Universidade de Aveiro Departamento de Biologia
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Uso de bacteriófagos na inactivação de bactérias patogénicas num sistema de aquacultura

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Use of bacteriophages on the inactivation of pathogenic bacteria in aquaculture system

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Microbiologia, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar do Departamento de Biologia da Universidade de Aveiro.

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palavras-chave

Terapia fágica, bacteriófagos, bactérias patogénicas, comunidade bacteriana, aquacultura.

resumo

A importância crescente da aquacultura a nível mundial contribui para compensar a progressiva redução das populações naturais de peixe. Contudo, o facto de várias pisciculturas sofrerem, frequentemente, grandes perdas económicas, devido a infecções causadas por microrganismos patogénicos, incluindo bactérias multiresistentes, torna urgente o desenvolvimento de estratégias menos lesivas para o ambiente. A Terapia fágica surge como uma potencial e emergente alternativa ao uso de antibióticos e outros antimicrobianos. O principal objectivo deste trabalho consistiu na avaliação da eficácia da terapia fágica para inactivar bactérias patogénicas de peixes em pisciculturas de regime semi-intensivo, sendo que para isso foram efectuados diversos estudos prévios.

A dinâmica sazonal das comunidades virais e bacterianas foi seguida em amostras de água da piscicultura Corte das Freiras, tendo-se identificando as principais bactérias patogénicas e avaliado o nível de contaminação fecal. O número total de vírus foi determinado por microscopia de epifluorescência e a abundância relativa das principais bactérias patogénicas determinada por FISH (*Fluorescent in situ hibridization*). A dinâmica sazonal da comunidade bacteriana foi avaliada por 16S rDNA DGGE (*Denaturing Gradient Gel Electrophoresis*). Uma vez que *vibriosis* e *photobacteriosis* representam duas das principais causas de mortalidade nos peixes em pisciculturas, a diversidade da comunidade bacteriana do género *Vibrio* também foi avaliada por DGGE. O nível de contaminação fecal foi avaliado através da quantificação do teor de coliformes fecais e de enterococos fecais, usando o método das membranas de filtração. Para o estudo da cinética da interacção bactéria-bacteriófago usou-se a infecção cruzada. Para aplicar com sucesso a terapia fágica é importante ter informações sobre a sobrevivência dos fagos e o seu impacto ecológico na estrutura da comunidade bacteriana após adição dos fagos das principais bactérias patogénicas de peixe. Os resultados obtidos mostram que o número total de vírus na água da aquacultura é bastante elevado, variando entre 6.1 x 10^{9} particulas L^{-1} e 1.0 x10^{10} particulas L^{-1}. O número total de bactérias permanece praticamente constante, contudo os grupos específicos de bactérias variam significativamente ao longo dos períodos de amostragem. No caso dos indicadores bacterianos, foi observada uma clara variação sazonal, com os níveis mais elevados de poluição fecal registados em Outubro de 2007 e os mais baixos registados em Maio 2009. A análise de fragmentos de 16S rDNA por DGGE sugere que a estrutura da comunidade bacteriana varia sazonalmente, verificado-se uma maior diversidade na estação mais quente. No caso do género *Vibrio* a análise por DGGE sugere também uma variação sazonal na comunidade bacteriana pertencente a este género, ainda que não tão evidente. Os resultados de infecção cruzada sugerem que, com excepção do fago de *Aeromonas salmonicida* , os fagos inoculados nas principais bactérias patogénicas de peixe apresentam um largo espectro de infecção do hospedeiro. A sobrevivência dos fagos na água da aquacultura variou, sendo que o fago de *Aeromonas salmonicida* sobreviveu aproximadamente 3 meses, enquanto o fago de *Vibrio parahaemolyticus* sobreviveu cerca de 15 dias. A adição dos fagos à comunidade bacteriana não conduziu a uma variação significativa na estrutura da comunidade bacteriana. Em conclusão, como o teor de vírus e a sobrevivência dos fagos na água da aquacultura são elevados e a adição dos fagos específicos das bactérias patogénicos tem um baixo impacto ecológico na estrutura da comunidade bacteriana natural, a terapia fágica pode ser aplicada com sucesso na inactivação das bactérias patogénicas de peixes. Além disso, a infecção múltipla pelos fagos aumenta a capacidade de inactivação de bactérias patogénicas. No entanto, como a densidade e a estrutura das comunidades bacterianas totais e patogénicas varia sazonalmente, torna-se necessário ter este facto em consideração aquando da escolha dos fagos a utilizar para terapia fágica.

keywords

Phage therapy, bacteriophages,fish pathogenic bacteria, bacterial communities,aquaculture

abstract

Due to the increasing importance of aquaculture for the compensation of progressive worldwide reductions in natural fish stocks and to the fact that several fish farming plants often suffer from heavy financial losses due to the development of infections caused by microbial pathogens, including multidrug resistant bacteria, more environmentally-friendly strategies to control fish infections are urgently needed to make the aquaculture industry more sustainable. Phage therapy is an emerging and potential viable alternative to antibiotics and other antimicrobials.

The main target of this work was to evaluate the use of phage therapy to inactive fish pathogenic bacteria in aquaculture systems, thus, several preliminary studies were developed. In this work the seasonal dynamics of viral and bacterial communities of the aquaculture system Corte das Freiras was followed, the main pathogenic bacteria were identified and the level of faecal contamination in the aquaculture was evaluated. The total number of viruses was determined by epifluorescence microscopy and the relative abundance of specific bacterial groups of was accessed by FISH (Fluorescent In Situ Hybridization). The seasonal dynamics of bacterial community structure was evaluated using 16S rDNA DGGE (Denaturing Gradient Gel Electrophoresis). As vibriosis and photobacteriosis represent two of the main causes of fish mortality in fish farms, the diversity of the fish pathogens belonging to the *Vibrio* genus was also assessed by DGGE. The level of faecal contamination was evaluated through the quantification of faecal coliforms and faecal enterococci, using the membrane filtration method. The kinetics of pathogenic bacteria-phages interaction was also studied in laboratory, using cross infection. In order to apply phage therapy successfully, it is important to know the survival of the phage and the ecological impact of phage therapy in the diversity of bacterial communities after the additions of phages of the main fish pathogenic bacteria. The results obtained show that the number of viruses in the aquaculture water was high, varying between 6.1 x 10^9 cells L^{-1} and 1.0 x10^{10} cell L^{-1}. The number total bacteria was almost constant over the year, but the specific bacterial groups varied significantly during the sampling period.A clear seasonal variation in bacterial indicators was observed, with the highest values of faecal bacteria occurring in October 2007 and the lowest in May 2009. The 16S rDNA DGGE results showed that bacterial community structure varied seasonally, showing a higher diversity during the warm season. The diversity of the *Vibrio* genus also showed a seasonal variation, but not so clear. The cross infection results showed that, with the exception of *Aeromonas salmonicida* phage, the phages inoculated on the main fish pathogenic bacteria displayed a large spectrum of host infection. The survival of the phages was variable; the *Aeromonas salmonicida* phage survived in aquaculture water during approximatelly three months and the phage of *Vibrio parahaemolyticus* survived only during fifteen days. Phage addition to the bacterial community did not result in a significant variation in bacterial community structure. In conclusion, as the level of viruses and survival of these phages are high in water from the aquaculture system and the addition of specific phages of pathogenic bacteria has a low ecological impact on the structure of the natural bacterial community, suggests that phage therapy can be a successful approach to inactivate fish pathogenic bacteria. Moreover, the occurrence of multiple infections by these phages improve their potential to inactive fish pathogenic bacteria. However, as the density and structure of total and pathogenic bacterial communities varied seasonally, it is necessary to take in consideration this variation when specific phages are selected for phage therapy.

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List of Acronyms and Abbreviations

µL	Microliter
µM	Micromolar
h	hour
mL	Milliliter
dNTP	Deoxynucleoside triphosphates
DMSO	Dimethyl sulfoxide
DGGE	Denaturing gradient gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
FISH	Fluorescent in situ hybridization
UFC	Colonies forming units
PBS	Phosphate buffer system
EDTA	Ethylenediamine tetraacetic acid
ANOVA	Analysis of variance
BSA	Bovine serum albumin
M	Molar
PFU	Plaque forming units
DNA	Deoxyribonucleic Acid
TSA	Tryptone soy agar
TSB	Tryptic Soy Broth
Na_2EDTA	Ethylene diamine tetracetic acid disodium
rDNA	Recombinant Deoxyribonucleic Acid
W/v	Weight/volume

V	Volt
PCR	Polymerase Chain Reaction
L	Liter
DAPI	4,6□diamino□2□fenil indol
V/v	Volume/volume

Chapter 1 – Introduction

1.1 Bacteriophages

1.1.1. Discovery of bacteriophages

Since the discovery of phages by Frederick Twort in 1915 and independently in 1917 by Felix d' Herelle, phages have become a relevant research field. D' Herelle pioneered two important areas of phage research: he reported that bacteriophages have potential to kill bacteria that cause diseases in humans, plants or animals at early stages and developed phage therapeutic agents in the pre-antibiotic era (Sulakvelidze *et al*, 2001).

With the recent development of antibiotic resistance within the microbial population, the need for new antibacterial and alternative strategies to control microbial infections has become an urgent necessity, leaving way for phage therapy to reborn as a valid option for treating bacterial infections. Lytic phage kill bacteria via mechanisms that differ from those of antibiotics, and therefore, can be considered as antibacterials with "novel mode of action", a concept desired for all new antibacterial agents (Sulakvelidze *et al*, 2001).

1.1.2. Properties of bacteriophages

Based on the new viral classification system proposed by Raoult and Forterre (2001), a prokaryotic virus can be defined as a capsid-encoding organism that is composed by proteins and nucleic acids, self-assembles in a nucleocapsid that uses a ribosome-encoding prokaryotic organism for the completion of its life cycle (Raoult *et al*, 2008). The bacteriophages (phages) present a variety of different morphological types, but the majority displays a capsid, collar and tail (Figure 1.1). According to the latest phage counts, tailed phages comprise at least 4950 viruses (Ackermann, 2000), polyhedral, filamentous and pleomorphic phages comprise only about 190 viruses (Ackermann, 2003). The head (or capsid) is a protein shell often in the shape of an icosahedron; it contains the viral genome that usually comprises a double-strand (ds) DNA molecule (Ackermann, 2003; Hanlon, 2007). Though most phages contain dsDNA, there are small phage groups with ssDNA, ssRNA, or dsRNA (Hanlon, 2007). The tail may have a contractile structure to which six tail fibres are usually connected. At the tips of the fibres, the presence of receptors allows the recognition of attachment sites on the bacterial cell surface. In phages that do not display tail or tail fibres, other attachment mechanisms are present (Hanlon, 2003).

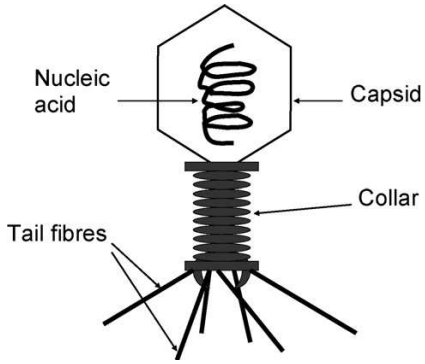

Figure 1.1: Diagrammatic representation of a typical bacteriophage (extracted from Hanlon, 2003).

1.1.3. Classification of bacteriophages

Bacteriophage taxonomy is based on their shape and size as well as on their nucleic acid (figure 1.2) (Ackermann, 2003). The ICTV or International Committee for Taxonomy of Viruses presently classifies viruses into three orders, 61 families, and 241 genera (M.H.V, 2000). Bacteriophages constitute one order, 13 families and 30 genera (Table 1.1).

Table 1.1: Basic properties of bacteriophages and their classification (Ackermann, 2003)

Shape	Nucleic acid	Order and families	Genera	Examples	Characteristics
Tailed	DNA, ds, L	Caudovirales	15		
		Myoviridae	6	T4	Tail contractile
		Siphoviridae	6	λ	Tail long, noncontractile
		Podoviridae	3	T7	Tail short
Polyhedral	DNA, ss, C	Microviridae	4	φX174	
	ds, C, T	Corticoviridae	1	PM2	Complex capsid, lipids
	ds, L	Tectiviridae	1	PRD1	Internal lipoprotein vescicle
	RNA, ss, L	Leviviridae	2	MS2	
	ds, L, S	Cystoviridae	1	φ6	Envelope, lipids
Filamentous	DNA, ss, C	Inoviridae	2	fd	Filaments of rods
	ds, L	Lipothrixviridae	1	TTV1	Envelope, lipids
	ds, L	Rudiviridae	1	SIRV1	Resembles TMV
Pleomorphic	DNA, ss, C, T	Plasmaviridae	1	L2	Envelope, lipids, no capsid
	ds, C, T	Fuselloviridae	1	SSV1	Spindle, lipids, no capsid

Legend: C, circular; L, linear; S, segmented; T, superhelical; 1, single-stranded; 2, double-stranded.

As for the other viruses, phages families are chiefly defined by nature of nucleic acid and particle morphology. There are no universal criteria for genera and species. The ICTV uses every available property for classification and has adopted the "polythetic species concept", meaning that a species is defined by a set of properties, some of which may be absent in a given member (M.H.V, 1990). Taxonomic names of orders, families, and genera are typically

constructed from Latin or Greek roots and end in *-virales*, *-viridae*, and *-virus*, respectively. Most icosahedric, filamentous, and pleomorphic phages have latinized names. So far, tailed phage genera have only vernacular names (e.g., "T4-like viruses").

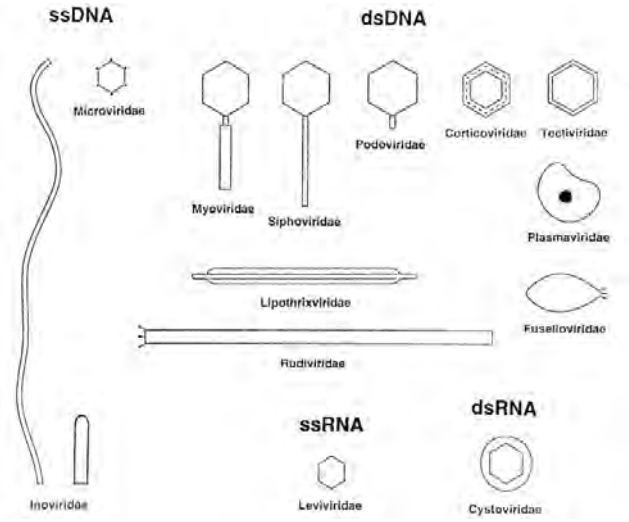

Figure 1.2 - Schematic representation of major phage groups (extracted from Ackermann, 2003).

1.1.4. The Phage Life Cycle

Viruses can interact with their hosts in two major and distinctive ways, the lytic and lysogenic cycles of infection and more sporadically through pseudolysogeny (Figure 1.3) (Almeida *et al*, 2009). During the life cycle of a prokaryotic virus several phases common to all viruses can be identified: adsorption, separation of nucleic acids from protein coat, expression and replication of the nucleic acids, virion assembly, release and transmission (Duckworth, 1987).

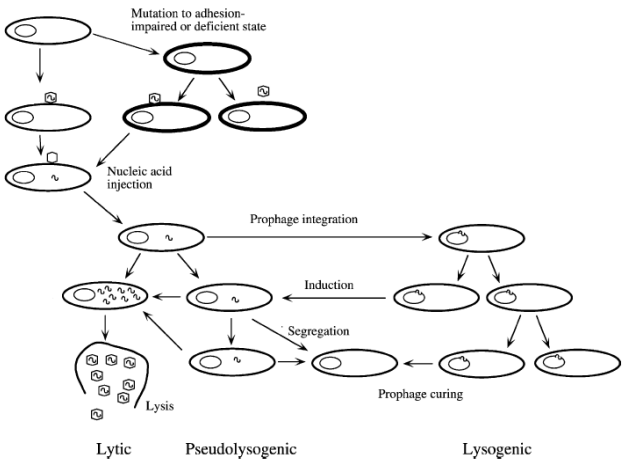

Figure 1.3: General phage life cycle. The model is adopted from Weinbauer (2004)

In the life cycle of a typical lytic bacteriophage. the virus encounters its bacterial host during random motion and attaches via specific receptor sites (Figure 1.4) that may be any one of a wide variety of cell surface components, including protein, oligosaccharide, teichoic acid, peptidoglycan and lipopolysaccharide (Lenski, 1988; Hanlon, 2007). In some cases the attachment sites might be present on the cell capsule, flagella or even conjugative pili (Hanlon, 2007). At start, the attachment is reversible but then becomes irreversible, being followed by transfer of phage genetic material into the host cell. Depending on the morphology of the virus, the phage genome can be injected into the bacterial cell using different mechanisms that often involve contraction of the tail and formation of a hole within the bacterial cell wall. The base of the phage DNA is chemically modified in order to protect itself from attacks by cellular restriction and nuclease enzymes. The viral genome is then transcribed by the host cell RNA polymerase, producing early mRNA that has the effect of taking over the metabolic machinery of the bacterium, redirecting its metabolic processes to the manufacture of new virus components. These components are then assembled into complete virions (Hanlon, 2007).

