Universidade de Aveiro Departamento de Biologia
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Terapia Fágica - Uma Nova Tecnologia para Depuração de Bivalves

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Phage Therapy - A New Technology for Depuration of Bivalves

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Professora Doutora Maria Adelaide de Pinho Almeida, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro Almeida, e Professor Doutor Jesús López Romalde, Professor Catedrático da Universidade de Santiago de Compostela, e da Professora Doutora Maria Leonor Nunes, Investigadora no Centro de Investigação Marinha e Ambiental

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o júri

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palavras-chave

Terapia fágica, Variação sazonal bacteriana, Depuração, *Escherichia coli*, *Salmonella* Typhimurium,*Cerastoderme edule*, segurança alimentar

Resumo

De forma a reduzir a transmissão de agentes patogénicos através do consumo de bivalves, devem ser desenvolvidas novas tecnologias a associar à depuração, tal como a terapia fágica. A eficácia da utilização de fagos para controlar infeções bacterianas tem sido relatada em diversos estudos. No entanto, relativamente à utilização da terapia fágica para inativar bactérias patogénicas durante a depuração de bivalves, existe apenas um estudo preliminar, feito sem circulação de água (contrariamente ao que acontece na depuração industrial).

O sucesso da combinação destas duas tecnologias depende da compreensão detalhada da dinâmica sazonal e espacial da comunidade bacteriana total, incluindo bactérias associadas a doenças devido ao consumo de bivalves e indicadores de qualidade microbiológica da água, nas zonas de produção de bivalves. Durante o Verão, a comunidade bacteriana total apresentou uma maior complexidade e aumento da abundância dos principais grupos de bactérias patogénicas, indicando que deste pode ser um período crítico onde a terapia fágica deve ser aplicada. No entanto, devido ao impacto das fontes de poluição antropogénicas e outras fontes de contaminação, a terapia fágica poderá ter que ser aplicada também durante a estação fria.

Cinco novos fagos foram isolados e caracterizados para controlar *Salmonella* Typhimurium (phSE-1, phSE-2 e phSE-5) e *Escherichia coli* (phT4A e EC2A), a fim de avaliar a sua potencial aplicação durante a depuração de bivalves.

Os ensaios *in vitro* demonstraram que o uso de fagos individuais (phT4A e EC2A), pode ser uma alternativa eficaz para o controlo de *E. coli*, em particular, quando combinados em cocktail. A depuração num sistema estático com MOI 1 usando os fagos phT4A e EC2A, foi a condição em que se obteve melhores resultados (diminuição ~2.0 log CFU/g) em berbigões artificialmente contaminados. Quando berbigões naturalmente contaminados foram tratados em sistema estático com as suspensões de fagos individuais e cocktails de fagos, foram obtidas reduções semelhantes na concentração de *E. coli* (diminuição ~0.7 log UFC/g). Quando os berbigões naturalmente contaminados foram depurados com o fago phT4A com circulação de água, a concentração de bactéria foi reduzida mais rapidamente que na ausência de fagos.

Os resultados dos ensaios *in vitro* mostraram igualmente que o controlo de *S.* Typhimurium com fagos phSE-5 e phSE-2 e o cocktail destes dois fagos foi eficiente. A aplicação do fago phSE-5 e cocktail (phSE-2/phSE-5) inativou eficazmente *S.* Typhimurium em berbigões contaminados artificialmente (redução de 1.7 UFC/g com o fago phSE-5 e 0.7 log UFC/g com o cocktail phSE-2/phSE-5) e em berbigões naturalmente contaminados (redução de 0.9 log UFC/g para ambos para a suspensão simples e para o cocktail) durante a depuração em sistema estático, especialmente quando são usadas suspensões do fago phSE-5 a uma MOI baixa. Os berbigões artificialmente contaminados também foram mais eficazmente descontaminados durante a depuração com circulação de água na presença do fago phSE-5 que quando foi usada apenas depuração sem adição de fagos (i.e. o processo convencional de depuração).

Este é o primeiro trabalho em que foi testado o uso de fagos durante a depuração de berbigões naturalmente contaminados e contaminados artificialmente em sistemas com circulação de água, tal como acontece na depuração industrial de bivalves, provando assim que esta tecnologia poderá ser transposta para a indústria. Os resultados obtidos usando fagos de *E. coli* e de *S.* Typhimurium mostraram que a combinação da terapia fágica e depuração melhora a segurança microbiana dos bivalves para consumo humano, melhorando a eficiência de descontaminação. Além disso, esta abordagem também permite reduzir o tempo necessário para a depuração e consequentemente, os custos a ela associados. No entanto, a seleção e caracterização dos fagos e determinação da MOI mais adequado para ser utilizado durante a terapia fágica, é essencial para o sucesso da terapia fágica no controlo de bactérias patogénicas.

keywords Phage therapy, Bacterial seasonal variation, Depuration, Bacteriophages, *Escherichia coli*, *Salmonella* Typhimurium, *Cerastoderme edule*, food safety

Abstract

In order to reduce the infections by microbial pathogens through the consumption of bivalves, it is essential to develop alternative approaches to the conventional depuration practices. One new promising approaches is to combine the depuration of bivalves with phage therapy. The use of phages to control bacterial infections has been reported across numerous fields by many researchers. However, relatively the combination of depuration and phage therapy to eliminate pathogenic bacteria in bivalves there is only one study, and this study did not replicate industrial depuration procedures.

The successful combination of those two technologies depends on a detailed understanding of the seasonal and spatial dynamics of the overall bacterial communities, including the bacteria implicated in bivalves-related illness and the indicators of microbiological water quality, in the harvesting areas. During the summer, the total bacterial community presented high complexity and an increase of abundance of the main pathogenic bacteria, indicating that this season is the critical time frame when phage therapy should be applied. However, due to the impact anthropogenic and other sources of contamination, phage therapy could be necessary also during the cold season.

Five new phages were isolated and characterized to control *Escherichia coli* (phT4A and ECA2) and *Salmonella* Typhimurium (phSE-1, phSE-2 and phSE-5) in order to evaluate their potential application during depuration.

The *in vitro* assays indicated that the use of phages individually (phT4A and EC2A) or combined in cocktails (phT4A/EC2A), can be an effective alternative to control of *E. coli*, particularly if combined in a phage cocktail. Depuration in static seawater at MOI 1 with phage phT4A and ECA2 revealed to be the best condition (decreased of the 2.0 log CFU/g) in artificially contaminated cockles. When naturally contaminated cockles were treated in static seawater with single phage suspensions and the phage cocktail, similar decreases in the concentration of *E. coli* (decreased of the 0.7 log CFU/g) were achieved. When naturally contaminated cockles were depurated using conventional practices with phage phT4A, bacterial concentration was reduced sooner.

The assays in vitro, demonstrated that the control *S*. Typhimurium with phages phSE-5 and phSE-2 and with these two phages combined in a cocktail was efficient, paving way for the *in vivo* studies. The efficiency of bacterial inactivation with single phage suspensions of phSE-5 and phSE-2 leaded to further *in vivo* studies to control of *Salmonella* in bivalves. The application of phage phSE-5 and phage cocktail phSE-2/phSE-5 can be successfully employed to inactivate *S.* Typhimurium (reduction of 1.7 log CFU/g for phSE-5 and 0.7 log CFU/g for phSE-2/phSE-5 in artificially contaminated cockles and reduction of 0.9 log CFU/g for both in naturally contaminated cockles) during depuration in static system, especially if phSE-5 phage is used individually and if a low MOI is employed. Using conventional depuration practices in the presence of phage phSE-5, bacterial concentration is more quickly and efficiently reduced in artificially contaminated cockles.

