are integrated into bacterial genome. Lysogenic viruses are normally induced to undergo some lysis in the presence of MMC resulting in decreased growth of host bacteria compared to the control. After the treatment with MMC, growth curves (Figure 4-1) for each of the bacterial strains showed that 9 out of 10 bacterial strains demonstrated prophage induction, but isolate Ah5 did not show any sign of induction in its growth curve (Figure 4-1 [e]). This observation confirms that the 9 positive bacterial strains contained prophages in their genome, which indicated the presence of lysogenic induction within the bacterial genome. This assay was conducted to test the suitability of the experimental bacterial strains in isolating lytic

bacteriophage. However, more specific tests, such as search for the presence of integrase genes are needed to confirm lysogeny.

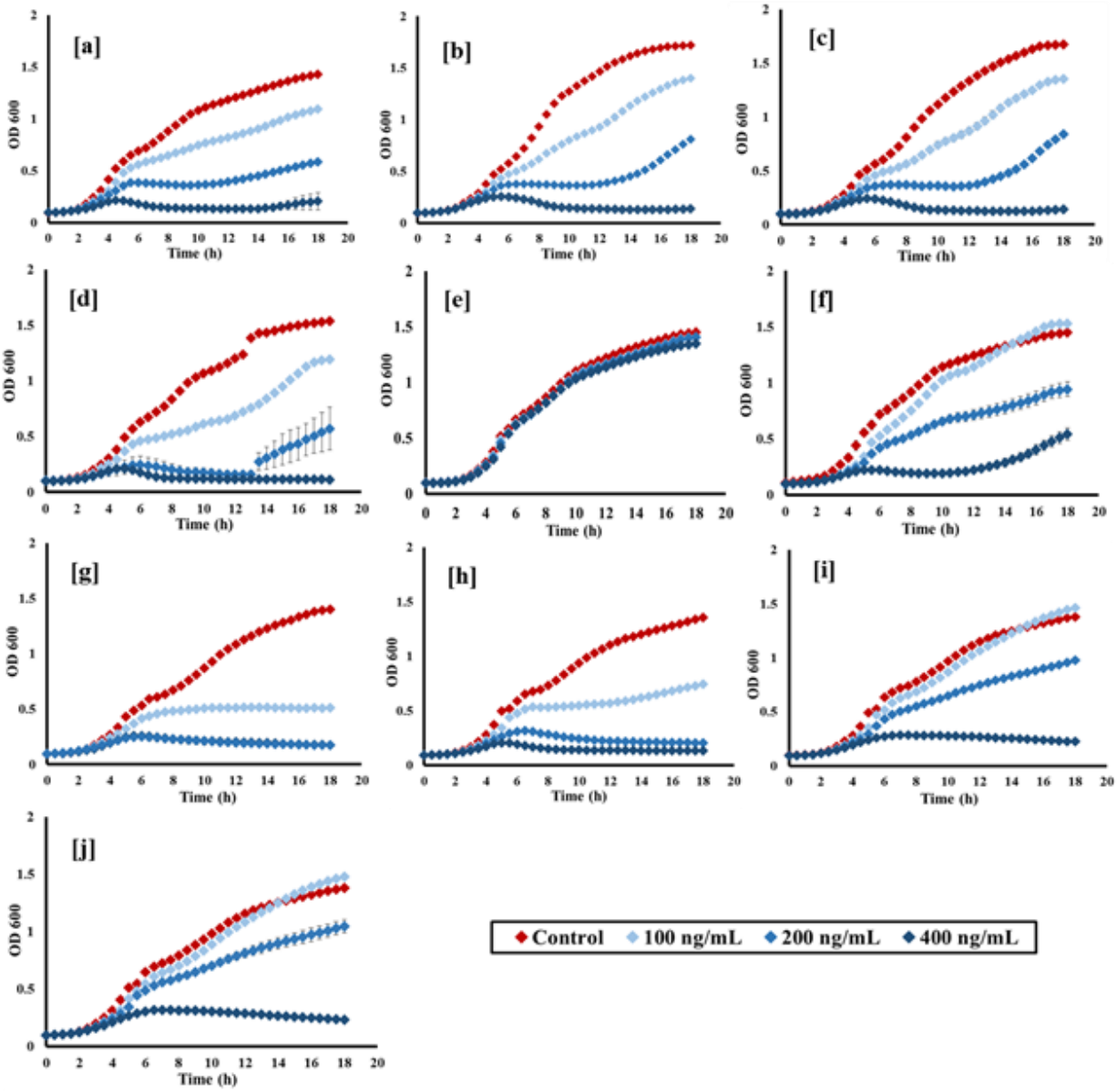

Figure 0-1. Growth profile of ten *A. hydrophila* treated with different concentration of MMC compared with the control group. [a] – Ah1; [b] – Ah2; [c] – Ah3; [d] – Ah4; [e] – Ah5; [f] – Ah6; [g] – Ah7; [h] – Ah8; [i] – Ah9 and [j] – Ah10. Growths are mean of triplicate observations of OD_{600}. Error bars represent standard error of mean. Some error bars are too small to be visible.

4.3.2. Host specificity of the isolated bacteriophages

Bacteriophages isolated from five different water samples were subjected to spot tests against all ten *A. hydrophila* bacterial strains (Ah1, Ah2, Ah3, Ah4, Ah5, Ah6, Ah7, Ah8, Ah9, and Ah10) to determine their host range. After incubation, the plates were examined

for complete clearing (lysis) within the applied spot, indicating the presence of a lytic phage that killed the host bacterium. If some turbidity within the plaques occurred, the phage is most likely temperate and therefore unlikely to be as effective for disease control. Samples were found to respond differently with different *A. hydrophila* host strains. The result for clear plaque formation by the bacteriophages on the ten *A. hydrophila* strains are presented in Table 4-2.

Out of 13 positive plaque formations observed, four bacteriophages (Ah6_G, Ah9_PP, Ah10_Se and Ah10_S – nomenclature based on their place of origin and bacterial strain) were selected to proceed with further experiments. These four bacteriophages were chosen to cover the range of phage sources, host susceptibilities and adequate phage titres (pfu/ml). The initial titre of these four bacteriophages is presented in Table 4-3.

Table 0-2. Bacteriophage host specificity against ten experimental *A. hydrophila* host strains. (√) indicates positive result (clear plaque formation)

Source of phage	**Strains**									
	Ah1	**Ah2**	**Ah3**	**Ah4**	**Ah5**	**Ah6**	**Ah7**	**Ah8**	**Ah9**	**Ah10**
Sewage (Se)									√	√
Bolivar (PP)	√								√	√
Bolivar (S)	√								√	√
Bolivar (C)									√	√
Glenelg (G)						√			√	√

Table 0-3. Titre of the selected bacteriophages and their plaque diameter

Bacteriophage	**Titre (pfu/ml)**	**Titre (Log_{10} pfu/ml)**	**Phage plaque diameter**
Ah6_G	1.27×10^9	9.10	0.5 mm
Ah9_PP	2×10^9	9.30	0.1 mm
Ah10_Se	1.8×10^9	9.26	0.5 mm
Ah10_S	1.4×10^9	9.15	0.5 mm

4.3.3. Identification of bacteriophages

4.3.3.1. Morphological observation

Transmission Electron Microscopy (TEM) was used to characterise and classify (Murphy, 1995, Ackermann, 2007, Fauquet et al., 2005) the selected bacteriophages based on their morphological features. The morphological view of the four bacteriophages in TEM images showed that they are all non-enveloped viruses. Three of four bacteriophages (Ah6_G, Ah10_Se and Ah10_S) were long tailed phages whereas Ah9_PP was short tailed. All four bacteriophages fall into the order *Caudovirales* and the presence of an

icosahedral head and long non-contractile tail would place three bacteriophages (Ah6_G, Ah10_Se and Ah10_S) in the *Siphoviridae* family. Another phage Ah9_PP possesses an icosahedral head and a short tail belongs to the viral family *Podoviridae* (Figure 4-2). Table 4-4 illustrates morphological description of four isolated bacteriophages and their respective families where they belong to.

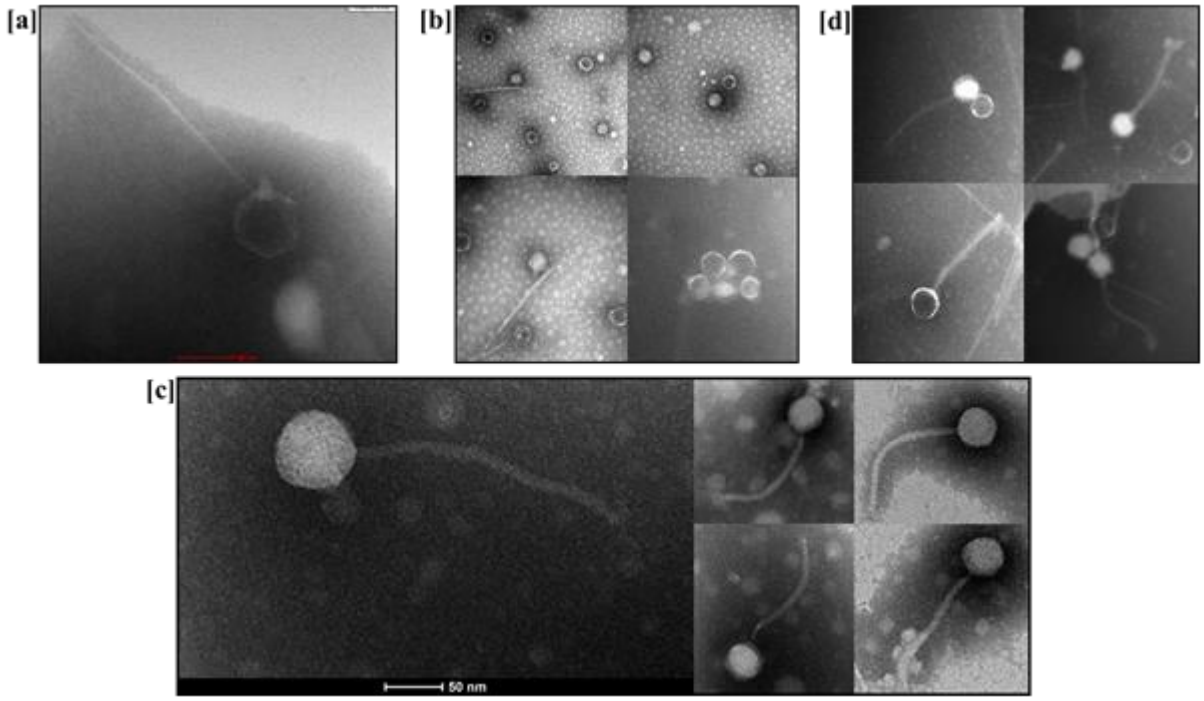

Figure 0-2. Transmission electron microscope view of four bacteriophages isolated and used in this study. [a] – Ah6_G; [b] – Ah9_PP; [c] – Ah10_Se and [d] – Ah10_S.

4.3.3.2. Organisation of nucleic acids of the isolated bacteriophages

The nucleic acids extracted from each of the four bacteriophages were treated with S1 Nuclease which is capable of cutting single stranded (ss) DNA or RNA. After S1 Nuclease treatment, gel electrophoresis did not reveal any small fragments of nucleic acid indicating the phage genome is double stranded (ds). Treatment of phage nucleic acids with RNase A did not reveal any fragments of nucleic acid, indicating the phage nucleic acid is DNA. As both of these enzymes failed to digest the bacteriophage nucleic acid, the four bacteriophages must possess double stranded DNA. Subsequently restriction endonuclease, BamHI digestion was performed on the phage nucleic acid. After BamHI digestion the agarose gels exhibited bands greater than 10 kb in size indicating each of the phages have genomes exceeding 10 kb of ds DNA (Figure 4-3).

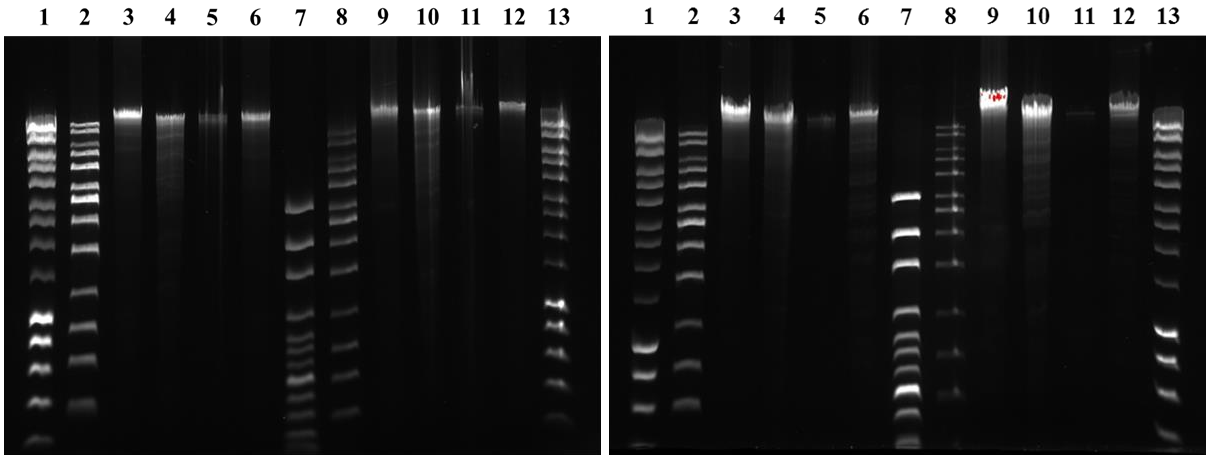

Figure 0-3. Gel image of the nucleic acids of the four isolated bacteriophages after digestion with three different enzymes (S1 Nuclease, RNase A and BamHI). [A] – Enzyme treated nucleic acids from bacteriophages Ah6_G and Ah9_PP. Lane 1 and 13 – hyper ladder; lane 2 and 8 – 1 kb molecular marker; lane 7 – 100 bp molecular marker; lane 3 – untreated Ah6_G phage nucleic acid; lane 4 – Ah6_G nucleic acid digested with S1 Nuclease; lane 5 – Ah6_G nucleic acid digested with RNase A; lane 6 – Ah6_G nucleic acid digested with BamHI; lane 9 – untreated Ah9_PP phage nucleic acid; lane 10 – Ah9_PP nucleic acid digested with S1 Nuclease; lane 11 – Ah9_PP nucleic acid digested with RNase A; lane 12 – Ah9_PP nucleic acid digested with BamHI. [B] – Enzyme treated nucleic acids from bacteriophages Ah10_Se and Ah10_S. Lane 1 and 13 – hyper ladder; lane 2 and 8 – 1 kb molecular marker; lane 7 – 100 bp molecular marker; lane 3 – untreated Ah10_Se phage nucleic acid; lane 4 – Ah10_Se nucleic acid digested with S1 Nuclease; lane 5 – Ah10SeG nucleic acid digested with RNase A; lane 6 – Ah10_Se nucleic acid digested with BamHI; lane 9 – untreated Ah10_S phage nucleic acid; lane 10 – Ah10_S nucleic acid digested with S1 Nuclease; lane 11 – Ah10_S nucleic acid digested with RNase A; lane 12 – Ah10_S nucleic acid digested with BamHI.

Table 0-4. Morphological description of the four bacteriophages as well as the families where they belong to depending on the morphology and their genomic material

Bacteriophage	Structure	Size (nm)	Nucleic acid	Family
Ah6_G	a. Head: Icosahedral b. Tail: Long, non-contractile and thin	a. Head diameter: 60-67 nm b. Tail length: 200-240 nm	dsDNA	*Siphoviridae* (Head: about 60 nm; tail: about 150 nm)
Ah9_PP	a. Head: Icosahedral b. Tail: short	a. Head diameter: 56-66 nm b. Tail length: 17-20 nm	dsDNA	*Podoviridae* (Head: About 63 nm; tail: about 17 nm)
Ah10_Se	a. Head: Icosahedral b. Tail: Long, non-contractile and thin	a. Head diameter: 73-77 nm b. Tail length: 235-265 nm	dsDNA	*Siphoviridae* (Head: about 60 nm; tail: about 150 nm)
Ah10_S	a. Head: Icosahedral b. Tail: Long, non-contractile and thin	a. Head diameter: 75-86 nm b. Tail length: 220-260 nm	dsDNA	*Siphoviridae* (Head: about 60 nm; tail: about 150 nm)

4.3.4. Lytic ability of the selected bacteriophages

When each of the bacterial strains were grown in TSB broths, they demonstrated typical bacterial growth curves (OD_{600} vs time) as observed in the control plots. As soon as the respective bacteriophages were added to the bacterial cultures, limited growth was observed as lysis of the hosts was greater than the rate growth of the bacteria (Figure 4-4). These growth curves confirm the lytic ability of the four selected bacteriophages to grow in their host cells and subsequently kill the host bacteria.

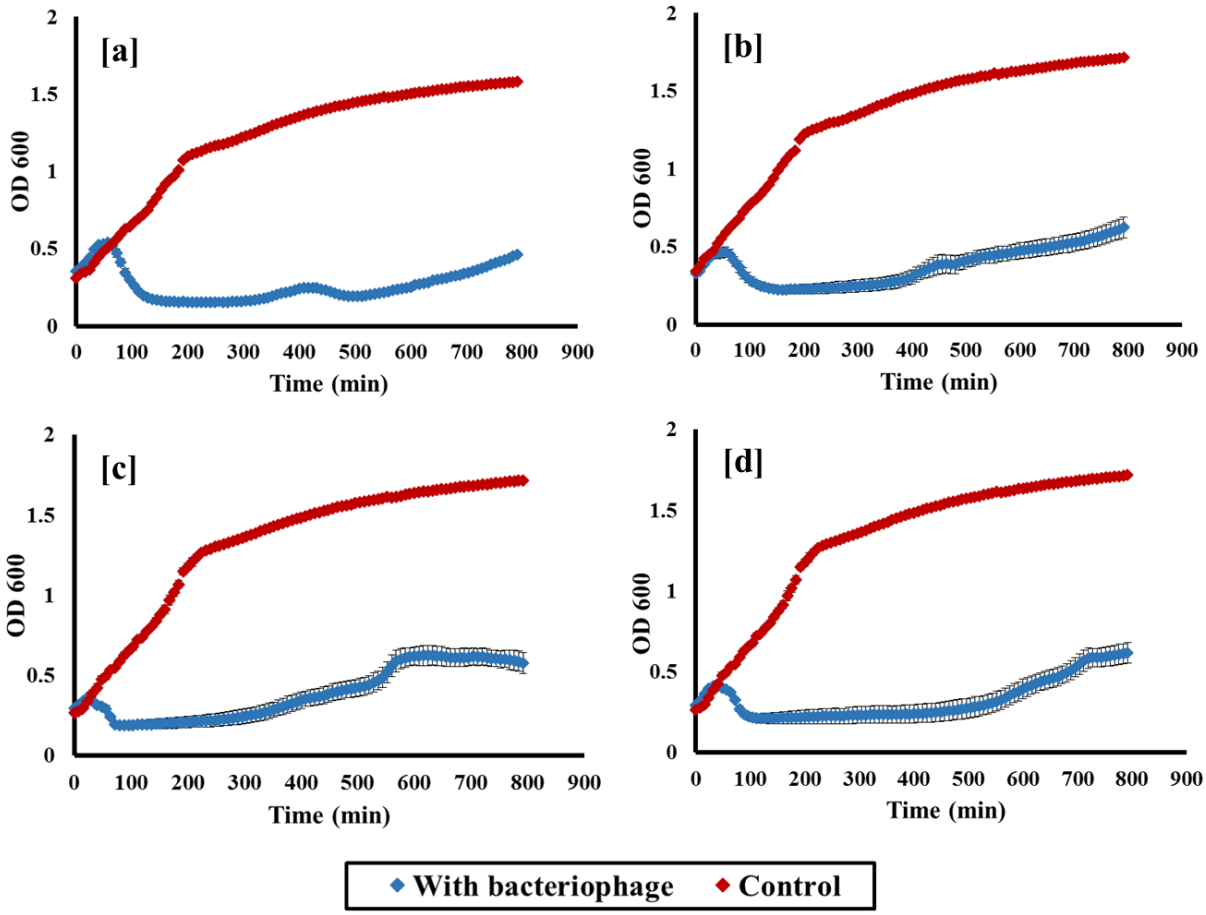

Figure 0-4. Bacterial growth curves of cultures infected with bacteriophages compared with the control group. [a] – Ah6 bacterial strain infected with Ah6_G; [b] – Ah9 bacterial strain infected with Ah9_PP; [c] – Ah10 bacterial strain infected with Ah10_Se; and [d] – Ah10 bacterial strain infected with Ah10_Se; and [d] – Ah10 bacterial strain infected with Ah10_S. Growths are mean of triplicate observations of OD_{600}. Error bars represent standard error of mean. Some error bars are too small to be visible.

4.4. Discussion

Bacteriophage therapy is gaining interest in aquaculture industries as a tool to control bacterial infections in an attempt to limit the widespread use of antibiotics. Studies on bacteriophages against *A. hydrophila* are of interest, with isolation and characterization of these phages as a first step to transition to use it as a therapeutic in aquaculture. In the present study we isolated some lytic *A. hydrophila* bacteriophages from different water sources (sewage and raw waste water effluent) and characterized them with a view to using them in controlling aeromoniasis in teleost fish.

To test that the bacterial hosts used in this study are suitable for isolation of bacteriophages, a prophage induction assay was carried out. Mitomycin C (MMC) is a

known chemical agent which is used for the prophage induction in bacteria. It is used to assess the occurrence of lysogenic integration of the viral genome within the bacterial genome (Silva et al., 2014b, Weinbauer and Suttle, 1999). It has been reported previously that a lysogenized bacterial host body tends to protect itself from infection with another closely related bacteriophage (Seed, 2015). Thus, phages which showed lytic plaques in this study were not the same as the lysogenic phages carried in their host genome. Moreover, it is well known that lytic bacteriophages (capable of producing clear plaque on agar plate) are more suitable to use for therapeutic purpose (Rao and Lalitha, 2015). Therefore, in this study environmentally available bacteriophages producing clear plaques were identified and selected for further study.

Bacteriophages can be isolated from anywhere in the environment where their respective host bacteria are present (Nakai and Park, 2002). A complex microbiota commonly resides in sewage water as it is sourced from different contamination-prone places including hospital drainage water (Piracha et al., 2014). This study also isolated phages from sewage and different waste water samples. Some other studies however utilised both fresh and marine water sources such as river water or sea water for the isolation of *A. hydrophila* bacteriophages (Cao et al., 2019, Easwaran et al., 2016, Jun et al., 2013). Another study described the isolation of *A. hydrophila* bacteriophage (AHP-1) from a mixture of tissue samples of a Crucian carp fish (Chandrarathna et al., 2020).

The first bacteriophages active against *Aeromonas* sp. were first reported by Bradley (1965). After that, the isolation and characterization of *A. hydrophila* bacteriophages have been described in a number of studies (Wu et al., 1981, Chow and Rouf, 1983, Merino et al., 1990a, Merino et al., 1990b, Cao et al., 2019, Yuan et al., 2018, Kazimierczak et al., 2019, Shen et al., 2012, Wang et al., 2016, Hoang et al., 2019). However, few studies described the protective abilities and *in vivo* application of these bacteriophages in fish (Akmal et al., 2020, Jun et al., 2013, Le et al., 2018, Hsu et al., 2000).

The present study isolated and evaluated the bacteriophage host range against ten different *A. hydrophila* strains. Wu et al. (1981) isolated eight bacteriophages active against one *A. hydrophila* but they provided detailed information on only one of these phages and their study did not include any description of the classification of their bacteriophage. Chow and Rouf (1983) reported two different bacteriophages Aeh1 and Aeh2, which were isolated from two different strains of *A. hydrophila*. In their study

phage Aeh1 was found to be active against eleven different *A. hydrophila* strains; whereas Aeh2 infected only its original host strain. Subsequently in this study Ah6_G, Ah9_PP, Ah10_S were found to infect three different *A. hydrophila* strains and Ah10_Se was found to infect only two. Later in 1990, two more *A. hydrophila* bacteriophages PM2 and PM3 were reported by Merino and co-workers (Merino et al., 1990a, Merino et al., 1990b). They found bacteriophages PM2 and PM3 were active against sixteen and seven different *A. hydrophila* host strains respectively. Another study reported by Jun et al. (2013) also used a wide range of bacterial isolates from different water sources. In contrast to these studies, some more recent studies on *A. hydrophila* isolation performed by Yuan et al. (2018), Cao et al. (2019) and Akmal et al. (2020) described the use of only one *A. hydrophila* strain for phage isolation. Nevertheless, to obtain higher bacteriophage efficacy in therapeutic purpose, it is recommended to use a cocktail/mixture of bacteriophages isolated from a wider range of bacterial isolates (Burrowes et al., 2011, Lu and Koeris, 2011).

The bacteriophages described in this study were lytic as they exhibited clear plaques of 0.1–0.5 mm diameter on TSB agar plates at 28 °C. In contrast to the phages in this study, some *A. hydrophila* bacteriophages have also been reported to be able to exhibit plaques at a higher temperature (37 °C) (Merino et al., 1990a, Merino et al., 1990b). However, it would be of interest to investigate further about the thermal stability of the isolated bacteriophages as it could be useful for bacteriophage application in different environments as well as to determine phage shelf life.

Endonuclease digestion with RNase A, S1 Nuclease and BamHI was conducted in this study to classify the bacteriophages based on their molecular characteristics and to determine their molecular size. Some previous studies (Phumkhachorn and Rattanachaikunsopon, 2010, Vinod et al., 2006) reported that *Siphoviridae* bacteriophages possess double stranded DNA and are larger than 10 kb. The enzyme digestion in this study also revealed similar results which confirms their family as *Siphoviridae*. The genome size of the podovirus described in the present study is consistent with the studies done by Wang et al. (2016), Cao et al. (2019) and Kazimierczak et al. (2019).

Transmission electron microscope (TEM) was used in this study to classify the bacteriophages based on their morphological analysis. The phage Ah6_G, Ah10_Se and Ah10_S morphologies described in this study are consistent with the morphology of

Siphoviridae phages described by Yuan et al. (2018) and Akmal et al. (2020). The TEM images of phage Ah9_PP revealed that this phage possesses an icosahedral head with a short tail (more like T7 phage). The head morphology and diameter (56-66 nm) and tail length of this phage is consistent with the phages belonging to the *Podoviridae* family (Murphy, 1995). A recent study by Kazimierczak et al. (2019) described five new *Podoviridae* phages specific for *A. hydrophila*. Other two *A. hydrophila* podoviruses were reported by Wang et al. (2016) and Cao et al. (2019). However, the isolated *A. hydrophila* bacteriophages of the present study require further genomic analyses to confirm their position as novel bacteriophage in the bacteriophage group.

4.5. Conclusion

This study reports the characteristics of bacteriophages that can kill a fish pathogen (*Aeromonas hydrophila*). The findings from this study provide a preliminary data for use in developing a bacterial control strategy for *Aeromonas* sp. infection using bacteriophages. However, more detailed characteristics at genomic level of the isolated bacteriophages are still required in order to understand phage-host interactions as well as for the assessment of the protective effect of lytic bacteriophages in designing future therapeutic models. The isolated bacteriophages in this study showed strong lytic potential against different strains of *A. hydrophila*, thus providing a sound basis for investigating their therapeutic potential for the control of *A. hydrophila* infection in fish.