After construction and assembly of new phage particles within the host cell, it remains the problem of their release in the environment. Almost all dsDNA phages develop enzymes that attack the bacterial peptidoglycan, such as lysozymes that target sugar bonds, endopeptidases that target peptide linkages or amidases that act on amide bonds (Fischetti, 2005). These lytic enzymes, usually termed muralytic enzymes or endolysins, are produced within the cytoplasm but require another enzyme to cross the cytoplasmic membrane before reaching their substrate. This enzyme is called a holin and it disrupts the membrane allowing the lysin to degrade the peptidoglycan (Young *et al*, 2000; Fischetti, 2005). Thus, the timing of cell lysis and release of phage progeny is controlled by the holin. Depending on their morphology, some phages are capable of escaping from the host cell without causing destruction of the host. These phages usually present a filamentous morphology and have no relevance in phage therapy. Between the adsorption phase and the lysing of the host cell and viral progeny release there is a period of time known as the latent period.

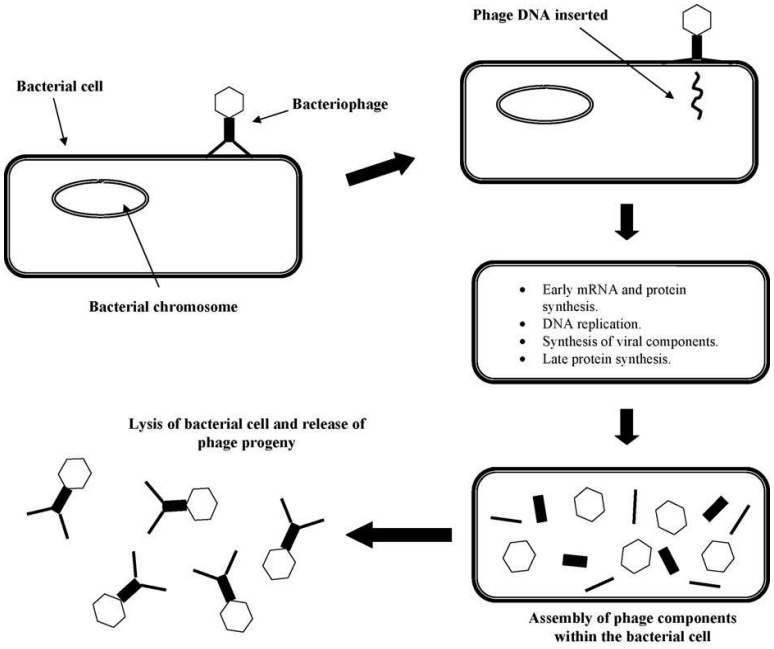

Figure 1.4: Lytic cycle of a bacteriophage (Hanlon, 2007).

The lytic cycle is sometimes overcome, with phages integrating their DNA into the host cell DNA. The phages that display this behavior are called temperate phages and the bacterial cells are then termed lysogenic. While bacterial DNA replicates, the phage DNA replicates simultaneously, thus each daughter cell will contain the viral DNA (known as prophage). Cells may undergo several rounds of division but, occasionally, spontaneous lysis occurs, and progeny phage is released. External factors can also induce lysis of lysogenic cells such as treatment with mutagenic agents or exposure to ultraviolet light (Hanlon, 2007). Some temperate phages such as Mu can switch between lysogeny and lytic growth due to variation on of high temperature and stationary phase (Ranquet *et al*, 2005). The prophage directs the synthesis of a repressor protein that blocks the transcription of its own genes and also those of closely related bacteriophages (Hanlon, 2007). The presence of a prophage can therefore confer upon a bacterial cell some sort of immunity to infection by other phages. Lysogenic bacteria may possess other advantages in terms of the acquisition of genes conferring pathogenicity or increased virulence.

When a prophage escapes regulation by the repressor, its DNA is cut free allowing it to engage in the lytic cycle. However, excision of prophage DNA is often imprecise and bacterial genes adjacent to the prophage DNA may be incorporated into the infectious phage DNA and then transferred to subsequent host cells. This process is known as transduction and is responsible for horizontal gene transfer, including those related to antibiotic resistance, from one bacterial cell to another (Hanlon, 2007).

The predominance of temperate phages on the marine environment is not clear, but Freifelder (1983) reported that more than 90% of known bacteriophages were temperate (Freifelder, 1983), while other authors (Ackermann, 1987; Jiang *et al*, 1994, Cochran *et al*, 1998) suggested that only around 50% of bacterial strains contained inducible prophages. Temperate phages are not suitable candidates for phage therapy since they may not immediately destroy bacteria.

Pseudolysogeny, also known as false lysogeny is described as a phenomenon where there is a constant production of phage in the presence of high host cell abundance (Ackermann, 1987). In this situation the phage lysis does not result in total host death and the abundance of phage remains in a state where he coexists with exponential host growth. Thus, in pseudolysogenic infection, bacteriophage can either proceed with lytic infection or enter a dormant intracellular phase (Wommack *et al*, 2000), in which case the phage genome does not integrate into host cellular replicons. Under these conditions, host cells do not provide enough energy in order for phages to enter in a true lysogenic or lytic condition.

1.1.5. Bacteriophages in the marine environment

The world of bacteriophages is currently in a period of renaissance due to new capabilities in metagenomic sequencing and due to the isolation of diverse novel virus-host systems. The reawakening of interest in prokaryotic viruses began in the mid 1990s as a consequence of their extraordinary abundance, namely in the marine environment and of the unchallenged acceptance of the fact that viruses represent the greatest pool of genetic diversity in the planet (Angly *et al*, 2006; Culley *et al*, 2006; Allen *et al*, 2008) Viruses are the simplest life forms in aquatic systems, yet they play a crucial role in the regulation aquatic processes. Their enormous abundance (10^{10} -10^{11} particles L^{-1}) (Almeida *et al*, 2001; Weinbauer, 2004; Suttle, 2007) and seemingly infinite diversity provides the vital clues to the true function of viruses.

Most of marine viruses are bacteriophages that kill bacteria (Weinbauer, 2004) playing a significant role on the bacterial communities (Suttle, 2007).

Viral lysis in aquatic systems removes 20-40 % of the bacterial standing stocks each day (Suttle, 1994) and can be as effective as grazing by protist to destroy bacteria (Almeida *et al*, 2001; Weinbauer, 2004; Suttle, 2005). Consequently, viral lysis has an importante role on the biogeochemical cycles (Bergh *et al*, 1989; Fuhrman, 1999) .Moreover, as virus are host specific, they influence bacterial composition of aquatic communities (Almeida *et al*, 2001)

Bacteriophages in the marine environment contain dsDNA genome (Weinbauer, 2004) and belong mainly to the Caudoviridales order (about 96%) and to the families Myoviridae, Siphoviridae and Podoviridae (Yoshida *et al*, 2006). The metagenomic analysis confirmed the dominance of dsDNA tailed phages in marine viral communities (Breitbart *et al*, 2002; Breitbart

et al, 2003; Breitbart *et al*, 2004). The same study also showed that a large number of sequences (6% of the total) corresponded to ssDNA phages belonging to Microviridae family (Angly *et al*, 2006), a previously overlooked group, due to exclusion of ssDNA viruses by amplification and cloning procedures. RNA phages are also present in the marine environment (Alcântara *et al*, 1995; Dore *et al*, 2000; Griffin *et al*, 2000) but in a recent metagenomic analysis of coastal waters no RNA phages were detected (Culley *et al*, 2006). However, the marine environment has been shown to be a reservoir of previously unknown RNA viruses, with 98% of RNA viruses belonging to positive-sense ssRNA viruses (Culley *et al*,2006) and the predominant hosts of marine RNA viruses being eukaryotes (Almeida *et al*.,2009).

1.2 Aquaculture

Aquaculture of aquatic animals and plants culture (Pillay *et al*, 2005). Usually, aquacultures are located in offshore areas where is salmon and trout are usually grown or in coastal or inland waters where a variety of fish such as catfish, yellowtail, tilapia and seabream are raised. Aquaculture system that are fully enclosed or have recirculating mechanisms have the advantage of being land based and not having to be in close proximity to the sea, avoiding infections caused by wasteborn diseases (Sapkota *et al*, 2008).

Aquaculture provides nearly one-third of the world's seafood supplies and is one of the fastest growing agricultural sector (Almeida *et al*, 2009). The high increase on aquaculture production makes this sector the sector even more attractive. The annual production has tripled during the last 15 years, from 16.8 million tons in 1990 to 52.9 million tons in 2005 (Figure 1.5) (FAO and Fishery Information Data and Statistics Unit, 2005). This behavior is predicted to continue, with total annual global fish production (wild and farmed) expected to increase steadily, from 129 million tons in 2000 to 172 million tons by 2015, accounting aquaculture for as much as 73% of the total increase (Sapkota *et al*, 2008)

A potential problem to this expansion is that aquacultures often suffer from heavy financial losses (Flegel *et al*, 2006; Saksida *et al*, 2006), due to the development of infections caused by microbial pathogens, including multidrug resistant bacteria that are easily transmitted through water and therefore able to infect a great variety of fish species. Thus, new approaches, as phages therapy, to reduce the impact of infections are welcome.

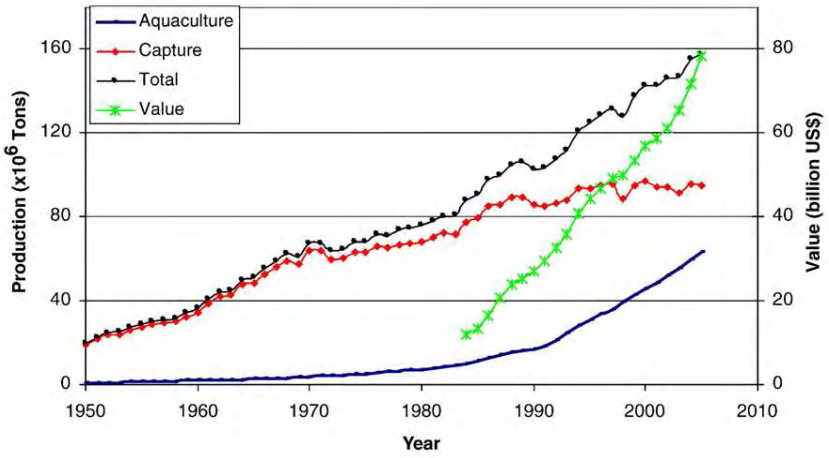

Figure 1.5 - Global trends in annual seafood production, 1950–2003 (FAO, 2005).

1.2.1. Fish Farming Diseases

Although there are many biological agents like bacteria, viruses, protists and helminths, oomycetes and fungi that threaten cultured fishes, bacterial diseases represent the main problem in the expansion of aquaculture industry (Alderma *et al*, 1996; Shao, 2001; Wahli *et al*, 2002).

Two broad groups of bacteria of public health significance infect culture fish: those naturally present in the environment - the indigenous microflora (e.g., *Photobacterium damselae, Vibrio anguillarum, V. vulnificus, Aeromonas hydrophila, Aeromonas. salmonicida*) and those introduced through environmental contamination by domestic animals and/or human wastes – non-indigenous microflora (e.g., Enterobactereaceae such as *Salmonella* sp. and *Escherichia coli*) (Muroga *et al*, 1987; Huss, 1994; Fukuda *et al*, 1996; Iida *et al*, 1997, Nakai *et al*, 1999; Nakai *et al*, 2002). Although a great number of pathogenic bacteria die in environmental water, a high number of these remain on the skin and in the guts of fish and can cause a health risk to consumers. Vibriosis and photobacteriosis (formerly pasteurellosis) are the main diseases of marine and estuarine fish, both in natural and commercial production systems throughout the world, occurring only occasionally in freshwater fish. These diseases can cause significant mortality in fish that might ultimately lead to total death in infected facilities, becoming responsible for the most outbreaks of fish farming plants. The vibriosis and photobacteriosis are caused by bacteria of the same family, the Vibrionaceae. Vibrosis is caused by species of *Photobacterium* (namely *Photobacterium damselae* subsp. *damselae*, formerly *Vibrio damselae*) and *Vibrio* (namely *V. anguillarum, V. vulnificus, V. alfinolyticus, V. parahaemolyticus* and *V. salmonicida*) ewhile photobacteriosis is caused by *P. damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*), an highly pathogenic bacteria that does not seem to have host specificity, infecting potentially an ample range of fish species (Toranzo *et al*, 1991;. Noya *et al*,1995) This pathogen was responsible for high mortality rates in cultured populations of seabass (*Dicentrarchus labrax*) and seabream (*Sparus aurata*) on several countries from the

Mediterranean area such as France, Italy, Spain, Greece, Portugal, Turkey, Malta, Israel and Croatia (Thyssen *et al*, 2001). Nowadays, photobacteriosis continues to be a severe problem in intensive cultures of different fish species in the Mediterranean area and in Japan. Vibrionaceae species are also known to cause disease in humans, most often following the consumption of contaminated aquaculture products. Other bacteria as *Rickettria* (Fryer *et al*, 1996), *Aeromonas salmonicida* (Bernoth *et al*, 1997), *Edwardsiella tarda* (Nakatsugawa *et al*, 1983; Mekuchi *et al*, 1995), *Cytophaga marina* (formerly *Flexibacter maritimus*) (Kusuda *et al*, 1982; Wakabayashi *et al*, 1984), *Flexibacter* sp (Hamaguchi *et al*, 1993) , *Flavobacterium psychrophilum* and *Pseudomonas plecoglossicida* are also important fish pathogens, some of them capable of infecting a wide range of fish (Park *et al*, 2000).

Viruses infection also cause heavy losses in aquaculture industry (Munn, 2006; Saksida *et al*, 2006), but the risk of human infection due to fish consumption is low because viruses causing fish disease are not pathogenic to humans (Almeida *et al*, 2009). Viruses infecting commercial fish have been intensively studied and have been shown to encompass a wide range of viral families, including Irodovirus, Rhabdovirus, Birnavirus, Nodavirus, Reovirus, and Herpesviruses (Muroga, 2001; Suttle, 2007). An important fact is that some of these viruses present broad host ranges and seem to circulate between marine and freshwaters, making the transmission of the virus to new areas a serious threat (Meyers *et al*, 1999; Skall *et al*, 2005).

A few helminth species also represent a concern in the aquaculture industry (WHO, 1995) causing diseases as trematodiasis, cestodiasis and nematodiasis, but also create conditions for bacterial infections to start (WHO, 1995). Infections by aquatic Oomycetes are considered the second cause of infection, after bacteria, namely in freshwater systems (Ogbonna *et al*, 1991; Gieseker *et al*, 2006).

Farmed fish live in a potential unfavorable environment, which can explain the high incidence of diseases in aquaculture plants. Relatively to wild ones, the great density they live in can enhance the transference of pathogens between individuals (Almeida *et al*, 2009). Other factors such as overfeeding, high temperature and fast growth to cultivate fish as soon as possible in fish farming plants also contribute to higher disease rates (Almeida *et al*, 2009). The presence of sick, moribund and dead fish in closed waters also increases the risk of pathogens transference because they are only removed with higher water renewal rates (Weiss, 2002).

1.2.2 Preventive Measures in Fish Farming Plants

Preventing the occurrence of aquaculture diseases is the ideal way and the most cost-effective measure in aquaculture. The success of prevention is always dependent on diverse variables, specially the fish farming conditions and feed quality that can lead to the need of using effective biocontrol techniques to reduce infections (Almeida *et al*, 2009). Other factors

can also accentuate the problem, includinglow microbiological quality of aquaculture waters (high levels of faecal indicators of water quality) (Huss ,1994 Howgate *et al*, 1997;Almeida *et al*,2009); low stability of environmental conditions (e.g., increased temperatures, salinity changes, oxygen depletion, high organic loads) that can lead to the expansion of the disease, often by weakening the innate defense systems of the fish (Defoirdt *et al*, 2007); high densities of fish stocks, which can induce disease transference, decreasing the resistance to infection (Defoirdt *et al*, 2006); the different stages of the fish life cycle are susceptible to infections (Alderman,1999); increasing problems with resistance against antibiotics in common pathogenic bacteria and the concern about spreading of antibiotics in the environment (Defoirdt *et al*, 2006); low efficiency of chemotherapeutic agents that are effective against bacteria and oomycetes against endospores and zoospores (Alderman,1999); and the fact that few drugs are licensed for fisheries use (Kusuda *et al*, 1998; Muroga, 2001).

Vaccination

Following the idea of prevention, vaccination would be the ideal method to prevent infectious diseases, but commercially available vaccines are still very limited in the aquaculture field (Reed *et al*, 1996; Romalde *et al*, 2002; Arijo *et al*, 2005).

Vibrosis and pasteurelosis have been already controlled to a great extent through the use of vaccines (Press *et al*, 1995; Reed *et al*, 1996; Romalde *et al*, 2002; Arijo *et al*, 2005). A vaccine against the *Vibrio spp*. has proven to be effective especially when administered by injection in European Salmonid aquaculture. *Photobacterium damsela piscicida* was controlled with a survival rate of 75% when applying a toxoid-enriched whole-cell vaccine to fish with 0.5-2.0 g. Another successful attempt was the prophylactic immunization for other bacterial diseases of farmed fish, namely against *Aeromonas salmonicida* and *Yersinia ruckeri* (Press *et al*, 1995). However, there are still no commercially available vaccines against two other important bacterial fish diseases: bacterial kidney disease and rickettsial septicaemia. The nature of the immunity acquired by fish through vaccination has not yet been clarified, namely in the cure of fish at larval age (Reed *et al*, 1996). Other problem is the impossibility of vaccinating fish larvae that are the most susceptible to infection, due to their small size and to the fact that they do not develop specific immunity (Vadstein, 1997).

The development of vaccines for fish viral diseases is even more difficult than for bacterial diseases. The unsuccess is due to killed virus residual virulence in the target species, virulence for other fish species, persistence in the treated fish and the fear that the virus might revert to virulence (Almeida *et al*, 2009).