To our knowledge, this is the first report of a depuration trial using phages in the artificially and naturally contaminated cockles using industrial depuration procedures, proving that this technology can be ported into the bivalves industry. The obtained results using phages of *E. coli* and *S.* Typhimurium indicated that combining phage therapy with depuration procedures enhance bivalve microbial safety for human consumption by improving decontamination efficiency. Moreover, this approach also displays the advantage of reducing the time required for depuration and consequently its associated costs. However, the selection and characterization of appropriate phages and the most adequate multiplicity of infection to be used in phage therapy is a critical stage to achieve a successful phage-mediated control of pathogenic bacteria.

Content

List of Abbreviations

ANOVA	Analysis of variance
ANOSIM	Analysis of similarity
BGE	Brilliant green agar
CDS	Coding Sequences
DAPI	4, 6-diamidino-2-phenylindole
CFU	Colony forming units
DGGE	Denaturing gradient gel electrophoresis
EOP	Efficiency of plating
FIL	Flesh bivalve and intra-valvular liquid
FISH	Fluorescent *in situ* hybridization
GRAS	Generally recognized as safe
MDS	Multidimensional scaling
MOI	Multiplicity of infection
MPN	Most probable number
PBS	Phosphate Saline Buffer
PCR	Polymerase Chain Reaction
PFU	Plaque forming units
TSA	Tryptone Soy Agar
TSB	Tryptic Soy Broth

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Thesis Outline and Objectives

Thesis Outline

The present work is focused on the potential use of phage therapy to inactivate bacterial implicated in seafood-related illness, during depuration of the bivalves. Production of bivalve shellfish had a fast economical growing and is an essential part of the diet of many populations. Bivalves are filter feeders that accumulate food particles and small organisms by circulating large volumes of seawater through their system; consequently, microorganisms, including human pathogens, become absorbed along with nutrients and accumulate in their bodies. Bivalves are consumed raw or lightly cooked; therefore, contaminated bivalves are potential vectors for human pathogens. Depuration is a method applied to eliminate human pathogens from live bivalves, but some pathogenic microorganisms are known to be resistant to this process and remain in bivalves after depuration. In order to reduce the risk of infections by microbial pathogens through the consumption of bivalves, it is essential to develop alternative approaches to conventional depuration practices. One of the most promising approaches is to combine bivalve depuration with phage therapy.

This document is divided into seven chapters. Chapter 1 is an in-depth literature review serving as a basis for the following experimental work, described in following chapters (Chapters 2 - 6). Chapter 7 resumes the main conclusions of the experimental work performed and Chapter 8 lists the references used in the present document.

Chapter 1 aims to provide a revision about depuration method and its limitations, applications of phage therapy and main aspects to consider for apply phage therapy during depuration. In the first part is presented a description of the bacteria implicated in seafood-related illness and the preventive measures used to control microbial contamination. It is also introduced the limitations associated to the depuration method. In the second part, the application of an innovative treatment, phage therapy, to control bacterial contamination in aquaculture is described. The main aspects to be considered for the application of this new technology are enumerated, namely: the effect of the incidence and seasonal variation of bacterial implicated in seafood-related illness in phage therapy efficiency, the selection of appropriate bacteriophages and the most adequate multiplicity of infection to be applied in

phage therapy, the bacterial/phage interaction kinetics. Other aspects as the emergence of phage-resistant mutants are also considered in this chapter.

Chapter 2 Evaluate the seasonal dynamics of the composition of bacterial communities, including the bacterial implicated in seafood-related illness, and the sanitary quality bacterial indicators, in the water of two differently classified (statutes B and C) harvesting zones at the Ria de Aveiro (Portugal).

Chapter 3 Evaluate the efficiency of two new phages of *E. coli* (phT4A and ECA2), individually or combined in cocktails, to control *E. coli* using different MOIs (1 and 100) *in vitro* assays. Describes the isolation and characterization in terms of genome analysis, host range, latent period, burst size and adsorption to the host of the new phages. The development of phage-resistant mutants was also evaluated after exposition to single phage suspensions and to phage cocktails**.**

Chapter 4 Describes the application of two new phages of *E. coli* (phT4A and ECA2), individually or combined in a cocktail, to control *E. coli* in natural and artificially contaminated cockles in static water and during depuration mimicking industrial procedures currently employed using naturally contaminated bivalves

Chapter 5 Evaluates the efficiency of three new phages of *S.* Typhimurium (phSE-1, phSE-2 and phSE-5), individually or combined in cocktails of two or three phages, to control *S.* Typhimurium growth in *in vitro* assays. Describes the isolation and characterization in terms of genome analysis (phage phSE-2 and phSE-5), survival in marine environment, host range, latent period, burst size and adsorption to the host of the new phages. The development of phage-resistant mutants was also evaluated after exposition to single phage suspensions and to phage cocktails.

Chapter 6 Describes the application of phSE-5 individually or combined in a cocktail (phSE-2/phSE-5), to control *S.* Typhimurium in natural and artificially contaminated cockles in static water and during depuration mimicking industrial procedures currently employed using artificially contaminated bivalves.

Chapter 7 Describes the main conclusions obtained in the experimental work and perspectives for future work.

Objectives

The main objective of this study was to develop an effective alternative to decontaminate bivalves by combining two techniques, depuration and phage therapy.

The specific objectives can be summarized as:

- Evaluation of the seasonal and spatial dynamics of the overall bacterial communities, namely the bacterial implicated in seafood-related illness and the indicators of microbiological water quality, in harvesting areas.
- Isolation and characterization of phages to control *S.* Typhimurium and *E. coli* (indicators of the depuration efficiency and bacterial implicated in seafood-related illness).
- Selection of an optimal protocol for viral inactivation of bacteria under different conditions (different concentrations and single phage suspensions or phage cocktails).
- Selection of appropriate phages individually or combined in a cocktail to control *S.* Typhimurium and *E. coli* during traditional depuration of bivalves.
- Development of a protocol for control *S.* Typhimurium and *E. coli* in artificially/naturally contaminated cockles, using phages during depuration in static system.
- Development of a protocol using phages during depuration mimicking industrial procedures currently employed, using artificially/naturally contaminated cockles.

Chapter 1. Introduction

1.1. Bivalves importance: economic and nutritional

Bivalve shellfish are an essential part of the diet of many populations and their commercial value has risen dramatically worldwide (Oliveira et al., 2011a). The bivalves are still of great importance in the human diet, being a source of essential nutrients and providing a protein content of high biological quality (Bernardino, 2000; Fauconneau et al., 2002; Murchie et al., 2005; Sapkota et al., 2008), and their nutritional value varies among bivalve species (Oliveira et al., 2011a).

The global tendency in the growth of human consumption of seafood and the need for its increased production will persist as the human populations expand. In recent years, the culture of bivalve molluscs has rapidly expanded and became more important for the aquaculture sector worldwide. Some bivalves are wild captured, while their majority is originated from aquaculture using seeds either produced from populations collected at natural breeding areas or from hatcheries of propagation production. However, the aquatic environment is becoming over-exploited and as a consequence of over-catching, the depletion of stocks is leading to the reduction of natural shellfish beds and to the need of human intervention in its production. The outcome is the development of artificial bivalve shellfish production and exploitation by the food industry (Hernroth et al., 2002). Aquaculture has been increasing exponentially and has became one of the fastest-growing food industries, especially in Asia (Defoirdt et al., 2004; FAO, 2006; Sapkota et al., 2008).