Chapter 5. Whole genome sequencing of four selected *Aeromonas hydrophila* bacteriophages

5.1. Introduction

Aeromonas hydrophila, an opportunistic, Gram negative, ubiquitous, rod shaped, non-spore forming, facultative anaerobic bacterium is known to cause various diseases in different aquatic organisms and can be readily isolated from a number of fresh and saline aquatic habitats, human foods and from drinking water (Alavandi et al., 1998, Pablos et al., 2010, Wang et al., 2016, Anand et al., 2016). The most common diseases caused by this pathogen are: motile *Aeromonas* septicaemia (MAS), haemorrhagic septicaemia, red sore disease, tissue swelling, scale protrusion and different ulcerative diseases which result in high mortality rates in aquatic organisms within aquaculture industries worldwide (Wang et al., 2016, Jun et al., 2015, Easwaran et al., 2016). *A. hydrophila* have also been reported to exhibit a number of virulence factors e.g. cytotoxin production in the environment and in humans (Easwaran et al., 2016, Roges et al., 2020, Wu et al., 2019). To combat *A. hydrophila* infection in aquaculture, commercial antibiotics have been used extensively which has caused the emergence of multidrug resistance within this species (Jun et al., 2010, Cao et al., 2012). The emergence of multidrug resistance has driven the search for alternative strategies to control bacterial infections in aquaculture. Among these strategies, bacteriophage (or phage) therapy is considered a promising technology (Abedon et al., 2011).

Phages are unique amongst the alternative antimicrobial agents as they are environment friendly and host specific, eventually killing the host pathogen with minimal impact on other non-target bacteria or the host animal. They replicate only with the presence of their host bacterium (Li et al., 2012, Petrovski et al., 2012). They have already become well accepted worldwide and reported as a potential antimicrobial agent against a number of bacterial pathogens (Zhu et al., 2010). Phages can be a tool for the detection of different food and animal borne bacterial pathogens from the environment (Easwaran et al., 2016). Recently research on phage therapy against *A. hydrophila* has become a focus (Le et al., 2018). Some successful applications of *A. hydrophila* phages for therapeutic purposes against virulent strains in experimental fish models have been reported (Jun et al., 2013).

Other than therapeutic potential, phages play a key role in various ecological processes by structuring and balancing natural bacterial community, by maintaining genetic diversity of the bacteria (Wang et al., 2004, Mann, 2005, Weinbauer and Rassoulzadegan, 2004).

With the advancement of understanding phage genomics, some questions have arisen regarding the co-existence of phages, their hosts and their evolution. Some potential drawbacks associated with the use of phages has also come into focus. Due to lysogenic conversion, phages have been reported to transfer resistance and virulence genes between bacterial pathogens (Modi et al., 2013, Vincent et al., 2017). Hence, phage genomic investigations would aid to fully understand the mechanism of how phages interact with bacteria, the properties and functions of the phage genomes and their potential in expressing targeted virulent genes. However, despite the abundance and diversity of phages within the biosphere, only a small fraction of phage genomes are available in public sequence databases (Vincent et al., 2017). Therefore, research is still being carried out to better understand phage-bacteria interaction and the potential of phages to control bacterial infection in aquatic organisms.

Recently whole genome sequencing of phages has become a pivotal tool in analysing and understanding phage biology. Whole genome sequencing serves as a reliable platform to understand and compare the genes responsible for both beneficial and detrimental characteristics (Blattner et al., 1997). Phage genome analysis can help select the appropriate phage to use for prophylactic and therapeutic purpose (Carrias et al., 2011). The small size and relatively simple structure of phage genomes makes it simpler to carry out genome analysis and genetic alterations (Zhu et al., 2010, Hatfull, 2008). Before the work described herein, 29 complete genomic sequences of phages specific for *A. hydrophila* had been reported and deposited in GenBank (Yuan et al., 2018, Akmal et al., 2020, Bai et al., 2019, Kazimierczak et al., 2019, Wang et al., 2021, Cheng et al., 2021, Jin et al., 2020, Rai et al., 2020).

For developing a therapeutic tool using bacteriophages to combat *A. hydrophila* infection in fish (with silver perch as a model), four phages were isolated from different waste water treatment plants and sewage tanks and differentiated based on their nucleic acid digestion with restriction enzymes; transmission electron microscopic images and host susceptibility. Their original nomenclature, based on place of origin and bacterial strain, was Ah6_G, Ah9_PP, Ah10_Se and Ah10_S. Of the four phages, Ah6_G, Ah10_Se and Ah10_S were initially classified within the *Siphoviridae* family because of their long non-contractile tail and capsid diameter, while Ah9_PP was classified as belonging to *Podoviridae* family based on TEM images.

In order to fully understand the genetic basis and phylogenetic affiliation of these bacteriophages, a full genome sequence analysis is helpful. Therefore, whole genome sequencing was conducted to understand their relationships, adaptive evolution, biodiversity, potential virulence and presence of any toxin genes. The results presented here demonstrate that we have isolated two types of phages, one completely novel.

5.2. Materials and methods

5.2.1. Bacteriophages

The DNA of the four isolated bacteriophage samples (Ah6_G, Ah9_PP, Ah10_Se and Ah10_S) described in the Chapter 4 were used for the present study. The details of the phage isolation, purification and preparation are described in the section 3.2.1 of chapter 3.

5.2.2. Bacteriophage propagation

The propagation or enrichment of four phages using PEG precipitation was followed by the methods described by Castro-Mejía et al. (2015) and Dong et al. (2013) with some modifications. Initially 10 ml of each phage stock solution was mixed with their respective overnight grown *A. hydrophila* strain (OD_{600} 0.2) and then incubated at 28 °C for 30-60 min at 110 rpm. The cultures were then centrifuged at 3200 ×*g* for 10 min at 4 °C in 50 ml Falcon tubes. The supernatants were discarded and the pellets resuspended in 300 ml fresh TSB medium. The cultures were then grown in a shaker incubator for 16-20 h at 28 °C and 150 – 180 rpm. Following incubation the cultures were centrifuged at 17,700 ×*g* in a Beckman centrifuge at 4 °C for 10 min. The supernatants (300 ml) were filtered through 0.45 µm syringe filters, precipitated by adding 75 ml (1:4) of PEG solution and incubated overnight at 4 °C. The next day, the phage mixtures were centrifuged at 17,700 ×*g* in a Beckman centrifuge at 4 °C for 10 min. Supernatants were discarded, the pellets resuspended in 100 ml PBS, and precipitated by adding 25 ml (1:4) of PEG solution. The mixtures were incubated for 2 h at 4 °C. After incubation the phage mixtures were centrifuged at 17,700 ×*g* in a Beckman centrifuge at 4 °C for 10 min and the supernatants discarded. The pellets were resuspended in a total of 20 ml PBS and precipitated by adding 5 ml (1:4) of PEG solution. After 1 h of incubation at 4 °C, the cultures were centrifuged at 3200 ×*g* for 10 min at 4 °C and the pellets resuspended with

same volume (or less) of PBS to obtain the final precipitated phage solution. The phage titre was estimated by serially diluting the phage preparations 10-fold in PBS.

5.2.3. Bacteriophage genomic DNA extraction and purification

The intact bacteriophage genome was extracted for whole genome sequencing. The DNA was extracted and purified using QIAamp® DNA Mini Kit (QIAGEN) to obtain high yield DNA concentration. The DNA was separated by agarose gel electrophoresis using a 0.8% agarose gel in Tris Borate EDTA (TBE) buffer at 90 V. DNA concentration was checked using a NanoDrop® Spectrophotometer (ND-1000) and Agilent Technologies 2200 TapeStation. Phage DNA was further purified using GeneRead Size Selection Kit (QIAGEN) to obtain the specific DNA length.

5.2.4. Sequencing

Illumina Nextera libraries were made from each DNA samples and sequenced on an Illumina MiSeq.

5.2.5. Bioinformatics analyses

The 2x 100nt Illumina short reads were *de novo* assembled with Unicycler (Wick et al., 2017), which uses Spades for assembly (Bankevich et al., 2012) and Pilon for polishing (Chen et al., 2021). Genomes (scaffolds) were visualized and separated using Bandage (Wick et al., 2015). The genomes were compared to all known sequences using BLAST, looking for short regions of similarity. Genomes showing a high and extensive level of similarity to phage Ahp1 (KT949345) were compared to each other and to Ahp1 using LASTZ, to identify extended regions in common. Genomes showing very little similarity to any other known sequence were compared to each other using LASTZ.

All genomes were annotated using tools on the PATRIC website (Wattam et al., 2014). To identify protein coding sequences (CDSs) missed by the PATRIC tools and to compare CDSs from similar phage, in-house R scripts (R-3.3.3) were used.

The genomes were uploaded to NCBI database and contain the BioProject accession number PRJNA803069.

5.3. Results

5.3.1. Short read assembly using Illumina

When genomes were assembled from each sample using Unicycler and compared to all known sequences, it became apparent that two different types of phage were present. All four samples contained an Ahp1-like circular phage, but two samples, Ah10_Se and Ah10_S, also contained a novel linear phage. The Ahp1-like genomes ranged in length from 41.4 to 47.4 kb, and the novel phage from 61.2 to 61.3 kb (Table 5-1).

Table 5-1. Summary details of assembled bacteriophages

Sample	Bacteriophage	Category	Length (bp)	No. of contigs	Identity with Ahp1
Ah6_G	Ah6_G_C	Ahp1-like circular phage	47,360	9	85.6 %
Ah9_PP	Ah9_PP_C	Ahp1-like circular phage	41,428	1	85.3 %
Ah10_Se	Ah10_Se_C	Ahp1-like circular phage	41,859	13	85.8 %
Ah10_S	Ah10_S_C	Ahp1-like circular phage	44,947	12	85.4 %
Ah10_Se	Ah10_Se_L	Novel linear phage	61,173	1	
Ah10_S	Ah10_S_L	Novel linear phage	61,337	1	

5.3.2. Ahp1-like bacteriophages

The four circular Ahp1-like phages were found to be similar to each other and to Ahp1 over most of their genomes. Their identity (%) with Ahp1 is shown in Table 5-1. Alignments of each phage against Ahp1 are presented in Figure 5-1. And the details of the four Ahp1-like phage contigs and their coverage are presented in Table 5-2.

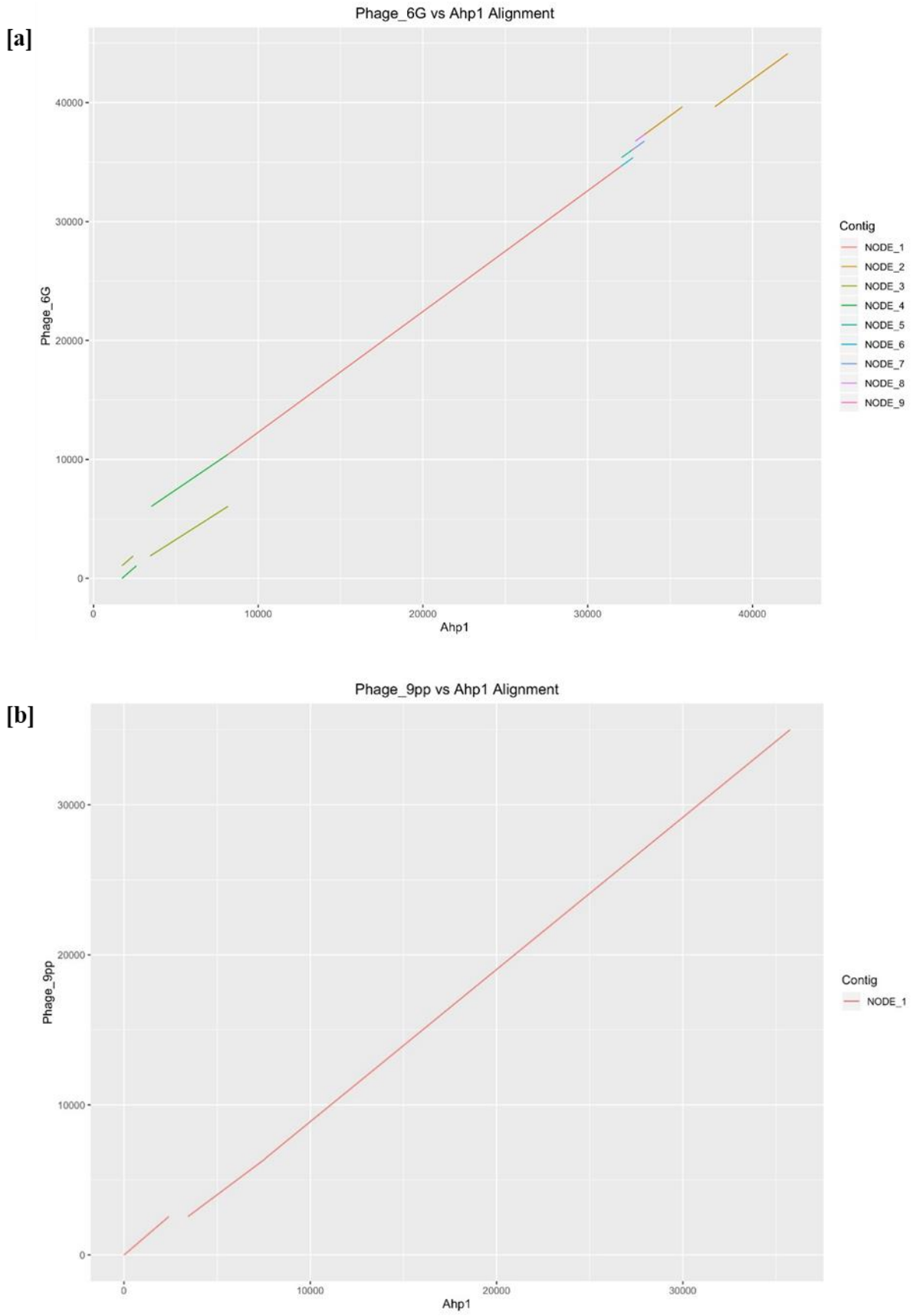

Figure 5-1. Sequence plots showing the alignments of phage Ah6_G and Ah9_PP against phage Ahp1 ([a] & [b] respectively).

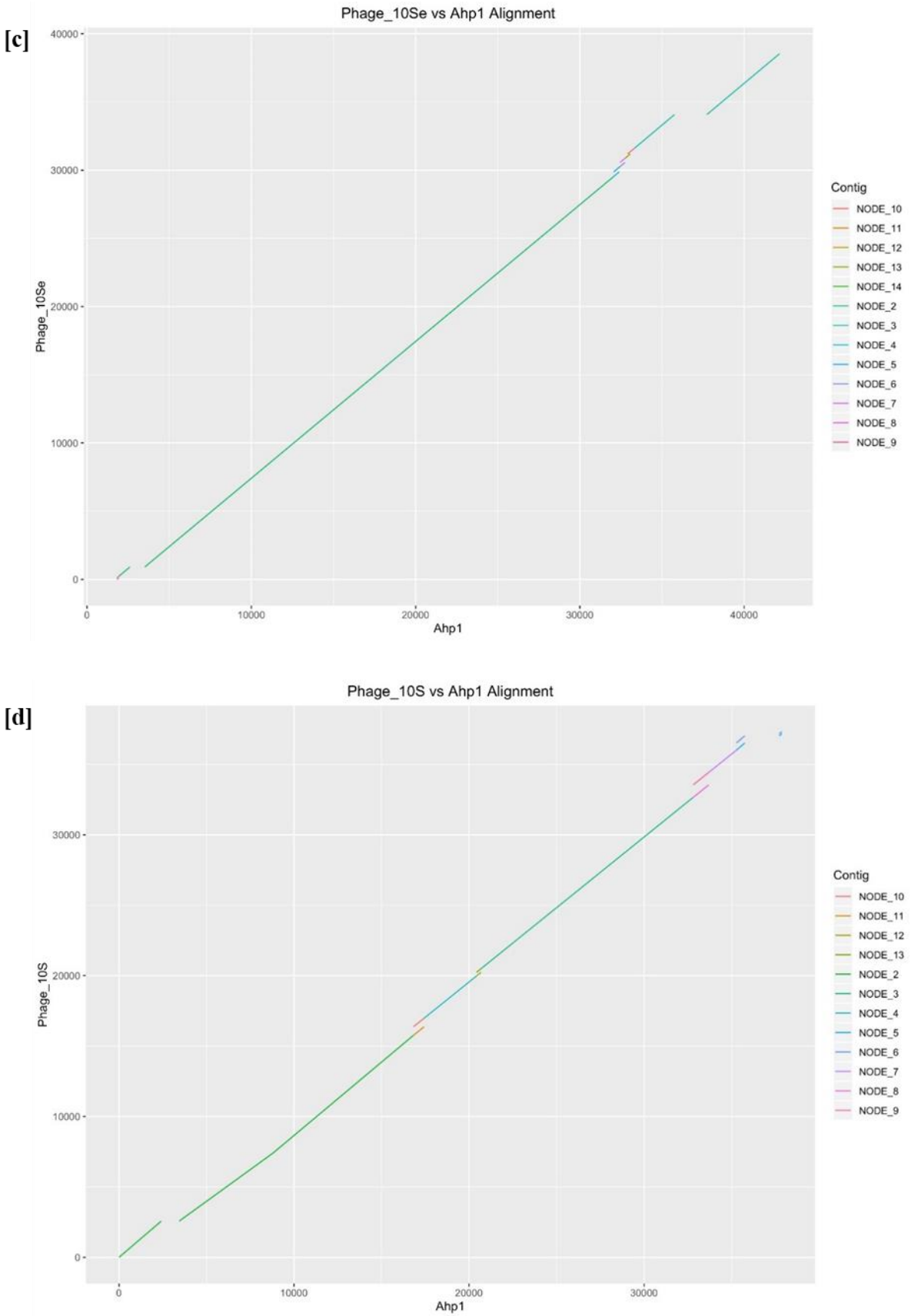

Figure 5-2. Sequence plots showing the alignments of phage Ah10_Se and Ah10_S against phage Ahp1 ([c] & [d] respectively).

Table 5-2. Contigs of the four Ahp1-like phages and their length and coverage

Sample	Bacteriophage	Contig	Length	Coverage
Ah6_G	Ah6_G_C	NODE1+	24,240	1.00
	Ah6_G_C	NODE2+	9,850	1.80
	Ah6_G_C	NODE3+	5,304	0.24
	Ah6_G_C	NODE4+	5,287	0.77
	Ah6_G_C	NODE5+	716	0.90
	Ah6_G_C	NODE6+	716	0.39
	Ah6_G_C	NODE7+	551	0.91
	Ah6_G_C	NODE8+	551	0.48
	Ah6_G_C	NODE9+	145	1.36
Ah9_PP	Ah9_PP_C	NODE1+	41,428	1.00
Ah10_Se	Ah10_Se_C	NODE2+	29,157	14.46
	Ah10_Se_C	NODE3+	10,055	26.42
	Ah10_Se_C	NODE4+	355	10.08
	Ah10_Se_C	NODE5+	355	9.29
	Ah10_Se_C	NODE6+	308	7.37
	Ah10_Se_C	NODE7+	308	9.65
	Ah10_Se_C	NODE8+	283	8.51
	Ah10_Se_C	NODE9+	267	15.01
	Ah10_Se_C	NODE10+	241	4.05
	Ah10_Se_C	NODE11+	166	8.33
	Ah10_Se_C	NODE12+	166	10.29
	Ah10_Se_C	NODE13+	145	17.90
	Ah10_Se_C	NODE14+	53	18.75
Ah10_S	Ah10_S_C	NODE2+	20,891	40.13
	Ah10_S_C	NODE3+	12,167	39.68
	Ah10_S_C	NODE4+	2,988	36.52
	Ah10_S_C	NODE5+	1,901	10.53
	Ah10_S_C	NODE6+	1,900	38.95
	Ah10_S_C	NODE7+	1,580	38.03
	Ah10_S_C	NODE8+	890	28.33
	Ah10_S_C	NODE9+	890	13.36
	Ah10_S_C	NODE10+	609	21.45
	Ah10_S_C	NODE11+	609	17.82
	Ah10_S_C	NODE12+	261	21.19
	Ah10_S_C	NODE13+	261	20.32

5.3.3. Novel linear bacteriophages

The two novel phages were found to be similar – Ah10_Se_L is 97.1% identical to Ah10_S_L and Ah10_S_L is 96.8% identical to Ah10_Se_L.

The CDS analysis of the two novel phages revealed that the coding sequence (CDS) counts were 75 and 73 for Ah10_Se_L and Ah10_S_L phages respectively. The functions of these CDSs are largely unknown (hypothetical proteins) with a few encoding proteins responsible for different phage structures (Table 5-3).

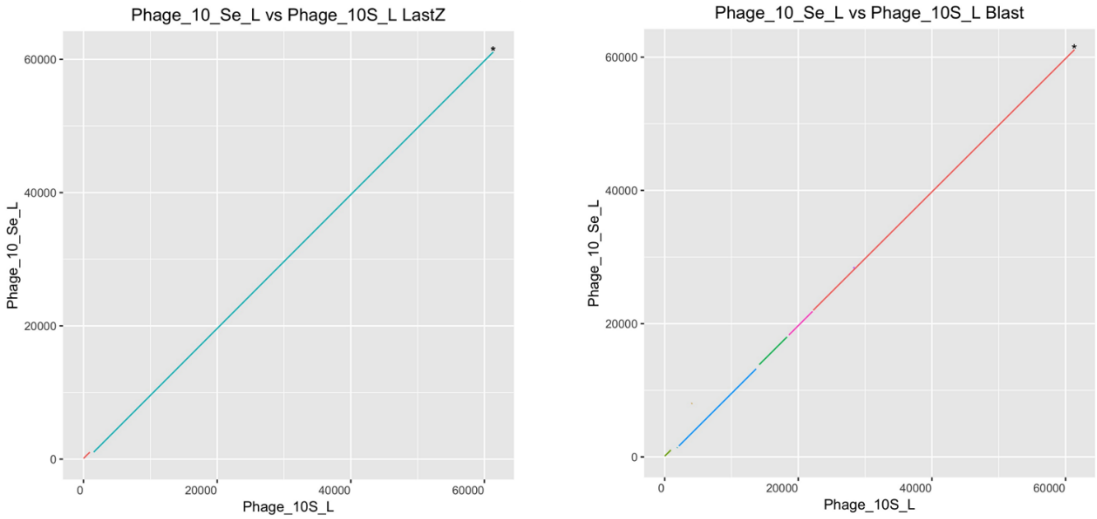

Figure 5-3. LASTZ alignments of phage Ah10_Se_L vs Ah10_S_L (* marks end of sequences).

Table 5-3. CDS functions

Function	**Count**
Hypothetical protein	67
Phage protein	2
ABC-type Fe3+-hydroxamate transport system, periplasmic component	1
DNA polymerase I	1
Magnesium and cobalt efflux protein CorC	1
Phage capsid and scaffold	1
Phage head, head-tail preconnector protease C / Phage head, scaffolding domain Nu3	1
Phage head, terminase DNA packaging protein A	1
Phage tail, component Z	1
putative DNA adenine methylase	1

5.3.4. BLAST results for each CDS of Ah10_Se_L and Ah10_S_L phages

5.3.4.1. Pairwise comparison

Matching CDSs across the novel genomes using protein function as a unique identifier was not possible because very few had an assigned protein function. Therefore, to find matching CDSs between the genomes, all CDSs for one phage were compared to all CDSs for the other by using BLAST, a total of 5475 comparisons. Examples of these BLAST results are given in Table 5-4.

For each CDS, there is usually only one comparison that shows high similarity across the majority of their sequence. For example for Ah10_S_L CDS 426.peg.1, only Ah10_Se_L CDS 427.peg.2 shows homology over more than a few percent of its length. This approach allowed the identification of the CDSs of two novel phages that are homologous to each other. Further examples of highly similar comparisons are given in Table 5-5.

Table 5-4. BLAST results for each CDS*

Query genome	Subject genome	Query sequence ID	Subject sequence ID	Identity	Length	Mismatch	Gaps	Query start	Query end	Subject start	Subject end
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.2	88.6%	656	75	0	1	656	1	656
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.48	94.4%	18	1	0	515	532	1196	1179
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.48	93.3%	15	1	0	75	89	458	472
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.48	100.0%	12	0	0	149	160	905	916
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.3	100.0%	15	0	0	359	373	700	686
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.3	77.4%	31	7	0	528	558	843	873
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.3	90.0%	20	1	1	277	295	1285	1266

Query function	Subject function	Query length	Subject length	Query full length	Subject full length	Query fraction	Subject fraction	Fraction product
ABC-type Fe3+-hydroxamate transport system	Hypothetical	656	656	657	657	0.998	0.998	0.997
ABC-type Fe3+-hydroxamate transport system	Hypothetical	18	18	657	1422	0.027	0.013	0.000
ABC-type Fe3+-hydroxamate transport system	Hypothetical	15	15	657	1422	0.023	0.011	0.000
ABC-type Fe3+-hydroxamate transport system	Hypothetical	12	12	657	1422	0.018	0.008	0.000
ABC-type Fe3+-hydroxamate transport system	Hypothetical	15	15	657	1428	0.023	0.011	0.000
ABC-type Fe3+-hydroxamate transport system	Hypothetical	31	31	657	1428	0.047	0.022	0.001
ABC-type Fe3+-hydroxamate transport system	Hypothetical	19	20	657	1428	0.029	0.014	0.000

* Not all columns shown, some truncated, some removed

Table 5-5. Top BLAST results for each CDS

Query genome	Subject genome	Query sequence ID	Subject sequence ID	Identity	Length	Mismatch	Gaps	Query start	Query end	Subject start	Subject end
Ah10_S_L	Ah10_Se_L	426.peg.1	427.peg.2	88.6%	656	75	0	1.0	656	1	656
Ah10_S_L	Ah10_Se_L	426.peg.2	427.peg.3	70.4%	1443	359	32	1.0	1409	1196	1179
Ah10_S_L	Ah10_Se_L	426.peg.3	427.peg.4	90.1%	496	47	2	1.0	495	458	472
Ah10_S_L	Ah10_Se_L	426.peg.4	427.peg.5	90.6%	438	41	0	1.0	438	905	916
Ah10_S_L	Ah10_Se_L	426.peg.5	427.peg.6	87.0%	679	86	2	1.0	678	700	686
Ah10_S_L	Ah10_Se_L	426.peg.6	427.peg.7	91.5%	199	17	0	1.0	199	843	873
Ah10_S_L	Ah10_Se_L	426.peg.7	427.peg.8	99.3%	1062	7	0	1.0	1062	1285	1266

Query function	Subject function	Query length	Subject length	Query full length	Subject full length	Query fraction	Subject fraction	Fraction product
ABC-type Fe3+-hydroxamate transport system	Hypothetical	656	656	657	657	0.998	0.998	0.997
Hypothetical	Hypothetical	1409	1409	1998	1428	0.705	0.987	0.696
Hypothetical	Hypothetical	495	495	495	495	1.000	1.000	1.000
Hypothetical	Hypothetical	438	438	438	438	1.000	1.000	1.000
Hypothetical	Hypothetical	678	678	678	678	1.000	1.000	1.000
Hypothetical	Hypothetical	199	199	201	201	0.990	0.990	0.980
Hypothetical	Hypothetical	1062	1062	1062	1062	1.000	1.000	1.000

5.3.4.2. Consolidation of CDS BLAST comparisons

There are 77 unique CDSs across the two linear phages. Table 5-6 shows a simplified comparison between the linearly arranged CDSs of two phages, highlighting differences. Out of 71 shared CDSs, 68 CDSs have > 80% identity over > 98% of their lengths while the remaining 3 CDSs are less similar over a shorter length. These remaining 3 CDSs, not only the sequences are substantially different lengthwise but also they do not have much protein matching at all (Table 5-7), given the fact that they are effectively the same protein but they may have been diverged from their ancestral protein coding sequences.