Similar to what occurs with bacteria, the difficulty in developing anti-viral vaccines is due to the fact that these diseases occur primarily at the fish fry age and it is difficult to inject these small animals (Almeida *et al*, 2009). Until now, only a recombinant DNA based vaccine for infectious pancreatic necrosis virus was approved (Christie *et al*, 1997).

Chemotherapy

Although the chemotherapy has been shown to be a rapid and effective method to treat or prevent infections, the more frequently used chemotherapeutic agents are often responsible for the development of drug resistant microbial strains (Almeida *et al*, 2009).

The recursive and massive use of antibiotics in aquaculture systems, in order to prevent the spread of disease, has contributed to the development of resistant strains on bacteria. As a consequence, antibiotics are frequently not effective in disease treatments. A possible reason might be the fast replication of microorganisms that can induce quick mutations that become predominant on the microbial population, helping the microbe to survive even in the presence of antibiotics (Almeida *et al*, 2009). In fact, in marine environment more than 90% of bacterial strains are resistant to more than one antibiotic and 20% are resistant to five, at least (Martinez, 2003).

Besides antibiotic treatment can became ineffective, the excessive use of this kind of treatment can become a serial threat to human and environment health (Alderman, 1996; Nakai *et al*, 1999). It has been showed that antibiotic resistances that have emerged and/or evolved in the aquaculture environments can be transmitted by horizontal gene transfer to bacteria of the terrestrial environment, including animal and human pathogens. This fact can bring alterations to the normal human gut microflora and generate problems like allergy and toxicity (Cabello, 2006).

This massive use of antibiotics in the aquatic field, especially in fish farms has been detected around the world (Almeida *et al*, 2009). Holmstrom *et al* (2003) reported that in 76 shrimp farmers interviewed in Thailand, 56 used antibiotics.

In Malasia, a study on retail fish showed that *Aeromonas* sp. isolated from fish were resistant to three or more antibiotics (Radu *et al*, 2003). In the Philippines, oxytetracycline, oxolinic acid, chloramphenicol, furazolidine, nitrofurans, erythromycin and sulfa drugs are commonly used to treat bacterial diseases (Nakai *et al*, 2002). Chemotherapy is also widely practiced in South America and Europe where only a limited number of legislated antibiotics are allowed, such as amoxacillin, ampicillin, chloramphenicol, erythromycin, florfenicol, flumequine, oxolinic acid, oxytetracycline, nitrofurazone, sulphadiazine-trimethoprim and tetracycline (Toranzo *et al*, 1991; Bakopoulos *et al*, 1995; Sano, 1998). The small number of legislated antibiotics allowed is explained by the emergence of antibiotic resistant pathogens that have resulted in banning or

restriction of some kinds of antibiotics. This situation implies the development of alternative approaches to antibiotics to control bacterial pathogens.

Other biocides such as malachite green and formaldehyde are also extensively used in aquaculture industry to treat protozoal and fungal infections. Malachite green is a triarylmethane dye that is highly effective against important protozoal and fungal infections (Hoffman *et al*,1974; Alderman,1985; Schnick, 1988), but it is well known that malachite green is environmentally persistent and produces a wide range of acute toxic effects on various fish species and certain mammals (Srivastava *et al*, 2004) The risks that this biocide brings to the consumers of treated fish (Alderman *et al*,1993) are enormous and includes effects on the immune, respiratory and reproductive systems due to its genotoxic, carcinogenic and mutagenic properties (Srivastava *et al*, 2004). Despite the use of this dye has been banned in 2002 in several countries, but is still being used in countries with lack of legislation (Almeida *et al*, 2009).

Other biocide used as disinfectant for prophylaxis of fish eggs and in first stages of larval development of fish is formaldehyde (Gieseker *et al*, 2006; Khodabande *et al*, 2006). Some successful studies were made using sodium chloride, formalin and iodine on carp eggs, leading to smaller mortality in infected adult fish (Gieseker *et al*, 2006; Khodabande *et al*, 2006). The main problem of this compound is the significant environmental impact and the carcinogenic risk to mammals, causing harmful effects on human health (Gieseker *et al*, 2006). Like formaldehyde and other antibiotics, its use is discouraged, limited or even banned in several countries (Magaraggia *et al*, 2006).

In this way, to reduce the risk of development and spreading of microbial resistances and to control fish diseases in aquaculture, alternative strategies must be developed to allow the use of reasonably cost effective and more environmentally friendly methods.

1.3. Phage Therapy: an Alternative to Antibiotics

As bacterial diseases are the major problem in the expanding aquaculture, phage therapy represents a potentially viable alternative to antibiotics and to other antibacterial compounds to inactivate indigenous and non-indigenous pathogenic bacteria in fish farming plants (Almeida *et al*, 2009).

1.3.1 Advantages of Phage Therapy over Chemotherapy

When comparing phage therapy to chemotherapy, there are a great number of advantages on using phage therapy instead of chemotherapeutic agents on the environment. (1) Phages have

specific target, that is, they are specific to a single species or even strain of bacteria, causing much less damage to the normal intestinal fish flora and to natural non-target bacteria. (2) The use of phages limits the development of resistance on bacteria, especially due to their higher mutation and replication rate when compared to antibiotics. The fact that phage co-evolves with their host bacteria also makes the discovery of new phages easier than developing new antibiotics Thus, even if bacteria acquire phage resistance, new mutant phage that acts lytically can be used against such bacteria (Matsuzaki *et al*, 2003). Other possibility to avoid the emergence of resistance strains during phage treatment is to prepare a mixture of different strains of phages. (3) Limited impact, unlike antibiotics, phages are self-replicating as well as self-limiting. They replicate exponentially as bacteria and decline when bacteria number decreases. Depending of the form of application, a single dose may be sufficient. (4) Regulatory approval, since phages are naturally occurring and very abundant, there may be substantially fewer problems involved in obtained regulatory approval for their use. (5) Environmental resistance; phages have contact with its pathogens on its natural arrangement, being permanently in contact with their hosts. (6) Efficient technology; the use of new therapies based on phages lead to a more flexible, fast and inexpensive technology than other that use antibiotics.

1.3.2. Phage Therapy and Its Clinical Applications

Before the development and massive use of antibiotics, it was suggested that bacterial infections could be prevented and/or be dealt with the administration of phages. At the beginning of 20th century, some studies involving human beings and animals were made, culminating in 1940 with the development of phage preparations by the Eli Lilly Company, in order to treat illnesses caused for example by *Staphylococcus*, *Streptococcus*, *Escherichia* and other pathogenic bacteria (Sulakvelidze *et al*,2001). But just after phage discovery, d'Herelle used phages to treat dysentery, in what was probably the first attempt to use bacteriophages therapeutically (Sulakvelidze *et al*, 2001). In this particular case, the phage preparation was ingested by d'Herelle and other hospital interns to test its effects before administering it in the next day to a 12-year-old boy with severe dysentery (Sulakvelidze *et al*, 2001). This first therapeutic use of bacteriophages ended successfully with the boy recovering in a few days after a single administration of d'Herelle's anti-dysentery phage. This preparation continued to be efficiently applied to patients.

Despite these practical studies, the first reported and published application of phages to treat infectious diseases on humans came in 1921 from Richard Bruynoghe and Joseph Maisin (Bruynoghe *et al*, 1921). In this particular case, bacteriophages were used to treat staphylococcal skin disease, being applied into and around surgically opened lesions, with the

infection ceasing within 24 to 48 hours. With other promising studies of phage therapy applied to medicine (Rice, 1930; Schless, 1932; Stout, 1933), and the continuous research by d'Herelle, that treated thousands of people having cholera and/or bubonic plague in India (Summers, 1999), several companies began active commercial production of phages against various bacterial pathogens.

The majority of studies demonstrating the efficacy of phage therapy in clinical settings came from research groups in Eastern Europe and the former Soviet Union and were published in non-English journals (Sulakvelidze *et al*, 2001). Some of the reported work is decribed in Table 1.2, where phages were applied on different kind of infections including the treatment of cerebrospinal meningitis in a newborn (Stroj *et al*, 1999), skin infections caused by *Pseudomonas*, *Staphylococcus*, *Klebsiella*, *Proteus*, and *E. coli* (Cislo *et al*, 1987), recurrent subphrenic and subhepatic abscesses (Kwarcinski *et al*, 1994).

More recently, studies with mice and farm animals by Smith *et al.* (1977) showed that phages could be used for both treatment and prophylaxis against bacterial infection (Smith *et al*, 1997). The success of these studies led to an increased interest of phage therapy applications, especially in the West where researchers have been studying the effect of phages on antibiotic-resistant bacteria capable of causing human infections. Other experiences with mice and guinea pigs reported the utility of phages in preventing and treating experimental disease caused by *Pseudomonas aeruginosa* and *Acinetobacter infections.* Soothill (1992, 1994) demonstrating no histological changes after administration of phage preparation. Infections caused by *Klebsiella ozaenae, Klebsiella rhinoscleromatis scleromatis* and *Klebsiella pneumonia* have also been successfully treated using bacteriophages (Soothill, 1992; Soothill, 1994).

Phase I/II clinical trials with human patients are currently being conducted and by 2011 it can reasonably be envisioned that phage treatments would be into phase III or in clinical use (Comeau *et al*, 2008).

Table 1.2: Clinical applications of Phage Therapy in Poland and Soviet Union. (Sulakvelidze *et al*, 2001)

References	Infections	Etiologic agents	Comments
Babalova *et al*.	Bacterial dysentery	*Shigella*	*Shigella* phages were successfully used for prophylaxis of bacterial dysentery.
Bogovazova *et al* (1968)	Infections of skin and nasal mucosa	*K. ozaenae, K. hinoscleromatis, and K. pneumoniae*	Adapted phages were reported to be effective in treating *Klebsiella* infections in all of the 109 patients.
Cislo *et al*. (1992)	Suppurative skin infections	*Pseudomonas, Staphylococcus, Klebsiella, Proteus, and E. coli*	Thirty-one patients having chronically infected skin ulcers were treated orally and locally with phages. The success rate was 74%.
Ioseliani *et al*. (1987)	Lung and pleural infections	*Staphylococcus, Streptococcus, E. coli, and Proteus*	Phages were successfully used together with antibiotics to treat lung and pleural infections in 45 patients.
Kochetkova *et al*.(1980)	Postoperative wound infections in cancer patients	*Staphylococcus and Pseudomonas*	A total of 131 cancer patients having postsurgical wound infections participated in the study. Of these, 65 patients received phages and the rest received antibiotics. Phage treatment was successful in 82% of the cases, and antibiotic treatment was successful in 61% of the cases.
Kucharewicz-Krukowska and Slopek (1989)	Various infections	*Staphylococcus, Klebsiella, E. coli, Pseudomonas, and Proteus*	Immunogenicity of therapeutic phages was analyzed in 57 patients. The authors concluded that the phages immunogenicity did not impede therapy.
Kwarcinski *et al*.(1994)	Recurrent subphrenic abscess	*E. coli*	Recurrent subphrenic abscess (after stomach resection) caused by an antibiotic-resistant strain of *E. coli* was successfully treated with phages.
Litvinova *et al*. (1978)	Intestinal dysbacteriosis	*E. coli and Proteus*	Phages were successfully used together with bifidobacteria to treat antibiotic-associated dysbacteriosis in 500 low-birth-weight infants.
Meladze *et al*. (1982)	Lung and pleural infections	*Staphylococcus*	Phages were used to treat 223 patients having lung and pleural infections, and the results were compared to 117 cases where antibiotics were used. Full recovery was observed in 82% of the patients in the phage-treated group, as opposed to 64% of the patients in the antibiotic- treated group.
Miliutina and Vorotyntseva (1993)	Bacterial dysentery and salmonellosis	*Shigella and Salmonella*	The effectiveness of treating salmonellosis using phages and a combination of phages and antibiotics was examined. The combination of phages and antibiotics was reported to be effective in treating cases where antibiotics alone were ineffective.
Perepanova *et al*.(1995)	Inflammatory urologic diseases	*Staphylococcus, E. coli, and Proteus*	Adapted phages were used to treat acute and chronic urogenital inflammation in 46 patients. The efficacy of phage treatment was 92% (marked clinical improvements) and 84% bacteriological clearance)
Sakandelidze and Meipariani (1974)	Peritonitis, osteomyelitis, lung abscesses, and postsurgical wound infections	*Staphylococcus, Streptococcus, and Proteus*	Phages administered subcutaneously or through surgical drains in 236 patients having antibiotic-resistant infections eliminated the infections in 92% of the patients.
Sakandelidze (1991)	Infectious allergoses (rhinitis, pharyngitis, dermatitis, and conjunctivitis)	*Staphylococcus, Streptococcus, E. coli, Proteus, enterococci, and P. aeruginosa*	A total of 1,380 patients having infectious allergoses were treated with phages (360 patients), antibiotics (404 patients), or a combination of phages and antibiotics (576 patients). Clinical improvement was observed in 86, 48 and 83% of the cases, respectively.
Slopek *et al*.(1983)	Gastrointestinal tract, skin, head, and neck infections	*Staphylococcus, Pseudomonas, E. coli, Klebsiella, and Salmonella*	A total of 550 patients were treated with phages. The overall success rate of phage treatment was 92%.
Stroj *et al*. (1999)	Cerebrospinal meningitis	*K. pneumoniae*	Orally administered phages were used successfully to treat meningitis in a newborn (after antibiotic therapy failed).
Tolkacheva *et al*. (1981)	Bacterial dysentery	*E. coli and Proteus*	Phages were used together with bifidobacteria to treat bacterial dysentery in 59 immunosuppressed leukemia patients. The superiority of treatment with phage-bifidobacteria over antibiotics was reported.
Weber-Dabrowska *et al* (1987).	Suppurative infections	*Suppurative infections*	Orally administered phages were used to successfully treat 56 patients, and the phages were found to reach the patients' blood and urine.
Zhukov-Verezhnikov *et al* (1978).	Suppurative surgical infections	*Staphylococcus, Streptococcus, E. coli, and Proteus*	The superiority of adapted phages (phages selected against bacterial strains isolated from individual patients) over commercial phage preparations was reported in treating 60 patients having suppurative infections.

1.3.3. Phage Therapy and Its Fish Farm Application

Phage therapy is currently being applied all over the world in several investigation studies and for various usages, including the utilization of bacteriophages on wastewaters treatment and as biocontrol agents in food (Leverentz *et al*, 2001; Goode *et al*., 2003; Huff et al, 2005), plants (Flaherty *et al*, 2000), and cyanobacterial blooms. On the other hand, on the aquaculture field, studies using phages were made with the main purpose of identifying the specific bacteriophages to use in bacterial typing schemes and to characterize the bacteriophages properties, including their potential role in virulence. It is, however, commonly accepted that phage therapy can be applied as a preventive measure on fish farms, but few attempts have been made to use phages towards preventing bacterial infections in fish (Nakai *et al*, 1999; Park *et al*,2000; Park *et al*, 2003) The results of these few studies using phages specific to *Lactococcus garvieae* and to *P . plecoglossicida*, pathogens of yellowtail and of ayu (*Plecoglossus altivelis*), respectively, suggest that phages could be useful for controlling bacterial infections of fish (Nakai *et al*, 1999; Park *et al*,2000; Nakai *et al*, 2002; Park *et al*, 2003).

Nakai *et al.* (1999) describes the *in vivo* survival of *L. garvieae* bacteriophages and the potential of the phage for controlling experimental *L. garvieae* infection in yellowtail (Nakai *et al*, 1999). On this experiment phages were administered by injecting phage infected cells into the spleen or intestine and also by phage-impregnated food. The results showed a high survival rate on phage-injected fish after challenge with *L. garvieae* (survival rate 100% of 20 yellowtail), when compared to uninjected fish (survival rate 10% of 20 yellowtail). Simultaneous administration of live *L. garvieae* and phage enhanced the recovery of the phage from the fish organs. Protection was also obtained in yellowtail receiving phage-impregnated feed. Another interesting result showed that orally administered phage was detected at high plaque-forming units from the intestines and spleens of the phage-treated fish until 48 hours after administration. This study demonstrated that fish infection with *L. garvieae* can be prevented by administrating anti-*L. garvieae* phage, proving the utility of phages to control this kind of disease. The use of bacterial cells as a protector or vehicle did not influence the curative effect of phage. In other experiments, Nakai used two types of bacteriophages specific to *P. plecoglossicida*, the causative agent of bacterial hemorrhagic ascites disease in these fish species (Park *et al*, 2000). Diseased fishes showed the presence of infected cells on its kidneys during all experiment time, while fish that were oral administrated with *P. plecoglossicida* phage showed lower mortality rates (22.5% vs. 60%). These results indicate that orally administered phage can be expected to kill bacterial cells in internal organs, as well as bacterial cells in the intestine, which means that phage therapy can be effective at the systemic infection stage. Another interesting observation was the obtainance of phage isolates that were *P. plecoglossicida*-specific but not strain specific, which suggests that a single phage strain or a

few phage strains could provide effective phage therapy. The fish infection was obtained via oral route, suggesting that the intestine is an important portal of entry for the pathogen, and the narrow host range of phage should be an advantage in phage treatment because the phage does not harm the normal intestinal microflora (Nakai *et al*, 1999).