Freshwater finfish represented half of global aquaculture production (54%) and molluscs were the second largest aquaculture product produced worldwide (24%) (FAO, 2009). In 2010, global fish production by capture and aquaculture was 148 million tons of which 128 million tons were used for food. Bivalve molluscs represented almost 10% of the total world fishery production, but 24% in volume (on about 4.6 kg per capita,

subdivided into 1.7 kg of crustaceans, 0.5 kg of cephalopods and 2.4 kg of other molluscs) and 14% in value of the total world aquaculture production. The oysters culture, particularly *Crassostrea gigas*, dominates the global production of molluscs (FAO, 2006). The Manila clam (*Ruditapes philippinarum*), the Yesso scallop (*Patinopecten yessoensis*), the blue mussel (*Mytilus edulis*) and the blood cockle (*Anadara granosa*) are also widely produced species (Figure 1.1). Crustaceans come next in relevance, in terms of production, represented mostly by penaeid shrimps and grapsid crabs (FAO, 2009, 2006).

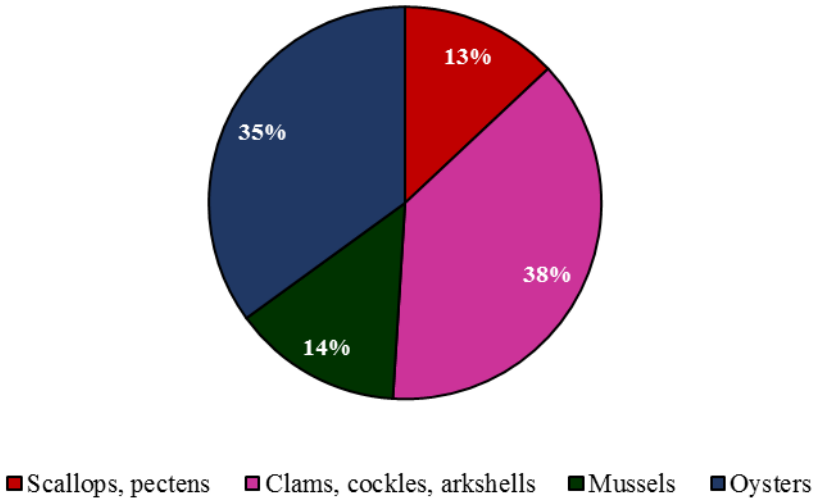


Figure 1.1 World aquaculture production the bivalve molluscs (adapted from: FAO, 2009).

1.2. General characteristics of bivalves

Bivalvia, with about 7500 species, includes animals with two shell valves such as mussels, oysters, scallops and clams. The shell has several functions: it acts as a skeleton for the attachment of muscles, it protects against predators, and it helps to keep mud and sand out of the mantle cavity in burrowing species. Shell main component its calcium carbonate and is formed by the deposition of crystals of this salt in an organic matrix of the protein, conchiolin. Three layers make up the shell: (1) a thin outer periostracum of horny

conchiolin, often much reduced due to mechanical abrasion, fouling organisms, parasites or disease, (2) a middle prismatic layer of aragonite or calcite, a crystalline form of calcium carbonate, and (3) an inner calcareous (nacreous) layer, that is either of dull texture or iridescent mother-of-pearl, depending on the species (Gosling, 2003).

The cockles (*Cerastoderme edule*) are filter feeders, using their gills to capture particulate food such as phytoplankton from the water. The cockles burrow into the protection offered by sand, mud or gravel using the foot. Contact is maintained with the surface by way of siphons that extend from the posterior end of the animal. The water flows in through the inhalant siphon, through the gills, where filtering of suspended food particles takes place, and exits through the exhalant siphon (Gosling, 2003) (Figure 1.2).

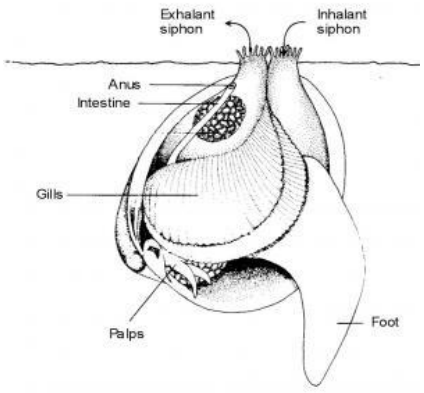


Figure 1.2 C. *edule* in sediment (adapted from: http://www.gopi.org.nz/the-inlet/natural-history/cockles-of-the-inlet/)

The digestive tract of typical bivalves consists of an oesophagus, stomach, and intestine. A number of digestive glands open into the stomach, often via a pair of diverticula, secreting enzymes to digest food in the stomach and including cells that phagocytose food particles, digesting them intracellularly. In filter-feeding bivalves, an

elongated rod of solidified mucus referred to as the "crystalline style" projects into the stomach from an associated sac. Cilia in the sac cause the style to rotate, winding in a stream of food-containing mucus from the mouth, and churning the stomach contents. This constant motion propels food particles into a sorting region at the rear of the stomach, which distributes smaller particles into the digestive glands and heavier particles into the intestine. Waste material is consolidated in the rectum and voided as pellets into the exhalent water stream through an anal pore (Figure 1.3). Feeding and digestion are synchronized with diurnal and tidal cycle.

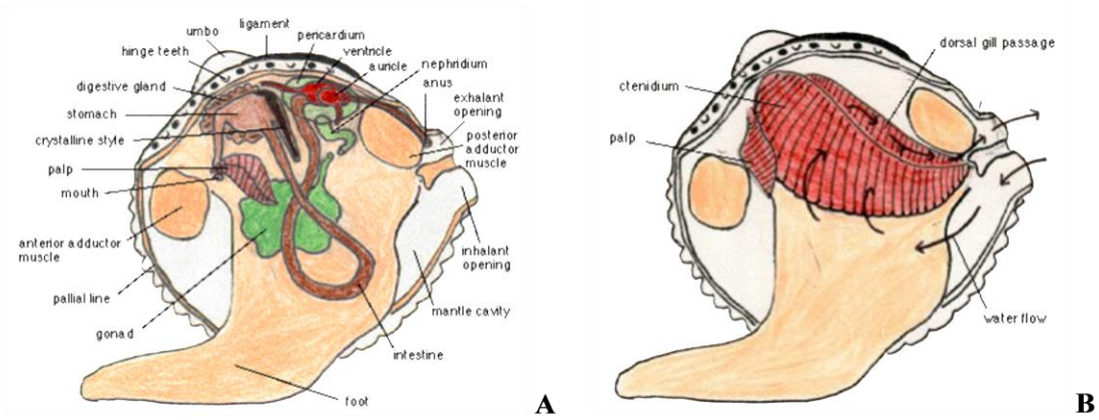


Figure 1.3 Anatomy of a typical bivalve. (A) Cross section of the bivalve, (B) Water flow through bivalves.

(adapted from: http://bio.classes.ucsc.edu/bioe122/molluscs/bivalve/bivalvia.html)

1.3. Microbial contamination and human health

Bivalve molluscan shellfish feed by filtering large volumes of seawater and accumulating food particles from their surrounding environment. When that environment is contaminated by sewage, shellfish will also accumulate human pathogenic bacteria and viruses during filter feeding and present a health risk when consumed raw or only lightly

cooked (WHO, 2010). The biological agents involved in seafood contamination consist of bacteria, viruses, parasites and microalgae (biotoxins producers) (Table 1.1), which can cause illnesses ranging from mild gastroenteritis to life-threatening diseases (Amagliani et al., 2012; Defossez and Hawkins, 1997; Dunphy et al., 2006; Huss et al., 2000; Lees, 2000; Oliveira et al., 2011a) . Foodborne disease is a public health problem which comprises a broad group of illnesses. Among them, gastroenteritis is the most frequent clinical syndrome which can be attributed to a wide range of microorganisms (Molnar et al., 2006). The risk of human intoxications is linked to the ingestion of bivalves contaminated with chemicals and biotoxins. The contaminants that are present in the environment and consequently are part of the normal biota can be considered natural causes for shellfish-derived illnesses (Shumway and Rodrick, 2009) while others can be human-generated before or after shellfish harvesting. Pre-harvesting microbial contamination (occurring naturally or as a result of human activities) includes a wide variety of viruses and pathogenic bacteria (Huss et al., 2000; Lees, 2000).