Two pairs of CDSs (CDS 24/75 and 32/76) are similar in position but show little sequence similarity. However, CDSs 24/75 do share 12 identical amino acids at their 5′ ends and CDSs 32/76 share 5 of the last 6 amino acids at their 3′ ends (Table 5-8).

Ah10_Se_L CDS 74 is the most 5′ CDS in this genome and appears truncated, suggesting the assembly may not be complete. It did not have a cognate in Ah10_S_L. Examination of the Ah10_S_L sequence revealed 38 bp of homology to the 3′ end of Ah10_Se_L CDS 74, 13 bp from the 5′ end of the genome. This suggests that either the CDS in Ah10_S_L has been truncated or like Ah10_Se_L, the assembly is not complete (Table 5-9).

Ah10_Se_L CDS 77 comprises 126 bp and located at about 1/3 position along the genome. This CDS did not have a cognate in Ah10_S_L, but comparison of DNA sequences revealed a perfect match in the appropriate position in Ah10_S_L (Table 5-10). It is unclear why the PATRIC tools failed to report this CDS. This increases the number of matching CDSs to 72.

Table 5-6. BLAST comparisons between the CDSs of Ah10_S_L and Ah10_Se_L

CDS ID	**Function**	**Ah10_S_L ID**	**Ah10_Se_L ID**	**Ah10_S_L Length***	**Ah10_Se_L Length***
CDSID 1	Hypothetical	426.peg.1	427.peg.2	218	218
…	…	…	…	…	…
CDSID 23	Hypothetical	426.peg.23	427.peg.24	111	111
CDSID 24	Hypothetical	426.peg.24		169	
CDSID 75	Hypothetical		427.peg.25		225
CDSID 25	Hypothetical	426.peg.25	427.peg.26	77	77
…	…	…	…	…	…
CDSID 31	Hypothetical	426.peg.31	427.peg.32	118	118
CDSID 32	Hypothetical	426.peg.32		98	
CDSID 76	Hypothetical		427.peg.33		101
CDS ID 33	Hypothetical	426.peg.33	427.peg.34	226	226
…	…	…	…	…	…
CDSID 73	Hypothetical	426.peg.73	427.peg.75	61	61
…	…	…	…	…	…
CDSID 74	Hypothetical	Truncated	642.427.peg.1		51
CDSID 77	Hypothetical	Present	642.427.peg.43		41

*Protein length

Table 5-7. Comparison of the 3 matching CDSs of the two linear phages showing less similarity

Query function	Subject function	Query length	Subject length	Query full length	Subject full length	Query fraction	Subject fraction	Fraction product
Hypothetical	Hypothetical	1409	1409	1998	1428	0.705	0.987	0.696
Hypothetical	Hypothetical	987	987	1389	1461	0.711	0.676	0.480
Hypothetical	Hypothetical	114	114	114	222	1.000	0.514	0.514

Table 5-8. CDSs details of the matching pairs (CDS ID 24/75 and 32/76)

Genome	CDS ID	PEG	Start	Stop	Strand	Length	Protein
Ah10_S_L	24	426.peg.24	13658	14167	+	510	MSTNAVIALTPEALNVLFPEGSEARVELQ…
Ah10_Se_L	75	427.peg.25	13184	13861	+	678	MSTNAVIALTPESVGHTLKGMYRSFQQCG…
Ah10_S_L	32	426.peg.32	18314	18610	+	297	…VAINAAIKDVIDKEGL
Ah10_Se_L	76	427.peg.33	18008	18313	+	306	…DRAWAAVRTAIDEEGL

Table 5-9. Analysis of CDS 74 in Ah10_Se_L

Genome	CDS ID	PEG	Start	Stop	Strand	Length	Protein
Ah10_Se_L	74	427.peg.1	1	156	+	156	ALAYEKAAWNTSDFVRSCYFFFKQKTAYE…

Query	Query ID	Subject	Identity	Length	Mismatch	Gaps	Subject start	Subject end	Subject strand
Ah10_Se_L	427.peg.1	Ah10_S_L	92.1%	38	3	0	13	50	+

Table 5-10. Analysis of CDS 77 in Ah10_Se_L

Genome	CDS ID	PEG	Start	Stop	Strand	Length	Protein
Ah10_Se_L	77	427.peg.43	24392	24517	+	126	MPSGKGPGLRKAVPPTTTDTKKDKKKDKQRKDEK VSPDSKG…

Query	Query ID	Subject	Identity	Length	Mismatch	Gaps	Subject start	Subject end	Subject strand
Ah10_Se_L	427.peg.43	Ah10_S_L	100%	126	0	0	24617	24742	+

5.3.4.3. Summary of the two linear phage CDS comparison

Overall these results can be summarized as:

- 72 CDSs are matched in the two linear phages
  - DNA identity and length of matches vary in some cases but are substantial
- 1 CDS was missed in Ah10_S_L because it is at the genome 5′ end
  - 5′ prime end of CDS appears to be truncated in Ah10_S_L
  - 38 bp match with 100% DNA identity
- 4 CDSs occupy approximately the same position but do not match
  - CDS 24/75 share 12 amino acids at their 5′ prime ends
  - CDS 32/76 share 5-6 amino acids at their 3′ prime ends.

5.4. Discussion

The present study examined complete genome sequencing of phages from four bacteriophage samples to investigate their genomic characteristics and similarity to known phages.

The assembly results showed that all four bacteriophages are similar to the genome sequence of Ahp1, but unexpectedly two bacteriophage samples, Ah10_Se and Ah10_S, also carried a novel linear phage. The complete genome sequencing of these four phages confirmed the fact that they possess dsDNA which was consistent with the results obtained from restriction enzymes digestion carried out earlier (Chapter 4).

From the restriction enzyme digestion and Transmission Electron Microscopy conducted in the preliminary experiments (Chapter 4), it was found that all phage samples Ah6_G, Ah10_Se and Ah10_S belong to the *Siphoviridae* family, whereas the feature phage Ah9_PP matches with *Podoviridae* family. The complete genome sequence of these four phages confirmed that the DNA sizes are consistent with the measurements of TEM and restriction enzyme digestion (Chapter 4).

A summary of the genomic features of *A. hydrophila* phage sequenced till date is collated in Table 5-11. Researchers have mainly used different Illumina platforms for sequencing and assembled the reads using various assembly tools (Table 5-11). Illumina sequencing is widely used despite its limitation of producing short reads that result in piecemeal genome assembly. Long read technologies such as those of Pacific Bioscience and

Oxford Nanopore may offer an alternative approach for sequencing phage genome as this technology can read the full length of these phage genomes, obviating the issues with short read assembly. However, Oxford Nanopore sequencing has a much higher error rate than Illumina that appears to contain systematic errors, so hybrid assembly of both ONT and Illumina reads using a tool like Unicyler may provide the best quality genomes at a reasonable cost. Alternatively, Pacific Biosciences reads are somewhat more accurate but are more expensive (Wick et al., 2017).

In the previous reports, the genome size range of *A. hydrophila* phages of *Siphoviridae* family were observed to be approximately 112-115 Kb (Table 5-11). In contrast, the present study reports the genome size of phages Ah6_G, Ah10_Se and Ah10_S to be 47.36 Kb, 61.17 Kb and 61.34 Kb respectively. Hence, the sizes of *Siphoviridae* phage genomes observed in the present study are smaller than the other similar phages found in the literature. However, the genome size of *Podoviridae* phage Ah9_PP was found to be 41.43 Kb which is similar to the most of the *Podoviridae* phage genomes reported to date (Table 5-11).

All *A. hydrophila* phages belonging to *Siphoviridae* family reported so far contain linear dsDNA (Akmal et al., 2020, Bai et al., 2019, Yuan et al., 2018). However, in the present study, it was observed that the phage Ah6_G, which is a member of *Siphoviridae* possesses circular dsDNA. The *Podoviridae* phage Ah9_PP analysed in this study is a circular dsDNA phage in contrast to other *Podoviridae* which are mostly linear dsDNA phages, except MJG which has a circular genome (Cao et al., 2019) (Table 5-11).

For the two novel linear phage most of the encoded protein products have never been described before and their CDSs are listed as hypothetical, with only a few sufficiently similar to those present in databases to identify them (Table 5-3). Therefore, it was not possible to determine which CDSs are shared by the two novel phages, based on function. Therefore, a pairwise comparison was conducted using DNA BLAST to compare all the CDSs between the genomes of Ah10_Se_L and Ah10_S_L. The comparison revealed that most of the CDSs are homologous, but there are also substantial differences between them. These two phages were isolated from waste water samples from two different places. The fact that some CDSs are missing and/or do not match suggests the two phages may have originated from the same ancestor but that they have diverged substantially.

The ONT analysis revealed that the long read lengths were consistent with that of the Illumina short reads. When further attempt to partition the long reads were made to compare between the short read genomes, not enough reads were found to be partitioned. Therefore, no further work was carried out using ONT in the present study.

Table 5-11. *Aeromonas hydrophila* bacteriophages sequenced to date

Name	Family	Type	Size (bp)	Lytic/ lysogenic	DNA type	Sequencing and assembly methods	Accession no.	Reference
Aeh1	*Myoviridae*	T4-like	233234	Lytic	ds, Linear	Sanger sequencing.	NC005260	(Petrov et al., 2006)
CC2	*Myoviridae*	T4-like	231743	Not reported	ds, Linear	454 GS-FLX Titanium Sequencing System (Roche).	JX123262	(Shen et al., 2012)
pAh6-C	*Myoviridae*		53744	Lytic	ds, Circular	Sanger sequencing and Next Generation Sequencing System (NGS: Ion PGM 314 sequencer.	KJ858521	(Jun et al., 2015)
AH1	*Myoviridae*		221116	Lytic	ds, Linear	IonTorrent Sequencing, GS *de novo* assembler version 2.6.	MG250483	Unpublished
50AhydR13PP	*Myoviridae*		164983	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179476	(Kazimierczak et al., 2019)
60AhydR15PP	*Myoviridae*		165795	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179477	(Kazimierczak et al., 2019)
85AhydR10PP	*Myoviridae*		47194	lysogenic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179479	(Kazimierczak et al., 2019)
13AhydR10PP	*Myoviridae*		47828	lysogenic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179470	(Kazimierczak et al., 2019)
14AhydR10PP	*Myoviridae*		48335	lysogenic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179471	(Kazimierczak et al., 2019)
CF8	*Myoviridae*		238150	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using SPAdes version 3.7.1.	MK774614	(Rai et al., 2020)
PS1	*Myoviridae*		237367	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using SPAdes version 3.7.1.	MN032614	(Rai et al., 2020)
PS2	*Myoviridae*		240447	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using SPAdes version 3.7.1.	MN453779	(Rai et al., 2020)
Ahp2	*Myoviridae*		47331	Lytic	ds, Linear	Illumina sequencing assembled with ABySS version 1.3.7.	KX455876	(Wang et al., 2021)

Name	Family	Type	Size (bp)	Lytic/ lysogenic	DNA type	Sequencing and assembly methods	Accession no.	Reference
AhyVDH1	*Myoviridae*		39175	Lytic	Linear	Illumina Hiseq assembled with SPAdes version 3.5.0.	MT276994	(Cheng et al., 2021)
AhSzq-1	*Siphoviridae*	*T5virus*	112558	Not reported	ds, Linear	Illumina HiSeq1500 sequencer, *de novo* assembly using SOAP-denove.	MG676224	(Yuan et al., 2018)
AhSzw-1	*Siphoviridae*	*T5virus*	115739	Not reported	ds, Linear	Illumina HiSeq1500 sequencer, *de novo* assembly using SOAP-denove.	MG676225	(Yuan et al., 2018)
Akh-2	*Siphoviridae*		114901	Lytic	ds, Linear	Illumina HiSeq1500 sequencer, *de novo* assembly using Platanus version 1.2.2.	MK318083	(Akmal et al., 2020)
MJG	*Podoviridae*	*Sp6virus*	45057	Lytic	ds, Circular	Illumina MiSeq platform, assembled with SPAdes version 3.9.0.	MK455769	(Cao et al., 2019)
Ahp1	*Podoviridae*	phiKMV-like	42167	Lytic	ds, Linear	Illumina Solexa technology.	KT949345	(Wang et al., 2016)
22PfluR64PP	*Podoviridae*	T7virus	40822	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179472	(Kazimierczak et al., 2019)
25AhydR2PP	*Podoviridae*		42696	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179473	(Kazimierczak et al., 2019)
71PfluR64PP	*Podoviridae*	T7virus	40582	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179475	(Kazimierczak et al., 2019)
67PfluR64PP	*Podoviridae*	T7virus	40748	Lytic	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179478	(Kazimierczak et al., 2019)
98PfluR60PP	*Podoviridae*		74361	Not classified	ds, Linear	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179480	(Kazimierczak et al., 2019)
CF7	*Podoviridae*		42439	Not reported	ds, Linear	Illumina sequencing assembled with SPAdes version 3.7.1.	MF683623	Unpublished
ZPAH7	*Autographiviridae*		30791	Not reported	ds, Linear	Illumina sequencing assembled with MicrobeTrakr plus version 0.9.1.	MH992513	Unpublished
Name	**Family**	**Type**	**Size**	**Lytic/**	**DNA type**	**Sequencing and assembly**	**Accession**	**Reference**

		(bp)	lysogenic		methods	no.	
ZPAH7B	*Autographiviridae*	30791	Not reported	ds, Linear	Illumina sequencing assembled with MicrobeTrakr plus version 0.9.1.	MK330684	Unpublished
62AhydR11PP	Unclassified	43755	Probably lysogenic	Unclassified	Illumina MiSeq sequencing, *de novo* assembly using CLC Genomic Workbench 7.5.	MH179474	(Kazimierczak et al., 2019)
HX-3		4941513	Lytic	Circular	Illumina Hiseq combined with third-generation sequencing.	CP046954	(Jin et al., 2020)
Ah6_G	*Siphoviridae*	47360	Lytic	ds, Circular	Illumina short reads and Oxford Nanopore long reads were assembled with Unicycler and Canu respectively.		This study
Ah9_PP	*Podoviridae*	41428	Lytic	ds, Circular	Illumina short reads and Oxford Nanopore long reads were assembled with Unicycler and Canu respectively.		This study
Ah10_Se	*Siphoviridae*	61173	Lytic	ds, Linear	Illumina short reads and Oxford Nanopore long reads were assembled with Unicycler and Canu respectively.		This study
Ah10_Se	*Siphoviridae*	41859	Lytic	ds, Circular	Illumina short reads and Oxford Nanopore long reads were assembled with Unicycler and Canu respectively.		This study
Ah10_S	*Siphoviridae*	61337	Lytic	ds, Linear	Illumina short reads and Oxford Nanopore long reads were assembled with Unicycler and Canu respectively.		This study
Ah10_S	*Siphoviridae*	44947	Lytic	ds, Circular	Illumina short reads and Oxford Nanopore long reads were assembled with Unicycler and Canu respectively.		This study

5.5. Conclusion

The present study reports the analysis of the genome sequences of six new *A. hydrophila* bacteriophages and a preliminary study of their genomic structure. The phages form two groups: each sample carried an Ahp1-like phage but two samples carried a novel linear phage. This preliminary study will serve as a basis on which to study their diversity, evolutionary relationship and adaptation within their respective environment as well as with their hosts. Additionally, the lytic ability of these four phages along with the genomic information presented here may also facilitate the diagnostic and therapeutic applications of phages in the aquaculture sector.

Further research is required to understand the functions of the genes and compare them with both related pathogens as well as with other microbial genomes to discover and explore evolutionary relationship.

Chapter 6. Effect of temperature and freeze-drying on the storage efficacy of selected *Aeromonas hydrophila* bacteriophages

6.1. Introduction

Bacteriophages (or phages) are a group of naturally occurring prokaryotic viruses which can infect specific target bacteria, subsequently either, replicating within the host body and killing the host bacteria (lytic bacteriophages), or, by replicating themselves as prophages within their host bacterial genome (lysogenic bacteriophages) (Colom et al., 2015, Golshahi et al., 2008, Clark and March, 2006, Monk et al., 2010). Bacteriophages are natural predators of bacteria and cannot harm human or animal cells. These organisms are highly host specific and metabolically inactive without the presence of their specific host bacteria (Jepson and March, 2004). Bacteriophages are one of the most abundant living entities in the biosphere and can be found wherever their respective host body is present. They are approximately ten times higher in number than the bacteria and can cause mortality in 10 – 80% of the bacterial community in a habitat (Hanlon, 2007, Jończyk et al., 2011, Edwards and Rohwer, 2005, Lin et al., 2010).

A phage particle consists of nucleic acid (DNA or RNA) encapsulated within a protein coat, called a capsid and in some cases a tail fibre which helps the phage to attach to bacterial cell surface receptors (Clark and March, 2006, Hawkins et al., 2010, Haq et al., 2012a). Over the past few decades, the therapeutic potential of bacteriophages has gained immense public and scientific attention due to concerns about the deleterious effects of antibiotic resistance. Some attributes of bacteriophages such as, relatively less complex structure, host specificity without harming the commensal microbiota, rapid replication rate and well-defined organizational skills in terms of combating bacterial infection make them a potential candidate for treating bacterial infections (Alfadhel et al., 2011, Hawkins et al., 2010, Storms et al., 2012, Merabishvili et al., 2013, Puapermpoonsiri et al., 2010, Nilsson, 2014, Nieth et al., 2015). The application of bacteriophages has been reported to be useful in a number of fields including molecular biology, human vaccine production, food packaging and agricultural industries, combating diseases in both humans and animals, diagnostic applications, as well as in recombinant protein biotechnology (Kakasis and Panitsa, 2019, Caflisch et al., 2019, Moghadam et al., 2020, Nikolich and Filippov, 2020).

So far a number of bacteriophage therapy trials have been performed using various animal models to determine their potential as an alternative to antibiotic treatment for combating bacterial infections (Furfaro et al., 2018, Hawkins et al., 2010). However, the

use of bacteriophages for therapeutic purpose has a number of challenges. For example, phages can be physically unstable and can also undergo conformational denaturation because of certain physical and chemical stresses such as pH, temperature, salinity and ions (Rosner and Clark, 2021). These factors can affect phage stability, viability or shelf life by altering their structural or genomic material (Vandenheuvel et al., 2015, Malik et al., 2017, Tovkach et al., 2012). Therefore, there is a need to better understand and characterize phage stability in a variety of environments in order to develop robust phage storage and delivery systems for use in a variety of applications.

Bacteriophages act as living organisms in the presence of their suitable host and therefore, they have inherent variability. It can be difficult to deal with living entities as various factors can cause deterioration or death as while handling them prior or during storage or after retrieval (Clark, 1962). Phages can be stabilized in various types of aqueous solutions containing buffers, sugars (e.g. sucrose and mannitol), peptides, salt and/or protein. Stabilizers help to maintain structural integrity of bacteriophages for longer periods of time (Ahiwale et al., 2013). Moreover, low temperature is also another key factor to secure long term bacteriophage storage (Ackermann et al., 2004, Ahiwale et al., 2013, Jepson and March, 2004, Jończyk et al., 2011, Olson et al., 2004). This study focused on the effect of different temperature on the long term storage of experimental phages because temperature is one of the important key factors that determines the survival of viruses in the environment (Leung et al., 2017, Vinner et al., 2017, Richards and Malik, 2021).

Another well-established method of securing long term bacteriophage shelf life is freeze drying or lyophilisation. Freeze-drying removes water from the environment (surrounding a biological molecule) under vacuum conditions by means of sublimation and desorption (Abdelwahed et al., 2006) and thus provides stability from denaturation during long term storage (Andya et al., 2003), because biomolecules are more stable in solid state compared to the aqueous form (Amorij et al., 2008). It is one of the most common methods to extend phage storage life and phage formulations for therapeutic and clinical purposes (Alfadhel et al., 2011, Puapermpoonsiri et al., 2010, Manohar and Ramesh, 2019, Ly et al., 2019, Merabishvili et al., 2013). However, knowledge about survival rates and structural integrity of bacteriophages resulting from freeze drying is limited (Amorij et al., 2008, Ma et al., 2008). For this reason, there is much interest in the effectiveness of

cryoprotectants (freezing stabilizers) such as polyethylene glycol (PEG), hydroxypropyl methylcellulose (HPMC), different sugars (for example trehalose, mannitol and sucrose) during freeze drying (Alfadhel et al., 2011, Puapermpoonsiri et al., 2010, Marton et al., 2021). Among the sugars, disaccharides such as trehalose and sucrose have been found to be the most commonly used cryoprotectants and shown to be the most effective stabilizers to protect phages from desiccation and thermal stress during the freezing and subsequent lyophilisation process (Schwegman et al., 2005, Wang, 2000, Merabishvili et al., 2013, Malik et al., 2017, Vandenheuvel et al., 2014). The advantages of sucrose are, less hygroscopic, does not have internal hydrogen bonds (Figure 5-1) allowing for flexible formation of hydrogen bonds with the capsid proteins, very low chemical reactivity, and, a higher glass transition temperature (Tg) (Abdelwahed et al., 2006). By comparison, trehalose forms a more homogenous amorphous matrix (Figure 6-1) than sucrose which forms a highly heterogeneous crystalline matrix during the freeze-drying process (Malferrari et al., 2014). The present study investigated the use of only higher concentrations (0.5 M) of sugars to test bacteriophage stability after freeze drying. The study performed by Puapermpoonsiri et al. (2010) showed that the activity of lyophilised bacteriophage depends on the moisture content of the freeze-dried cakes. Cakes containing high concentrations of cryoprotectants have low amounts of residual moisture content and therefore are more efficient in terms of the drying process which takes place during lyophilisation.

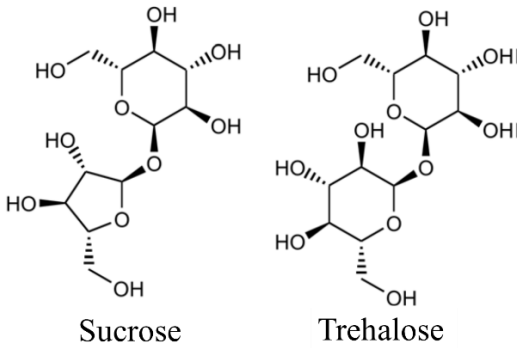

Figure 0-1. Molecular structure of sucrose and trehalose adapted from Malferrari et al. (2014).

The bacteriophage plaque assay described in this study is a quantitative analysis to enumerate the lytic abilty of the individual bacteriophages. In a lytic cycle, each single phage adsorbs onto a host cell, replicates inside the cell and after cell lysis, the daughter

phages are released and each then adsorbs onto a new bacterium, thus producing the plaque (clear zone) on the plate. Each plaque represents a single bacteriophage host cell infection resulting from the co-incubation of these phages with their respective viable bacterial host cells. Serial dilution of each bacteriophage sample was performed to enable accurate counting of plaques following the initial overnight co-incubation. This may sometime result in the loss of bacteriophage activity over time (Puapermpoonsiri et al., 2009). Nevertheless, the spot plaque assay provides relatively quick and simple titration results. This type of assay is suitable for estimating phage titre in studies which require a number of complex experiments, as it does not require the use of an excessive number of plates (Kutter, 2005).

This study investigates the stability and shelf life of four fresh *Aeromonas hydrophila* bacteriophage suspensions compared to freeze dried phage preparations stabilised with a number of cryoprotectants under different temperature conditions. These investigations were performed to determine the method which would secure the longest shelf life for the four experimental bacteriophages. Therefore, the main focus of this study was i) to compare the efficacy of bacteriophage solutions after storing them in different temperature in the presence of cryoprotectants; and ii) to compare freeze-dried phage efficacy with cryoprotectants with a view to securing their long term storage life.

6.2. Materials and methods

6.2.1. Bacteriophage stock solution

Four isolated *Aeromonas hydrophila* bacteriophages Ah6_G, Ah9_PP, Ah10_Se and Ah10_S were used in this study to determine their long-term storage ability in the presence of cryoprotectants and after freeze drying (lyophilisation). The bacteriophage stock solution contained a mixture of equal volume (1:1) of PBS and SM buffer prepared as described in section 3.2.3 of chapter 3.

6.2.2. Sugar stock solution

The sugar stock solutions were prepared using the two cryoprotectant sugar stabilizers trehalose dihydrate and sucrose (Sigma) at concentrations of 1 M and 5 M for both of the sugars. The sugars were dissolved in equal volume (1:1) of sterile PBS and SM buffer mixture then stored at room temperature (25-27 °C) for further use.

6.2.3. Preparation of bacteriophage-sugar solutions for storing as aqueous solution

One ml of each bacteriophage-sugar solution was prepared with 100 µl of sugar stock solution (final sugar concentrations of 0.1 M and 0.5 M), 300 µl of bacteriophage stock solution and 600 µl of PBS and SM (1:1) buffer solution. These aliquots were prepared in 1.5 ml microcentrifuge tubes (Adelab Scientific). Five different solutions were made for each of the four bacteriophages – 0.1 M and 0.5 M trehalose and sucrose solutions respectively and a control solution that contained no cryoprotectant (just additional buffer). Three batches of separate triplicate samples were prepared for all the different types of bacteriophage-sugar solutions to store at three different temperatures. Each of the five different solutions were immediately stored in freezer boxes at 4 °C, -20 °C and -80 °C for further stability testing. The stability of the samples was monitored by determining the lytic activity in pfu/ml (titre) of the bacteriophages using the plaque spot assay at weekly intervals starting from the day the samples were prepared for first 8 weeks and then monthly until the end of the experiments. The spot assay technique used for monitoring phage stability is described in section 3.2.2 of chapter 3. Spot assays were executed separately for all of the triplicate samples.

6.2.4. Bacteriophage spot assay

A. hydrophila bacterial strains (described in section 3.1.1 of chapter 3.), specific to the four different bacteriophages, were initially grown in TSB media at 28 °C overnight, which were then used to make bacterial lawns on TSB agar plates. To determine the bacteriophage titre (pfu/ml), each bacteriophage-sugar sample was serially diluted in TSB (10-fold) and then 10 µl aliquots of each dilution were plated onto the respective bacterial lawn of the TSB agar plate. The plates were incubated overnight in an inverted position at 28 °C. The following day, bacteriophage titre (pfu/ml) was determined by counting the plaques formed.

6.2.5. Preparation of Bacteriophage-sugar solutions for freeze-drying (lyophilisation)

Bacteriophage-sugar solutions for lyophilisation were prepared using bacteriophages and the two sugar stabilizers sucrose and trehalose (Sigma) at a final concentration of 0.1 M and 0.5 M. The sugars were dissolved in equal volume (1:1) of PBS and SM buffer mixture. These stock solutions were stored at room temperature for further use. For

lyophilisation bacteriophage-sugar solutions were dispensed in 1.5 ml microcentrifuge tubes (Adelab Scientific). Each tube for lyophilisation contained 500 µl of bacteriophage-sugar stock solution (150 µl bacteriophage stock diluted in 300 µl PBS-SM buffer solution and 50 µl sucrose or trehalose solution). Two batches of separate triplicate samples were prepared and stored at 4 °C and room temperature (25-27 °C).