In other experiments with the ayu fish, a combination of the previously isolated phage together with specific phage against *P. plecoglossicida*. The mortality rate of fish that received one of the phages varied from 53.3 to 40.0%, decreasing to 20.0% when the mixture of the two phages was used, increasing to 93.3% in the control fish receiving no phages, demonstrating the therapeutic effect of phages in natural infections (Park *et al*, 2003). In a field trial, when phage-impregnated feed was administered to ayu in a pond, where the disease occurred naturally (fish mortality about 10 Kg d^{-1};ca. n=900 d^{-1}), fish daily mortality decreased at a constant level (5 % per day) from days 3 to 15, reaching a steady state at about 6 Kg mortality d^{-1} (ca. n=300 d^{-1}) thereafter, corresponding to a one-third reduction in relation to natural conditions. The fact that no phage-resistant organisms or phage-neutralizing antibodies were detected on the fish treated with phages proved the success of phage therapy treatment (Park *et al*, 2003).

Additional studies have been conducted to demonstrate the efficacy of bacteriophage combination has a preventive measure on fish farms (Imbeault *et al;* 2006). Imbeault *et al.* (2006) studied the interaction between *A. salmonicida* and a bacteriophage to treat furunculosis in brook trout (*Salvelinus fontinalis*). The results obtained showed that more than one phage could infect *A. salmonicida* and that mutants resistant to one phage were sensitive to other phages. Resistant bacteria had a shorter generation time than the original strain and the success of their replating was very low. After the first plating, more than 25% of the mutants seemed to revert to the original strain phenotype and the totality was sensitive to three or more phages.

Besides some successful results, other experiments have not shown the same behavior on fish when applying phage therapy. The attempt to control furunculosis of Atlantic salmon (*Salmo salar*) caused by *A. salmonicida* showed that fishes injected with bacteriophages immediately after challenge, died at a significant slower rate than those that were not treated with phages (Verner-Jeffreys *et al*, 2007). Phage treatment 24 hours after the challenge resulted in a mortality rate similar to the rate observed in un-treated fish. The results for Atlantic salmon were not as good as other described before, but the fact that there were no safety problems associated with the approach must also be highlight.

More recently, Walakira *et al.* (2008) isolated two lytic bacteriophages specific for *Edwardsiella ictaluri* that cause enteric septicemia of catfish (Walakira *et al*, 2008). Each *E. ictaluri* strain tested was susceptible to phage infection with variable efficiencies but with no evidence of lysogeny and with no plaques detected on other bacterial species, demonstrating

their potential use as biotherapeutic and diagnostic agents associated with enteric septicemia of catfish.

Studies using bacteria that infect fish farm plants and can also be responsible for serious infections on humans were recently reported. This group includes bacteria of the Vibrio family such as *Vibrio campbelli*, *V. parahaemolyticus*, *Vibrio cholerae*, *V. vulnificus* and the luminescent Vibrio *harveyi*. A host range of seven phages from hatchery and creek water of aquaculture systems specific to *V. harveyi* were characterized and it was shown that all the phages were highly lytic against *V. harveyi*, displaying different lytic spectra for the large number of isolates tested (183 isolates) (Shivu *et al*, 2007). These phages lysed 15 to 65% of the strains. Six of the seven phages have a broad lytic spectrum and could be potential candidates for biocontrol of the *V. harveyi* in aquatic systems. None of the phages were able to infect other *Vibrio* species. Another interesting result using lytic phages against *V. harveyi* on shrimp farm water, showed significant higher survival rates when phage was applied even when comparing to results obtained on treatments with daily addition of antibiotics (5 mg L^{-1} oxytetracyclin and 10 mg L^{-1} kanamycin) (Vinod *et al*, 2006). The results showed that antibiotic treatment led to initial reduction of luminous bacteria after forty-eight hours when bacteria proliferate to about 10^6 PFU mL^{-1} (survival of 40%), but when using specific phages against *V. harveyi* the luminous bacteria were not detected throughout the seventeen day study period (survival of 86% in the phage treated tank). The phages used in this study did not carried virulence genes. The results suggest that bacteriophages have potential for biocontrol of *V. harveyi* in hatchery systems (Vinod *et al*, 2006).

In table 1.3 these and other important reports on phage therapy applied to aquaculture systems are summarized.

Table 1.3: Experiences of phage therapy applications to aquaculture (Almeida *et al*, 2009).

Reference	Phage	Bacteria	Treated fish/shellfish	Phage application	Effects
Bogovazova et al., 1991	*Klebsiella pneumoniae* bacteriophage	*Klebsiella*	BALB/c mice	Intraperitoneal, intravenous or intranasal administration	Rescue of generalized *Klebsiella* infection
Barrow *et al.*, 1998	Bacteriophage R isolated from domestic sewage	*E. coli H247 (O18:K1:H7)* (bacteremic)	Chickens and calves	Intramuscular (10^2-10^6 PFU) and intracranial (10^8 PFU) inoculation of chickens; oral and intramuscular inoculation of calves with $3x10^{10}$ PFU)	Protection against morbidity and mortality
Cao *et al.*, 2000	*Helicobacter pylori* M13 recombinant phage	*Helicobacter pylori*	BALB/c mice	Oral administration of 10^7 PFU	Reduction of stomach colonization by *Helicobacter pylori*
Flaherty *et al.*, 2000	*Xanthomonas campestris* pv. *vesicatoria* specific H-mutant bacteriophages	*Xanthomonas campestris* pv. *vesicatoria*	"Sunbeam" tomato *Lycopersicon esculentum*	Foliar applications of 10^8 PFU/mL phage suspensions	Reduction of bacterial spots and increase in fruit weight.
Leverentez *et al.*, 2001	*Salmonella* – specific phages	*Salmonella enteritidis*	Fresh-cut fruit (melons and apples)	Direct application of 5 x 10^7 PFU/mL phage suspension on fruit slices	Reduction of *Salmonella* concentration by 2.5-3.5 logs on melon but not on apple.
Biswas *et al.*, 2002	*Enterococcus* phages ENB6 and C33 isolated from raw sewage	Vancomycin-resistant *Enterococcus faecium* (agent of VRE bacteremia)	BALB/c mice	Intraperitoneal injection of 3 x 10^8 PFU	Complete rescue of bacteraemia in 48 hours
Nakai & Park 2002	Siphoviridae isolated from diseased fish and sea water in fish culture cages.	*Lactococcus garvieae*, formerly *Enterococcus seriolicida*	Yellowtail *Seliora quinqueradiata* and Ayu *Plecoglossus altivelis*	Oral administration of phage-impregnated feed or intraperitoneal injection	Protective/curative effect (increase in the survival rate).
Goode *et al.*, 2003	*Salmonella enteritidis* phage types P125589, phage 29C and transducing lambdoid phage P22, HTint, isolated on *S. enterica* serovar Enteritidis from sewage; *Campylobacter jejuni* phage 12673	*Salmonella enterica* serovar Enteritidis and *Campylobacter jejuni*	Chicken skin	Surface spreading with 10^3 PFU/cm^2	Reduction by 2 log units in bacterial abundance over 48 h.
Jado *et al.*, 2003	Phage-coded lysins (enzybiotic): Pal amidase and/or Cpl-1 lysozyme	Antibiotic-resistant *Streptococcus pneumoniae* 541, serotype 6B	BALB/c mice	Intraperitoneal injection of 1 mg/mL (110 000 U/mg) enzyme solutions.	Rescue of bacteraemia and prevention of death in 72 hours.
Hagens *et al.*, 2004	Genetically engineered non-replicating, non-lytic filamentous phage Pf3R obtained from phage Pt1 isolated from river water using PAO1 as the host.	*Pseudomonas aeruginosa*	BALB/c mice	Intraperitoneal inoculation with 10^6-10^8 PFU	Higher survival rate and reduced inflammatory response after 12-24 hours.
Fiorentin *et al.*, 2005	*Salmonella enteritidis* lytic phages CNPSA 1, CNPSA 3, CNPSA 4	*Salmonella enteritidis*	Chicken cuts (thighs and drumsticks)	Immersion in 10^9 CFU/mL bacteriphage suspensions	Reduction of *Salmonella enteritidis* counts in treated chicken cuts.
Huff *et al.*, 2005	*Escherichia coli* phages SPR02 and DAF6	*Escherichia coli* iso lated from poultry	Broiler chickens	Injection in the air sac with 10^4 or 10^8 PFU/mL phage suspensions and bird spraying with phage suspensions.	Decreased bird mortality.
Toro *et al,* 2005	*Salmonella* – specific bacteriophages	*Salmonella typhimurium*	Chicken	Oral administration.	Reduction in *Salmonella* counts in cecum and ileum treated chickens.
Wills *et al,* 2005	Bacteriophage LS2a	*Staphylococcus aureus* strain 2698 (abscess forming)	New Zealand White rabbits	Subcutaneous injection with 2 x 10^9 PFU	Prevention of abscess formation
Verner-Jeffreys *et al,* 2007	*Aeromonas salmonicida* phages O, R and B	*Aeromonas salmonicida* subsp. *salmonicida*	Atlantic salmon *Salmo salar*	Injection (1.9 x 10^8 PFU/fish), oral administration (1.88×10^5 PFU/g) and bath (1.04×10^5 PFU/mL)	Lower rate mortality but similar absolute mortality. No protection was offered by any of the bacteriophage treatments.
Watanabe *et al* , 2007	Phage strain KPP10 isolated from a highly polluted river using *P. aeruginosa* strain PA20 as the host.	*Pseudomonas aeruginosa* strain D4 (agent of gut-derived sepsis)	ICR mice	Intraperitoneal inoculation with 10^{10} PFU	Higher survival rate and reduced inflammatory response after 24 hours.

1.3.4. Disadvantages of phage treatment

Phage therapy is not seen as a consensual and fully established alternative to antibiotics. Therapeutic phages have at least some theoretical advantages over antibiotics, and phages have been reported to be more effective in treating bacteria infections (Inal, 2003). However, identified problems of phage therapy must not be discarded. Some of the more discussed ones are the development of antibodies after repeated treatment with phages or, for example, against phages used to treat enteric pathogens.

The problem of antibodies, which could presumably be overcome by theuse of cocktails of phages, is not deemed as important as clearance from the bloodstream by the spleen, which is much more rapid. Other problems might include the rapid uptake and inactivation of phages by the spleen and the contamination of therapeutic phage preparations with endotoxin from bacterial debris (Inal, 2003).

These concerns on phage therapy treatments are being somehow addressed, with encouraging results. For example, when purifying phages prepared from bacteria using cesium chloride centrifugation, the contamination levels with endotoxin and exotoxin released during lytic growth (Merril *et al*, 1996) were highly diminish (by over 100-fold). Although elimination of phages by the reticuloendothelial system can also be avoided by selecting for phage variants (at least in mice) able to circulate for a long time after intraperitoneal administration, by a natural selection process (the so-called “serial passage technique”) (Merril *et al*,1996) this would not be practical and could be overcome by further administration of phages.

Furthermore, despite the effectiveness of phages in destroying bacteria *in vitro*, little is known about how they behave *in vivo*, in particular in the human body.

A consensus objection to the use of phage is its excessive narrow host range in comparison with, for example, antibiotics. Such fact is probably due to the presence or absence of the attachment antigen but also by potentially greater restriction problems resulting from variations in DNA restriction patterns between different isolates of the same organism (Barrow *et al*, 1997). Another approach is to search for phages that attach to the surface virulence determinants. These are generally shared by many or all relevant strains and phage-resistant bacteria may be attenuated, as indicated previously

The possibility of using a combination of phages against the major strains of bacteria was already tested by Smith (Smith *et al*, 1983) that developed a phage pool for enterotoxigenic *E. coli* strains. One advantage of using more than one phage for each strain is that, should mutants arise that are resistant to a particular phage and are still virulent, a second phage to which the strain is susceptible is available. This approach has been used successfully in the *E. coli* diarrhoea work (Smith *et al*, 1983).

Other undesired effect arise when phages do not lyse bacteria but remain as genetic material in the cells in a state of lysogeny. Such phages can code for undesirable features of bacteria, such as toxin production (Barrow *et al*, 1997) but they can be avoided. In some infections, the invading bacteria are thought to become primarily intracellular within the host. It is quite likely that infections with such pathogens, including *Salmonella, Brucella, Yersinia* and the mycobacteria, would not be amenable to this sort of approach.

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Chapter 2

Evaluating seasonal dynamics of bacterial communities in marine fish aquaculture: a preliminary study before applying phage therapy

Abstract

The increasing problems with antibiotic resistance in common pathogenic bacteria and the concern about the spreading of antibiotics in the environment due to medical treatments in humans, animal farms and fish-farming plants, bring the need to find new methods to control fish pathogens. Phage therapy represents a potentially viable alternative to antibiotics and other antimicrobials. The use of phage therapy in aquaculture requires, however, a detailed understanding of bacterial communities, namely of fish pathogenic bacteria, and the awareness of various novel kinetics phenomena not known in conventional drug treatments. The knowledge of the structure of bacterial communities in aquaculture systems is relevant and must be considered in order to test the suitability of phage therapy to control pathogenic bacteria of fish. Therefore, in this study the seasonal dynamics of the overall bacterial communities, microbiological water quality and disease-causing bacteria were followed in a marine aquaculture system of Ria de Aveiro (Portugal). Analysis of the water samples by means of denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments suggested that the bacterial community structure varied seasonally, showing a higher complexity during the warm season. The main fish pathogenic bacteria, assessed by DGGE targeting the *Vibrio* genus, showed less remarkable seasonal variation, with new dominating populations appearing mainly in the spring. Bacterial indicators, faecal coliforms and enterococci were enumerated by the filter-membrane method, using selective culture media. A clear seasonal variation was observed for bacterial indicators with the highest values in October. The relative abundance of specific groups of bacteria was accessed by fluorescence in situ hybridization (FISH), using 16S rRNA-targeted probes. The FISH results showed that the specific groups of indigenous and non-indigenous bacteria varied during the study period and that the non-indigenous *Enterobactereaceae* family was the most abundant group followed by *Vibrio* and *Aeromonas*. The *Pseudomonas* genus was the less abundant group. As the density and structure of total and pathogenic bacterial communities varied seasonally, it is necessary to take in consideration this variation when specific phages are selected for phage therapy. The seasonal variation detected in this study of the main fish pathogenic bacteria in terms of density and diversity implicates a careful monitoring of water along the year in order to select the suitable phages to inactive bacteria.

Keywords: phage therapy, aquaculture, viruses, bacterial community, faecal pollution, seasonal variation

Introduction

The increasing importance of aquaculture to compensate the progressive worldwide reduction in amount and quality of natural fish populations has contributed to aquaculture becoming one of the fastest growing productive sectors, providing nearly one-third of the world's seafood supplies. However, the growth and even survival of the aquaculture industry is threatened by uncontrolled bacterial diseases that cause extensive losses (Flegel, 2006; Saksida *et al*, 2006).

The main biological agents that cause food-borne disease are bacteria, viruses, parasites, oomycetes and to a lesser extent moulds (Almeida *et al*, 2009). Parasite-related food safety concerns in aquaculture are limited to a few helminth species, and are largely focused on communities where consumption of raw or inadequately cooked fish is a cultural habit (WHO, 1995). Human viral diseases caused by the consumption of fish appear to present a low risk, because viruses that are pathogenic to fish are not pathogenic to man.

Bacterial diseases are a major problem in the expanding aquaculture industry (Alderman *et al*, 1996; Shao *et al*, 2001; Wahli *et al*, 2002). The level of contamination of aquaculture products will depend on the environment and the bacteriological quality of the water where the fish is cultured. There are two broad groups of bacteria of public health significance that contaminate products of aquaculture: those naturally present in the environment – indigenous microflora (e.g. *Aeromonas hydrophila*, *Clostridium botulinum*, *Vibrio parahemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus* and *Listeria monocytogenes*) and those introduced through environmental contamination by domestic animals excreta and/or human wastes – non indigenous microflora (e.g. *Enterobactereaceae* such as *Salmonella*, *Shigella*, *Escherichia coli*) (Muroga *et al*, 1987; Huss, 1994; Fukuda *et al*, 1996; Iida *et al*, 1997, Nakai *et al*, 1999; Nakai and Park, 2002). This faecal material can be originated from point source discharges such as raw sewage, storm water, effluent from wastewater treatment plants and industrial sources (Seurinck *et al*., 2005). In addition, non-point source discharges such as agriculture, forestry, wildlife and urban run-off can also impair water quality (Seurinck *et al*., 2005).

Vibriosis and photobacteriosis (formerly pasteurellosis) are primarily diseases of marine and estuarine fish, both in natural and commercial production systems throughout the world, occurring only occasionally in freshwater fish. Both diseases can cause significant mortality in fish, reaching values of up to 100% in infected facilities, being currently responsible for the most outbreaks of fish farming plants. Vibriosis and photobacteriosis are caused by bacteria from the family Vibrionaceae. Vibriosis is caused by species from the genera *Photobacterium* (namely *Photobacterium damselae* subsp. *damselae*, formerly *Vibrio damselae*) and *Vibrio* (namely *V. anguillarum*, *V. vulnificus*, *V. alfinolyticus*, *V. parahaemolyticus* and *V. salmonicida*). Photobacteriosis is caused by *P. damselae* subsp. *piscicida* (formerly *Pasteurella*

piscicida) that is a highly pathogenic bacterium that does not seem to have host specificity, infecting an ample range of fish species (Toranzo *et al*, 1991; Noya *et al*, 1995). Other bacteria as *Aeromonas salmonicida*, *Rickettsia*-like bacteria, *Cytophaga marina*, *Flavobacterium psychrophilum* and *Pseudomonas plecoglossicida* are also important groups of fish pathogens, affecting a variety of fish species from diverse geographical aquatic environments (Almeida *et al*, 2009).