Table. 1.1 Hazards associated with bivalve mollusc consumption (FAO, 2008)

Class of hazard		**Contaminant**
Infections	Bacteria	*Salmonella* spp., *Shigella* spp., *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Vibrio cholerae*, *Campylobacter* spp., *Listeria monocytogenes*
	Viruses	Noroviruses, Hepatitis A virus, Enteroviruses, Adenoviruses
Intoxications	Chemical	Heavy metals: including Mercury (Hg), Cadmium (Cd), Lead (Pb). Organics: Dioxins, Polychlorinated Biphenyls (PCBs), Polycyclic Aromatic Hydrocarbons (PAHs), pesticides
	Biotoxin	Paralytic shellfish poisoning (PSP), Diarrhetic shellfish poisoning (DSP), Amnesic shellfish poisoning (ASP), Neurotoxic shellfish poisoning (NSP)

Pathogenic bacteria associated with seafood can be categorized into three general groups: bacteria which are normal components of the marine or estuarine environment

(indigenous bacteria), enteric bacteria which are present due to faecal contamination (non-indigenous bacteria) and bacterial contamination during processing (Feldhusen, 2000; Reilly and Kaferstein, 1997). Examples of indigenous bacteria of the marine or estuarine environment are *Vibrio cholerae, Vibrio parahaemolyticus, Vibrio vulnificus, Clostridium botulinum* and *Aeromonas hydrophila*. In the case of non-indigenous enteric bacteria resulting from faecal contamination, *Salmonella* spp., pathogenic *Escherichia coli*, *Shigella* spp., and *Campylobacter* spp., and *Yersinia enterocolitica* can be found. Regarding bacteria introduced during processing, *Bacillus cereus, Listeria monocytogenes, Staphylococcus aureus* and *Clostridium perfringens* are some examples (Feldhusen, 2000). The majority of reported seafood associated illnesses has undefined etiologies and is often assumed to be viral. While viruses are frequently the cause of seafood-related infections, hospitalizations and deaths are especially and generally related with bacteria (Butt et al., 2004; Potasman et al., 2002). Most of the infectious outbreaks cause acute symptoms and a few, generally associated with Noroviruses, Hepatitis A viruses and *V. vulnificus* infections, can be fatal (Mesquita et al., 2011; Potasman et al., 2002). *Vibrio* species account for 20% of all outbreaks of disease (Butt et al., 2004; Normanno et al., 2006; Ripabelli et al., 1999). Other bacteria pointed as the etiological agent of shellfish poisoning are *Plesiomonas shigelloides, L. monocytogenes, E. coli* and *Campylobacter, Salmonella* and *Shigella s*pecies (Brands et al., 2005; Butt et al., 2004). Although these bacteria are commonly implicated in seafood diseases, they are only occasionally traced to seafood (Potasman et al., 2002). First, some of the bacteria are autochthones, existing in the fresh water and estuaries. Naturally occurring bacteria are the most often cited causative agents of disease and death related to shellfish consumption (Croci et al., 2002; Huss et al., 2000; Wittman and Flick, 1995). Indeed, contamination by autochthone bacteria represents an

additional challenge in achieving edible bivalves. Second, the most frequent clinical syndrome (self-limiting gastrointestinal symptoms as gastroenteritis) can be related to a wide range of etiological agents and their laboratory identification requires several laborious tests (Molnar et al., 2006).

Shellfish-associated infections with *Salmonella* spp. used to be a significant problem in Europe and North America but occur less often now. Shellfish-associated gastrointestinal illness due to *Shigella* spp. and *Campylobacter* spp. has been reported from the USA but not from other countries. Illnesses due to *L. monocytogenes* have so far only been linked to the consumption of smoked bivalves (specifically mussels) and not those consumed live or cooked without being smoked (FAO, 2008).

The increased consumption of bivalves worldwide has been accompanied by numerous reports of infectious seafood outbreaks from almost all continents. The most commonly implicated bivalves are oysters, followed by clams and mussels, since they are also the most consumed bivalves (Potasman et al., 2002).

Viral diseases are usually associated with seafood such as bivalves. Adenoviruses, enteroviruses, noroviruses and hepatitis A viruses are the viruses that are more often linked to shellfish contamination (Lees, 2000; Muniain-Mujika et al., 2003) (Table 1.2).

Chemical hazards (pesticides, heavy metals and drug-residues) are usually associated with bivalves caught from polluted waters but, in general, are uncommon in commercially harvested shellfish. (Huss et al., 2000).

Biotoxins, produced by diatoms and dinoflagellates, also are a serious health problem. These toxins, usually linked to the unpredictable growth of those microalgae (microalgae blooms) are heat resistant, which means that even well-cooked bivalves might still present a risk to consumer safety. Accumulation of toxic marine algae in raw or light

cooked bivalve shellfish has been associated to Neurotoxic Shellfish Poisoning (NSP), Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Azaspiracid Poisoning (AZP) and Amnesic Shellfish Poisoning (ASP) occurrences (Botana, 2008; Huss et al., 2000).

Table 1.2 Microbial causes of bivalve shellfish-associated illness (adapted from: Butt et al., 2004; Oliveira et al., 2011).

Risk	Ethiology		Incubation period	Illness, symptoms and signs
Infection	Bacteria	*Salmonella* spp.	1-3 days	Gastroenteritis and Enteric (typhoid) fever. Diarrhea, fever, vomiting, abdominal cramps.
		Shigella spp.	24-28h	Diarrhea, fever, abdominal cramps.
		Enterotoxigenic *Escherichia coli*	1-3 days	Watery diarrhea, abdominal cramps, fever, vomiting.
		Campylobacter jejuni	2-5 days	Diarrhea, cramps, fever, vomiting
		Staphylococcus aureus	1-6 h	Nausea, vomiting, abdominal cramps, fever, vomiting
		Listeria monocytogenes	9-48 h 2-6 weeks	Listeriosis, septicaemia, central nervous system infections (meningitis), gastroenteritis, endocarditis, arthritis, encephalitis, osteomyelitis, pulmonary infections. Fever, muscle aches, nausea, diarrhea, violent or bursting headache and convulsions.
		Vibrio vulnificus	1-7 days	Wound infections, septicaemia, gastroenteritis. Vomiting, diarrhea, abdominal pain.
		Vibrio parahaemolyticus	2-48h	Wound infections, septicaemia, gastroenteritis. Nausea, abdominal cramps, watery diarrhea, vomiting
		Vibrio cholerae	24-72h	Epidemic and non-epidemic gastroenteritis. Profuse watery diarrhea, vomiting and dehydration causing death with hours.
		Aeromonas hydrophilla	Unknown	Gastroenteritis
		Plesiomonas shigelloides	20-24h	Gastroenteritis
		Clostridium botulinum	12-72h	Botulism, weakness, visual defects, death by respiratory paralysis, cardiac arrest
		Clostridium perfringens		Diarrhoea, seldom lethal
		Bacillus cereus		Vomiting, diarrhea
		Yersinia enterocolitica		Diarrhoea, vomiting, fever
	Viruses	Noroviruses	24-48h	Nausea, vomiting, watery large-volume diarrhea
		Hepatitis A virus	15-50 days	Diarrhea, dark urine, flu-like symptoms
		Enteroviruses	10-70 h	Nausea, vomiting, abdominal pain, malaise, headache, fever.
		Adenoviruses	10-70h	Nausea, vomiting, abdominal pain, malaise, headache, fever

1.4. Legislation for safeguarding consumers

The microbiological safety of bivalves as well as the suitability of coastal areas for growing and harvesting shellfish is directly related to the quality of the water in which they grow (Son and Fleet, 1980). However, water quality does not necessarily reflect the sanitary quality of shellfish harvested (Burkhardt et al., 1992).