6.2.6. Freeze drying (Lyophilisation)

The lyophilisation protocol using the Christ Alpha 2-4 LD freeze dryer (John Morris Scientific) involved firstly sealing each sample tube with Parafilm™ that was then punctured with a 200 µl plastic pipette tip to form 6-7 holes. This puncturing was done to aid in the sublimation of the bacteriophage-sugar solution. All samples were then placed inside 20 ml scintillation glass vials and cooled to and maintained at -20 °C for 30 min. In the meantime, 5 dry ice cakes were prepared and mixed with 20 ml of 95% ethanol in a thermal box to make a dry-ice slurry. The amount of dry ice used depended on the number of sample tubes being lyophilised. The sample tubes were then placed in the dry-ice slurry for 15 min. In the meantime, the freeze-drier chamber was pre-cooled to -89 °C. The frozen sample tubes in the dry-ice slurry were then loaded into the freeze-drier chamber (Figure 6-2). The frozen bacteriophage samples were dried for 12 h at -89 °C with a chamber pressure of 60 mbar. Following the completion of lyophilisation, the Parafilm™ was removed from the sample tubes; the lids were closed and stored in airtight containers at 4 °C and at room temperature (25-27 °C) for 12 months.

Figure 0-2. Freeze dryer with loaded samples.

6.2.7. Monitoring of bacteriophage titre

The titre of the samples was monitored by testing the lytic activity of the bacteriophages at weekly intervals starting from the day the samples were prepared, using the spot assay (section 3.2.2 of chapter 3). At each point of time, the samples were reconstituted in 500 µl of equal volume (1:1) PBS and SM buffer solution. Spot assays were executed separately for all of the triplicate samples.

6.2.8. Transmission Electron Microscopy

The freeze-dried bacteriophage solutions were reconstituted with 500 µl of PBS and SM buffer (1:1) and used to examine the morphology of the freeze-dried bacteriophages using transmission electron microscopy (TEM). Transmission Electron Microscopy (TEM) (Adelaide Microscopy) was carried out following the methods described in section 3.2.4 of chapter 3.

6.2.9. Statistical analysis

Log_{10} values of bacteriophage titres were assessed by the two-way ANOVA test and Bonferroni Post hoc to estimate any statistical differences of the sample means whilst looking at three independent variables (time, temperature and concentration) at a time using IBM SPSS Statistics 25 program. Statistical significance was defined as $p<0.05$.

6.3. Results

6.3.1. Effect of temperature on bacteriophage storage when stored in solution form

The spot assay was conducted to determine the bacteriophage titre, which gives an estimation of the number of plaque forming units (pfu) present in each sample (Figure 6-3). This assay gives an indication of the lytic activity of the bacteriophages and whether they are still active against the target bacteria.

The titres (pfu/ml) of four bacteriophages (Ah6_G, Ah9_PP, Ah10_Se and Ah10_S) in three different storage temperatures, 4 °C, -20 °C -80 °C were compared in the presence and absence (control) of cryoprotectants to determine the effect of temperature on the shelf life of the four bacteriophages. The results presented in this study are the value of mean and standard error calculated from three individually prepared samples. The bacteriophage titre values were presented as $\log_{10}$ (pfu/ml) values for practical purposes.

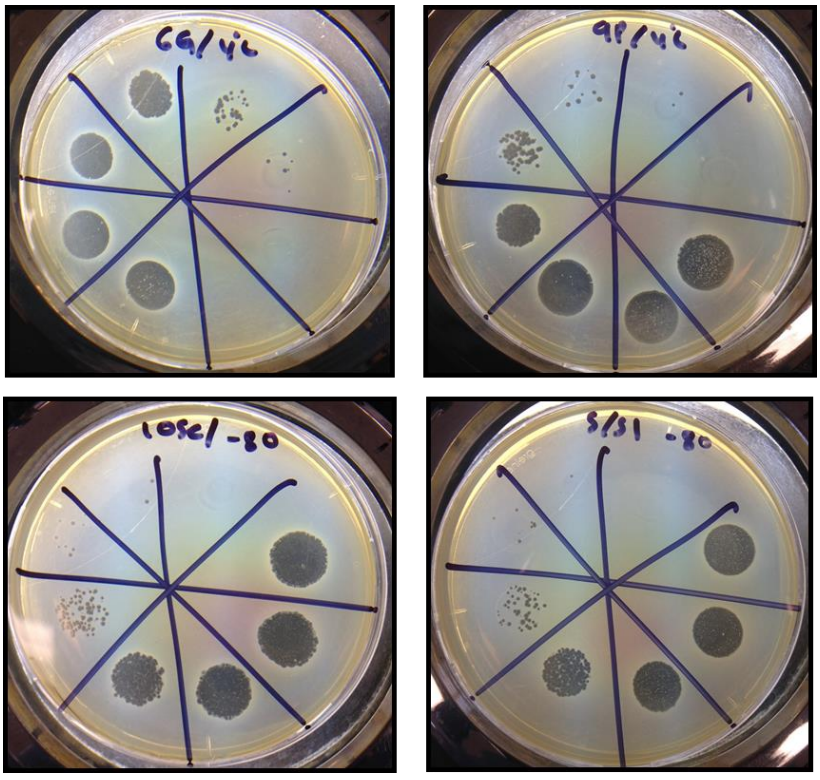

Figure 0-3. Spot assay of four bacteriophages (Ah6_G, Ah9_PP, Ah10_Se and Ah10_S).

6.3.1.1. Effect of temperature without cryoprotectants (control) – bacteriophage in solution

The titres of four bacteriophages Ah6_G, Ah9_PP, Ah10_Se and Ah10_S were compared when stored at three different temperatures for up to 56 weeks without sugar stabilizers (cryoprotectants) (Figure 6-4). For bacteriophage Ah6_G, Ah9_PP, Ah10_Se and Ah10_S, no statistically significant change ($p>0.05$) in phage titre were observed over 56 weeks at 4 °C and -80 °C. However, there was an approximate 0.4 $\log_{10}$ pfu/ml decrease for phage Ah6_G at both temperatures; 0.2 $\log_{10}$ pfu/ml increase and 0.3 $\log_{10}$ pfu/ml decrease for phage Ah9_PP stored at 4 °C and -80 °C respectively; 0.1 $\log_{10}$ pfu/ml and 0.2 $\log_{10}$ pfu/ml decrease for phage Ah10_Se at 4 °C and -80 °C respectively and 0.5 $\log_{10}$ pfu/ml and 1.2 $\log_{10}$ pfu/ml increase for phage Ah10_S at 4 °C and -80 °C respectively. On the other hand, for phage Ah6_G and Ah9_PP, there was a statistically significant reduction ($p<0.05$) in mean phage $\log_{10}$ titre at -20 °C; whereas phage Ah10_Se and Ah10_S did not show any statistically significant reduction in their mean $\log_{10}$ titre at -20 °C. For phage Ah6_G and Ah9_PP, the reduction in their mean $\log_{10}$ titre was from 9.3 to 0 and 9.02 to 0 respectively. For phage Ah10_Se and Ah10_S, the reduction in their mean $\log_{10}$ titre was from approximately 9.5 to 7.9 and 8.5 to 7.8 respectively. However, these results were not statistically significant ($p>0.05$).

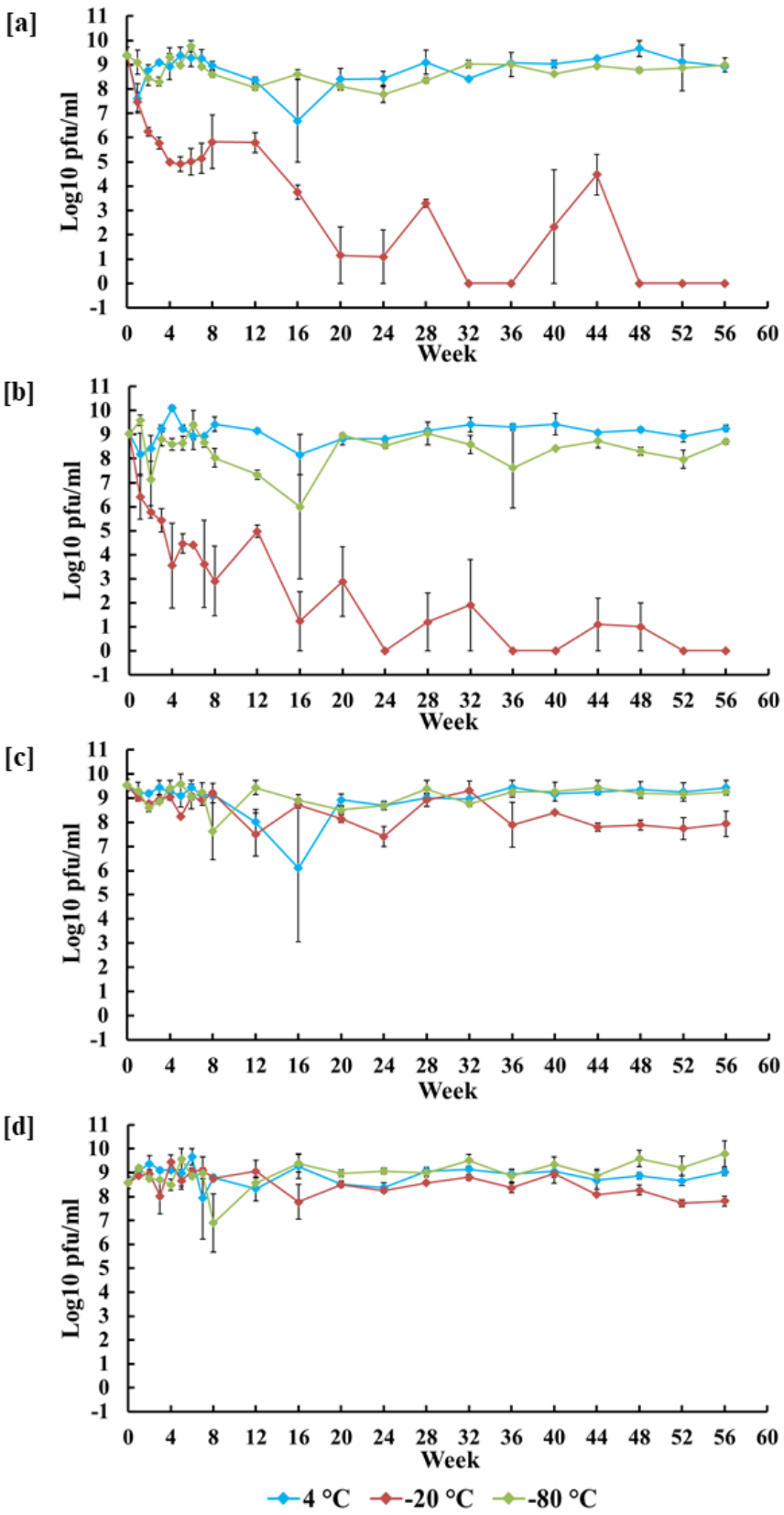

Figure 0-4. Stability of four bacteriophage solutions at three different temperatures without cryoprotectants; [a] bacteriophage Ah6_G; [b] bacteriophage Ah9_PP; [c] bacteriophage Ah10_Se and [d] bacteriophage Ah10_S. Error bars represent standard error of mean. Some error bars are too small to be visible.

6.3.1.2. Effect of temperature in the presence of sugars (cryoprotectants) – bacteriophage in solution

Subsequently, the protective effects of two sugars (sucrose and trehalose) and their two final concentrations (0.1 M vs 0.5 M) were investigated on phage stability (mean $\log_{10}$ pfu/ml) when stored at 4 °C, -20 °C and -80 °C. At 4 °C and -80 °C bacteriophages Ah6_G and Ah9_PP were stable without cryoprotectants (controls). In the presence of 0.1 M and 0.5 M trehalose or sucrose at these same temperatures there was no demonstrable change with the inclusion of the cryoprotectants, therefore, the data is not presented here (see Appendix figures 9-1 and 9-2). This was not the case at -20 °C and the effect of cryoprotectants is shown in Figure 6-5.

For phage Ah6_G, in the presence of 0.5 M sucrose, there was no significant reduction in its titre but when 0.1 M sucrose was used the titre declined from 9.18 $\log_{10}$ pfu/ml to 6.63 $\log_{10}$ pfu/ml indicating 0.5 M sucrose provides greater stability at -20 °C (Figure 6-5). When trehalose was added as a cryoprotectant at 0.5 M concentration, the phage titre declined from 9.03 $\log_{10}$ pfu/ml to 6.47 $\log_{10}$ pfu/ml which is significantly different from what was recorded with 0.5 M sucrose. In addition, the phage stability in 0.1 M trehalose was not as good as that achieved with 0.1 M sucrose but the difference was not significant.

In case of phage Ah9_PP, the inclusion of either sucrose or trehalose provided better phage protection compared to the control. For both sucrose and trehalose, 0.5 M concentrations were better than 0.1 M, but there were no significant differences between the 0.5 M sugars, or between the 0.1 M sugar solutions. Neither solute was better than the other.

The percentage loss in phage viability shown in Table 6-1 supports the storage results of Ah6_G and Ah9_PP phage when stored at -20 °C.

Table 6-1. Ah6_G and Ah9_PP phage titre trends after 56 weeks when stored at -20 °C

Bacteriophage	**Sugar**	**Titre (Log_{10} pfu/ml)**		
		Initial (0 week)	**After 56 weeks**	**Percentage loss of viability**
Ah6_G	Control	9.38	0	100 %
	0.1 M Sucrose	9.18	6.63	27.8 %
	0.5 M Sucrose	9.32	8.49	8.9 %
	0.1 M Trehalose	8.34	5.09	39.1 %
	0.5 M Trehalose	9.03	6.47	28.3 %
Ah9_PP	Control	9.03	0	100 %
	0.1 M Sucrose	9.38	5.26	43.9 %
	0.5 M Sucrose	9.32	7.10	23.8 %
	0.1 M Trehalose	8.91	5.10	42.8 %
	0.5 M Trehalose	9.07	4.59	49.4 %

Furthermore, for both Ah10_Se and Ah10_S phages, no changes were observed ($p>0.05$) in their mean titres over 56 weeks while stored at 4 °C, -20 °C and -80 °C, regardless of sugar type (sucrose and trehalose) or concentration (0.1 M and 0.5 M) (see Appendix figures 9-3 and 9-4).

From the above data it is apparent that Ah6_G and Ah9_PP phages are significantly more stable while stored at -20 °C in the presence of sucrose or trehalose compared to the control (without cryoprotectants). The results also demonstrated that Ah6_G with the addition of sucrose (both 0.1 M and 0.5 M) were more stable compared to that of the trehalose when stored at -20 °C. For phage Ah9_PP, higher concentrations of both sucrose and trehalose have been found to be more effective over 56 weeks. Conversely, the addition of sucrose and trehalose did not produce any noticeable titre changes for phages Ah10_Se and Ah10_S while stored at 4 °C, -20 °C and -80 °C.

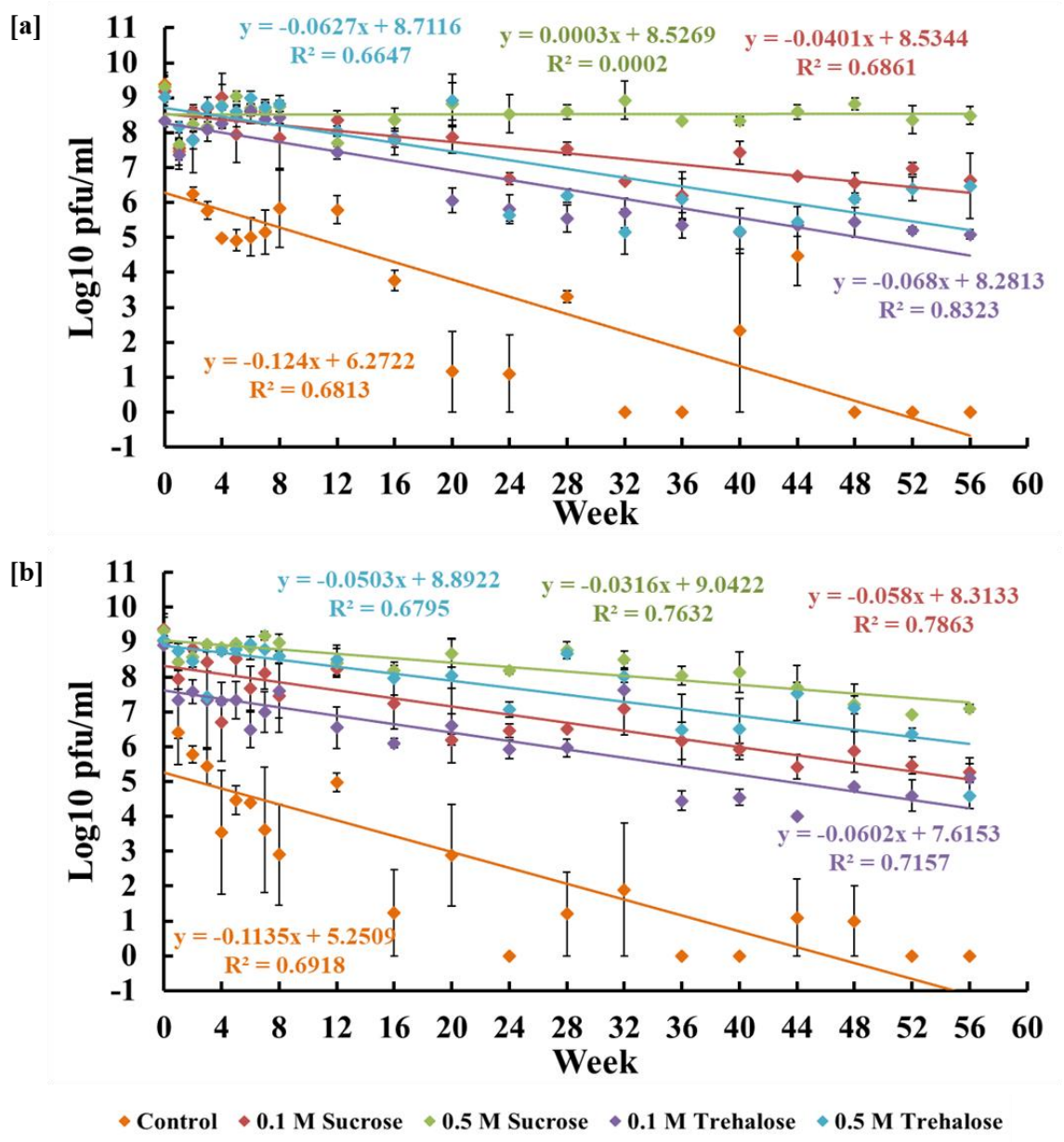

Figure 0-5. Stability of Ah6_G and Ah9_PP bacteriophage solutions at -20 °C with cryoprotectants; [a] – Ah6_G phage titre at -20 °C; [b] – Ah9_PP phage titre at -20 °C. Error bars represent standard error of mean. Some error bars are too small to be visible.

6.3.2. Effect of freeze-drying on bacteriophage storage

Titre (pfu/ml) of four freeze dried bacteriophages (Ah6_G, Ah9_PP, Ah10_Se and Ah10_S) were compared in the presence and absence (control) of cryoprotectants/stabilizers while stored at two different temperatures: 4 °C and room temperature (RT: 25-27 °C), to determine the stability of the bacteriophages.

Table 6-2. Freeze dried bacteriophage titre trend before and immediately after freeze-drying

Bacteriophage	**Sugar**	**Titre (Log_{10} pfu/ml)**		
		Initial (before FD)	**After FD**	**Percentage loss of viability**
Ah6_G	Control	9.38	8.48	9.6 %
	0.5 M Sucrose	9.32	8.05	13.6 %
	0.5 M Trehalose	9.03	8.26	8.5 %
Ah9_PP	Control	9.03	7.80	13.6 %
	0.5 M Sucrose	9.32	7.57	18.8 %
	0.5 M Trehalose	9.07	7.50	17.3 %
Ah10_Se	Control	9.53	8.11	14.9 %
	0.5 M Sucrose	8.68	8.78	0 %
	0.5 M Trehalose	8.63	8.40	2.7 %
Ah10_S	Control	8.58	8.06	6.1 %
	0.5 M Sucrose	9.05	8.70	3.9 %
	0.5 M Trehalose	8.54	8.78	0 %

Table 6-2 shows the decreases in mean phage titre immediately after freeze drying. The results for freeze dried phage stored at 4 °C are shown but the results are the same when immediately stored at room temperature, as might be expected. Without cryoprotectant (control) the percentage loss in phage viability varied from 6.1% - 14.9% for the four phages. Compared to the control, addition of 0.5 M sucrose and 0.5 M trehalose reduced the rapid titre decline immediately after freeze drying, however it was only for Ah10_Se and Ah10_S that any significant protection was noted with sucrose and trehalose. For the other two phages the sugars did not significantly improve the survival during the freezing drying process.

The TEM imaging of four bacteriophages is represented in Figure 6-6. The figure illustrates the morphology of four bacteriophages immediately after freeze drying and showed that for all four tailed bacteriophages without cryoprotectant, the tails became separated from the head due to the freeze-drying stress.

Subsequent to the freeze-drying process the stability of the phages was monitored over 44 weeks at both 4 °C and RT: 25-27 °C (Figures 6-7 and 6-8). Lines of best fit were determined and the slopes calculated which varied between 0.07 and -0.0174 as summarised in Table 6-3.

By comparing the slopes it is apparent that in the presence of sucrose or trehalose, the phages are more stable when stored at 4 °C compared to room temperature. Furthermore,

the presence of sucrose or trehalose provides better stability than the control at either temperature.

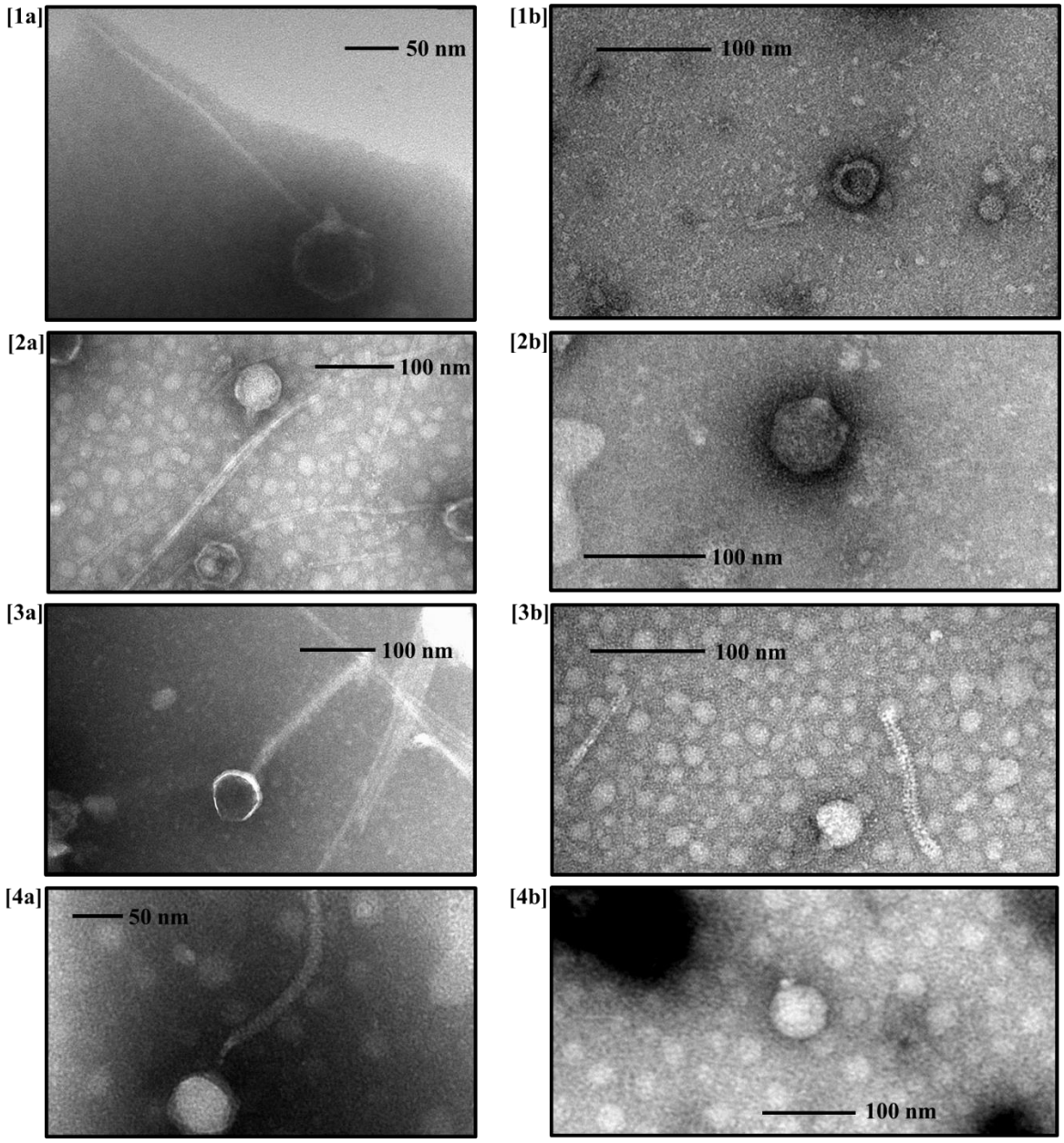

Figure 0-6. Transmission electron microscope (TEM) view of four bacteriophages before [a] and after [b] freeze drying. [1a & 1b] – Ah6_G; [2a &2b] – Ah9_PP; [3a & 3b] – Ah10_Se and [4a & 4b] – Ah10_S.

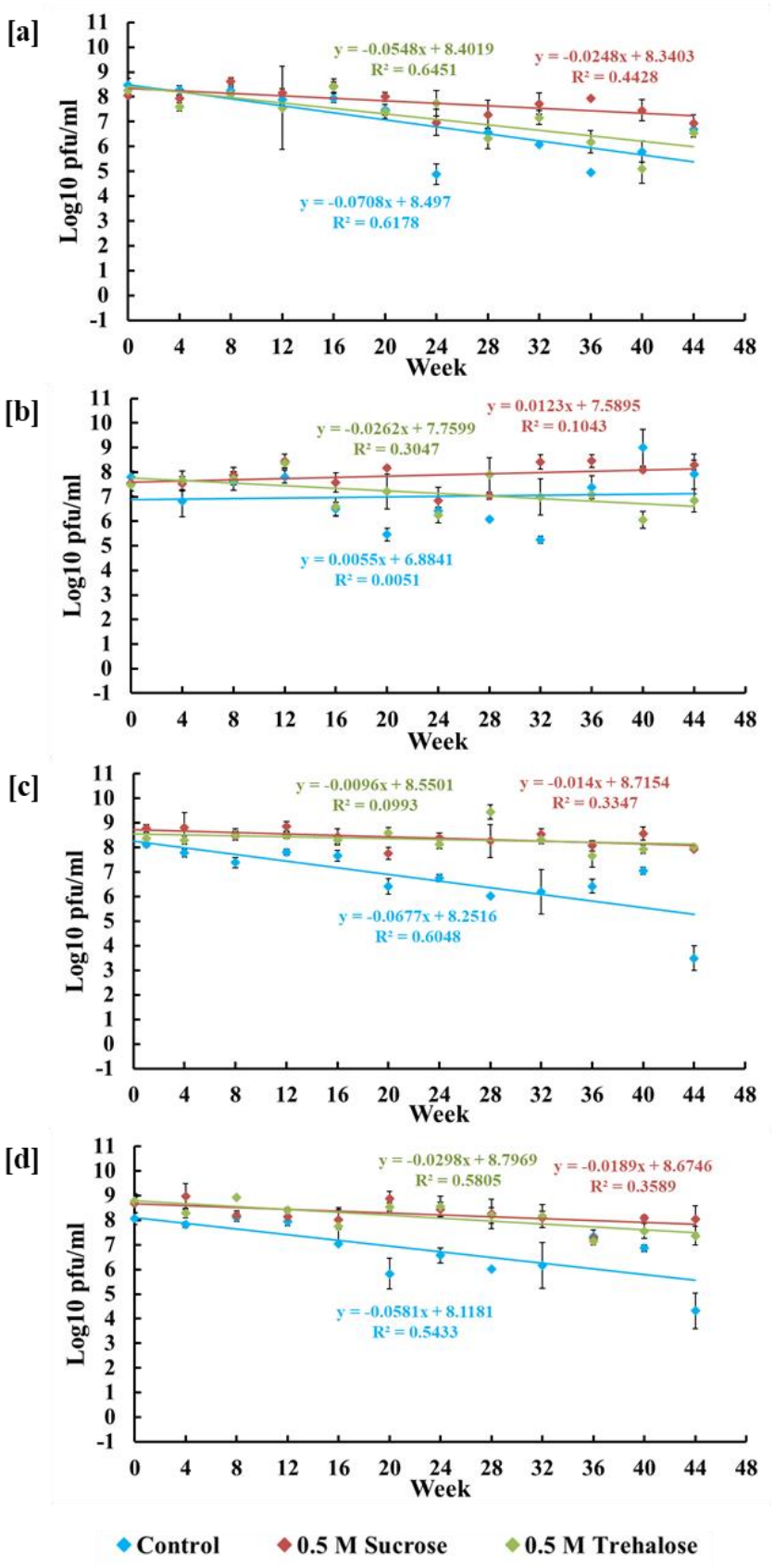

Figure 0-7. Freeze dried Ah6_G, Ah9_PP, Ah10_Se and Ah10_S bacteriophage titres when stored at 4 °C with cyoprotectants. Error bars represent standard error of mean. Some error bars are too small to be visible.