Due to the fast worldwide emergence of antibiotic-resistant bacterial pathogens in human, medicine, agriculture and aquaculture, regulators are severely limiting antibiotic usage. Phage therapy represents a potentially viable alternative to antibiotics and other antimicrobials. When compared to other methods based on the direct addition of antibiotic/disinfectant into the aquaculture systems, phage therapy present lower risk for fish (Nakai and Park, 2002, Inal *et al*, 2003; Clark *et al*, 2006), inactivating pathogenic bacteria and avoiding fish contamination. The safety of the phage therapy approach is additionally increased by the fact that phages do not induce the selection of resistant bacterial strains (Inal *et al*, 2003; Clark *et al*, 2006; Almeida *et al*, 2009). The possibility to replace at least some of the currently used chemical agents working as antimicrobials (e.g., antibiotics bronopol, hydrogen peroxide, formalin), which are potentially dangerous for the fish and consumers and invariably cause some degree of environmental pollution, adds further value to the phage therapy approach by minimizing the risk of undesired side effects and allowing a less risky repetition of the treatment. However, the success of phage therapy depends on the level of faecal contamination and on the knowledge of the main pathogenic bacteria, having into account their seasonal variation. The main purpose of this work was to follow the seasonal dynamics of viral and bacterial communities of aquaculture systems, identifying the main pathogenic bacteria groups and evaluating the level of faecal contamination. The total number of viruses was also determined in order to evaluate the availability of viruses that will be isolated and used in phage therapy

Material and Methods

STUDY AREA

This study was conducted in the aquaculture system Corte das Freiras located in Ria de Aveiro (Figure 2.1). Ria de Aveiro is an estuarine system situated on the north-western coast of Portugal. Fish semi-intensive culture for human consumption is an important economic activity in Ria de Aveiro.

Figure 2.1: Ria de Aveiro with the aquaculture systems Corte das Freiras (A)

SAMPLING

Water samples were collected two hours before low tide, in mild weather conditions, from a culture tank of *Sparus aurata* (gilthead bream). Samples from surface water were taken directly into sterile glass bottles and kept cold and in the shade during transport to the laboratory where they were processed within the next 1-2 hours.

In a first phase it was quantified the main pathogenic bacteria of fish in water samples collected on five dates: April 2007, October 2007, December 2007 and February 2008. In a second phase, the seasonal dynamics of the bacterial community structure and the total viral number were evaluated on, November 2008, January 2009, March 2009, May 2009 and July 2009. The bacterial indicators (faecal coliforms and enterococci) were analyzed during the all study period, on nine different dates: October 2007, December 2007, February 2008, June 2008, November 2008, March 2009, May 2009 and July 2009.

WATER PROPERTIES

Temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter. Dissolved oxygen was also determined in the field with a WTW OXI 96 oxygen meter equipped with a WTW BR 190 stirrer. pH was measured in the laboratory, at 25ºC, with a pH probe (Orion, Model 290 A).

EVALUATION OF THE SEASONAL DYNAMICS OF BACTERIAL COMMUNITY STRUCTURE AND OF *VIBRIO* GENUS.

Triplicate sub-samples of 300 mL were filtered by 0.22 µm-pore-size filters (Poretics Products Livermore, USA). Collected cells were resuspended in 2 mL of TE buffer [10 mM Tris HCl, 1 mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] and centrifuged. After resuspension in 200 mL TE, 1 mg mL^{-1} lysozyme solution was added to induce cell lysis and incubated at 37ºC for 1 hour, according to the procedure described by Henriques *et al* (2004) .DNA extraction was performed using the genomic DNA purification kit (MBI Fermentas, Vilnius, Lithuania). DNA was resuspended in TE buffer and stored at –20ºC until analysis. The yield and quality of DNA were checked after electrophoresis on 0.8 % (w/v) agarose gel.

PCR amplification of an approximately 400 bp 16S rDNA fragment (V6-V8) was performed using the primer set F968GC and R1401 (Nubel *et al*., 1996) .The reaction was carried in a Multigene Gradient Thermal Cycler from MIDSCI. The 25 µl reaction mixture contained approximately 50 to 100 ng of extracted DNA, 1x PCR buffer (PCR buffer without Mg Cl_2: PCR buffer with K Cl_2, 1:1), 275 Mm $MgCl_2$, 0.2 mM of each nucleotide, 0.1 µM of each primer, 1U of Taq Polymerase (all reagents purchased from MBI Fermentas, Vilnius, Lithuania). Acetamide (50%, 0.5 µM) was also added to the reaction mixture. The protocol included a 4 min initial denaturation at 94ºC, 34 cycles of 95ºC for 1 min, 53ºC for 1 min and 72ºC for 1 min 30 s, and a final extension for 7 min at 72ºC.

The diversity of the *Vibrio* genus was analyzed after amplification of bacterial DNA using the primers Vib-F (727) 5'-AGG CGG CCC CCT GGA CAG A-3' and Vib-R (1423) 5'-ARA CTA CCY RCT TCT TTT GCA GC-3'. Each PCR reaction mixture contained: 1x PCR buffer, 0.2 mM d-NTP's, 25 mM $MgCl_2$, 0.5% DMSO; 0.2 µM of each primer, 2.5 units Taq, 50 ng of the DNA template and 13.25 µL of distilled deionized water to a total reaction volume of 25 µL. Thermal cycling was as follows: 1 cycle of 7 min at 94 ºC; 30 cycles of 1 min at 94 ºC, 1 min at 56 ºC and 1 min at 72 ºC and a final extension of 72 ºC for 10 min. PCR products were checked using standard agarose gel electrophoresis and ethidium bromide staining (Sambrook *et al*., 1989).

DGGE was performed with the CBS System (CBS Scientific Company, Del Mar, CA, USA). PCR products were loaded onto 6-9% polyacrylamide gel in 1xTAE buffer (20 mmol/L Tris, 10 nmol/L acetate, 0.5 mmol L^{-1} EDTA pH 7.4). The 6-9 % polyacrylamide gel (bisacrylamide:acrylamide = 37.5:1) was made with a denaturing gradient ranging from 32 to 60%. Electrophoresis was performed at 60ºC for 16 h at 150 V. Following electrophoresis, the gels were incubated for silver staining. The solutions used were 0.1% (v/v) ethanol plus 0.005% acetic acid for fixation, 0.3 g silver nitrate for staining, freshly prepared developing solution containing 0.003% (v/v) formaldehyde, 0.33% NaOH (9%), and finally, 0.75% sodium

carbonate solution to stop the development. GelCompar 4.0 program (Applied Maths) was used to analyze bacterial community profiles of the images of DGGE gels as described by Smalla *et al*. (2001).

QUANTIFICATION OF BACTERIAL INDICATORS OF FAECAL CONTAMINATION

Samples were analyzed for bacterial indicators, faecal coliforms (FC) and faecal enterococci (FE). Faecal coliforms and enterococci were enumerated by filter-membrane method using selective culture media, m-FC medium (Difco Laboratories) and m- KF (Difco Laboratories), respectively. Faecal coliforms were incubated at 44.5 ºC for 24 hours and enterococci incubated at 37ºC for 48 hours. The results were expressed as colonies forming units for one hundred milliliters (CFU 100 mL^{-1}).

QUANTIFICATION OF THE MAIN PATHOGENIC BACTERIA OF FISH

Samples were filtered through 0.22 μm polycarbonate filters (GE Osmonics), fixed with 4 % paraformaldehyde for 30 min and rinsed with PBS 1X and MilliQ water. The filters were stored at room temperature until hybridization. The specific groups of bacteria were accessed by *Fluorescent in situ hybridization* (FISH) using 16S rRNA target probes (Amann *et al*, 1990). The probe Eub338-II-III was used to quantify bacteria belonging to the Domain Bacteria. The bacteria belonging to the non-indigenous *Enterobacteriacea* family and to the *Vibrio*, *Aeromonas* and *Pseudomonas* genera were detected with the specific probes ENT183, VIB572a, AERO1244 and Pae997, respectively.

For each probe, three filter sections were placed on a Parafilm-covered glass slide and overlaid with 30 μL hybridization solution with 2.5 ng μL^{-1} of probe. The hybridization solution contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% SDS, and the optimum concentration of formamide for each probe [32]. Filters were incubated in sealed chambers at 46 °C for 90 min. After hybridization, filters were washed for 20 min at 48°C in wash solution (20 mM Tris-HCl pH 7.4, 5 mM ethylenediaminetetraacetic acid, 0.01% SDS, and the appropriate concentration of NaCl) (Pernthaler *et al*., 2001) . Rinsed and dried filter pieces were counterstained with 2 μg mL^{-1} 4', 6-diamidino-2-phenylindole (DAPI) and mounted with Vectashield and Citifluor (1:4). Besides the relative abundance, DAPI staining also provided a measure of total microorganisms. Samples were examined with a Leitz Laborlux K microscope equipped with the appropriate filter sets for DAPI and CY3 fluorescence. At least 15 fields were counted for each of the three replicates.

QUANTIFICATION OF TOTAL VIRAL NUMBER

Total viral number was determined by epifluorescence microscopy after staining with SYBR gold (Noble and Fuhrman, 1998). Water samples were previously filtered by 0.2 μm pore-size polycarbonate membrane filters (Poretics Products Livermore, USA) with vacuum pressure 150 mm Hg. One milliliter of 0.2μm filtered water was filtered through a 0.02 μm pore size AI_2O_3 Anodisc membrane filter at approximately 20 kPa vacuum. The Anodisc membrane was filtered to dryness, removed with forceps with the vacuum still on, and laid the sample side up on the drop of the Sybr gold at 2x solution for 15 min in the dark. The Anodisc filter was mounted on a glass slide with a drop of 50% glycerol, 50% phosphate buffered saline (PBS, 0.05 M Na_2HPO_4, 0.85% NaCl, pH 7.5) with 0.1 % p-phenylenediamine. For each filter, 10 to 20 fields were selected randomly and a total of at least 200 viruses were counted on an epifluorescence microscope (Leitz Laborlux K microscope) equipped with the appropriate filter for SYBR gold (Noble and Fuhrman, 1998).

STATISTICS

Total viral and bacterial numbers were tested for normality (Kolmogorov–Smirnov test) before the comparison of means. Parametric analysis of variance (ANOVA) was performed, providing that data was normally distributed and that the variance of group means was homogeneous (Levene test).

RESULTS

WATER PROPERTIES

The range of values of the parameters that describe water properties are summarized in Table 2.1. In the aquaculture system, salinity range varied between 35.7 in October 2007 and 16.7 in December 2007. Temperature oscillated from 10.8°C in November 2007 to 20.8°C in June 2008 and the dissolved oxygen ranged between 1.55 mg L^{-1} in July 2009 and 6.01 mg L^{-1} in April 2006.

Table 2.1: Physical characterization of the water in the aquaculture system during the study period.

Sampling dates	Salinity	Temperature (ºC)	Dissolved Oxygen (mg L^{-1})	pH
April 2007	32.1	17.7	6.01	7.38
October 2007	35.8	16.9	4.64	7.90
December 2007	16.7	13.4	2.78	8.02
February 2008	31	16	3.41	809
June 2008	31.4	20.8	5.65	7.70
November 2008	29.5	10.8	2.87	8.05
January 2009	19.7	12.8	2.67	7.98
March 2009	31	13.8	2.43	8.09
May 2009	31.4	17.5	2.70	8.19
July 2009	32.8	20.1	1.55	8.02

EVALUATION OF THE SEASONAL DYNAMICS OF BACTERIAL COMMUNITY STRUCTURE AND OF *VIBRIO* GENUS.

Bacterial community structure was examined by comparing DGGE profiles of 16S rDNA fragments during the different sampling moments (Figure 2.2). Reproducibility of PCR amplification and DGGE was confirmed by similar results obtained for the three sub-samples analyzed at each date (Figure 2.2).

DGGE profiles revealed a complex and specific pattern of bands (Figure 2.2) in aquaculture water samples as well as seasonal differences in the structure of bacterial communities, with a higher diversity during the warm season. Bray-Curtis similarity index for bacterial community ranged between ~25% and ~97%, varying widely between sampling moments. Cluster analysis of the band patterns obtained from DGGE analysis (Figure 2.3) revealed the occurrence of weak similarities (as low as 25%) between the bacterial community structures in water samples collected in the different months. The bacterial community in the water sample collected in March showed the lowest value of similarity (~25%) with those detected in the remaining sampling periods. For the other months, the bacterial communities present in the water samples collected in November and January showed the highest similarity index (> 85%).

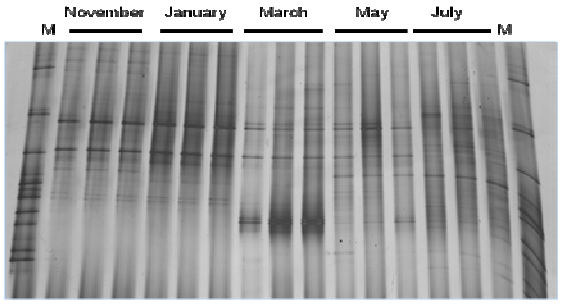

Figure 2.2: Seasonal DGGE profile of 16S rDNA of bacterial communities of the aquaculture system.

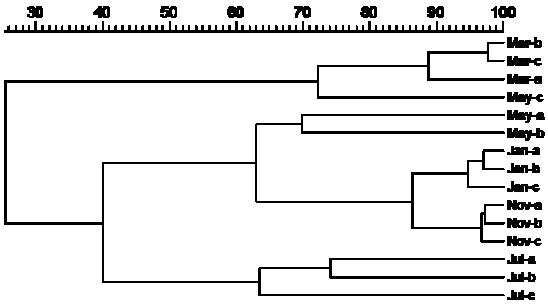

Figure 2.3: Dendrogram generated from the pattern of bands obtained by DGGE (figure 2.2) of aquaculture water.

Bray-Curtis similarity index for *Vibrio* ranged between ~80% and ~96%, varying between sampling moments (figure 2.5). Cluster analysis of the band patterns obtained from DGGE analysis (Figure 2.4) revealed that the diversity of *Vibrio* in water samples collected in November showed the lowest similarity value (<80%) with the remaining sampling moments. The samples displaying the highest similarity values (~92%) were those collected in the months of January and March.

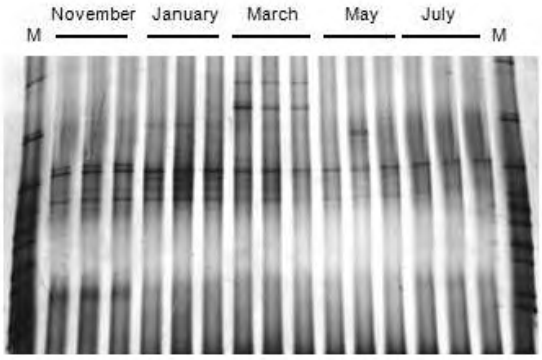

Figure 2.4: Seasonal DGGE profile of *Vibrio* 16S rDNA.in an aquaculture system

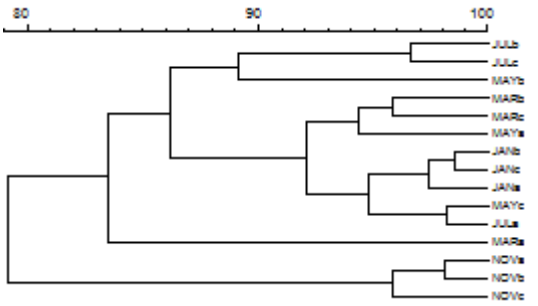

Figure 2.5: Dendrogram generated from the pattern of bands obtained by DGGE (figure 9) of aquaculture water.

QUANTIFICATION OF BACTERIAL INDICATORS OF FAECAL CONTAMINATION

The results of the number of faecal coliforms and enterococci presented in the aquaculture system are represented in Figure 2.6. The concentrations of faecal coliforms were, in general, slightly higher than those of for enteroccoci. The differences observed for faecal coliforms and enterococci densities during the sampling period were significant (ANOVA< 0.05).

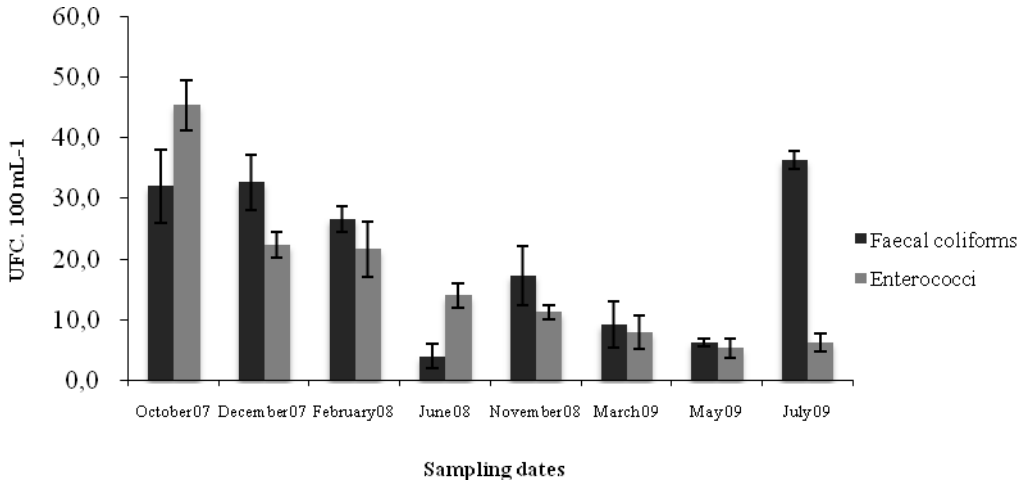

Figure 2.6: Seasonal variation of faecal coliforms and enterococci in the aquaculture system.