Human health problems associated with bivalve shellfish are well recognized worldwide. The fact that outbreaks of infection continue to occur despite apparently adequate control measures highlights the role of microbial contamination of bivalves (Potasman et al., 2002). Numerous factors contribute to the existence of shellfish associated diseases outbreaks. Problems regarding monitoring shellfish growing areas and post-harvest contamination of the product (during handling storing, processing, labeling and shipping), as well as the lack of consumer education and public awareness are on the basis of diseases outbreaks (Potasman et al., 2002; WHO, 2010). The control of the risk of disease requires an integrated management and monitoring of the shellfish growing areas together with post- harvest product purification and processing (WHO, 2010).

The microbiological safety of bivalves destined for human consumption in member states of the European Union (EU) is covered by Council Regulation 853/2004 and 854/2004 (EC Regulations, 2004a). Briefly, capture/production areas for bivalves in the EU are ranked according to the levels of *E. coli* present in 100 g of flesh bivalve and intra-valvular liquid (FIL) and quantified through a 5-tubes 3-dilution most probable number (MPN) test. Based on the classification it will be determined if the shellfish can be sent for direct consumption or must be treated before commercialization (Lees, 2000). The harvesting zones are grouped in four classes, from A through D (Table 1.3). Bivalves originating from an area classified as “A” display less than 230 MPN of *E. coli* per 100 g

of FIL. Consequently, these bivalves do not require any post-harvest treatment to reduce microbiological contamination. Bivalves originating from an area classified as "B" must not exceed in 90% of sampled specimens with 4600 MPN *E. coli* per 100 g of FIL, with the remaining 10% of specimens not exceeding 46000 MPN *E. coli* per 100 g of FIL. Bivalves originated from an area classified as "C" must not exceed the limits of MPN test of 46.000 *E. coli* per 100 g of FIL. These bivalves must be relayed or cooked by an approved method (EC Regulations, 2005, 2004a). Where *E. coli* concentrations exceed 46.000 *E. coli* per 100 g of FIL (category D), harvesting is forbidden.

Table 1.3 European classification of bivalves growing areas according of *E. coli* (Lees, 2000).

Category	**MPN of *E. coli* per 100g of seafood**	**Treatment required**
A	≤ 230	Direct human consumption
B	]230, 4600]	Depuration or relaying, to meet category A
C	]4600, 46000]	Protracted relaying to meet category A Relaying to meet category B and depuration
D	> 46000	Harvesting prohibited

The final product is sealed, labelled for traceability any commercialized giving distributors and consumers the confidence of a safe certified product (Lees, 2000; Shumway and Rodrick, 2009).

1.5. Purification methods

Sanitary regulations rely on bacterial indicators of sewage contamination to classify shellfish harvesting waters and to estimate the efficiency of purification methods (Murchie et al., 2005). These purification procedures, used to reduce anthropogenic or natural

microbial contamination of bivalve molluscs, have been used since the 1920s and are now extensively used worldwide (Lees, 2000). Unhealthy harvested bivalves purge contaminants when transferred into clean natural shellfish beds (relaying) or into tanks (depuration) (Shumway and Rodrick, 2009). Depuration systems now in use worldwide include processes where water is static or changed in batches through to systems where seawater is flashed through continually or recycled through a sterilizer (WHO, 2010). Depuration consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores, and activated oxygen) or physically (UV irradiation) disinfected water to allow purification under controlled conditions (Otwell et al., 1991; Roderick and Schneider, 1994). The purification rate is positively related to body weight, clearance rates per individual are higher in bivalves with higher body weight (Troost et al., 2009). This process of depuration usually occurs in 2 days (Lees, 2000). Relaying consists of transferring contaminated harvested bivalves to cleaner areas allowing self-purification in the natural environment for longer periods, at least two months for category C shellfish, according to EU standards (Lees, 2000). Purification processes are based on the assumption that if by filtering polluted water shellfish can become contaminated, they may also purge the contaminants by filtering clean water. Thus, microbial depuration decreases the risk for potential infections due to shellfish consumption. However, it is already well documented that a number of pathogenic microorganisms still persist in depurated bivalves (FAO, 2008; Martínez et al., 2009; Rong et al., 2014). Depuration has been applied to most bivalve shellfish species sold live including oysters, clams, mussels, cockles and scallops (WHO, 2010). After depuration, bivalves may be destined for consumption if they have a level of less than 230 *E. coli* in 100 g of flesh bivalve and intra-valvular liquid (FIL), and absence of *Salmonella* sp. (FAO, 2008).

The efficiency of depuration is related, mainly, with bivalve's size, siphoning activity, and physiological conditions (Jones et al., 1991; Richards, 1988). The type and quantity of initial contamination is also related with depuration efficiency, as more contaminated bivalves require longer depuration times and different microorganisms respond differently to the purification process. Artificially contaminated molluscs depurate more rapidly than naturally contaminated molluscs in the environment (Croci et al., 2002; Jones et al., 1991; Richards, 1988). Different rates of elimination also occur when bivalves are contaminated with individual or several bacteria (Son and Fleet, 1980). The design of the system, the quality of the seawater used, the way the system works combined with the work of the operator, and the provision of the right physiological conditions for the shellfish for depuration time are factors affecting the effectiveness of depuration.

1.5.1. Depuration - practical considerations

It is essential to create the correct physiological conditions for the shellfish depuration in order to allow normal filter-feeding and avoid mortalities. These conditions are outlined in the following sections.

1.5.2. Water disinfection

Water quality is a critical factor that influences the shellfish depuration process. The water should be filtered or disinfected by ultraviolet light, chlorine, chlorine-containing compounds, ozone or other means to prevent it from becoming a source of bacterial contamination.

Filtration is a process of removing impurities, such as particles and bacteria, from water based on the pore size of membrane filters. Certain bacteria and viruses can persist in

seawater making it a source of contamination during shellfish depuration, particularly in a re-circulating system, if the water is not filtered or sterilized during the process. Both flow-through and re-circulating depuration systems can be equipped with a filtration device (Bozianis, 2014).

Chlorination was the first form of disinfection to be used in depuration systems in 1914. The disinfection capability of chlorine is well known, although bacteria are more susceptible than enteric viruses (WHO, 2010). Chlorine can be added directly to seawater to eliminate or reduce populations of bacteria. However, the presence of chlorine in seawater may be unfavorable for the growth and affect the water-filtering activity of shellfish (Bozianis, 2014).

Ozone has been used for many years in Europe for treating water for depuration processes, as this compound reacts rapidly with organic matter. Therefore, seawater intended to be used in combination with ozone for shellfish depuration, should be filtered first to remove most of the organic materials before being mixed with ozone to allow ozonated water to exhibit strong bactericidal properties (Bozianis, 2014). Unlike chlorine, ozone has the advantage of not imparting taste or odours to the shellfish neither affecting their appearance (WHO, 2010).

Iodophor disinfections in depuration systems has been carried out in Italy. Re-circulating systems using 0.1 to 0.4 mg iodophor/litre of tank water produced rapid reductions in the bacterial content of the shellfish without unduly affecting the activity of the shellfish or their organoleptic quality. However iodophors are not in common use in shellfish purification systems (WHO, 2010).