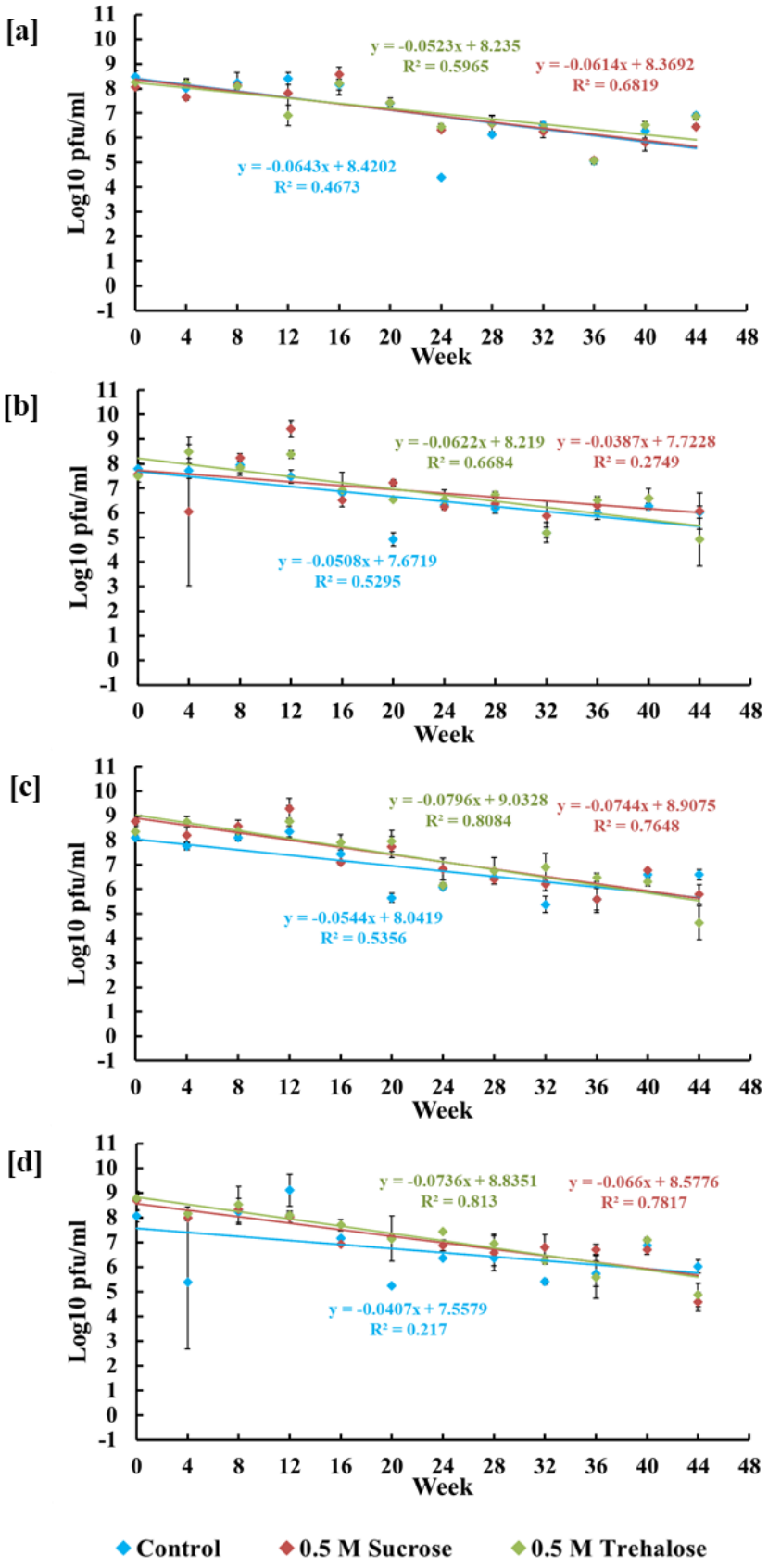

Figure 0-8. Freeze dried Ah6_G, Ah9_PP, Ah10_Se and Ah10_S bacteriophage titres when stored at room temperature (RT: 25-27 °C) with cyoprotectants. Error bars represent standard error of mean. Some error bars are too small to be visible.

Table 6-3. Die off rates of four freeze dried bacteriophages with cryoprotectants

Bacteriophage	Sugar	Die off rates (slopes of lines of best fit)	
		4 °C	RT: 25-27 °C
Ah6_G	Control	-0.0708	-0.0643
	0.5 M Sucrose	-0.0248	-0.0614
	0.5 M Trehalose	-0.0548	-0.0523
Ah9_PP	Control	0.0055	-0.0508
	0.5 M Sucrose	0.0123	-0.0387
	0.5 M Trehalose	-0.0262	-0.0622
Ah10_Se	Control	-0.0677	-0.0544
	0.5 M Sucrose	-0.014	-0.0744
	0.5 M Trehalose	-0.0096	-0.0796
Ah10_S	Control	-0.0581	-0.0407
	0.5 M Sucrose	-0.0189	-0.066
	0.5 M Trehalose	-0.0298	-0.0736

6.4. Discussion

To determine the shelf life of the four *Aeromonas* phages, suspensions of phage were stored at 4 °C, -20 °C and -80 °C. These studies have demonstrated that over a 56-week period storage at 4 °C or -80 °C, insignificant loss of viability occurred for each of the four phages tested. However, at -20 °C, Ah6_G and Ah9_PP, both showed significant loss of viability without a cryoprotectant. The inclusion of sucrose (0.1 M or 0.5 M) or trehalose (0.1 M or 0.5 M) provided better stability than control (without cryoprotectant) but with the exception of Ah6_G in the presence of 0.5 M sucrose at -20 °C none of these conditions were as good as storage at 4 °C or -80 °C. Studies by Yan et al. (2021) and Tabare et al. (2021) also showed the presence of cryoprotectants (trehalose, mannitol and L-leucine) provided better stability of the spray-dried phages while stored at different temperature conditions.

The observation in this present study is consistent with the statement given by Ackermann (2004) and Puapermpoonsiri et al. (2010) for all four phages stored at both 4 °C and -80 °C as the phages did not show any significant decrease in their titre stability. Three of the phages (Ah6_G, Ah10_Se and Ah10_S) are long tailed but there were no differences in stability with the short tailed phage Ah9_PP, although previous studies have indicated tailed phages are generally more stable (Ackermann, 2004, Puapermpoonsiri et al., 2010).

When phages were stored at -20 °C, Ah10_Se and Ah10_S, both tailed phages, were stable for 56 weeks even without cryoprotectants. However, Ah6_G and Ah9_PP (which

is short tailed) were both unstable at -20 °C with or without cryoprotectants. This is not surprising as previous research has indicated that slow cooling to -20 °C leads to the breakdown of macromolecular structures due to the formation and growth of ice crystals. As the cooling continues, the ice crystals grow at the expense of pulling water from within the phage cell and as a result the phage struggles to maintain its macromolecular structure (Gould, 1999). The addition of cryoprotectants such as sucrose or trehalose is designed to minimize the damage to the phages during freezing and extended cold storage (Moraes de Souza et al., 2021, Gonzalez-Menendez et al., 2018). In the present study, sucrose was marginally better than trehalose, but neither were able to stabilize Ah9_PP for the 56-week trial. A similar result was reported by Olson et al. (2004), where they looked at the effect of different temperatures on the storage viability of MS2 virus and found that the titre of MS2 showed a significant decay when stored at -20 °C.

The present study has demonstrated all four phages were stable at -80 °C for up to 56 weeks which is similar to (Golec et al., 2011) studies on tailed phage. Furthermore, Ackermann (2004) indicated that the *Aeromonas* phage they studied were stable for up to 5 years at -80 °C. Another study (Jepson and March, 2004), demonstrated similar results to the present findings and stated that phage when stored as stock solution, was more stable at 4 °C.

The four *Aeromonas* phages were freeze dried in buffer solution (control), 0.5 M sucrose and 0.5 M trehalose and stored at 4 °C or room temperature (RT: 25-27 °C). The initial process of freezing the phage suspended in buffer (control) using dry ice and alcohol, then drying under vacuum led to an immediate decrease in phage viability varying between 6-15% of the original titre (approx. $\log_{10}9$). When sucrose or trehalose were incorporated there was little cryoprotection detected, probably because the phages were snap frozen in the mixture of dry ice and alcohol.

The effectiveness of sucrose or trehalose to stabilise the freeze-dried suspensions over 44 weeks at either 4 °C or room temperature has shown some degree of protection compared to the control, as measured by the die off rates, with 0.5 M sucrose the most effective treatment. These studies with 0.5 M sucrose and 0.5 M trehalose are similar to those reported by Merabishvili et al. (2013), who showed that sucrose and trehalose at a concentration of 0.5 M works more effectively as cryostabilizer during freeze-drying and no more than 1 $\log_{10}$ decrease was observed after 27 months at 4 °C in their study. The

loss of phage stability without cryoprotectants observed in this study has also been shown by Jepson and March (2004), Puapermpoonsiri et al. (2010). Freeze drying data obtained in the present study demonstrated that both freezing and drying had a negative impact on the phage titre. Freeze drying stresses such as, solute concentration, ice crystal formation, changes in pH, etc. can weaken the stability of a biological entities (Merabishvili et al., 2013, Wang, 2000). Another study showed titre reduction of the phages during atmospheric sprayed freeze-drying in the presence of different concentrations of trahalose and mannitol (Ly et al., 2019). The effectiveness of cryoprotectants to overcome the problem described here has also been observed by Abdelwahed et al. (2006), Merabishvili et al. (2013), Manohar and Ramesh (2019) where the inclusion of cryoprotectants increased storage stability. In general, the die off rates are smaller when the freeze-dried preparations are stored at 4 °C rather than room temperature.

The research so far is not consistent with respect to the impact "stabilizers" have on phage surviving freeze drying and then storage. The role of sucrose or trehalose is to reduce free water and hence speed up the rate of drying to protect the phages as shown by Puapermpoonsiri et al. (2010) and also demonstrated in this study. Only 0.5 M sucrose and 0.5 M trehalose were investigated but other studies have demonstrated different optimal concentrations for different phage types (Merabishvili et al., 2013, Puapermpoonsiri et al., 2009, Carne and Greaves, 1974, Cox et al., 1974, Davies and Kelly, 1969).

Therefore, for the purpose of this study freeze drying in 0.5 M sucrose and storage at 4 °C is most likely to provide the best storage conditions.

From the observation and when the lines of best fit are compared for each of the phages in buffer (control) at 4 °C or -80 °C and freeze-dried phages in the presence of 0.5 M sucrose stored at 4 °C (Figure 6-9), there is not a lot to be gained by any one process.

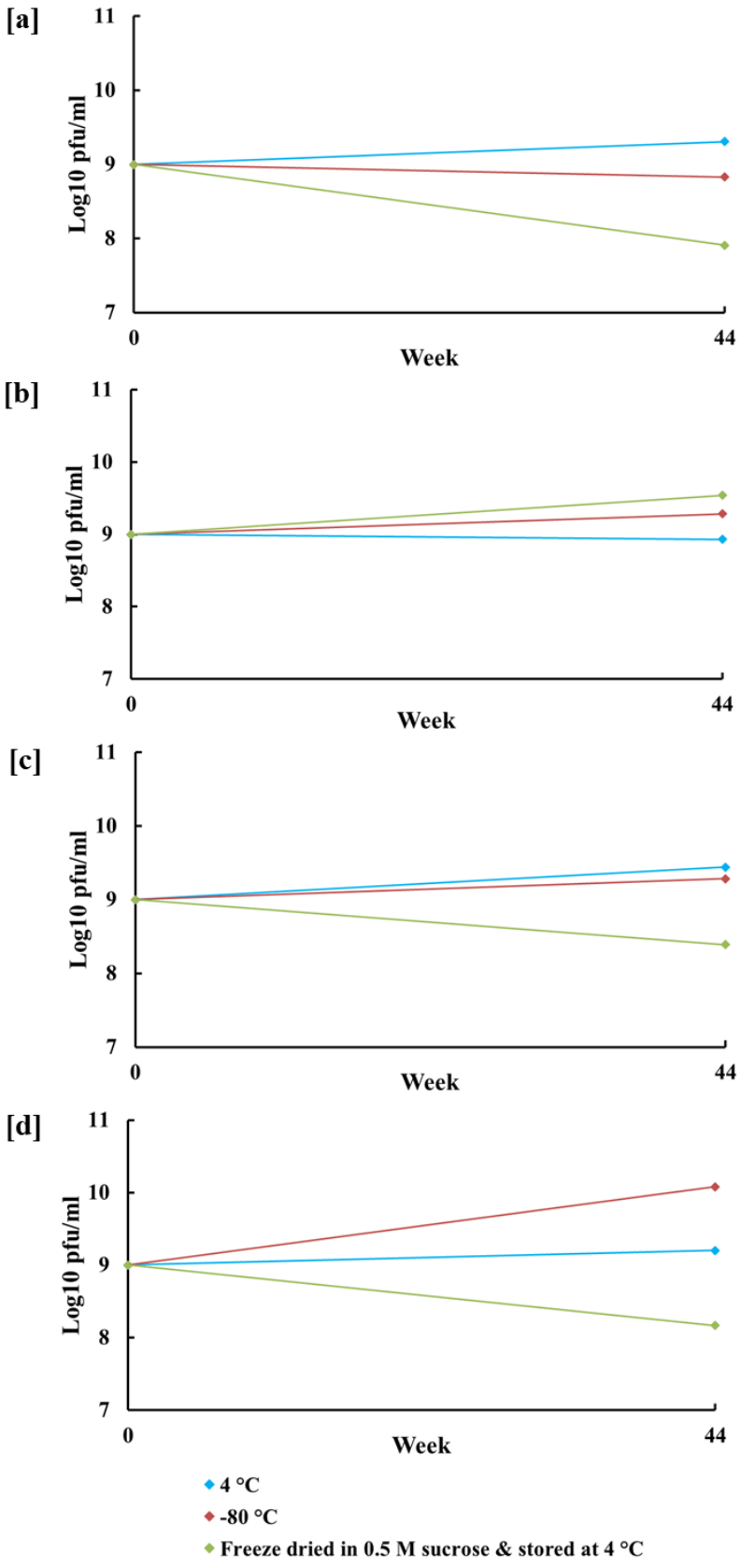

Figure 0-9. Normalized plots of [a] – Ah6_G, [b] – Ah9_PP, [c] – Ah10_Se and [d] – Ah10_S bacteriophages based on phage data stored at 4 °C (control); at -80 °C (control) and when freeze dried in 0.5 M sucrose and stored at 4 °C.

However, for short term storage of these *Aeromonas* phage, storage of phage in solution at 4 °C has been shown to be adequate. For preservation and long-term storage frozen storage at -80 °C is most likely to offer the best recovery of infective phage as recommended by (Gould, 1999). But for the purpose of preparing phage for therapeutic studies where phage may need to be transported to aquaculture farms or similar, freeze dried phages will be beneficial since non-refrigerated transport may be required. Furthermore, freeze dried phage are more suitable for applications such as use in pharmaceuticals where novel formulations for oral administration or injection into humans or animals are required, or use for bath exposures in therapeutic applications.

Freeze drying procedures vary depending on the equipment design and phages from different families, even different phages witin a family show varying stability to physical or chemical stresses (Malik et al., 2017). Although the stability of the freeze dried bacteriophages is quite good, this may be enhanced by the introduction of a secondary drying cycle at elevated temperature (25 °C) as reported by Puapermpoonsiri et al. (2010). Therefore, the lysophilisation protocol should be adjusted, optimized and standardized for each different phage in order to obtain the best lyophilisation result for individual phages. On the other hand, according to Merabishvili et al. (2013), different authors noted the novelty of lyophilized bactriophages in terms of formulation but their shelf-life was scarcely considered and if considered then only for a couple of months of storage.

Regardless the above mentioned facts, previous published literature showed that both freezing and thawing of phages has a negative impact on their stability (Arakawa et al., 2001). Therefore, triplicates of all the individually prepared bacteriophage samples were kept at their respective storage tempeatures until the samples were needed for a spot assay. Hence, only one spot assay was performed from each stored vial/tube. Additionally, the same master stock solutions for each of the phages were used throughout the study. They were also stored at the same temperature to minimize discrepancies. All spot assays were performed under identical conditions to avoid any variability. Gould (1999) suggested that use of high titre phages are more feasible for the long term storage; therefore, present study also followed this approach and used high titres of bacteriophages for the stock to obtain more accurate results. Nevertheless, the type of assay employed in the present study and the condition of plating may create

differences in phage titre. Sometimes as an artifact the phage titre may rise markedly rather than falling according to the result expectations.

6.5. Conclusion

From the observation of the present study (Figures 6-6 and 6-7), it is evident that freeze drying of the four bacteriophages was not as efficient as storing them in solution at different temperatures with or without cryoprotectants as freeze drying resulted in a decrease in the mean titre for all four bacteriophages (Table 6-2).

Furthermore, the experimental bacteriophages were found to be more stable over 56 weeks while stored in solution at three temperatures (4 °C, -20 °C and -80 °C) in the presence of cryoprotectants (either sucrose or trehalose) compared to the control (without cryoprotectant).

Therefore, bacteriophages stored in solution at 4 °C were used in the fish trials described in the next chapter (Chapter 7).

Chapter 7. Investigation of bacteriophage efficacy for the treatment of *Aeromonas hydrophila* infection in Silver Perch (*Bidyanus bidyanus*)

7.1. Introduction

Aquaculture makes up a significant source of seafood worldwide contributing to approximately 46.8% of the world's seafood demand (FAO, 2018). Finfish are one of the major contributors to the aquaculture farming sector globally. As a vital constituent of the animal kingdom, fish reside in diverse environments and are prone to a number of bacterial pathogens. Compared to wild populations, there is a greater risk of developing infectious disease in farmed fish and factors contributing to this risk are the high stocking densities at which the animals are maintained for production, improper handling and hypoxia. Suboptimal conditions can reduce the immune defence response in fish against infectious diseases (Silva et al., 2016, Milla et al., 2010). For the maintenance of fish health and for the sustainability of the aquaculture industry, many management and control strategies to eradicate bacterial infection have been utilized (Kiron, 2012, Kreutz et al., 2014, Soliman et al., 2019, Sieiro et al., 2020). Nonetheless, pathogens are still responsible for the major losses in the fish farming sector (Verner–Jeffreys et al., 2007, Opiyo et al., 2018, Pepi and Focardi, 2021).

Since the 1980s, the therapeutic potential of bacteriophages to control bacterial infection has gained attention among the scientific community (Defoirdt et al., 2011, Phumkhachorn and Rattanachaikunsopon, 2010, Soliman et al., 2019). For more than six decades, bacteriophages have been the subject of studies and have been used extensively in Eastern Europe and in the former Soviet Union in different fields of human and animal medicine (Rao and Lalitha, 2015, Lu and Koeris, 2011). However, only a few studies have used bacteriophages for the control and prophylaxis of bacterial infection in the aquaculture sector (Colavecchio and Goodridge, 2018, Kowalska et al., 2020, Doss et al., 2017, Ramos-Vivas et al., 2021). The unique characteristics of phages to replicate exponentially within the bacterial host and their specificity to the target bacteria, without causing any harm to other microflora, have made them an attractive and potential candidate for therapeutic purposes in controlling pathogenic bacterial infections (Kutateladze and Adamia, 2010). Furthermore, the mode of action of bacteriophages is different from that of antibiotics, which makes them useful for the treatment of multidrug resistant bacteria as well (Nakai and Park, 2002). Recent studies of bacteriophage applications have proven the therapeutic value of bacteriophages in aquaculture (Karunasagar et al., 2007, Nakai and Park, 2002, Nakai et al., 1999, Park et al., 2000, Vinod et al., 2006, Park and Nakai, 2003, Shivu et al., 2007, Higuera et al., 2013). Many

studies have focused on the isolation and identification of *Aeromonas* sp. bacteriophages, but few have been done on the therapeutic application of bacteriophages against infection with an *Aeromonas* sp. pathogen (Imbeault et al., 2006, Verner–Jeffreys et al., 2007, Akmal et al., 2020).

This chapter describes results obtained from experiments to assess the potential of using the bacteriophages described earlier in this thesis to eliminate *Aeromonas hydrophila* infections in silver perch. Silver perch (*Bidyanus bidyanus*), a native fish of Australia, was used in this study as a model to test the therapeutic approach of bacteriophages against an *A. hydrophila* pathogen. The data from this fish trial could be used as a ground work for future studies using modified models and conditions to optimise the application of bacteriophages for the control of bacterial infections in aquaculture.

7.2. Materials and methods

7.2.1. Fish infection trial

7.2.1.1. Test fish

One hundred and fifty-nine fish (15-20 cm body length) were used in the fish infection trials. Fish were obtained from a local commercial aquaponics supplier (Aquaculture Advantage) in Adelaide, South Australia and transported to the aquaculture laboratory facility, Roseworthy Campus, The University of Adelaide for acclimatisation.

7.2.1.2. Controls for infection trials

Each fish from the control groups was injected intraperitoneally (IP) with of 0.1 ml per 10 kg fish volume of sterile Phosphate Buffered Saline (PBS) (8.0 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1000 ml deionized water).

7.2.1.3. Bacterial stock solution preparation

A single strain of *A. hydrophila* (Ah10) was selected for the infection trial. The preparation of the bacterial stock is described in section 3.3.1 of chapter 3. The bacterial inoculum in the concentration of approximately 6.7×10^7cfu/ml was used for the fish infection trials.

The fish infection trial was conducted in two different phases:

1. Preliminary pilot trial

2. Actual infection challenge trial

7.2.2. Preliminary pilot trial

A preliminary pilot study was conducted to investigate the pathogenicity of the bacterial isolate selected for study post injection into the experimental fish and to determine a suitable dose rate to be applied to the fish during the actual infection trial. The morbidity and mortality of fish were also observed during the trial.

7.2.2.1. Experimental system for the preliminary trial

Following the acclimatisation, 15 fish from the original 159 were randomly selected and then randomly assigned to 5 glass aquarium tanks (capacity – volume 65 l; dimension – 60 cm × 30 cm × 36 cm) (3 fish in each tank) and kept for one week to acclimatise to the laboratory conditions before starting the preliminary trial. Fish tanks were maintained with continuous aeration and flow through filters. Fish were fed on a regular basis with a commercial fish feed supplied by Aquaculture Advantage and tank water cleaned and changed regularly to maintain optimum water conditions. The optimum water quality parameters were maintained at: temperature: 22-23 °C; pH: 7.7- 7.9; dissolved O_2: 6 mg/l.

7.2.2.2. Protocol for determining dose to be used in actual challenge trial

For the pilot study each tank represented a treatment group (i.e. one control and four infection groups). The four infection groups were administered with serially diluted stock suspensions of *A. hydrophila* and the control was PBS (see below).

Fish preparation and sedation for the preliminary trial was undertaken utilising the same method described in the section 3.3.2 of chapter 3.

To prepare the treatment suspension, a bacterial stock suspension of *A. hydrophila* (Ah10) (6.7×10^7 cfu/ml) was serially diluted in PBS solution to four different dilutions (10^{-3}, 10^{-6}, 10^{-9}, and 10^{-12}). Fish from four infection groups were injected intraperitoneally (IP) with one of the four concentrations of bacteria and fish of control group were IP injected with PBS. Fish were maintained for six days to develop infection. On day seven post injection, all fish were euthanized humanely by emersion using Aqui-S overdose (approximately 175 mg/l) in tank water.

7.2.2.3. Fish organ collection and their bacterial counts

Fish organ collection and the assessment of bacterial loads was conducted following the same method as set out in the sections 3.3.3 and 3.3.4 of chapter 3 respectively.

7.2.3. Actual infection challenge trial

Following the preliminary pilot trial, a larger fish challenge trial was conducted to determine the efficacy of bacteriophage on the experimental fish model (silver perch).

7.2.3.1. Experimental system for the actual challenge trial

The remaining post acclimatised fish (144 fish) were randomly assigned to 21 different glass aquarium tanks (capacity – volume 65 l; dimension – 60 cm × 30 cm × 36 cm) (7 fish in each tank) and kept for one week to acclimatise to the laboratory conditions before starting the infection trial. Fish tanks were maintained with continuous aeration and flow through filters. Fish were fed on a regular basis with a commercial fish feed supplied by Aquaculture Advantage and tank water cleaned and changed regularly to maintain optimum water conditions. The optimum water quality parameters were maintained at: temperature: 22-23 °C; pH: 7.7- 7.9; dissolved O_2: 6 mg/l.

The large infection trial consisted of 7 treatment groups as showed in Table 7-1.

Table 7-1. Distribution of treatment groups among tanks

Groups	**Treatments**	**No. of tanks**	**No. of fish**
1. Control	1a. Triplicate	3	18 (6 in each tank)
2. Infected fish inoculated with 3 different doses of phage	2a. Bacteria + high phage dose: triplicate	3	21 (7 in each tank)
	2b. Bacteria + medium phage dose: triplicate	3	21 (7 in each tank)
	2c. Bacteria + low phage dose: triplicate	3	21 (7 in each tank)
3. Non-infected fish inoculated with 3 different doses of phage	3a. High phage dose: triplicate	3	21 (7 in each tank)
	3b. Medium phage dose: triplicate	3	21 (7 in each tank)
	3c. low phage dose: triplicate	3	21 (7 in each tank)

Tank selection of fish and the selection of treatment for each tank was randomised.

7.2.3.2. Bacterial stock solution for actual challenge trial

The concentration of the bacterial suspension to be used for the actual trial was determined from the preliminary pilot trial as 6.7×10^7 cfu/ml.