In general, the values of the faecal indicators decreased during the sampling period. The highest values of enterococci were obtained in October 2007 (45.33 UFC. $100mL^{-1}$) and the lowest in May 2009 (5.33 UFC. $100mL^{-1}$). Over the cold months (October, November and December) a clear decrease in the abundance of faecal coliforms was noticed, reaching its lower values on May and June. The highest values of faecal coliforms were obtained in July 2009 (36.33 UFC. $100mL^{-1}$) and the lowest in June 2008 (4.00 UFC. $100mL^{-1}$).

QUANTIFICATION OF MAIN PATHOGENIC BACTERIA OF FISH

The relative abundance of bacteria belonging to *Bacteria* domain as well as *Enterobacteriacea* family and *Vibrio*, *Aeromonas* and *Pseudomonas* genera detected by FISH is shown in Figures 2.7 and 2.8. The relative abundance of the *Bacteria* domain varied between 76.63 ± 1.05% in December 2007 and 95.40 ± 0. 97% in February 2008, relatively to the DAPI counts. The differences observed for total bacterial number were not significantly different during the sampling period (ANOVA, $p > 0.05$).

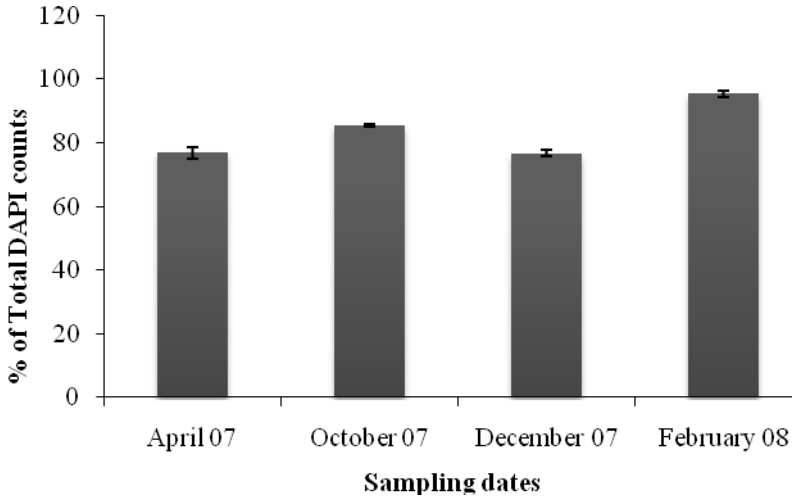

Figure 2.7: Relative abundance of the *Bacteria* domain detected by FISH in the aquaculture system.

The most abundant specific group of bacteria in October 2007 and February 2008 is the *Enterobacteriacea* family, whereas in December 2007 and April 2007 is the *Aeromona*s genera. The least abundant group over the different sampling dates was the *Pseudomonas* genera. On April 2007, the relative abundance of the four groups of fish pathogenic bacteria was similar (ANOVA, $p > 0.05$) but in the other months, a wide variation was observed in the relative abundance of the four studied groups (ANOVA, $p < 0.05$).

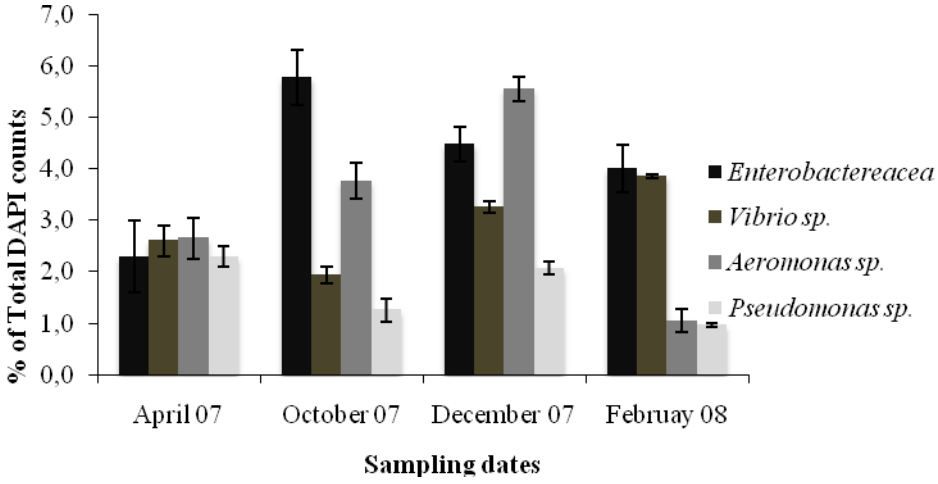

Figure 2.8: Relative abundance of *Enterobacteriacea* family, *Vibrio*, *Aeromonas* and *Pseudomonas* genera detected by FISH in the aquaculture system.

The relative abundance of *Enterobacteriaceae* family was higher in October 2007 (5.77± 0. 53%), decreasing over the winter and spring seasons and reaching its lower values on April 2006 (2.29±0.7%). *Aeromonas* presented the highest relative abundance values in December

2007 (5. 55 ± 0.23%) and the lowest on February 2008 (1.05±0.22%). An increase on the relative abundance of *Vibrio* was observed between October 2007 (1.94± 0.17%) and February 2008 (3.86± 0.04%) and the highest values of *Pseudomonas* were obtained in April 2007 (2.29 ±0.2%) and the lowest in February 2008 (0.96±0.04%).

QUANTIFICATION OF TOTAL VIRAL NUMBER

The results of the total viral number in the aquaculture system are represented in Figure 2.9. Viral abundance varied between 6.10 x 10^9 particles L^{-1} in May 2009 and 1.09 x10^{10} particles L^{-1} in March 2009. The total viral number at each moment of sampling was significantly different (ANOVA< 0.05).

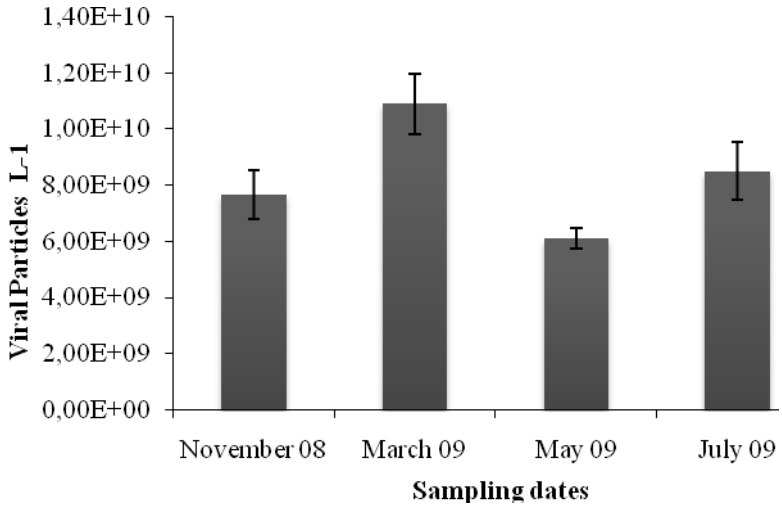

Figure 2.9: Seasonal variation of viruses in the aquaculture system.

Discussion

The success of phage therapy depends on the knowledge of the density of the main pathogenic bacteria, as well as of their seasonal variation. In general, the total bacterial numbers were fairly constant over the year, but the relative abundance of specific bacterial groups varied significantly during the sampling period. Among potentially pathogenic bacteria, *Enterobacteriaceae* were de most abundant, indicating that non-indigenous pathogenic bacteria are an importance source of contamination in this aquaculture system. In fact, the indicators of faecal contamination, faecal coliforms and enteroccocci, were present during all the sampling

period in the aquaculture system, confirming the importance of these bacterial groups in aquaculture. Although there is a rapid die-off of these enteric bacteria in managed farm fish, significant numbers of those bacteria remain on the skin and in the guts of fish and can cause a health risk to consumers (Almeida *et al*, 2009). *Aeromonas* and *Vibrio* genera that include *A. salmonicida*, causative agent of furunculosis (Bernoth, 1997), that is capable of infecting a wide range of host species, and members of the family *Vibrionaceae* that are currently responsible for the most outbreaks of fish farming plants, were also present at high concentrations in the aquaculture system (Toranzo *et al*, 1991; Noya *et al*, 1995). Consequently, phages of *Enterobacteriaceae, Aeromonas* and *Vibrio* groups must be considered in phage therapy.

The results of this study show that the bacterial community structure in general and, more specifically, that the diversity of the bacterial group most implicated in fish farming plants outbreaks (*Vibrio* genus) varied seasonally, indicating that it is necessary to take in consideration this variation when specific phages are selected to inactive fish pathogenic bacteria. Although the seasonal variation of the *Vibrio* genus was milder than that of the total bacterial community, the primers used to analyze the diversity of fish pathogens belonging to this genus (e.g. *V. anguillarum, V. vulnificus, V. alfinolyticus, V. parahaemolyticus and V. salmonicida*) also detect species from the *Photobacterium* genus (*P. damselae* subsp. *damselae*, formerly *Vibrio damselae*; and *P. damselae* subsp. *Piscicida*, formerly *Pasteurella piscicida)* that cause vibriosis and photobacteriosis (formerly pasteurellosis) that are primarily diseases of marine and estuarine fish, both in natural and commercial production systems throughout the world (Toranzo *et al*, 1991; Noya *et al*, 1995) .

The density of viral community in the water column of the aquaculture system (ranging from 6.10×10^9 - 1.09×10^{10} particles L^{-1}) could suggest a strong overall effect on the planktonic system and, eventually, a modulation of bacterial community. These values are in accordance with the ones obtained on other studies developed in the Ria de Aveiro (Almeida *et al*, 2007) where the total number of viruses varied between 0.1 to 3.7×10^{10} cells L^{-1}. Despite the fact that the abundance of bacteriophages was not determined, studies made on the same estuarine system suggest that the majority of the existing viruses are bacteriophages (Almeida *et al*, 2001). The number of viruses in the estuarine system is high, exceeding bacterial abundance by more than one order of magnitude (average 17.6) (Almeida *et al*, 2001). Moreover, the majority of the viruses are in the bacteriophage size-range and the patterns of viral density followed those of bacterial abundance, explaining 40 % of virioplankton variation. Experimental data of microcosm assays showed also that, in the Ria de Aveiro, bacterial lysis by viral infection is effective in reducing the density of bacteria below the values determined by predation, namely in brackish water (59% against 36% in the marine zone) (Almeida *et al*, 2001). Although the concentrations of bacteriophages in aquatic environments determined by PFU enumeration on

various bacteria hosts usually are in the order of 1000 to 10000 PFU L^{-1} Farrah, 1987; Moebus, 1987), in the Kiel Bight is up to 3.65 x 10^7 PFU L^{-1} (Ahrens,1971). Torrella and Morita (1979) used electron microscopy and estimated the total number of phage particles to be >10^7 L^{-1} in the seawater from Yaquina Bay, Oregon. Bergh *et al*. (1989) found abundances of 1 x 10^5 to 2.5 x 10^{11} phages PFU L^{-1} in various aquatic samples. These studies suggest that the concentration of phages in marine waters may be much higher than indicated by the concentrations obtained by culturing methods. Bergh *et al.* (1989) concluded that the high concentrations of bacteriophages in aquatic environments indicate that viruses may play important roles as agents of bacterial mortality and also control bacterial diversity in nature.

The seasonal variation of the overall bacterial community and of the disease-causing bacteria as well as of the indicators of microbiological water quality implicates a careful monitoring of water along the year in order to select the suitable phages to inactive fish pathogenic bacteria. The high content of virus in the aquaculture system suggests that it will be easy to isolate specific phages of fish pathogenic bacteria.

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Chapter 3

Bacteriophages with potential for inactivation of fish bacterial pathogens - effects on bacterial community structure

Abstract

Bacterial diseases can cause heavy losses in the fish production industries. The increasing problem of antibiotic resistance in common pathogenic bacteria and the concern about spreading of antibiotics in the environment, bring the need to find new methods to control fish pathogenic bacteria. Phage therapy represents a potentially viable alternative to antibiotics and other antimicrobials to inactivate pathogenic bacteria in aquaculture systems.

The use of phage therapy, however, requires an awareness of various novel kinetics phenomena not applied to conventional drug treatments. Therefore, in order to evaluate the use of phage therapy in aquaculture, preliminary studies are needed. The objective of this work was to determine the survival of *Aeromonas salmonicidae* and *Vibrio parahemolyticus* phages in marine water and to study the impact of these two viruses on the structure of the bacterial community of the aquaculture system. In order to evaluate the suitability of these viruses for phage therapy, their host specificity was also evaluated and compared with the specificity of other phages of six pathogenic bacteria isolated in the aquaculture system. The survival of the phages was determined after phage addition to filtered water samples through the quantification of phages by the soft agar overlay technique. The ecological impact of phage addition on the structure of bacterial community was evaluated using 16S rDNA DGGE. The host specificity of the phages was evaluated by spotting 10 µl of the marine phage suspension on confluent layers of the six host bacteria.

The survival period of the phages in marine water varied between 12 and 91 days, being the survival of *Aeromonas salmonicidae* phages clearly higher relatively to the other phage tested. The addition of specific phages of pathogenic bacteria had a low ecological impact on the structure of the natural bacterial community. The cross infection results showed that, with the exception of *Aeromonas salmonic*ida and *Escherichia coli* phage, the phages showed a large spectrum range of host pathogenic bacteria.

The results of this study show that the high survival of phages in marine water associated with the low impact of phages on total bacterial community structure and the high impact of specific phages on a large range of pathogenic bacteria, indicate that the phage isolated in this study can be successfully applied to inactivate fish pathogenic bacteria, even those resistant to antibiotics.

Keywords: Phage therapy, bacteriophages, fish pathogenic bacteria, aquaculture, bacterial community structure

Introduction

Aquaculture comprises all types of culture of aquatic animals and plants in fresh, brackish and marine environments (Pillay *et al*, 2005). Nearly one-third of the world's seafood supplies come from aquaculture industry, representing the fastest growing agricultural sector. Over the past ten years, aquaculture production has increased on average by 6 % per year (FAO, 2009). The production has increased from 8.7 million tons of fish in 1990 to 31.6 million tons in 2006 (FAO, 2009; FAO, 1998). Fish farming plants, however, often suffer from heavy financial losses (Subasinghe *et al*, 2001; Flegel, 2006; Saksida *et al*, 2006) due to the development of infections caused by microbial pathogens, including multidrug resistant bacteria that are easily transmitted through water and therefore able to infect a great variety of fish species.

Although vaccination is the ideal method to prevent infectious diseases, commercially available vaccines are still very limited in the aquaculture field (Reed and Francis-Floyd, 1996; Romalde, 2002; Arijo *et al*, 2005; Lin *et al*, 2006). This is partly due to the fact that many different kinds of infectious diseases occur locally in a variety of fish and shellfish species (Nakai and Park, 2002). On the other hand, chemotherapy is a rapid and effective method to treat or prevent bacterial infections, but frequent use of antibiotics has allowed drug-resistant strains of bacteria to develop. This problem can be particularly serious since few chemotherapeutic drugs are licensed for fisheries use (Muroga, 2001; Nakai and Park, 2002).

Bacterial diseases are a major problem in the expanding aquaculture industry (Alderman, 1996; Shao, 2001; Wahli *et al*, 2002). The level of bacterial contamination of aquaculture products by pathogenic bacteria will depend on the environment and the bacteriological quality of the water where the fish is cultured. There are two broad groups of bacteria of public health significance that contaminate products of aquaculture: those naturally present in the environment - the indigenous microflora (e.g. *Photobacterium damselae, Vibrio* sp, *Aeromonas hydrophila, Clostridium botulinum*) and those introduced through environmental contamination by domestic animals excreta and/or human wastes – non-indigenous microflora (e.g. *Enterobactereaceae* such as *Salmonella* sp., *Escherichia coli*) (Muroga *et al*, 1987; Huss, 1994; Fukuda *et al*, 1996; Iida *et al*, 1997, Nakai *et al*, 1999; Nakai and Park, 2002).

To reduce the risk of development and spreading of antibiotic resistant bacteria, other more environmentally friendly methods to control fish disease in aquaculture must be developed. In line with this idea, the use of bacteriophage therapy in aquaculture seems to be very promising. Though bacteriophages have been discovered over 80 years ago (Summers, 1999; Comeau *et al*, 2008) their potential application as an alternative to antibiotics has been recognized only recently (Angly *et al*, 2006; Culley *et al* 2006, Allen *et al*, 2008). Recent studies testify the use of bacteriophages as biocontrol agents in different areas (Toro *et al*, 2005; Flaherty *et al*, 2000; Withy *et al*, 2005). Although, phages of fish-pathogenic bacteria have been described

(Stevenson and Airdrie, 1984; Merino *et al*, 1990), there have been few attempts to use phages to control bacterial infections in fish (Park *et al*, 2000; Nakai and Park, 2002; Park and Nakai, 2003; Verner-Jeffreys *et al*, 2008). Phage therapy has several potential advantages over chemotherapy such as: specific target; limited resistance development, limited impact, unlike antibiotics, phages are self-replicating as well as self-limiting, simple regulatory approval, high resistance of phages to environmental conditions, technology flexible, fast and cheap (Inal, 2003; Clark and March, 2006). Despite phage specificity being seen as an advantage of phage therapy, Miller *et al* (2003) opposed this fact. In his study, a broad-host-range vibriophage, KVP40 was isolated from sea water, using *Vibrio parahaemolyticus* 1010 (EB101) as the indicator host. The host range of KVP40 extended over at least eight *Vibrio* sp. and one *Photobacterium* sp. (Miller *et al*, 2003.).