Ultraviolet irradiation is the preferred mean of disinfection for purification systems in several countries. It has an advantage over other means of disinfection in that it does not

alter the physical or chemical properties. UV light causes disruption of the DNA or RNA of the microbial cell which usually leads to lethal changes in the biochemical processes. Clearly, only the water passing through the UV unit is subjected to the disinfected action of the UV light and those organisms present within the shellfish will not be affected with this form of disinfection, unlike chlorination (WHO, 2010). However, the efficacy of UV radiation in inactivating bacteria in seawater can be influenced by particles suspended in the water. Therefore, it is recommended that a filter is installed with depuration systems, particularly for re-circulating systems so that any sand, mud and faeces released by the shellfish can be removed from the seawater before it is pumped through a UV sterilizer (Bozianis, 2014).

1.5.3. Temperature

Temperature is an important parameter to consider in the purification process according to the type of shellfish (FAO, 2008; Oliveira et al., 2011a). The metabolism of shellfish can be directly influenced by the temperature of their environment. Therefore water temperatures are required to be kept above a minimum level during depuration and these should be specified as part of the approval conditions (WHO, 2010). Lowering the temperature may help in keeping bivalves alive longer and maintain lower bacterial concentrations, however, this would also extend the period of time required for effective depuration, because removal contaminate in shellfish become less active (Oliveira et al., 2011a; Shumway and Rodrick, 2009). However, if the temperature becomes too high, then the dissolved oxygen level in the system may fall again, leading to a cessation of activity and potentially also shellfish mortalities (Shumway and Rodrick, 2009). This is likely to cause a significant increase in the turbidity of the water which in turn will reduce the

efficiency of the UV disinfection system (WHO, 2010). The shellfish themselves are also likely to be weakened by the process of spawning and, consequently, their depuration efficiency may be affected. Also, water volume and shellfish loading rates will affect the pH and the dissolved oxygen levels in the system. The number of bivalve layers in depuration recipients can promote increases in the microbial load as result of recontamination, obstruction of water flow and restrictions of shell opening (Richards, 1988).

1.5.4. Salinity

It is also necessary to provide seawater of the correct salinity range for the shellfish being depurated, and to take into account the salinity of the harvesting areas, as requirements vary according to species (WHO, 2010). The salinity of the seawater used for depuration of shellfish is of critical importance. Specifically, the salinity of the depuration processing water in which the shellfish are placed in, can affect the pumping rate and other physiological processes. Since the salinities of the depuration processing water may differ from the shellfish harvesting area, shellfish should be acclimated to their new seawater environment. The period of acclimation is variable but should be long enough to allow for adequate pumping activity to be restored. For reasons that still remain unclear, higher salinities seem to enhance the depuration process while lower salinities inhibit the process. It is recommended that the salinity of the depuration water does not vary more than 20% of that of the water where shellfish were harvested (Shumway and Rodrick, 2009).

1.5.5. Dissolved oxygen

Shellfish require oxygen to maintain normal physiological activities (Shumway and Rodrick, 2009; WHO, 2010). To facilitate normal shellfish activity, sufficient oxygen must be available in the water. Minimum dissolved oxygen levels of 50% saturation are recommended for purification systems, however, a well designed and operated system will maintain levels that are much higher than this (Shumway and Rodrick, 2009). Various factors influence the dissolved oxygen levels of the seawater in the depuration tank. These factors include: (1) the surface area of the seawater exposed to air, (2) the flow rate of seawater during depuration, (3) the number of shellfish to be depurated, (4) the physiological activity of the shellfish during depuration, (5) aeration, (6) the salinity and (7) temperature of seawater shellfish require oxygen to maintain used for depuration (Shumway and Rodrick, 2009; WHO, 2010). All these factors must therefore be carefully controlled during the purification process. The method of aeration must not disturb the normal activity of the shellfish, or the settlement of shellfish faecal material. In addition, the presence of small gas bubbles in the water may inhibit respiration of the shellfish by blocking gas exchange in the gill tissue (WHO, 2010)

1.5.6. Stocking

Shellfish must be loaded in the trays at a density that allows them the space to be able to function normally. They should be able to open as they would in the natural marine environment and carry out their normal filter-feeding activity. The density of shellfish will vary according to the species being depurated. The level of water above the shellfish should be sufficient to ensure that the shellfish remain immersed throughout of the depuration. The trays of shellfish are normally orientated so as to allow highly oxygenated

water to flow through them to the shellfish (providing oxygen and dispersing metabolic by-products) (WHO, 2010).

1.5.7. Shellfish to water ratio

The loading of bivalve shellfish for a given volume of water needs to be controlled, for maintain dissolved oxygen levels, ensure optimum shellfish activity and ensure that the build-up of metabolic by-products does not reach inhibitory levels. The capacity maximum the shellfish is specified in the conditions of each type of system. This will be dependent upon the type of system and the individual species concerned (WHO, 2010).

1.5.8. Water flow

It is essential to provide a sufficient and uniform flow of water throughout the system to maintain adequate levels of oxygen in the water and prevent the build-up of metabolic by-products which may inhibit normal shellfish activity. The flow of water in must not be so great as to prevent the settlement of faecal material or cause the disturbance of such material that has already reached the bottom of the tank (WHO, 2010).

1.5.9. Turbidity

Excessive levels of suspended particulate matter may influence the efficacy of depuration. Control of turbidity is important for two reasons. The turbidity affects the filtration rate of shellfish and reduces the penetration of UV light through the water, thereby reducing their ability to effectively purge themselves during depuration (Jackson and Ogburn, 1999). Secondly, if the turbidity is excessive, the gills of the shellfish may become clogged, again preventing effective depuration.

If the shellfish is disturbed directly by the effects of cascades, aeration or operator handling during the purification cycle, the purification should start again.

1.5.10. Limitations of depuration

This method was established for reducing fecal contamination in shellfish, such as *Salmonella* and *E. coli*, but is generally not effective against *Vibrio* spp. (FAO, 2008; Otwell et al., 1991). However, low temperature depuration was found to be effective in reducing *V. parahaemolyticus* in oysters by 2 – 3 log MPN/g (Phuvasate et al., 2012); although this process took several days. Additionally, the chemical treatments commonly used for seawater disinfection result in chemical hazards in seafood. Depuration has been shown to be ineffective in reducing a number of *Vibrio* species in bivalves and there are concerns that, if the salinity is in the right range (e.g. between 10 and 30 ppt) and the temperature is high enough (e.g. above 20 °C) an increase in the concentration of *Vibrio* spp. may occur during a depuration cycle (FAO, 2008). Chae et al. (2009) reported that during the depuration at 15ºC the concentration of *V. parahaemolyticus* and *V. vulnificus* in Gulf oysters (*Crassostrea virginica*) was reduced by 2.1 and 2.9 log MPN/g, respectively, after 48 h. More recently, other authors investigated depuration with refrigerated seawater at 5 ºC for reducing *V. parahaemolyticus* in the Pacific oysters and reported that the process reduced *V. parahaemolyticus* populations in Pacific oysters by >3.0 log MPN/g after 96 and 144 h (Su et al., 2010).

Studies on the removal of bacteria during depuration using bivalves artificially seeded with bacterial cultures tend to show a greater degree of removal than do studies using naturally contaminated shellfish (Croci et al., 2002; Jones et al., 1991; Richards,

1988). The use of such seeding in the investigation of depuration criteria or the validation of the effectiveness of commercial systems is thus questionable.

Research undertaken in northern Europe with Pacific oysters (*C gigas*) has shown that viruses are removed much more slowly during depuration than is *E. coli* (indicator of an efficient depuration). Even in properly designed and operated systems, approximately one-third of the starting concentration of viruses will remain after 2 days at 8 °C. At higher temperatures (e.g. from 18 to 21 °C) viruses are removed from the shellfish more quickly, however while most virus present will be removed after 5 – 7 days at such temperatures, some residual viral contamination may remain even when only moderately contaminated shellfish are depurated. Given that the infectious dose of these viral pathogens is thought to be low, this means that depuration cannot be regarded as a primary mitigation factor for them (FAO, 2008)

However, such reductions will obviously reduce the risk of illness to some extent and therefore it is necessary to optimize the design and operation of systems for the removal of pathogens and not to target these simply at the removal of bacterial indicators such as *E. coli.* Data on the depuration of mussels (*Mytilus* spp.) artificially seeded with Hepatitis A virus indicates that the depuration period needed for removal is also prolonged (FAO, 2008).