7.2.3.3. Bacteriophage stock solution preparation

The isolation, purification and preparation of bacteriophage stock has been previously described (section 3.2.1 of chapter 3). A mixture of four different bacteriophages (bacteriophage cocktail) was used for this trial. Equal volumes of four different phage stocks were mixed to make the bacteriophage cocktail. The phage cocktail titre was determined as 1.5×10^9 pfu/ml.

Three different concentrations of the bacteriophage cocktail were used to inoculate the fish. The bacteriophage cocktail was serially diluted (10-fold) in equal volume (1:1) of PBS and SM buffer solution up to 1:100000 (10^{-5}) dilution. High (approximately 10^9 pfu/ml), medium (approximately 10^6 pfu/ml) and low (approximately 10^4 pfu/ml) were injected into the fish corresponding respectively to treatment groups 2a, 2b, 2c and again for 3a, 3b, 3c.

7.2.3.4. Preparing the fish for challenge trial

Fish preparation and sedation for challenge trial were undertaken utilising the same method described in the section 3.3.2 of chapter 3.

7.2.3.5. Challenge trial

7.2.3.5.1. Infecting the fish with bacteria

The fish to be inoculated with *A. hydrophila* (Ah10) and then treated with bacteriophage (treatment groups 2a, 2b and 2c) were injected intraperitoneally (IP) with the pre-determined dose rate (0.1 ml per 10 kg fish of a suspension of 6.7×10^7 organisms per ml) of *A. hydrophila* bacteria. Fish were kept for six days to develop infection.

7.2.3.5.2. Treatment with bacteriophage

Six days after the bacterial infection initiation, each infected fish from treatment groups 2a, 2b and 2c (63 fish in total) were injected with the high, medium and low doses of bacteriophage cocktail solution respectively. Fish were injected intraperitoneally (IP) with the same volume rate (0.1 ml per 10 kg fish) used to infect with *A. hydrophila* bacteria.

The same day, the remaining 63 “non-infected” fish from 3a, 3b and 3c treatment groups were injected with high, medium and low doses of the bacteriophage cocktail solution respectively following the same techniques.

Three days after the bacteriophage inoculation, all fish from all treatment groups were euthanized humanely as described previously.

7.2.3.6. Fish organ collection

7.2.3.6.1. Fish organ (kidney and spleen) collection for bacterial counts

Fish organ collection and the assessment of bacterial loads was conducted following the same method as set out in the preliminary pilot trial section.

7.2.3.6.2. Fish organ collection for histopathology

The remaining kidney and spleen mass, other intestinal organs, gill, brain, muscle, eye from each fish were removed and fixed in 10% neutral buffered saline (formalin) for histopathological analyses. The formalin fixed organ tissues were then decalcified and processed using standard histological procedures and tissue sections were stained with haematoxylin and eosin (H&E) for microscopy and microphotography. One of each organs from each replicate of the treatments was examined microscopically for presence of pathogens and histopathology.

7.2.3.7. Fish blood collection

Blood samples were collected from the caudal vein of each fish with 25-gauge needles and 1 ml syringes (Adelab Scientific, South Australia) and placed in 1 ml heparinised tubes to avoid blood clotting. The blood tubes were then centrifuged at 20000 $\times g$ for 10 min at 4 °C and the blood plasma was removed and transferred to a new 1.5 ml microcentrifuge tube (Adelab Scientific) and stored at -80 °C for future analysis. One drop (10 µl) of blood from each fish was also used to streak onto TSA (Tryptic Soy Agar) and AMB (*Aeromonas* Medium Base, Edwards Group Pty Ltd, NSW, Australia) agar plates to detect bacterial growth. All agar plates were streaked in the same manner for consistency and incubated overnight at 28 °C. The next day all agar plates were checked for growth and colonies counted.

7.2.4. Lysozyme activity assay

To determine the effect of infection of silver perch with *A. hydrophila* and subsequent inoculation with bacteriophage on the immune system of the fish, blood samples were analysed to estimate lysozyme activity. Blood plasma of all 7 fish from each tank were pooled together to make one aliquot. The lysozyme activities were measured from triplicate pooled aliquots of each treatment group. An increase in lysozyme activity would normally suggest an increase in immune activity.

The lysozyme assay was carried out using a turbidimetric assay described by Milla et al. (2010) and Kreutz et al. (2011) with some minor modifications. This assay measures the lysis rate of *Micrococcus lysodeikticus* (*M. luteus*) (Sigma Life Science) bacteria with blood plasma lysozyme. Initially 96-well flat bottom microplates were loaded with fish blood plasma and phosphate buffer (Na_2HPO_4), pH = 6.2 (10 µl + 10 µl) and 180 µl *M. luteus* solution (0.6 mg/ml: 30 mg of *M. luteus* mixed with 50 ml phosphate buffer) at 25 °C. The difference in absorbance (optical density, OD = 450) was then measured with a Benchmark Plus^{TM} Microplate Spectrophotometer (BIO-RAD) after 0 time, 5 min, 10 min and 15 min intervals immediately after adding *M. luteus* into each microplate well. The differences were then used to calculate lysozyme activity expressed in units/ml of plasma. Each unit is defined as a reduction in the OD of 0.001 per min. The lysozyme assay is based on absorbance being a linear function of *M. luteus* concentration. The result was compared with the function of a standard. The standard was made of lysozyme extracted from chicken egg white (CEWL) (Sigma Life Science), with a specified activity of 40,000 units/mg. From the plate reading, the rate of change in absorbance at 450 nm (ΔA_{450} /min) during the linear stage of reaction was calculated, giving the following standard equation:

$y = mx + b$ (where $y = \Delta A_{450}$ /min; x = units of activity, equivalent to CEWL).

7.2.5. Blood total protein levels

Total protein in the blood samples was measured to determine total immunoglobulin (Ig) levels following the standard procedure for a Beckman Coulter AU480 Analyzer. Briefly 200 µl of each blood plasma sample contained in 1.5 ml microcentrifuge tubes (Adelab Scientific) was tested and the analyser automatically computed each determination

(results g/L) at the same time interval. The results were then analysed statistically to determine the differences between each treatment groups.

7.2.6. Statistical analysis

The blood lysozyme activity and immunoglobulin (Ig) levels were assessed by the one-way ANOVA test and Post hoc Tukey Test, at $p<0.05$ to estimate any statistical differences of the sample mean results whilst looking at one independent variable (dose) using IBM SPSS Statistics 25 program. Statistical significance was defined as $p<0.05$. Values given are mean ± SE from three replicates each.

For the evaluation of fish bioassay, bacterial counts (cfu/g) across the experimental tanks were subjected to Generalized Linear Model (Negative Binomial) Analysis ($P<0.10$) using SPSS Statistics 25 program. The percent coefficient of variation (%CV) between technical replicates of cfu/g counts were maintained <20%. The results from biological replicates were presented in box plots.

7.2.7. Ethics approval

All fish trial experiments were carried out following the animal ethics guidelines proposed by Animal Ethics Committee (AEC) and the ethics approval (AEC Project No. S-2015-201A) was obtained from the Animal Ethics Committee (AEC) of The University of Adelaide.

7.3. Results

7.3.1. Pilot study

During the acclimatisation period, one death was observed. A diagnostic investigation was undertaken including diagnostic microbiology. Identification and bacterial counts (cfu/g) in kidney and spleen samples were carried out. *Aeromonas* sp. was detected in samples from the fish which indicated either a latent infection or pathogenicity of the *Aeromonas* sp. given the fish were acclimatising prior to any experimentation.

During this pilot trial there were no clinical signs of morbidity or mortality in the experimental fish after the inoculation with the test *A. hydrophila* strain. This indicated that this specific strain of *A. hydrophila* was not significantly virulent for the experimental fish. Therefore, bacterial counts (cfu/g) from the kidney, spleen and blood

samples were used to determine the potential dose to use for the actual infection challenge trial. Bacterial counts from TSB and AMB agar plates showed only few bacterial colonies (<30) at the higher dilutions (10^{-6}, 10^{-9}, and 10^{-12}) and only the (10^{-3}) dilution resulted in adequate growth that could provide an accurate titre calculation. As a result of this finding, to obtain significant bacterial cell counts after the challenge trial, the higher dose (10^{-3}) was chosen to use for further fish infection trials.

7.3.2. *Aeromonas* sp. counts in kidney and spleen samples from the fish trial

From Figure 7-1, it can be seen that for the control group (treatment 1) 10^6 cfu/g *Aeromonas* sp. was present in both kidney and spleen tissues, indicating that the fish purchased from the supplier were already contaminated with *Aeromonas* sp. This compromised the study and interfered with assessing the effectiveness of the phage to control infection. The appearance of the colonies (in pure cultures) on TSA and AMB in all cases was consistent with *Aeromonas* sp.

From Figures 7-1 [a] and 7-1 [b], it can be seen that the bacterial counts were significantly reduced compared to the control, especially with the medium dose of phage. Furthermore, the reduction in bacterial count achieved with the medium dose (3b) was significantly greater than that achieved with the high or low doses, which also produced reductions in counts compared to the control and were significantly different ($p<0.10$).

A similar trend was also observed for the counts from the spleen tissues (Figures 7-1 [c] and 7-1 [d])

In the case of fish which were deliberately infected with *A. hydrophila* on top of the existing colonisation with *Aeromonas* sp. (treatments 2a, 2b and 2c), there were no significant differences in counts when challenged with any of the doses of phage, compared to the controls.

In addition to culturing kidney and spleen biopsies, blood cultures were also examined for bacterial counts on TSB and AMB. *Aeromonas* sp. was isolated from most fish in all treatments as seen in Table 7-2, but since this was done qualitatively, it is not possible to determine if the phage used in treatments 3a, 3b and 3c achieved any significant reductions in bacterial counts in the blood of the fish.

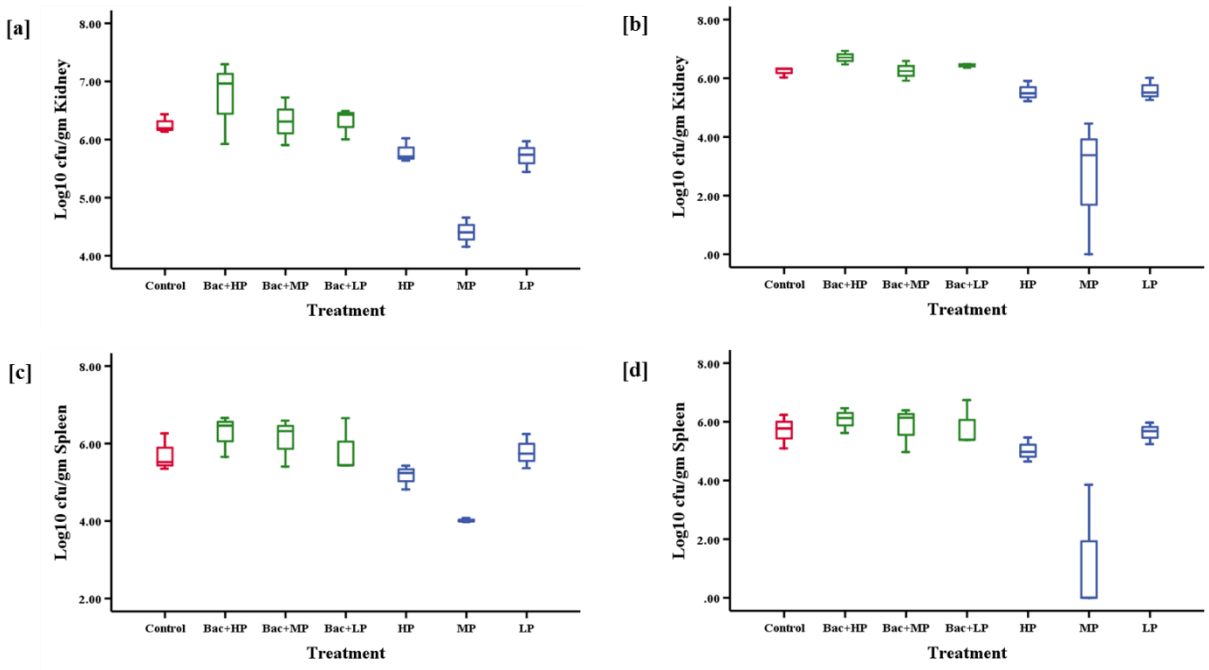

Figure 0-1. Effect of different doses (high, medium and low) of bacteriophage cocktail on silver perch infected with and controls without *A. hydrophila*. [a] and [b] – bacterial count per g of kidney when grown in TSA and AMB respectively; [c] and [d] bacterial count per g of spleen when grown in TSA and AMB respectively. The cfu/g counts were log normalized, box plots were prepared from the results of all biological replicates. The abbreviations on X axes are: 1 (Control) – no bacteria/no phage, 2a (Bac+HP) – *A. hydrophila* infected fish and treated with high dose of phage, 2b (Bac+MP) – *A. hydrophila* infected fish and treated with medium dose of phage, 2c (Bac+LP) – *A. hydrophila* infected fish and treated with low dose of phage, 3a (HP) – uninfected fish injected with high dose of phage, 3b (MP) – uninfected fish injected with medium dose of phage and 3c (LP) – uninfected fish injected with low dose of phage.

Table 7-2. Qualitative detection of *A. hydrophila* in blood culture of each fish from the experimental tanks in TSA and AMB plates

Treatment / Media	Fish 1		Fish 2		Fish 3		Fish 4		Fish 5		Fish 6		Fish 7	
	T	A	T	A	T	A	T	A	T	A	T	A	T	A
1 (Control)	√	√	√	√	√	√	√	√	√	√				
1 (Control)	√		√	√	√	√	√	√						
1 (Control)	√	√	√				√	√						
2a (Bac+HP)	√	√	√	√	√	√			√	√	√			
2a (Bac+HP)		√	√		√		√	√		√	√	√	√	√
2a (Bac+HP)	√	√		√	√	√	√	√	√	√	√	√	√	√
2b (Bac+MP)	√	√			√	√	√	√	√	√				
2b (Bac+MP)	√	√	√	√	√	√	√	√		√	√		√	√
2b (Bac+MP)			√	√	√				√	√	√	√	√	
2c (Bac+LP)	√	√	√	√	√	√	√	√	√	√	√	√		
2c (Bac+LP)	√	√	√		√	√	√	√	√	√				
2c (Bac+LP)	√	√	√	√	√	√	√	√	√	√	√	√		
3a (HP)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3a (HP)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3a (HP)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3b (MP)	√	√	√	√	√	√	√	√	√	√	√	√	√	√
3b (MP)	√	√	√	√	√		√	√	√	√	√	√	√	√
3b (MP)	√		√	√	√	√	√	√	√	√	√	√	√	√
3c (LP)	√	√	√	√	√	√	√		√	√	√	√	√	√
3c (LP)	√	√	√	√	√	√	√	√	√	√	√	√		
3c (LP)	√	√	√	√	√	√	√		√				√	√

Note: Tick (√) is the indication of positive *A. hydrophila* colony development on the plate. T denotes TSB and A denotes AMB; blanks indicate no colony. Each bacterial colony was biochemically tested (Gram staining, O/F test) to confirm *A. hydrophila* growth.

7.3.3. Effect of bacteria and bacteriophages on fish lysozyme activity and total immunoglobulin level

The blood of "non-infected" fish (treatment 3a) which was injected with a high dose of bacteriophage showed the highest lysozyme activity compared to the control or any of the other treatments (Figure 7-2 [a]). The observed increase in lysozyme activity could be due to the fish responding to the antigenic determinants on the phage and not in fact due to the pathogen, which would be consistent with observed results for "infected" fish.

When the "infected" fish were challenged with phage (treatments 2a, 2b and 2c), the lysozyme activity did not increase relative to the control (Figure 7-2 [a]) possibly because the fish had already developed some immunity to *Aeromonas* sp. from the endemic infection. This would also be consistent with the fact that no mortality was recorded after being further challenged with the bacteria.

In addition to that, the concentration of immunoglobulins in blood across all treatments showed no significant differences across the treatments (Figure 7-2 [b]).

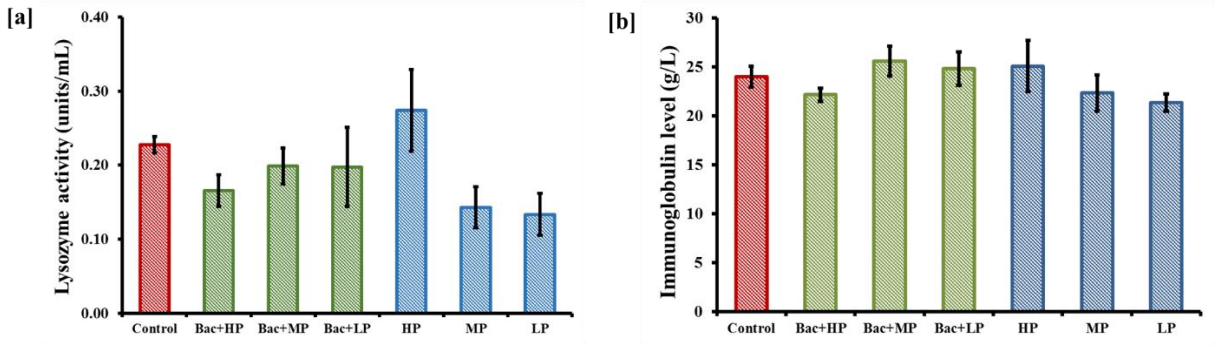

Figure 0-2. [a] – lysozyme activity and [b] – total immunoglobulin level measured on the plasma of experimental silver perch fish. The abbreviations on X axes are: 1 (Control) – no bacteria/no phage, 2a (Bac+HP) – *A. hydrophila* infected fish and treated with high dose of phage, 2b (Bac+MP) – *A. hydrophila* infected fish and treated with medium dose of phage, 2c (Bac+LP) – *A. hydrophila* infected fish and treated with low dose of phage, 3a (HP) – uninfected fish injected with high dose of phage, 3b (MP) – uninfected fish injected with medium dose of phage and 3c (LP) – uninfected fish injected with low dose of phage. Each bar represents the mean value from three determinations with the error bars of standard error of mean.

7.3.4. Fish histopathology

The histological alterations observed in fish organs are detailed in the Appendix table 9-1. A brief description of histological changes observed from each of the fish organs is as follows:

7.3.4.1. Gill

Some significant histological anomalies were observed in the gill samples of the experimental fish from different treatment groups. The common histological findings in the gills were congestion and bridging fusion of the secondary lamellae (in approximately 80% of the samples) which resulted from the hypertrophy and hyperplasia of the epithelial cells. Some generalised epithelial thickening was also observed across approximately 60% of the samples. Changes in the gills observed in approximately 20% of fish samples were found to be consistent with a monogenean infection. Presence of mild to moderate levels of monogeneans (unidentified gill flukes) and “blue bodies” (*Chlamydophila*) are shown in Figures 7-3 [a], 7-3 [b], 7-3 [c], 7-3 [d], 7-3 [e] and 7-3 [f].

In summary the pathological changes were interpreted as incidental. Gill infection due to the *Chlamydophila* and presence of the monogeneans (unidentified gill flukes) are indicative of the fish being infected at their place of origin before they were received at the University experimental system.

From the gill pathology of the observed fish did not show any signs of infection due to *A. hydrophila* which mainly manifests as dermal lesions, scale loss, haemorrhaging around the infected area, fin rot or necrosis (DPI, 2007).

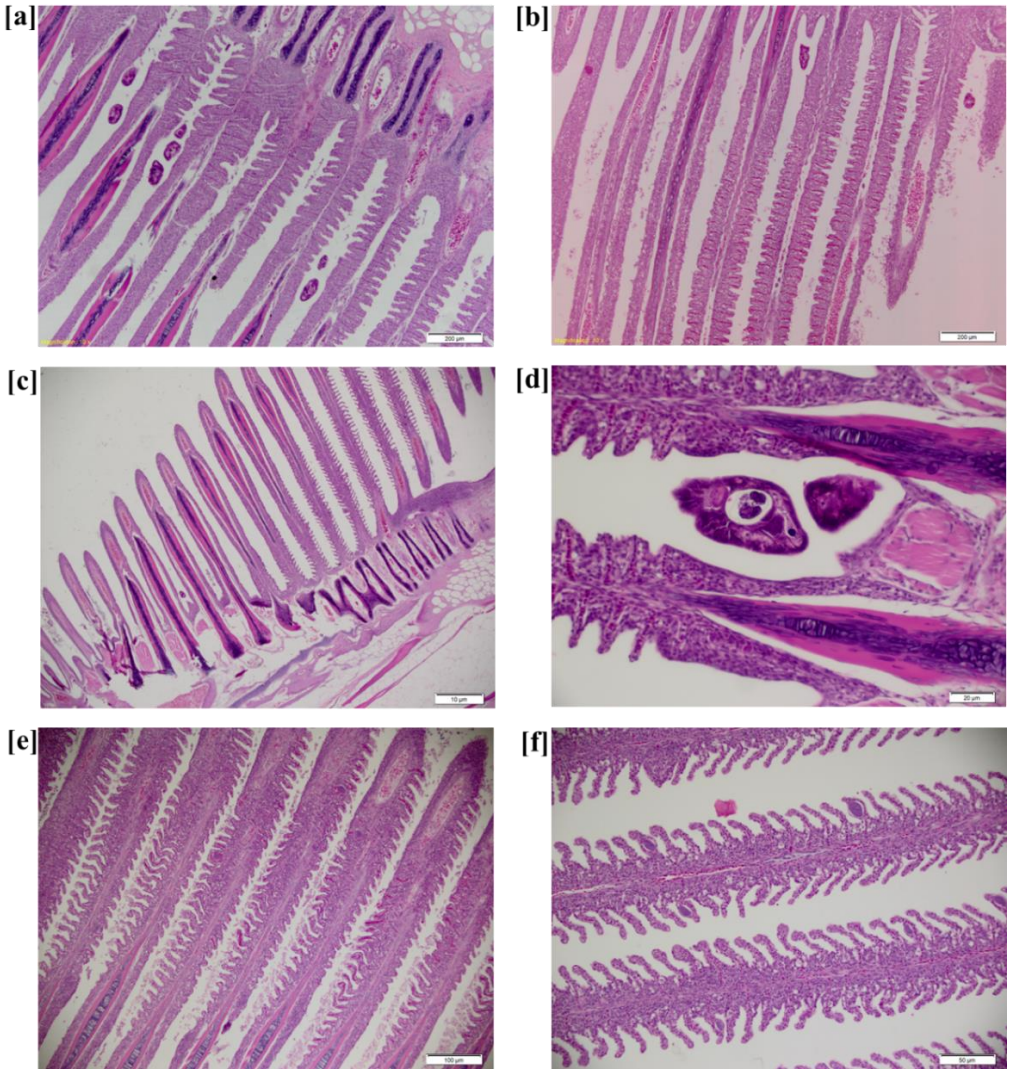

Figure 0-3. Histopathological analyses of gill tissue of silver perch; [a] – gill section showing monogenean infestation (bar – 200 µm); [b] – gill tissue showing bridging and laminar fusion with monogenean infestation (bar – 200 µm); [c] – gill section showing *Chlamydophila* (blue bodies) infection (bar – 50 µm); [d] – gill section showing bridging and spongiosis of the secondary lamellae (bar – 10 µm); [e] – gill section showing epithelial hyperplasia with mild secondary laminar fusion (bar – 100 µm); [f] – presence of flukes in the gill (bar – 20 µm).

7.3.4.2. Digestive organs (liver, stomach, intestine, pancreas)

Twenty percent of the liver tissue samples of all examined fish showed mild to generalized hepatocellular vacuolation and lipidosis (Figure 7-4 [a]). Otherwise, no significant histological findings were observed. Stomach tissue sample did not show any significant histological alterations. Zymogen depletion and acinar cell atrophy were the significant histological changes observed in approximately 10% of the pancreas tissue samples (Figure 7-4 [b]). The histological changes observed in approximately 20% of fish intestine tissue were mild to moderate lymphocytic infiltration in the lamina propria of the pyloric caeca (image is not shown).

The mild changes observed in the digestive tissues were not indicative of infection with *A. hydrophila* and are occasionally observed in fish with generalised luminal antigenic presence. Stress due to the handling of fish and water quality of the experimental tank meant fish were not eating properly, probably leading to the pathological changes observed in the liver and pancreas which are consistent with inappetence and mild cachexia.

There was mild focal granulomatous coelomitis observed in one fish indicating possible focal reaction to injected material (Figure 7-4 [c]). It was anatomically situated around the site of injection. No intralesional bacteria were observed.

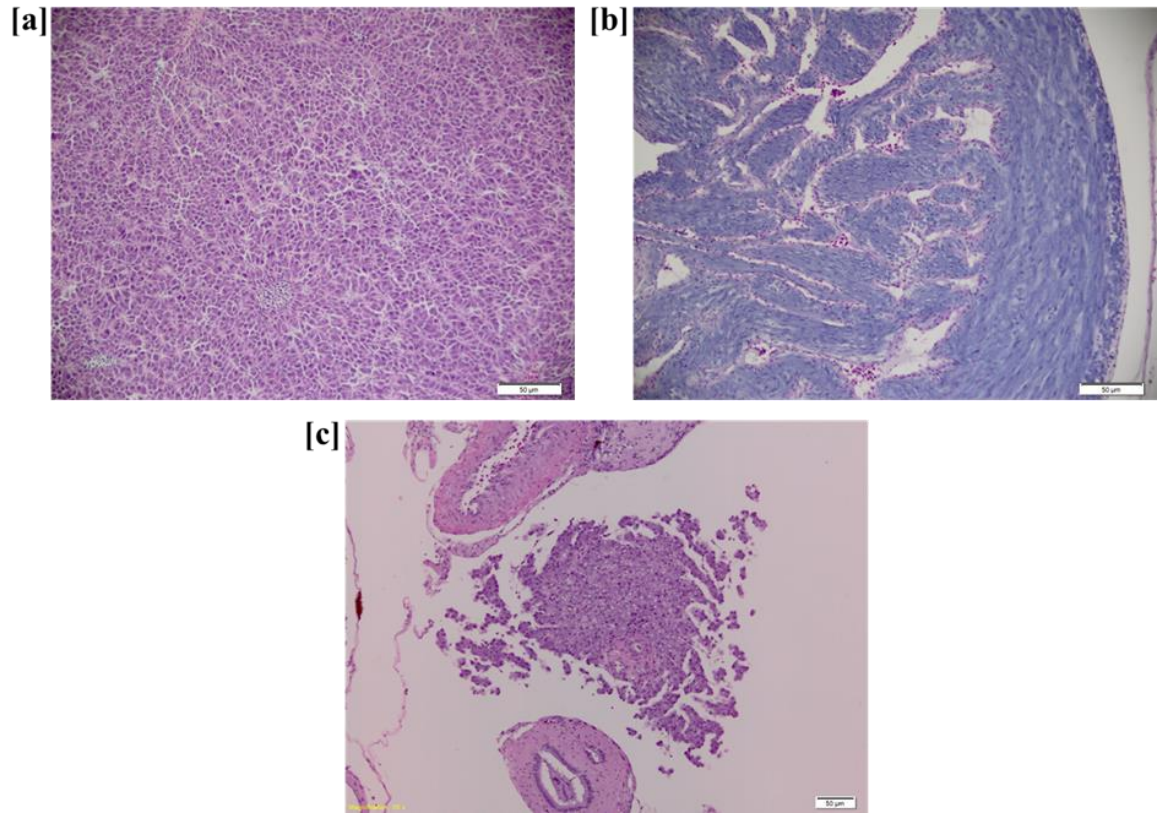

Figure 0-4. Histopathological analyses of digestive tissues of silver perch. [a] – liver section showing hepatocellular vacuolation (bar – 50 µm); [b] – pancreas section showing acinar cell atrophy and low zymogen (bar – 50 µm); [c] – section of the coelomic cavity near pancreas showing some areas of granulomatous coelomitis (bar – 10 µm).