The success of phage therapy to control pathogenic bacteria of fish depends on virus density, but also on their survival and viability in culture water of fish-farming plants. These enteric viruses can remain infective for long periods in the environment and have been reported to survive for up to 130 days in seawater and up to 120 days in freshwater and sewage, at 20 to 30°C (Moebus, 1992). However, virtually no formation is available on marine phage survival (Kapuscinski and Mitchell, 1980).

The success of phage therapy also depends on the effect that phages have on the bacterial community, namely when extensive and semi-extensive regimes are used. Although phages only infect specific bacterial species or even strains of bacteria, potentially causing less damage to the bacterial community structure of aquaculture waters, a careful environmental evaluation must be done before the implementation of a phage therapy procedure to inactivate bacteria in aquaculture systems. In semi-intensive and extensive aquaculture systems, non-pathogenic bacteria have a central role in the functioning and productivity of these ecosystems. Bacteria are the most important biological component involved in the turnover (transformation and remineralization) of organic matter in aquatic systems (Cho and Azam, 1990; Pomeroy *et al*, 1991). In coastal waters, heterotrophic bacteria may utilize as much as 40% of the carbon fixed by the primary producers (Cole *et al*, 1988; Cho and Azam, 1990; Ducklow and Carlson, 1992). Heterotrophic bacteria are particularly skilled for organic matter transformation. They hydrolyze dissolved and particulate organic matter and can use substrates of difficult degradation and even different allochtonous compounds as further sources of organic carbon. They convert dissolved organic carbon that would inevitably be lost to higher trophic levels (microbial loop). Through remineralization of organic matter, bacteria regenerate nutrients which are then use by primary producers (Simon and Azam, 1989). As a large fraction of bacteria is not culturable, thus not detected by traditional methods, molecular tools (as denaturating gradient gel electrophoresis -

DGGE) must be used to evaluate the effect of phage therapy on the general bacterial community structure.

The main objectives of this work were to isolate bacteriophages resistant to environmental conditions with potential to inactivate fish pathogenic bacteria, without majorbeffects on the bacterial community structure of aquaculture waters.

Material and Methods

STUDY AREA

This study was conducted in the estuarine system Ria de Aveiro (Figure3.1) located on the northwest Atlantic coast of Portugal (40°5´N, 8°8´W), where the semi-intensive culture of fish is an important economic activity. Water samples were collected in the aquaculture system Corte das Freiras, located near the city of Aveiro, which is subjected to contamination introduced by human wastes and where chemotherapy treatments are commonly applied.

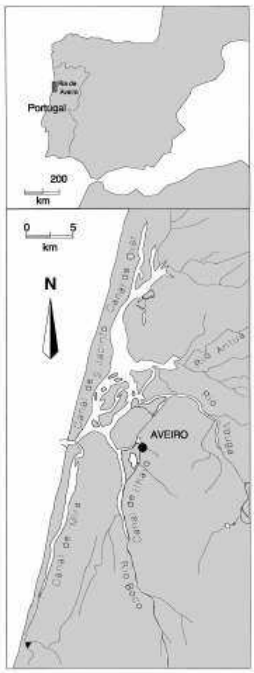

Figure 3.1: Ria Aveiro (Portugal).

SAMPLING

Samples were collected in a culture tank of *Spanus aurata* (gilthead bream), two hours before low tide water. Sampling was conducted with mild weather conditions. Samples from underlying waters were taken directly into sterile glass bottles and kept cold and in the shade during transport to the laboratory where they were processed within the next 1-2 h.

WATER PROPERTIES

Temperature and salinity were measured in the field using a WTW LF 196 Conductivity Meter. Dissolved oxygen expressed as the percentage of saturation, was also determined in the field with a WTW OXI 96 oxygen meter equipped with a WTW BR 190 stirrer. pH was measured in the laboratory, at 25ºC, with a pH probe (Orion, Model 290 A).

BACTERIA ISOLATION AND IDENTIFICATION

Three of the strains used in this study were isolated from water samples of the aquaculture system, using Tryptic Soy Agar (TSA; Difco) and Thiosulfate Citrate Bile Salts (TCBS; Difco), both supplemented with 1.5% NaCl. The plates were incubated aerobically at 25°C and at 37°C, during 3-5 days.The other three strains *Photobacterium damselae damselae, Photobacterium damselae piscicidae* and *Escherichia coli* used in this work were obtained in previous studies (Costa *et al*, 2008; Romalde, 2002).

Phenotypic identification was performed according to Bergey's Manual of Systematic Bacteriology (Garrity,1984). The tests used were: Cytochrome oxidase, Catalase and Tryptophanase activity, Carbohydrate Fermentation, Citrate, Oxidation/Fermentation of Glucose, Mobility, O/129 sensibility, growth at 42°C and at different NaCl concentrations. API 20E system evaluation was also applied to all isolates. After phenotypic identification, the isolates were identified by molecular techniques.

Strains identification confirmation was done by nucleotide sequence analyses. DNA was extracted with Fermentas Genomic DNA Purification kit (K0512), according to the procedure described by Henriques *et al* (2004). The 16S rDNA sequence was amplified with the universal primers 27F (5'- AGA GTT TGA TCC TGG CTC AG-3') and 1494R (5'-TAC GGT TAC CTT GTT ACG AC-3'). A reaction mixture of 25 µl was prepared containing 1 x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 4 % (vol/vol) bovine serum albumin (BSA - Sigma), , 0.1 µM primers , 1U Taq polymerase (Fermentas, Vilnius, Lithuania), and template DNA (ca. 10 ng). The amplification cycle was 94°C for 5min followed by 32 cycles of 94°C for 45s, 56°C for 45s, 72°C for 1:30min, with a final extension of 72°C for 10min. Amplification was confirmed by electrophoreses (1% agarose, 1x TAE buffer; 100V for 35min). The 16S rRNA gene sequences obtained from the isolated strains were sequenced using the primer 27F. Sequences were classified according to the Naive Bayesian rRNA Classifier (Version 1.0) of the Ribosomal Database Project II (http://rdp.cme.msu.edu/) and compared with different sequences available in the GenBank database using BLAST-N (http://www.ncbi.nlm.nih.gov/BLAST/).

CHARACTERIZATION OF BACTERIAL RESISTENCE TO ANTIBIOTICS

The resistance of fish bacterial pathogens (*Vibrio anguillarium*, *Vibrio parahemolitycus*, *Aeromonas salmonicida*, *Photobacterium damselae damselae, Photobacterium damselae piscicidae* and *Escherichia coli*) to antibiotics was determinate by the disk method (Oxoid) according to the manufacturer instructions. The antibiotics tested were: tetracycline, ciprofloxacin, erythromycin, chloramphenicol, amoxacillin + clavulanic acid, penicillin G, ampicillin, imipenem and neomycin. The diameter of the innibition zone was measured and compared with that indicated in the manufacturer intructions in order to classify the bacteria as sensitive or resistant to the antibiotics.

PHAGES ISOLATION AND SUSPENTION PREPARATION

Six pathogenic bacteria of fish (*Vibrio anguillarium, Vibrio parahemolitycus, Aeromonas salmonicida, Photobacterium damselae damselae, Photobacterium damselae piscicidae* and *Escherichia coli*) were used as hosts to produce phage suspensions in aquaculture water.

Five hundred milliliters of water was filtered sequentially by 3 µm and then 0.45 µm -pore-size polycarbonate membranes (Millipore). Filtered water was added of five hundred milliliters of Tryptic Soy Broth (Merck) with double concentration and one milliliter of bacterial culture. The mixture was left at ambient temperature (25 °C) for 12–18 hours and filtered through 0.45 µm pore-size membranes. The filtrate was tested for the presence of phages by inoculating 25 µl of it on lawns of bacterial culture prepared on tryptone soy agar (Merck). The plates were left at ambient temperature (25 °C) for 12–18 h and inspected for zones of clearing. Areas of clearing were cut out and inoculated in fresh specific bacterial culture in Tryptic Soy Broth (Merck) in order to obtain isolated phage plaque. The mixture was left at ambient temperature (25 °C) for 10-12 hours and filtered through 0.45 µm pore-size membranes (Millipore). Phage stocks were stored at 4°C with 1% of chloroform.

To determine the phage suspension titer the soft agar overlay technique was used (Adams, 1959). Phage suspensions were serially diluted in PBS and 500 µl of each phage dilution as well as 100 µl of fresh bacterial culture were added to 4 mL of molten soft agar tubes. The mixture was rolled between palms to enhance good mixing and overlaid on tryptone soy agar plates. After the plates were incubated at ambient temperature (25 °C) for 10–12 hours, the number of plaques was counted and the results expressed as plaque forming units per milliliter (pfu mL^{-1}).

DETERMINATION OF PHAGE HOST RANGE

To determine the phage host specificity, the soft agar overlay technique was used (Adams, 1959). Three hundred microliters of fresh bacterial culture were added to 4 mL of soft soy agar and overlaid on TSA plates. After solidification, 10 µl phage suspensions were inoculated on the plates. The plates were incubated at ambient temperature (25°C) over 10–12 hours to observe the presence /absence of phages plaques.

PHAGE SURVIVIAL DETERMINATION

The survival of *Aeromonas salmonicidae* and *Vibrio parahemolyticus* phages was tested in marine water. One hundred milliliters of water were filtered through 3 µm pore-size filters and then by 0.22 µm pore-size membranes (Poretics Products Livermore, USA). Filtered water was added of 50 µl of phage suspension. Phage titer was determined at time zero and at intervals of 72 h, using the soft agar overlay technique.

IMPACT OF PHAGES ADDITION ON BACTERIAL COMMUNITY STRUCTURE

150 mL of aquaculture water were added to each of nine erlenmeyers. Three of the erlenmeyers were added of phage suspensions: 100µl of stock with 6.15 x 10^9 PFU mL^{-1} (*Aeromonas salmonicidae*) or 250µl of stock with 1.7 x 10^{10} PFU mL^{-1} (*Vibrio parahemolyticus*). The other three erlenmeyers were added, respectively, of 100 or 250 µl of Tryptic Soy Broth (TSB) and 1% of chloroform and were used as controls (since the phages were preserved in TSB and chloroform). All the erlenmeyers were incubated at ambient temperature during 10 hours. After incubation, the samples were filtered through 0.2 µm pore-size filters.

For the extraction of bacterial DNA, 150 mL of samples from the different treatments were filtered through 0.22 µm pore-size membranes (Poretics Products Livermore, USA) and bacterial cells retained on the membranes were resuspended in 2 mL of TE buffer [10mM Tris HCl, 1mM ethylenediamine tetraacetic acid (EDTA), pH 8.0] and centrifuged. After resuspension in 200 mL TE, 2 mg mL^{-1} lysozyme solution was added to induce cell lysis and incubated at 37ºC for 1 hour according to the procedure described by Henriques *et al* (2004). DNA was resuspended in TE buffer and stored at –20ºC.

The DNA extracted was used to amplify 16 rDNA gene fragments, using a nested PCR approach. In the first PCR, the universal bacterial primers 27F and 1494R were used to amplify *ca.* 1450 bp of the 16S rDNA gene (Weisburg *et al.*, 1991). A reaction mixture of 25 µl was prepared containing 1 x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM deoxynucleoside triphosphates, 3.75 mM $MgCl_2$, 4 % (vol/vol) bovine serum albumin (BSA, Sigma) , 0.1 µM

primers synthesized by IBA , 1U Taq polymerase (Fermentas, Vilnius, Lithuania), and template DNA (*ca*. 10 ng). After 5 minutes of denaturation at 94 °C, 30 thermal cycles of 45 s at 94 °C, 45 s at 56 °C, and 1.5 minutes at 72 °C were carried out. A final extension step at 72°C for 10 min was performed to finish the reaction. One µl of the product of the first PCR was used as the template for a second PCR with bacterial DGGE primers F968-GC and R1401 (*ca*. 347 bp) according to Nubel *et al*. (1996). The reaction mixtures (25 µl) consisted of 1 µl template, 1 x PCR buffer (Fermentas, Vilnius, Lithuania), 0.2 mM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 4 % (vol/vol) acetamid (Fluka), 0.1 µM primers and 1 U DNA Taq polymerase (Fermentas). PCR products were checked using standard agarose gel electrophoresis and ethidium bromide staining (Sambrook *et al*., 1989).

Samples containing approximately equal amounts of PCR amplicons were analysed by DGGE, performed with a CBS System (CBS Scientific Company, Del Mar, CA, USA).
PCR products were loaded onto 6-9% polyacrylamide gel in 1xTAE buffer (20 mmol/L Tris, 10 nmol/L acetate, 0.5 mmol L^{-1} EDTA pH 7.4). The 6-9% polyacrylamide gel (bisacrylamide:acrylamide = 37.5:1) was made with a denaturing gradient ranging from 32 to 60%. Electrophoresis was performed at 60ºC for 16 h at 150 V. Following electrophoresis, the gels were silver stained. The solutions used were 0.1% (v/v) ethanol plus 0.005% acetic acid for fixation, 0.3 g silver nitrate for staining, freshly prepared developing solution containing 0.003% (v/v) formaldehyde and 0.33% Na OH (9%), and finally, 0.75% sodium carbonate solution to stop the development. GelCompar 4.0 program (Applied Maths) was used to analyze bacterial community profiles of the images of DGGE gels as described by Smalla *et al*. (2001)

Results

BACTERIA ISOLATION AND IDENTIFICATION

The three bacterial strains isolated from the aquaculture systems were gram-negative and were identified as *Vibrio anguillarium*, *Vibrio parahemolyticus* and *Aeromonas salmonicida*, based on phenotypic characteristics of this species (Table 3.1).

Table 3.1: Phenotypic identification of the *Vibrio anguillarium*, *Vibrio parahemolyticus* and *Aeromonas salmonicida.* (+ : Positive test, - : Negative test , R:Resistance and S: Sensitive).

Tests		Bacteria		
		Vibrio anguillarum	***Vibrio parahaemolyticus***	***Aeromonas salmonicida***
Gram Stain		-	-	-
Cytochrome Oxidase		+	+	+
Catalase Activity		+	+	+
Indol test		+	+	-
Methyl Red test		-	+	+
Citrate		+	+	-
Mobility test		+	+	-
Oxidation/Fermentation of Glucose		-	+	+
Growth at 42°C		+	+	-
Methyl Red test		-	+	+
TSB +NaCl	0%	+	-	+
	3%	+	+	+
	6%	-	+	+
	8%	-	+	+
	10%	-	-	+
Voges-Proskauer test		+	-	-
O/129 Sensibility	10µg	S	S	R
	150µg	S	S	R

The results of the analysis of the gene sequences rRNA 16S are presented in Table 3.2. The isolate 2A presented a homology of 99% with *Vibrio anguillarium,* the isolate 3-2 presented a homology of 99% with *Vibrio parahemolyticus* and the isolate 7-2 presented a homology of 99% with *Aeromonas* genus.

Table 3.2: Phylogenetic affiliation and percent similarity with the closest relative of rRNA gene clones of the bacterial isolates in aquaculture system.

	Classificação RDP[b] Classe/Ordem/Family	**Identidade BLAST-N**	**%**	**Nº de Accession[a]**
Bacteria 2A	Gammaproteobacteria Vibrionales Vibrionaceae Listonella	*Vibrio anguillarium*	99	X16895
3-2	Gammaproteobacteria Vibrionales Vibrionaceae Vibrio	*Vibrio parahemolyticus*	99	EF203212
7-2	Gammaproteobacteria Aeromonadales Aeromonadaceae Aeromonas	*Aeromonas salmonicida*	99	P000644

[a] Genebank access number of the most similar sequence
[b] RDP classification - Ribossomal Database Project

CHARACTERIZATION OF BACTERIAL RESISTANCE TO ANTIBIOTICS

The results of the resistance of fish bacterial pathogens (*Vibrio anguillarium*, *Vibrio parahemolitycus, Aeromonas salmonicida*, *Photobacterium damselae damselae*, *Photobacterium damselae piscicidae* and *Escherichia coli*) to antibiotics (tetracycline, ciprofloxacin, erythromycin, chloramphenicol, amoxacillin + clavulanic acid, penicillin G, ampicillin, imipenem and neomycin) are presented in Table 3.3.

Fish bacterial pathogens showed the highest resistance to antibiotics imipenem and neomycin, followed by ampicillin and erythromycin. All the bacteria were sensitive to Ciprofloxacin. *Vibrio parahemoliticus* showed the highest antibiotic resistance . *Vibrio anguillarium* and *Aeromonas salmonicidae* were the most sensitive bacteria to the tested antibiotics.

Table 3.3: Resistance of pathogenic bacteria antibiotics (R: resistant; S: sensitive and RI: intermediate resistance)

ANTIBIOTICS	*Vibrio anguillarium*	*Aeromonas salmonicida*	*Photobacterium damsela damselae*	*Photobacterium damsela piscicida*	*Vibrio parahemolyticus*	*E.coli*
Tetracyclin	S	S	S	RI	RI	S
Ciprofloxacin	S	S	S	S	S	S
Erythromycin	S	S	R	S	R	RI
Chloramphenicol	S	S	R	S	S	S
Amoxacillin + clavulanic acid	S	S	S	S	R	S
Penicillin G	RI	S	R	S	R	R
Ampicillin	S	RI	S	RI	RI	RI
Imipenem	R	R	R	R	R	R
Neomycin	RI	R	S	RI	R	S

PHAGES ISOLATION AND SUSPENTION PREPARATION

Using the isolated bacteria, phage suspensions of *Vibrio anguillarium, Aeromonas salmonicidae, Photobacterium damselae* subsp *damselae, Photobacterium damselae* subsp. *piscicida, Vibrio parahemoliticus* and *Escherichia.coli* were obtained with titers varying from $6.50x10^{8}$ to 3.00 $x10^{10}$ PFU/ml.