For reduce the risk of transmission of infections caused by microbial pathogens it is essential to develop alternative approaches. One of the most promising is the association of phages to the depuration process (Rong et al., 2014). This association will contribute to the improvement of the decontamination efficiency, likely reducing the time required for the depuration, and consequently, the production costs, with additional benefits in bivalve safety and quality.

1.6. Bivalve harvesting and production in Portugal

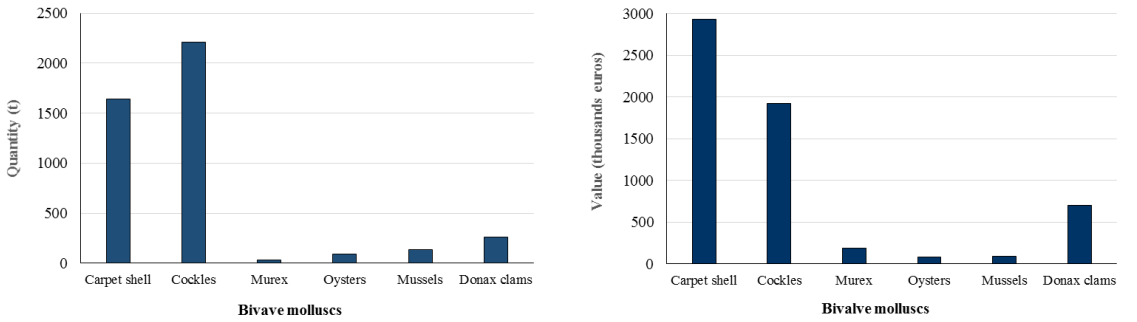
The exploitation of bivalves is an important activity in Portugal, with significant impact in the national economy. Seafood is an excellent natural resource which is common in the Portuguese diet and is in high demand by tourists. The production of bivalves is practiced from north to south of Portugal with significant impact in the national economy. It is worth of notice that Tagus estuary harbors the largest natural oyster bed of Europe (IPIMAR, 2008). In 2013, shellfish accounted for 50% of total production with carpet shell remaining as the most representative species, followed by mussels (1.640 tons) and cockles (INE, 2015).The main harvested bivalves were cockles, clams, razor, shells, mussels and oysters (Table 1.4).

Table 1.4 List of species of bivalve molluscs according to their interest to fisheries in Portugal (data from 2009) (adapted from: Oliveira et al., 2013).

Scientific	English name
Cerastoderma edule	Common edible cockle
Cerastoderma glaucum	Olive green cockle
Crassostrea angulate	Portuguese oyster
Crassostrea gigas	Pacific cupped oyster, Japanese oyster
Ensis ensis	Pod razor shell
Ensis silique	Sword razor shell
Mytilus edulis	Blue mussel
Mytilus galloprovincialis	Mediterranean mussel
Ostrea edulis	European flat oyster
Pharus legume	Bean solen
Ruditapes decussatus	Grooved carpet shell
Rudipates philippinarum	Japanese carpet shell
Solen marginatus	European razor clam
Spisula solida	Solid surf clam
Benerupis pullastra	Pullet carpet shell

In 2013, captured molluscs totalized 18504 tons, with an estimated value of 63065 thousands of euros. In 2013, cockles represented 11.9% of the national molluscs capture accounting for 1925 thousands of euros and carpet shell represented 8.9% corresponding to

a profit of 2930 thousand of euros were among the most produced species: dorax clams, mussels, oysters and murex, came next in quantity with 1.4% (706 thousands euros), 0.7% (93 thousands euros), 0.5% (79 thousands euros) and 0.2% (184 thousands euros) (Figure 1.4).



A B

Figure 1.4 Portuguese production in quantity (A) and value (B) during 2013 (adapted from: INE, 2015).

The control of salubrity of bivalve production areas (BPA) in Portugal is made by IPMA (Instituto Português do Mar e da Atmosfera). This Institute is responsible for the classification of these areas, determining if shellfish can be directly consumed or must suffer treatment(s) before commercialization (Lees, 2000) safeguarding public health. Portuguese production areas are defined in 17 estuarine zones and 9 coastal areas (IPIMAR, 2008). The areas that register the higher production of bivalves are the center of Portugal and the Algarve (southern Portugal) (Figure 1.5).

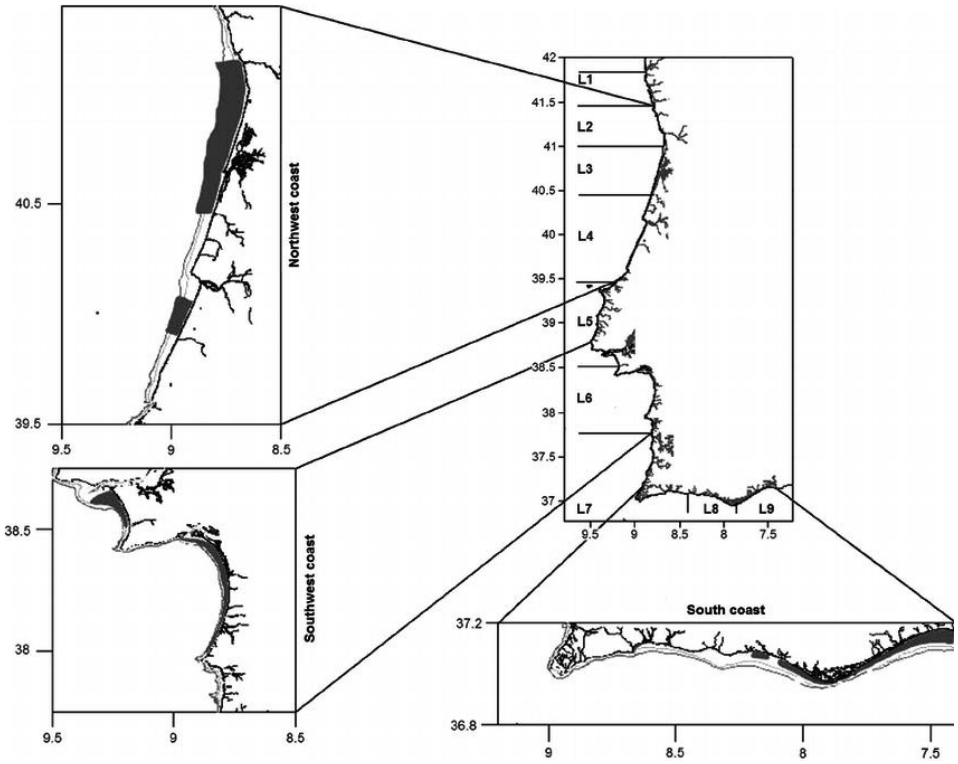


Figure 1.5 Distribution of bivalve beds (gray areas) and the bivalve production zones (L1 – L9) in Portugal (adapted from: Oliveira et al., 2014).

Ria de Aveiro is a coastal lagoon located in the western Atlantic margin of Portugal where commercialization of bivalves play an important socio-economic role (Pereira et al., 2014), especially the harvesting of cockle (*Cerastoderma edule*), which exceeds 1000 tons per year. This coastal lagoon currently has official bivalve capture/production areas classified either as B (Canal de Mira, Canal de S. Jacinto, Canal Principal and Esteiro dos Frades, Canal de Ovar, Cale da Moacha and Cale do Ouro) or C (Canal de Ílhavo, Canal do Espinheiro and Canal do Parrachil and Testada) (Figure 1.6).