7.3.4.3. Eyeball

Hyperplasia of the corneal epithelium or corneal thickening in 40% of the tissue samples (Figures 7-5 [a] and 7-5 [b]), congestion in the choroid plexus in approximately 40% of the tissue samples (Figures 7-5 [c], 7-5 [d] and 7-5 [e]) and mild macrophage infiltration in 20% of the tissue samples (Figure 7-5 [c]) were the most obvious histological changes observed in fish eyeball tissue samples.

These changes were not indicative of *A. hydrophila* infection but rather poor water quality and/or parasitic infestations.

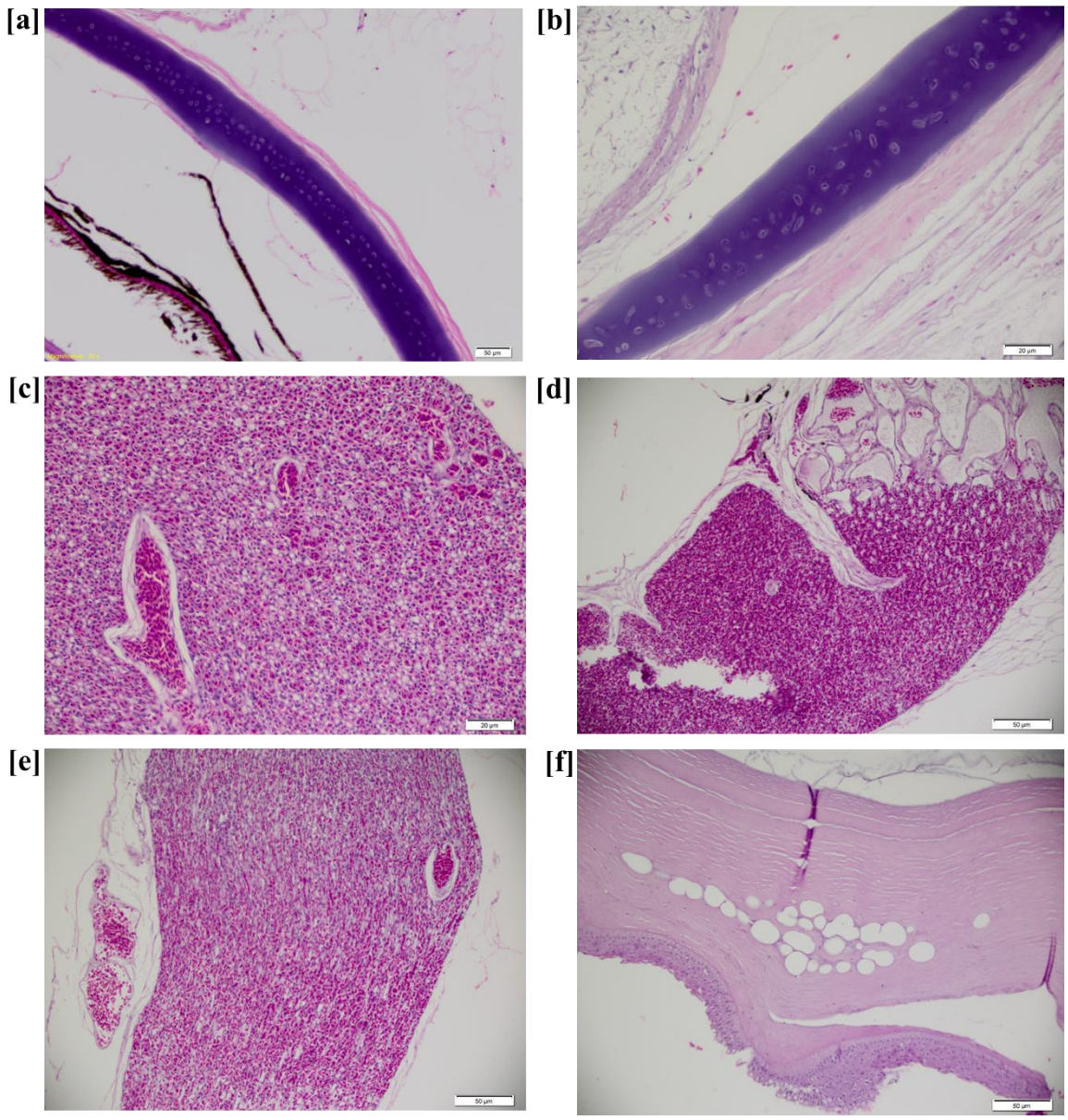

Figure 0-5. Histopathological analyses of eyeball of silver perch. [a] – eyeball section showing hyperplasia of the corneal epithelium (bar – 50 µm); [b] – eyeball section showing hyperplasia of the corneal epithelium and vacuolation in the corneal stroma (bar – 20 µm); [c] – eyeball section showing congested choroid plexus and macrophage infiltration (bar – 20 µm); [d] – eyeball section showing congestion in the choroid plexus (bar – 50 µm); [e] – eyeball section showing choroid plexus congestion (bar – 50 µm); [f] – eyeball section showing vacuolation of the corneal stroma (bar – 50 µm).

7.3.4.4. Brain

The significant histological changes observed in the fish brain tissues (40% of the samples) were moderate to multifocal vacuolation in the grey and white matter (Figures 7-6 [a] and 7-6 [b]), marked vascular congestion (in approximately 10% of the samples). Otherwise no significant findings were observed.

Vacuolation in the brain of most samples of fish is normally indicative of hyperammonemia, as a consequence of ammonia toxicity. This has not been observed in silver perch previously, although it has been reported in zebrafish (Wang et al., 2019). It is also observed as an artefact of fixation and processing of brain tissues from fish.

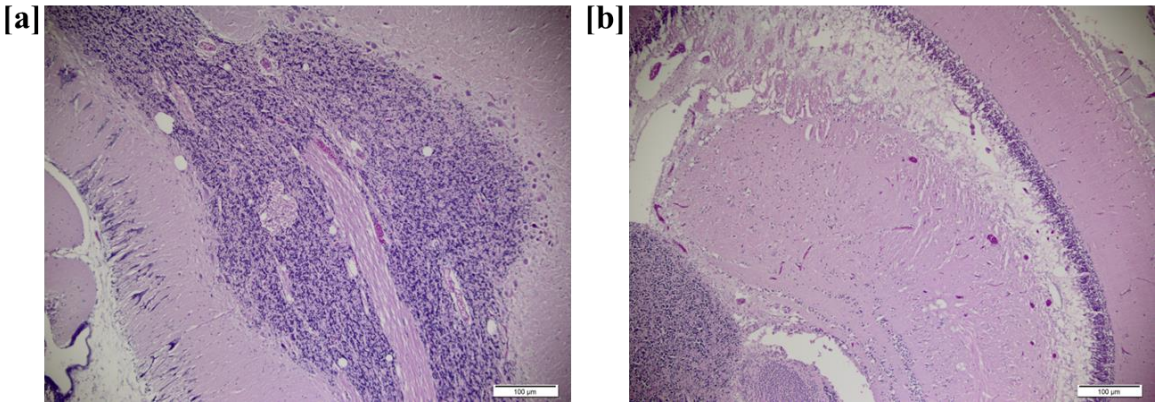

Figure 0-6. Histopathological analyses of brain tissue of silver perch. [a] – brain section showing vacuolation of the grey matter and optic lobe (bar – 100 µm); [b] – brain section showing vacuolation of the white matter (bar – 100 µm).

7.3.4.5. Skin

No significant changes were observed in the skin tissue of the experimental fish. No significant findings (NSF) were observed in approximately 60% of the samples. Mild to generalized epithelial thickening (Figures 7-7 [a], 7-7 [b], and 7-7 [c]) with some loss of goblet cells was observed in approximately 30% of the samples. These changes are more consistent with the fish being affected by their environment i.e. poor water quality and/or high stocking density rather than specifically relating to signs of *A. hydrophila* infection.

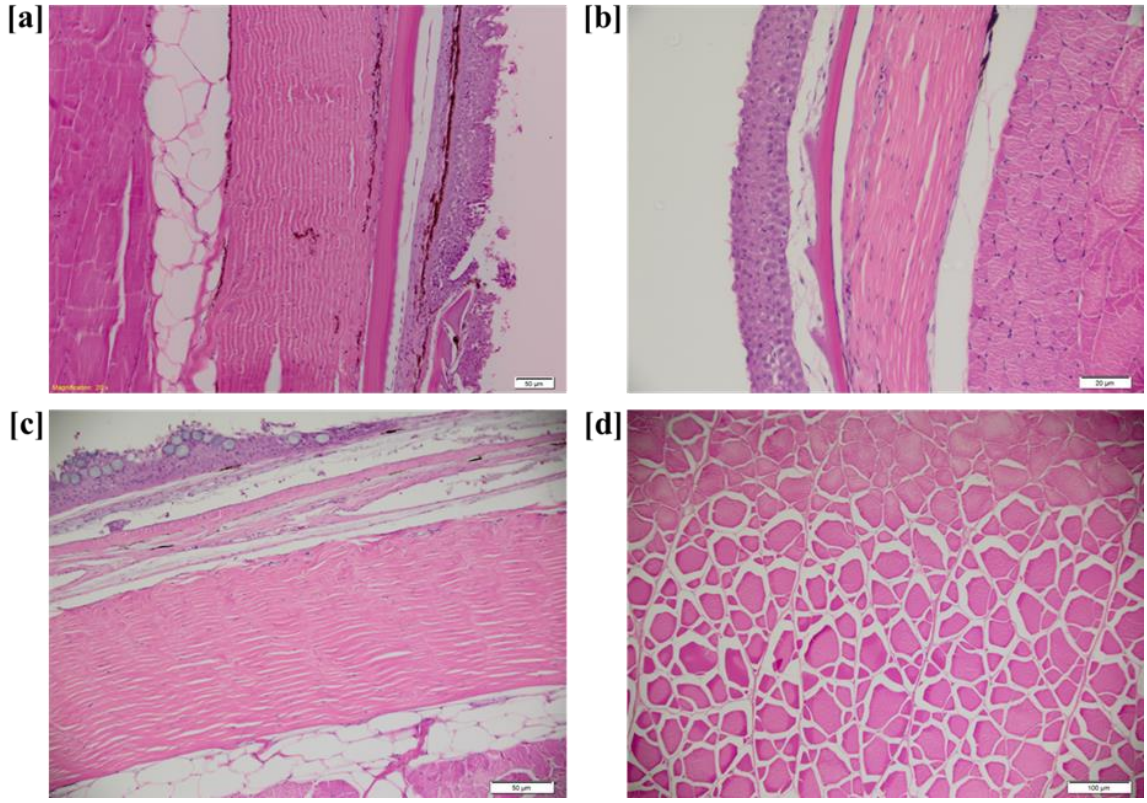

Figure 0-7. Histopathological analyses of skin and muscle tissue of silver perch. [a] – skin section showing epithelial thickening, hyperplasia and loss of goblet cells (bar – 50 µm); [b] – skin section showing epithelial thickening and loss of goblet cells (bar – 20 µm); [c] and [d] – skin section showing epithelial hyperplasia (bar – 50 µm) and muscle section (bar – 100 µm) respectively.

7.3.4.6. Heart

Minimal histopathological changes were observed in the heart tissues examined. Approximately 10% of the heart tissue sample showed dense and spongy ventricular walls which can be related to stress (image is not shown).

7.3.4.7. Spleen and kidney

Moderate to multifocal infiltration of brown to black melanomacrophage centres and marked congestion with depletion of lymphoid tissue were observed in the spleen samples (Figure 7-8 [a], 7-8 [b] and 7-8 [c]).

Because of the aims of the present study to investigate the effect of bacteriophage on bacterial counts most of the kidney and spleen samples were used for *A. hydrophila* bacterial re-isolation and colony (cfu) counts for the bacteriophage efficacy analysis. Therefore, only a few spleen samples were collected for histopathology.

Some randomly picked histopathology slides were subject to Gram staining to observe the presence of *Aeromonas* (Gram negative rods) in the tissue. Some Gram negative rods were observed in all the Gram stained slides.

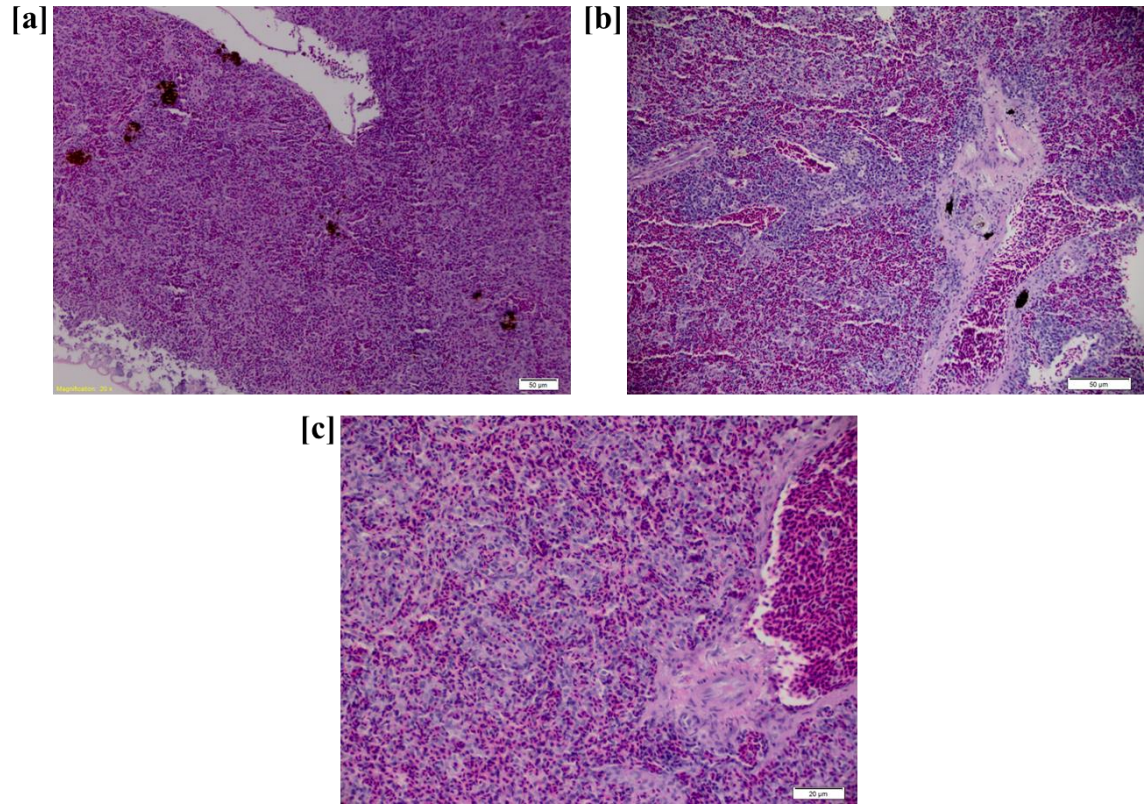

Figure 0-8. Histopathological analyses of spleen tissue of silver perch. [a] – spleen section showing multifocal, brown to black melanomacrophage centres (bar – 50 µm); [b] – spleen section showing ellipsoid disruption and presence of melanomacrophage centres (bar – 50 µm); [c] – spleen section showing generalized congestion, lymphoid depletion and ellipsoid disruption (bar – 20 µm).

7.4. Discussion

The present study aimed to examine the efficacy of four bacteriophages (which were described in chapter 4) with a view to developing a therapeutic tool to treat *A. hydrophila* infection in fish. For this purpose, an extensive challenge trial was designed using silver perch (*B. bidyanus*) as a model to evaluate the therapeutic potential of the experimental bacteriophages. The fish trial consisted of two different phases one of which was a preliminary pilot trial (consisting of 15 fish and 5 treatments) to assess the pathogenicity of the bacterial isolate and from there to determine the bacterial dose rate to be used in the second phase of trial (actual infection trial with 144 fish and 7 treatments). For the actual infection trial, the hypothesis was that *A. hydrophila* bacteriophage therapy will be useful

in reducing bacterial counts in the target organs (blood, spleen and kidney) within the fish body. There was also hope that a secondary assessment could be made in that the use of bacteriophage cocktail would reduce the morbidity and mortality in experimentally infected fish.

Initially fish were challenged with a single *A. hydrophila* strain (Ah10). Given the fact that lytic bacteriophages possess a very specific host range with no known impact on the environment (Gutiérrez et al., 2010, Jun et al., 2014) and to overcome challenges associated with phage resistance to bacteria (Levin and Bull, 2004, Nakai, 2010, Silva-Aciares et al., 2013, Vieira et al., 2012), a cocktail of four lytic bacteriophages was utilized in this study to counteract *A. hydrophila* infection. In addition, it has been found that a mixture or cocktail several bacteriophages is more effective in controlling bacterial infection and it is recommended that a phage cocktail be used for therapeutic purposes to overcome any bacteriophage resistance within the bacterial strain (Chan et al., 2013, Nieth et al., 2015, Mateus et al., 2014, Rao and Lalitha, 2015, Filippov et al., 2011, Chen et al., 2018). The results obtained from this challenge trial showed a significant treatment effect when averaged across the tanks and there was no difference between the bacterial counts from two different media (TSA or AMB) used for enumerating the reisolated *A. hydrophila* strain from kidney and spleen of the experimental fish. However, the overall result obtained was not sufficient to determine if the applied bacteriophage cocktail was effective in combatting *A. hydrophila* infection. It was clear that the fish were already sub-clinically colonised with *Aeromonas* sp. – although this was not realised at the start of the trial and that, they may have already developed immunity. As a result, after the infection, the number of reisolated Ah10 did not increase significantly because the fish may have already had immunity. For this reason, a comparison between the effectiveness of high, medium and low doses of bacteriophage could not be determined.

In the main study, administration of bacteriophage was via intraperitoneal (IP) injection as this type of administration has been proven to be a successful route of administration for immediate distribution of phages into the blood circulation system (Ryan et al., 2011, Verner–Jeffreys et al., 2007, Huff et al., 2003b). However, using this route of administration, each fish was sedated, injected, resuscitated and returned back to the tank which resulted in a variable degree of stress for the fish. Hence, there may be a need to reconsider the mode of bacteriophage administration. Moreover, from a practical

perspective it would not be feasible to inject individual fish in a commercial aquaculture system. Future studies need to be undertaken to determine how stable the bacteriophage would be in the aquarium water as an emersion preparation. This would mean that larger volumes of bacteriophage stock would need to be generated for this delivery of treatment. Another possible route of administration that could be considered would be *per os*. Phage impregnated feed may be viable for therapeutic purposes. Sick fish however, tend not to consume normal amounts of offered feed and thus reducing the chance that phage impregnated feed would be consumed at a high enough level to produce the desired clinical result. Moreover, the acidic pH of the upper gastrointestinal tract may also affect the therapeutic potentiality of orally consumed phages (Nakai and Park, 2002, Silva et al., 2014b, Nakai et al., 1999).

It is also difficult to control or determine an accurate treatment dose of phage per fish when it is delivered through the aquatic environment compared to treatment of land-based farm animals. Additionally, for bath exposure, a very large amount of phage suspension is required to deliver the appropriate treatment dose per fish and so this approach is not feasible in terms of associated labour and cost involved for large scale fish aquaculture system. Nevertheless, studies done by Silva et al. (2014b) found that the administration of bacteriophage to fish larvae in water showed effective results as phage could reach the specific infection sites and destroy *Vibrio* sp. residing within the fish and on the skin of fish larvae. They also suggested that the delivery method and the life stages of experimental fish were the most important factors in designing a successful bacteriophage based therapeutic strategy for aquaculture.

Many studies of bacteriophage therapy have reported promising results in contrast to those from the present study (Li et al., 2016, Martínez-Díaz and Hipólito-Morales, 2013, Karunasagar et al., 2007, Silva et al., 2014b, Vinod et al., 2006). The data presented here indicates that there may be some limitations to bacteriophage treatment for *A. hydrophila* infection in silver perch as the fish were already sub-clinically infected with *Aeromonas* sp. Therefore, future trials should include microbiological assessment of the fish before any trials start and if any pathogens are isolated – they should either be treated with antibiotics to clear the infection or search for phages effective against those colonising bacteria. Alternatively, bacteriophages may be better applied as an adjunctive therapy in conjunction with antibiotics, rather than using it as a total replacement to the antibiotic use. The *A. hydrophila* counts from fish kidney and spleen demonstrated the effect of

different doses (high, medium and low) of bacteriophages on experimental fish (uninfected and infected with *A. hydrophila*). A medium dose was found to be the most effective of the doses used in this study. This is possibly because of the fact that when phages were present in high concentrations the bacterial count was too low to demonstrate phage efficiency or when low dose was used there were insufficient phage to infect the bacteria present. Moreover, it is already reported that phages remain active as long as their specific host organism is present (Oliveira et al., 2012, Efrony et al., 2007). However, sometimes the phage replication rate can be less than the rate of phage loss which may limit bacteriophage infectivity in aquaculture systems (Levin and Bull, 2004, Martínez-Díaz and Hipólito-Morales, 2013).

Fish develop some immunologic responses to the presence of bacteriophages which they recognize as foreign entities (Pirisi, 2000, Sulakvelidze and Morris, 2001, Oliveira et al., 2012). To observe the fish immune response to bacteriophages, lysozyme activity and total immunoglobulin (Ig) levels of fish blood plasma from each treatment group were determined. It was predicted that due to the systemic administration of bacteriophages to the fish body, there would be an acquired immune response development. However, lysozyme activity and total immunoglobulin (Ig) levels did not indicate any significant changes in the immune response resulting from bacteriophage administration (i.e. there was no statistical difference between control and treated fish immune assay). This could be as a result of infection with environmental *Aeromonas* sp. prior to the commencement of the actual infection trial. This conclusion is reinforced with the finding that there was no significant difference in concentration of immunoglobulins in blood from fish across all treatments and controls (i.e. pilot and actual challenge trial) indicating the immune response had already been primed prior to the trial beginning. Therefore, the primary and possibly the secondary immune response had already occurred prior to the challenge with bacteria and bacteria plus bacteriophage. In contrast to this study, another study on shrimp suggested some changes occurred in immune response due to the IP injection (Alagappan et al., 2016). Some other assays (e.g. respiratory burst, phagocytic activity, ELISA) could have been used for detecting immune response within the fish. However, the size of the fish in this trial study did not allow sufficient blood samples to be taken for conducting additional immunological assays.

Fish tissue histopathology was conducted to observe any potential histological changes resulting from *A. hydrophila* infection and bacteriophage treatment. As a result of the

limited application of the infection model revealing morbidity and mortality differences between control cohorts and treated cohorts within the study, it was proposed that immune active tissues (i.e. kidney and spleen) could be targeted for histopathological analysis. A limiting factor for this approach was however imposed by the fact that these organs were also required for bacterial re-isolation and the fish were not big enough to enable adequate samples to be collected for histopathological analysis. Hence, the present study recorded changes in all of the fish organs accepting that there were only a few histopathological changes that are consistently associated with *A. hydrophila* infection in teleost fish and none recorded for this infection in silver perch.

As reported previously (Dymowska et al., 2012, Zepeda-Velázquez et al., 2015), the changes in fish gill tissue observed in this study, such as epithelial hyperplasia or structural loss are changes induced by poor water quality or potential toxins. The water quality of the present study was maintained within accepted parameters for silver perch. The presence of monogenean fluke infection and many of the gill structural pathologies were chronic and possibly present on arrival of fish from their original source.

The zonal atrophy, vacuolation of the hepatocytes and hepatocellular degeneration observed in some of the fish liver samples (Figure 7-4 [a]) are consistent with changes observed in previous *A. hydrophila* infection studies (Wang et al., 2017, Hongda and Lihua, 1996, Zepeda-Velázquez et al., 2015). The instances of granulomatous coelomitis observed (Figure 7-4 [c]) was interpreted to be associated with the site of injection (IP) and has been previously reported by Coscelli et al. (2014).

A number of histopathological changes observed in the trial fish, such as corneal epithelial hyperplasia, macrophage infiltration and congestion of the choroid plexus (Figure 7-5) and vacuolation in the grey and white matter in the brain (Figure 7-6) are indicative of response to poor water quality and in particular an indication of high levels of ammonia in the culture water. Given that the water quality was maintained throughout the acclimatisation and trial period at optimal levels it was suspected that the fish were subjected to water quality stress prior to admission to the study. It should be noted however that limited histopathology findings resulting from high ammonia level have been published for silver perch (Feldman et al., 2014). The conclusions made from the histopathological tissue organ analyses indicate that in the absence of commonly associated pathologies with *A. hydrophila* infection, the pathologies that were noted were

probably as a result of stress on the fish induced by the route of injection (at trial), handling and the water quality in the premises of the supplier of the fish.

A limitation of the study was the pre-existing colonisation of the fish with *Aeromonas* sp. In hindsight it would have been wise to test a representative sample of the fish before commencing the pilot study. This would have also allowed assessment of any pre-existing pathologies which may have resulted from pre-existing bacterial infection. In addition, the small size of the fish compromised selection of adequate samples for histopathology while still allowing enough material for bacterial counts. Therefore, factors like size of the fish or the use of "pathogen free" fish should be considered prior to designing any experiment for any aquaculture-based setting.

Compared to terrestrial settings, aquatic environments have additional obstacles for therapeutics or feed additives. Therefore, it is always challenging to design a practical, suitable and effective therapeutic approach for the aquaculture environment. Another important factor associated with the selection of suitable bacteriophages as therapeutic agents is to check whether they are carrying any toxin encoding genes which may enhance the pathogenicity of the host bacterium. Therefore, preparation and selection of appropriate methods of purifying phages to remove bacterial toxins should be considered in order to avoid secondary infections in phage therapy.

It has already been reported that phages are not always able to reach the site of infection in the animal host and thus may be effective only as a preventive strategy (i.e. reducing environmental bacterial loads) instead of working as a control measure for bacterial infection (Martínez-Díaz and Hipólito-Morales, 2013). Compared to antibiotic based applications, some clinical approaches for treatment of bacterial infections can be more effective with the use of bacteriophage mediated applications. To identify all the details of the potential of this therapeutic approach, more rigorous research is required for making the bacteriophage application more achievable for the prophylaxis and treatment of bacterial infection and to secure more suitable and practical ways to overcome bacteriophage based therapeutic obstacles.

7.5. Conclusion

For the successful control of infectious disease in fish and shellfish communities, it is of prime importance to emphasize a few attributes in phage therapy: adequate and well-

planned strategies should be developed for accurately identifying causative pathogens. In addition, adequate knowledge of the isolated bacteriophage (such as, lytic activity, lifespan, phage-host interaction, and their infection duration) is a key factor to be considered; proper understanding of the surrounding environment and pathogenic variation in different seasons is also important.

The results obtained from the present study can serve as a reference for future therapeutic approaches for teleost fish indicating the potential of bacteriophage in controlling bacterial infections. However, it is important to validate the potential of bacteriophages and the limitations associated with their use in culture conditions. Moreover, environmental and seasonal based research needs to be conducted in both semi-intensive and real scale environments in order to assess the potential of this bacteriophage control strategy in different time points.