DETERMINATION OF PHAGE HOST SPECIFITY

The results of phage specificity to different host bacteria are presented in Table 3.4.

With the exception of *Aeromonas salmonicidae*, the isolated phages showed a large spectrum of infection, infecting all the pathogenic bacteria tested (Table 3.4.). Although *E.coli* phages infected all tested hosts, *E.coli* was only infected only by its phage.

Table 3.4: Phage specificity to different bacteria. (+) Formation of phage plates and (-) No formation of phage plates

	FISH PATHOGEN BACTERIAL					
Specific Phages	***Vibrio anguillarium***	***Aeromonas salmonicida***	***Photobacterium damselae damselae***	***Photobacterium damselae piscicida***	***Vibrio parahemoliticus***	***E.coli***
Vibrio anguillarium	Positive Control	+	+	+	+	—
Aeromonas salmonicida	—	Positive Control	—	—	—	—
Photobacterium damselae damselae	+	+	Positive Control	+	+	—
Photobacterium damselae piscicida	+	+	+	Positive Control	+	—
Vibrio parahemoliticus	+	+	+	+	Positive Control	—
E.coli	+	+	+	+	+	Positive Control

PHAGE SURVIVIAL DETERMINATION

The results of the survival of the *Aeromonas salmonicida* and *Vibrio parahemolyticus* phages on marine water are represented in Figure 3.2 and 3.3. The pattern of phage survival was different for the two phages tested. The abundance of *A. salmonicidae* phages decreased by one order of magnitude in the first 15 days but, after reaching a plateau, that value was maintained up to 45 days. Afterwards, the phage titer decrease slightly up to 84 to 91 days.

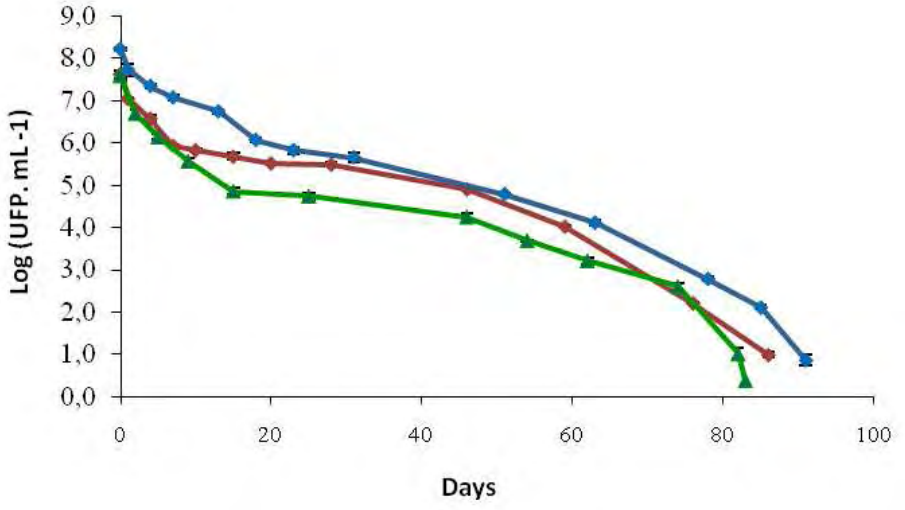

Figure 3.2: Survival of the specific phage for *Aeromonas salmonicidae*

Contrarily, the abundance of *V. parahemoliticus* phages decreased strongly during the incubation period, showing a survival period (12 to 16 days) much lower than the *A. salmonicidae* phage.

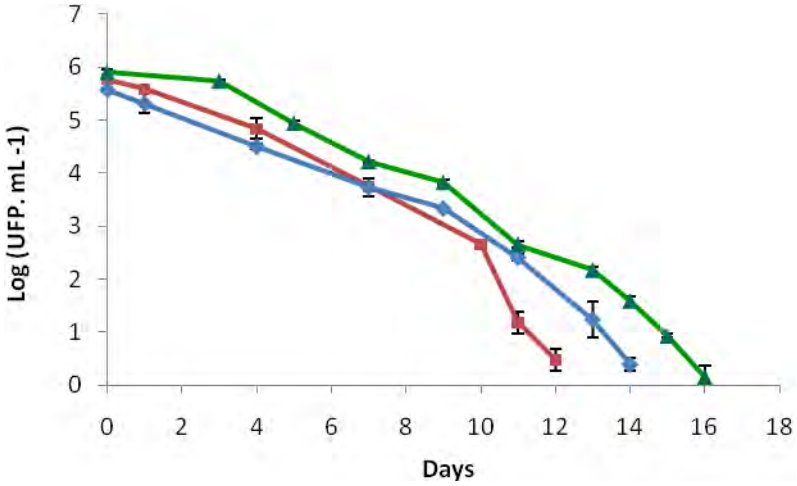

Figure 3.3: Survival of the specific phage for *Vibrio parahemoliticus*

PHAGE ADDITION EFFECTS ON THE BACTERIAL COMMUNITY STRUCTURE

The effect of the addition of the specific phage of *Aeromonas salmonicidae* and of *Vibrio parahemoliticus* in the structural diversity of the bacterial community of the aquaculture can be observed in Figure 3.5 and Figure 3.6. Addition of the phages did not significantly alter the band profile, indicating that the addition of the specific phages of *Aeromonas salmonicidae* and of *Vibrio parahemolyticus* did not result in significant effects on the bacterial community structure.

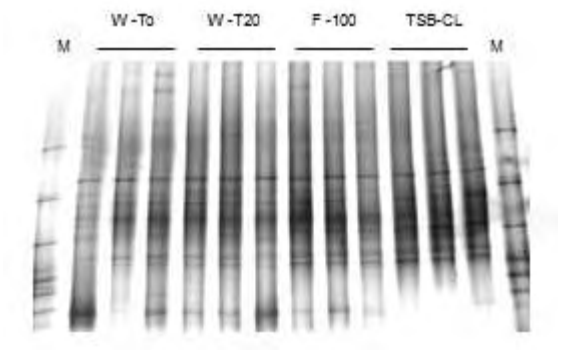

Figure 3.4: DGGE profile of 16S rDNA after *Aeromonas salmonicida* phage addition to bacterial community of the aquaculture system. (M –Molecular weights marker, W-T_0 – Water time zero; W-T_{20} - Water incubated during 10 hours; F-100 – Water added of 100 µl *Aeromonas salmonicida* phage and incubated during 10 hours; TSB-CL - Water added of chloroform and TSB medium).

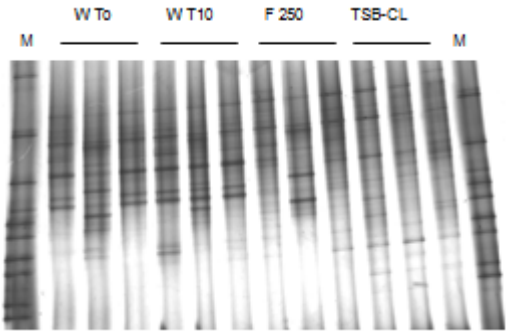

Figure 3.5: DGGE profile of 16S rDNA after *Vibrio parahemolyticus* phage addition to bacterial community of a aquaculture systems. (M –Molecular weights marker ;W-T_0 – Water time zero; W-T_{10} - Water incubated during 10 hours; F-250µl – Water added of 250µl *Vibrio parahemolyticus* phage and incubated during 10 hours; TSB-CL - Water added of chloroform and TSB medium).

Bray-Curtis similarity index ranged between ~24% and ~93%, varying widely between samples. Cluster analysis of the band patterns obtained from DGGE analysis of the experiment with *Aeromonas salmonicida* phages (Figure 3.7) revealed the occurrence of two groups that included F-100, W-T_{20} and W-T_0 and TSB-CL (control). Samples W-T_0, W-T_{20} and TSB-CL were closely grouped (similarity > 80%), but were separated from TSB-CL (similarity 75%).

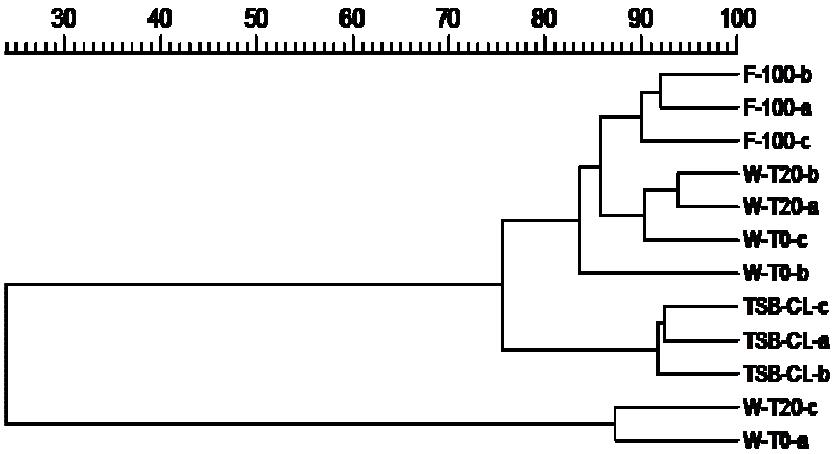

Figure 3.7: Dendrogram generated from the pattern of bands obtained by DGGE (Figure 3.5). (W-T_0 – Water time zero; W-T_{20} - Water aquaculture systems incubation during 10 hours; F-100 – Water and 100µl *Aeromonas salmonicida* phage incubation during 10 hours; TSB-CL - Water added of chloroform and TSB medium).

Cluster analysis of the band patterns obtained from DGGE analysis of the experiment with *Vibrio parahemolyticus* phages (Figure 3.8) revealed the occurrence of two main groups, one including F-250, WT_{10} and TSB-CL (control) with a similarity > 92%, and the other include WT_0 with a similarity > 86%.

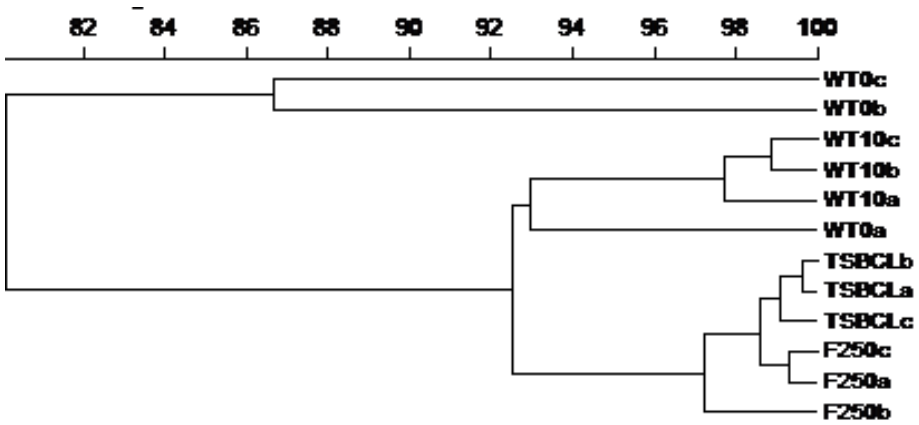

Figure 3.8: Dendrogram generated from the pattern of bands obtained by DGGE (Figure 3.6). (WT_0 – Water time zero; WT_{10} - Water aquaculture systems incubation during 10 hours; F250 – Water and 250µl *Vibrio parahemolyticus* phage incubation during 10 hours; TSBCL - Water added of chloroform and TSB medium).

Discussion

Fish contamination by pathogenic bacteria naturally present in the environment and by bacteria introduced through environmental contamination through domestic animals excreta and/or human wastes is a major problem on the expansion of aquaculture. As bacterial diseases are the major problem in the growing aquaculture industry, phage therapy represents a potential viable alternative to antibiotics and to other antimicrobial compounds to inactivate indigenous and non-indigenous pathogenic bacteria in fish farming plants. However, the success of phage therapy in aquaculture depends mainly on the phages selected to inactivate the fish pathogenic bacteria. The selected phages must remain viable in marine waters, infecting pathogenic bacteria but not altering significantly the non-pathogenic bacteria that have an important ecological role mainly in extensive and semi-intensive aquaculture regimes. The results of this study showed that phages of pathogenic bacteria can survive in marine water for up to three months, affecting a wide range of pathogenic bacteria without altering significantly the structure of the bacterial community. Unlike antibiotics, phages are self -replicating as well as self –limiting. They replicate exponentially as bacteria replicate and decline when bacterial numbers decrease. In this study it was possible to produce phages suspensions with high titers, up to 10^{10} PFU mL^{-1}, suitable to use in phage therapy, that declined when bacteria were removed, but maintaining their high concentrations even after three months in marine water. This indicates that phages isolated in this study are well adapted to the aquatic environment, surviving for long periods in marine waters at *in situ* temperatures and, consequently, unlike antibiotics, a single phage done may be sufficient to inactivate bacteria in fish farming plants.

Although it has been described that phages have specific targets, that is, they are specific to a single species or even strain of bacteria, causing less damage to the natural non-

target bacteria, the results of this study show that the majority of the strain-specific phages can inactivate pathogenic bacteria from different families. Phages of indigenous bacteria are responsible for the majority of the outbreaks in fish farms. *Photobacterium* and *Vibrio* infect the host family but also bacteria of other families, such as *Aeromonas*. Similar results were obtained by Miller et al (2003) that isolated a broad-host-range vibriophage, KVP40, from sea water using *Vibrio parahemolyticus* 1010 (EB101) as the indicator host range of KVP40 extended over at least eight *Vibrio* sp and one *Photobacterium* sp. The phage of the non-indigenous bacterium *Escherichia coli* also infected the main fish pathogenic bacteria of the Vibrionaceae family and *Aeromonas,* but their host *E. coli* was not infected by five of the phages. However, the specific phage of the indigenous *Aeromonas salmonicidae* infected only its host, the agent of furunculosis. The phages of the Vibrionaceae bacteria and of *E. coli* can be used to inactivate a broad range of different fish infections, but the phages of *Aeromonas salmonicida* can only be used to treat the furunculosis. As the isolated bacteria display resistance to a broad range of antibiotics, namely *Vibrio parahemolyticus*, and are infect by at least one of the tested phages, phage therapy is a potentially viable alternative to antibiotics, inactivating even bacteria resistant to seven different antibiotics.

Although the phages of *Vibrio parahemolytius* infect all the tested bacteria, they do not cause significant damage to the bacterial community structure. This fact suggests that *Vibrio parahemolytius* phages do not affect the non pathogenic bacteria, which are extremely important in extensive and semi-intensive aquaculture regimes, where part of the fish food results from organic matter transformed and /or produced by these bacteria.

In conclusion, as the survival of pathogenic bacterial phages is high in marine water of aquaculture systems and these have a low ecological impact on the structure of the natural bacterial community of the aquaculture systems, phage therapy can be successfully applied to inactivate fish pathogenic bacteria. Moreover, the wide host range of these phages improves their potential to inactivate fish pathogenic bacteria.

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Chapter 4 - Conclusions

This work has been presented in four chapters that include preliminary studies in order to evaluate the suitability of phage therapy to treat bacterial infections on aquaculture systems, that were then analyzed based on the experiment results obtained.

The main conclusions of this work are summarized in the following topics:

- The analysis of the density of viral community in water column of the aquaculture system (6.10×10^9 - 1.09×10^{10} cells L^{-1}), suggests that phage therapy can be a successful approach to inactive pathogenic bacteria.

- The total bacterial number revealed to maintain almost constant over the year, but with a significant variance for the specific bacterial groups during the sampling period.

- *Enterobactereacea* was the most abundant bacteria present in the aquaculture system. The presence of the indicators of faecal contamination during all the sampling period confirms the importance of the enteric bacterial group. This fact indicates that non-indigenous pathogenic bacteria are an important source of contamination in the aquaculture system.

- *Aeromonas* and *Vibrio* genera also presented high concentrations in the aquaculture, showing that indigenous pathogenic bacteria are also an important source of contamination in the aquaculture system.

- The high host range of the phages of *Enterobacteriaceae, Aeromonas* and *Vibrio* groups revealed that these phages could to be suitable for use in phage therapy treatments.

- The diversity of the bacterial group that is responsible for the most fish farming plants outbreaks (*Vibrio* genus) varied seasonally, indicating that the monitoring of its seasonal variation should be done when selecting the specific phages to inactive fish pathogenic bacteria.

- The phages of the Vibrionaceae bacteria and of *E.coli* can be used to inactive a broad range of different fish infections, but the phages of *Aeromonas salmonicida* can be used only to treat the furunculosis.

- Comparatively, the use of phages to inactive pathogenic bacteria instead of antibiotics bring many advantages such as the fact that phages are well adapted to the aquatic environment, surviving for long periods in marine waters at in situ temperatures and that a single phage dose may be sufficient to inactive bacteria in fish farming plants.

- The addition of phages of *Vibrio parahemolytius* and *Aeromonas salmonicida* represents a low ecological impact on the structure of the natural bacterial community, which is extremely important in extensive and semi-intensive aquaculture regimes.

As the survival of phages of pathogenic bacteria is high in marine water of aquaculture systems and these have a low ecological impact on the structure of the natural bacterial community of the aquaculture systems, phage therapy can be successfully applied to inactive fish pathogenic bacteria. Their wide host range is also an important fact that increases the potential of phage therapy to heal bacterial infections on aquaculture systems.