Conclusion and future direction

With the increasing concerns related to antibiotic resistance and issues associated with the presence of antibiotic residues within the aquatic food animals, bacteriophage therapy is now gaining attention as an alternative antibacterial control measure. Bacteriophages represent an excellent eco-friendly, cost effective and host-specific bacterial controlling device which specifically infects the bacterial cell by injecting phage genome into the host cells. Overall, the present study of four bacteriophages demonstrated a promising potential for use in reducing counts of a bacterial pathogen *A. hydrophila in vitro*. Part of the present study involved *in vivo* phage challenge trial in Silver Perch (*B. bidyanus*) as a model fish with a view to develop a phage therapeutic tool to treat *A. hydrophila* infection. The overall results of the trial warrants further investigation to determine the *in vivo* phage effectivity on experimental fish. The present study identified some issues which if resolved will improve future studies and facilitate successful results. While conducting the challenge trial, it could not be anticipated that the fish might already be sub-clinically colonised with *Aeromonas* sp. and that, they might have already developed immunity. Therefore, in future studies, the health status of the fish must be checked before the challenge trial and fish must also be investigated for any parasitic infestations. Ensuring the experimental tanks are operating optimally (e.g. controlling ammonia level, oxygenation) and maintaining appropriate stocking density are also pivotal factors to be considered in order to avoid the experimental fish getting stressed. Selection of pathogenic host bacterial strains (in this case *Aeromonas sp.*) for the challenge trial, screening of phage preparations to check for the presence of virulence genes and selecting only the phages negative for virulence factors also need to be considered prior conducting any challenge trial. Genotyping confirmation of the fact that the bacterial strain (in this case *Aeromonas sp.*) reisolated from the fish organs (e.g. kidney, liver, spleen) of the infected fish is the same as the one used for treatment also needs to be considered.

In the present study, a whole genomic investigation of four phage samples was conducted in order to know their genomic characteristics and to compare their similarity to known phages. Two novel phages were identified from the analysis. Following this analysis, more research can be done to understand the diversity of phages associated with each candidate bacterial pathogen, evolutionary relationships as well as to identify specific phages lacking an integration tool in their genome. This will aid to secure effective and safe use of phages as bactericidal tool in the aquaculture sector.

The phage storage efficacy investigations performed in the present study have added significant information to enable secure phage storage under different temperature conditions as well as in the lyophilised state. The data generated from the present study will be useful for commercial agencies to evaluate and secure phage shelf-life and to build up new protocols for long term storage of phage.

Overall, this study has demonstrated the promising potential of *A. hydrophila* bacteriophages to be used for bacterial therapeutic purpose as a ground for further studies in the field of phage therapy. Nevertheless, detailed evaluation and proper risk assessment prior phage implementation will aid better outcome in future.

Appendix

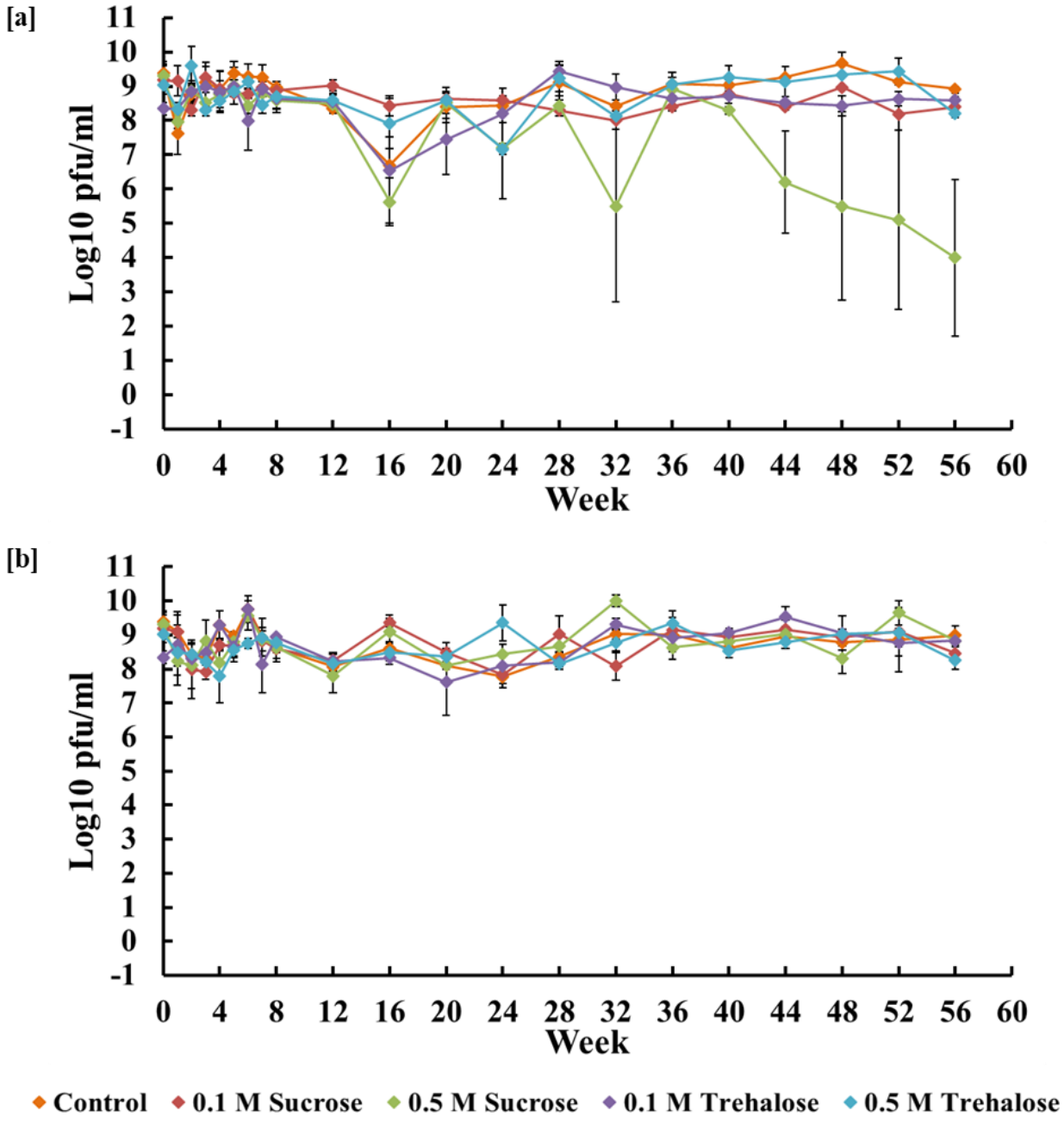

Figure 0-1. Stability of Ah6_G bacteriophage solution with cryoprotectants; [a] – at 4 °C and [b] – at -80 °C. Error bars represent standard error of mean. Some error bars are too small to be visible.

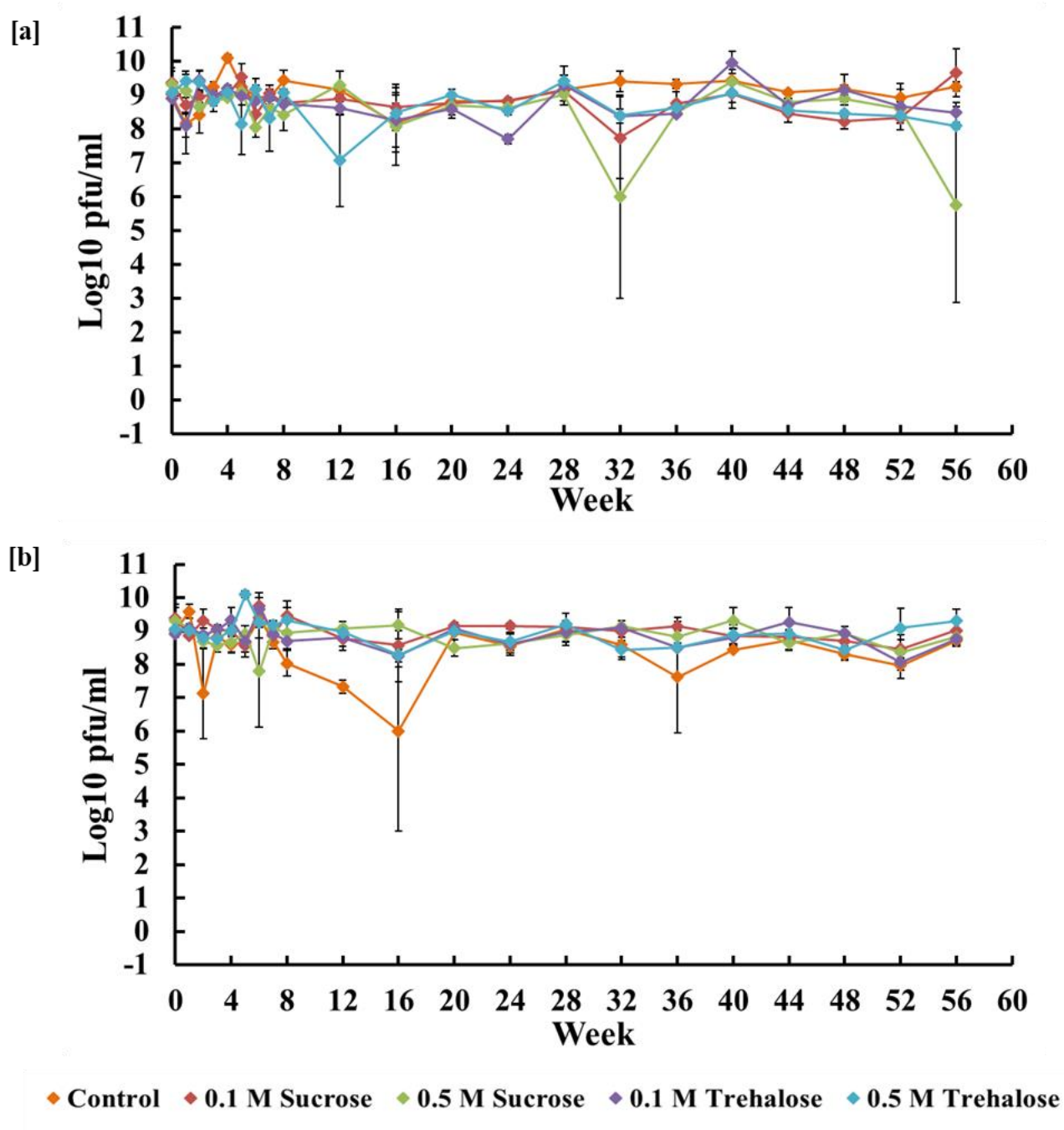

Figure 0-2. Stability of Ah9_PP bacteriophage solution with cryoprotectants; [a] – at 4 °C and [b] – at -80 °C. Error bars represent standard error of mean. Some error bars are too small to be visible.

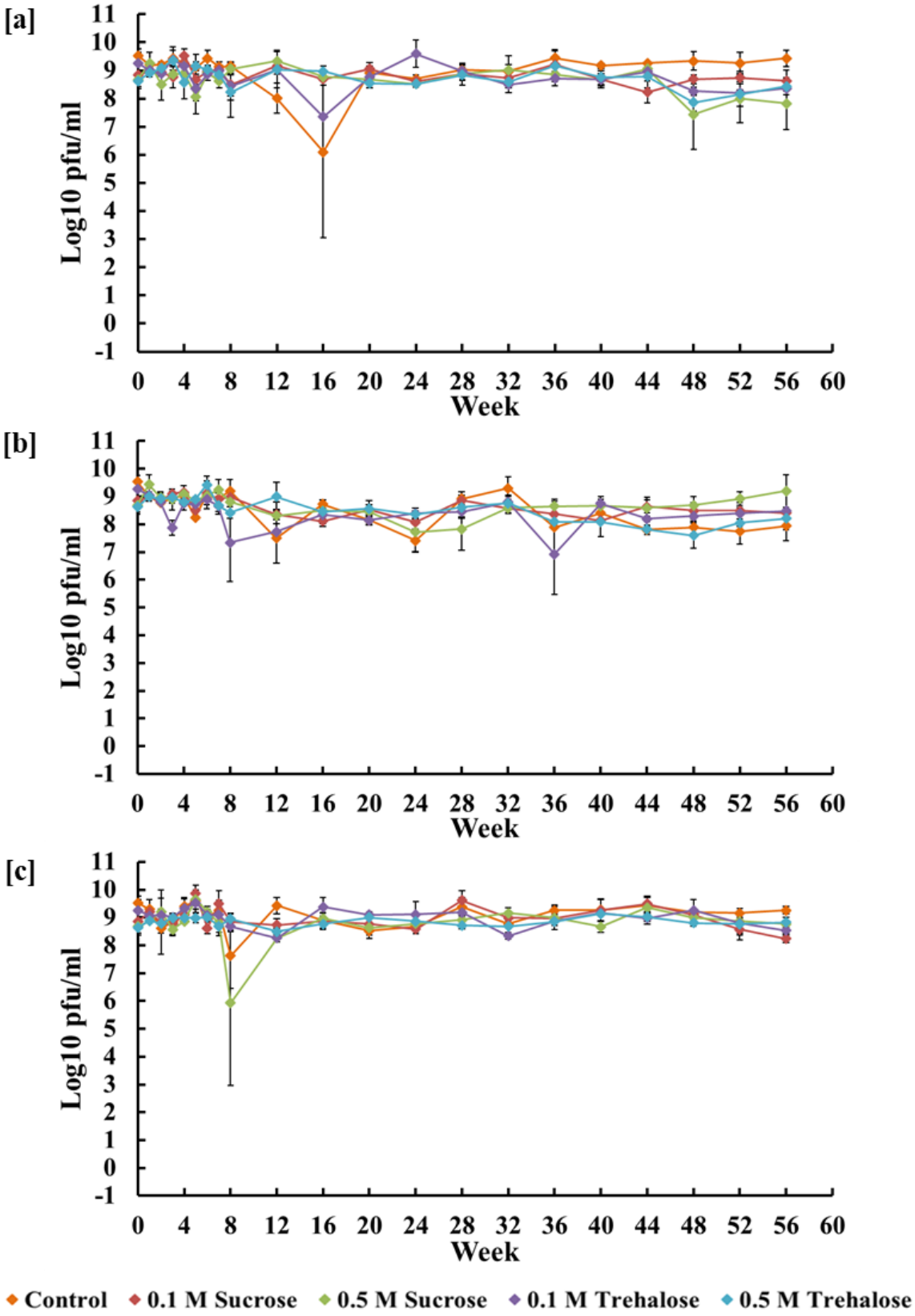

Figure 0-3. Stability of Ah10_Se bacteriophage solution at three different temperatures with cryoprotectants; [a] – at 4 °C; [b] – at -20 °C & [c] – At -80 °C. Error bars represent standard error of mean. Some error bars are too small to be visible.

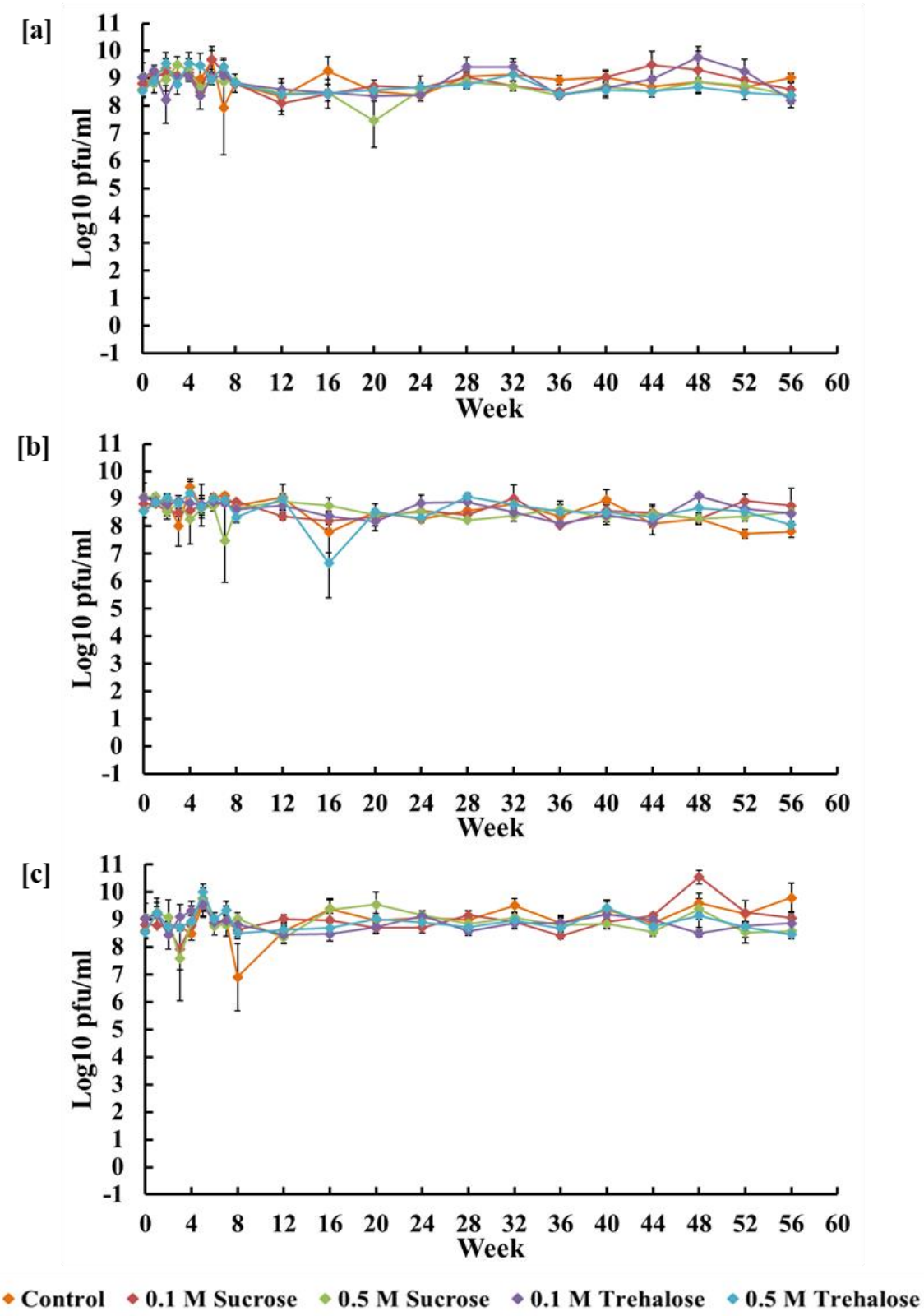

Figure 0-4. Stability of Ah10_S bacteriophage solution at three different temperatures with cryoprotectants; [a] – at 4 °C; [b] – at -20 °C & [c] – At -80 °C. Error bars represent standard error of mean. Some error bars are too small to be visible.

Table 0-1. Histopathological changes and characteristics of different fish organs from the fish belonging to each treatment and the control

Gill	Muscle (Skin)	Eyeball	Liver	Stomach	Pancreas	Intestine	Brain	Heart	Spleen	Kidney
Control										
• Multifocal thickening of the epithelium with bridging of the secondary lamellae • Spongiosis of the secondary lamellae • Branchitis (inflammation of the gill) • Presence of monogeneans	NSF	• Generalized hyperplasia of the corneal epithelium • Congestion of the choroid plexus	NSF	NSF	NSF	NSF	NSF	NSF	NE	NE
• Congestion of the secondary lamellae vessels, otherwise NSF	• Epithelial thickening of the skin with focal loss of goblet cells	• Thickening & hyperplasia of the corneal epithelium	• Diffused mild hepatocellular vacuolation	NSF	NSF	NSF	• Moderate vacuolation in the grey and white matter of the brain	• Dense ventricle wall of the heart, otherwise NSF	NE	NE
• Congestion of the secondary lamellae	NSF	• Congestion of the choroid plexus, otherwise NSF	• Generalized fatty vacuolation of the hepatocytes	NSF	NSF	NSF	NSF	NSF	NE	NE
Bac+ HP										
• Marked epithelial thickening • Vascular congestion • Marked bridging and laminar fusion across most of the sample	• Focal hyperplasia • Thickening of the epidermis with focal loss of goblet cells	• Focal thickening of the corneal epithelium and corneal matrix • Multifoca	• Focal random areas of hepatocellular lipidosis	NSF	NSF	• Moderate lymphocytic infiltration of the lamina propria	NSF	• Ventricular myocardium is dense, but normal	• Multifocal, brown to black MMC	NE

• Moderate to marked population of monogeneans (unidentified) within the gill as well as mild infestations of lymphocystis (chlamydophila like organisms) (blue bodies)		l cysts within the matrix of the cornea				• Villi atrophy in the pyloric caeca				
• Focal congestion • Generalized moderate to marked epithelial hyperplasia with mild secondary laminar fusion • Presence of chlamydophila (blue bodies)	• Generalized epithelial thickening • Loss of goblet cells • Focal spongiosis of the epithelium and hyperplasia	• Corneal epithelial thickening • Mild macrophage infestation of the choroid plexus (increasing number of macrophages)	NSF	NSF	NSF	NSF	NSF	NSF	NE	NE
• Thickening of the epithelium • Bridging fusion of secondary lamellae	• Generalized thickness of the epithelium • Loss of goblet cells • spongiosis	• Marked congestion of the choroid plexus • Hyperplasia, hypertrophy of corneal epithelium • Moderate infiltration of macrophage in the	• Near liver, where we injected, there are multi-focal areas of granulomatous coelomitis (injection reaction)	NSF	NSF	NSF	NSF	NSF	NE	NE

		choroid plexus							
Bac+ MP									
• Epithelial proliferation • Bridging fusion of the epithelium • Presence of monogeneans • Congestion in the secondary lamellae	• Mild epithelial thickening • Loss of goblet cells	NSF	NSF, Focal granulomatous in the coelom	NSF	NSF	• Epithelial sloughing in the pyloric caeca • Mild infestation in the lamina propria	• Vascular congestion • Spongiosis of the light and grey matter	NSF	NE	NE
• Generalized spongiosis of the secondary lamellae • Areas of congestion in the secondary lamellae	NSF	• Thickening and hyperplasia of the corneal epithelium • Corneal laminal separation with haemorrhage • Areas of multi vacuolation • Generalized congestion in the choroid plexus with macrophage proliferation (reactive choroid plexus)	• Diffused mild hepatocellular vacuolation	NSF	• Depletion of zymogen granules in the acinar cell of the pancreas (atrophy in the acinar cell)	• Mild lymphocytic infiltration in the lamina propria of the intestine and pyloric caeca	• Multifocal vacuolation of the white matter in the optic lobe • Some vacuolation in the brain stem as well	NSF	NE	NE

• Generalized epithelial thickening • Congestion and fusion of the secondary lamellae	NSF	NSF	• Generalized vacuolation of the hepatocytes	NSF	NSF	NSF	• Multifocal areas of vacuolation in both grey and white matter of the brain	NSF	NE	NE
Bac+ LP										
• Marked epithelial thickening • Marked bridging and laminar fusion across most of the sample • Generalized population of monogeneans (unidentified) within the gill as well as moderate infestations of lymphocystis (Chlamydophila like organisms) (Blue bodies)	NSF	NSF	• Zonal atrophy of the hepatocytes. • Occasional pale staining MMC	NSF	NSF	NSF	NSF	• Spongy myocardium, otherwise NSF	NE	NE
• Congestion of the secondary lamellae vessels	NSF	NSF	NSF	NSF	NSF	NSF	• Mild focal vacuolation of grey and white matter of the brain	NSF	NE	NE
• Generalized fusion and focal congestion of the secondary lamellae • Presence of flukes	• Hyperplasia of the skin	• Proliferation & hyperplasia of the corneal epithelium • Vacuolation of the	NSF	NSF	• Depletion of zymogen of acinar cell of pancreas	NSF	• Multifocal areas of vacuolation in both grey and white matter	NSF	NE	NE

		corneal stroma							
HP									
• Mild, generalized epithelial hyperplasia • Occasional focal congestions	NSF	NSF	• Dense, normal (NSF)	NE	NSF	NSF	NSF	NE	• Marked congestion with depletion of lymphoid tissue • Mild numbers of pale staining MMC • Mild ellipsoid disruption (more of a reactive spleen) • No bacterial infestation	• Interstitial lymphoid depletion • Occasional MMC
• Congestion and mild proliferation of the epithelium • Focal fusion of the epithelium	NSF	NSF	• Dense and compact, otherwise NSF	NSF	NSF	NSF	• Some vacuolation in the grey matter of the optic lobe	NSF	NE	NE
• Congestion of the secondary lamellae, otherwise NSF	• Thickening of the skin • Loss of goblet cells	• Moderate macrophage infiltration, vessel degeneration (necrosis) in the choroid plexus	• Diffused mild hepatocellular vacuolation	NSF	NSF	NSF	NSF	NSF	NE	NE
MP										
• Proliferated gill • Generalized reduction in the goblet cells • Thickening of the base (indication of higher turnover	NE	• Thick corneal epithelium (hyperplasia) • Hypertrophy in the	• Moderately fatty liver • Vacuolation of the hepatocytes • Occasionally small,	NSF	NSF	• Mild to moderate infiltration of lymphocytes in	NSF	NSF	NE	NE

rate of the epithelium) • Spongiosis of the epithelium • Focal, random congestion of the secondary lamellae vessels		corneal membrane (indication of any kind of irritation in the fish) • Moderately congested choroid plexus.	lightly staining MMC throughout the parenchyma			the lamina propria of the intestine and pyloric caeca			
• Moderate infection of chlamydophila (blue bodies) • Focal bridging of secondary lamellae • Spongiosis (significant lesions)	• Generalized epithelial thickening • Loss of goblet cells • Focal spongiosis of the epithelium and hyperplasia	• Marked congestion of the choroid plexus • Moderate infiltration of the macrophages	• Focal fatty infiltration	NSF	NSF	NSF	• Marked vascular congestion of the brain • Focal vacuolation of the grey matter, particularly in the optic lobe • Congestion of the pituitary gland • Focal myocardial degeneration with proliferation of mural macrophages in the atrium	NSF	• Marked generalized congestion • Lymphoid depletion • Small numbers of brown to black MMC • Mild ellipsoid disruption	NE
• Congestion of the vessel in the secondary	NSF	• Markedly congestion in the	• Diffused mild hepatocell	NSF	NSF	NSF	• Multifocal vacuolati	NSF	NE	NE

lamellae		choroid plexus	ular vacuolation				on of the white matter in the brain			
LP										
• Congestion, spongiosis of the epithelium • Lymphoid infiltrations in the epithelium • Branchitis (inflammation of the gill)	NSF	• Marked congestion of the choroid plexus	NSF	NSF	NSF	NSF	NSF	• Spongy • Moderate proliferation of mural macrophages in the atrium	NE	NE
• Epithelial proliferation and hyperplasia of the secondary lamellae • Bridging fusion of the secondary lamellae • Infestation of chlamydophila (blue bodies) and flukes (unidentified)	• Focal thickening of the epithelium with loss of goblet cells	NSF	• Diffused mild hepatocellular vacuolation	NSF	• Low abdominal fat levels • Atrophy in the acinar cell • Low zymogen	NSF	• Multifocal vacuolation in the white matter of the brain	NSF	NE	NE
• Generalized epithelial proliferation in the gill • Bridging and generalized secondary lamellae fusion	NSF	• Congestion of the choroid plexus	• No vacuolation, NSF	NSF	NSF	NSF	NSF	NSF	• Generalized congestion, lymphoid depletion throughout the spleen • Splenic ellipsoid intact • Low number of brown staining MMC	NE

*MMC – Melanomacrophage centres; *NSF – No significant findings; *NE – Not examine

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