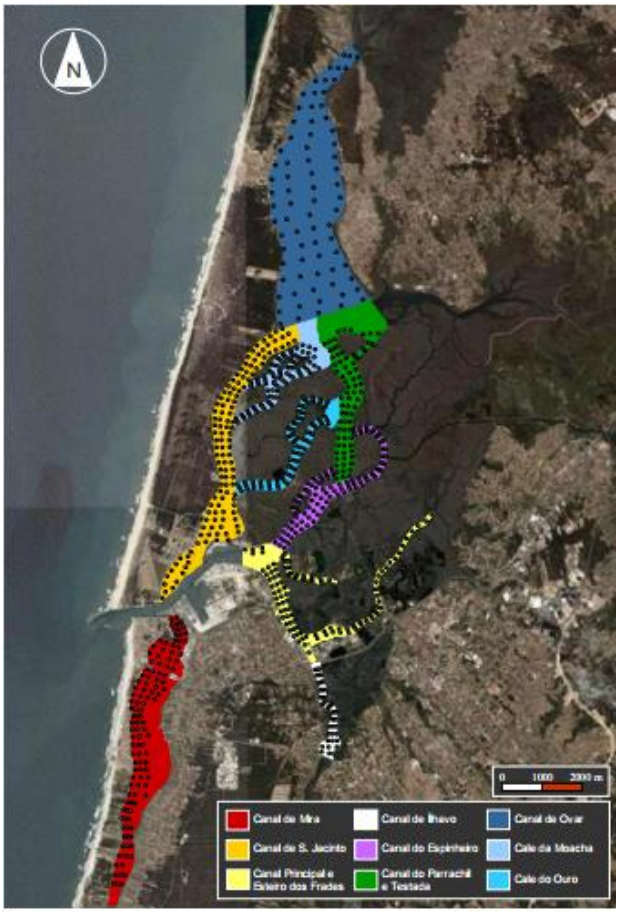


Figure1.6 Distribution of bivalve harvesting and production in Ria de Aveiro (adapted from: Pereira et al., 2014).

1.7. Phage therapy

Phage therapy consists in the utilization of bacteriophages, or phages, to inactivate pathogenic bacteria. Phages were discovered by the early 1920s and their infectious cycles were understood by that time, however, the literature of the past half-century regarding the possible therapeutic role of phages against infectious diseases is almost none. The poor understanding of mechanisms of bacterial pathogenesis and the nature of phage-host interactions, led to a succession of badly designed and executed experiments. This method of treatment was used to treat and prevent bacterial infection diseases in the former Soviet Union and Eastern Europe, however, was abandoned by the West in the 1940 with the

appearance of the antibiotics. The emergence of pathogenic bacteria resistant to antibiotics, including multiresistant bacteria, has recently motivated the western scientific community to reevaluate phage therapy as a valid option for the treatment of bacterial infections. In the time when bacterial resistance to antibiotics is increasing, the use of phages has different advantages, along with relatively few disadvantages. The great advances in scientific knowledge about the biology of these agents, can provide a better understanding of the true potential of phage therapy. Currently, the potential use of phage therapy in agriculture, veterinary biocontrol, food safety and in clinical treatment of human infections is also being studied (Balogh et al., 2010; Deresinski, 2009; Gill and Hyman, 2010; Kutter et al., 2010; Mahony et al., 2011; Mole and Maskell, 2001). Although today there are some approved applications of phage therapy in the environmental area, namely in food, agriculture and veterinary, to prevent and control bacterial infections, no application is approved for aquaculture and depuration of bivalves. However, there are already several laboratorial studies indicating that phage therapy is a promising alternative to control and prevent bacterial diseases and to prevent the spread of multiresistant bacteria in aquaculture (Almeida et al., 2009; Nakai and Park, 2002; Rong et al., 2014; Silva et al., 2016, 2014a). Relatively to the depuration of bivalves there is only one study (Rong et al., 2014).

In 2006, a major milestone in Western world phage history was achieved with the approval of the first phage-based product (ListShield^{TM}) (www.ebifoodsafety.com) to control *L. monocytogenes* in meat and received the highly desirable GRAS (generally recognized as safe) status for its use in all food products (Endersen et al., 2014; Hagens and Loessner, 2010). Phage preparations active against *E. coli* and *Salmonella* are also offered (www.omnilytics.com); some have approval for being sprayed, showered, or

nebulized on cattle and chickens respectively, prior to slaughter of the animals (Johnson et al., 2008; Sulakvelidze and Barrow, 2005). Moreover, phage preparations active against tomato and pepper pathovars of *Pseudomonas putida* (www.omnilytics.com), developed for treatment of plants against bacterial spot diseases, have been approved for use by the US Environmental Protection Agency (EPA) (Balogh et al., 2010). These recent developments highlight the fact that, besides the use of phage for direct addition to food, much effort has also gone into phage-based control of pathogens that can colonize plants or animals used in food production. It can be expected that many more phage products will appear on the market in the near to mid-term future.

1.7.1. Bacteriophages

Phages are viruses that infect only prokaryotes (bacteria and archaea) (Ceyssens and Lavigne., 2010; Skurnik and Strauch, 2006), resulting usually in propagative lyses (lytic cycle) or lysogenization (lysogenic cycle) of the infected cell (Azizian et al., 2013; Borie et al., 2014; Teng-hern et al., 2014). Lytic phages may be candidates for phage therapy, because they replicate fast within their hosts and lyse them (Azizian et al., 2013). They 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 and Forterre, 2008). They were independently discovered and described in 1915 by William Twort, and in 1917 by Felix d'Herelle that realized that they had the potential to kill bacteria (Sulakvelidze et al., 2001). They are the most abundant organisms in the biosphere and they are a ubiquitous feature of prokaryotic existence (total number estimated to be 10^{30}-10^{32}) (Bhardwaj et al., 2015; Clokie et al., 2011; Fuhrman, 1999). The presence of the phages in the biosphere is that phages co-

evolve with their host bacteria and provide the earth's ecological equilibrium in several environmental or ecological niches (Vos and Pirnay, 2015). The most of the phages are tailed phages, which accounts for 96% of all phages present on earth, belonging to the order Caudovirales (families *Myoviridae, Siphoviridae* and *Podoviridae*) (Hanlon, 2007). However, RNA phages are also present in the marine environment (Alcantara and Almeida, 1995; Cole et al., 2003; Doré et al., 2000; Grabow, 2001; Griffin et al., 2000)

1.7.2. Bacteriophages morphology

The phages present a variety of different morphological types, but the majority displays a capsid, collar and tail (Figure 1.7) (Hanlon, 2007). Phages contain a core nucleic acid encapsulated with a protein or lipoprotein capsid which is connected with a tail that interacts with various bacterial surface receptors via the tip of the tail fibers. The capsid is a protein shell often in the shape of an icosahedron and comprises the nucleic acid. The capsid has three important functions during the phage life cycle: (1) protect the phage genome during its extracellular phase, (2) enable the adsorption of the phage, fixing the virus to the host bacterium (in *Caudovirales*), and (3) the subsequent delivery of the phage genome into the host cytoplasm (Goodridge and Abedon, 2003). Phage genomes can be double-stranded DNA, single stranded DNA, or single-stranded RNA and can vary between 17 kb and 500 kb. The tail may or may not be a contractile structure to which six fibres are usually connected, containing receptors on their ends that recognize binding sites on the surface of the bacterial cell. However, not all phages have tails and tail fibers and in this situation other attachment mechanisms are present (Goodridge and Abedon, 2003; Hanlon, 2007).

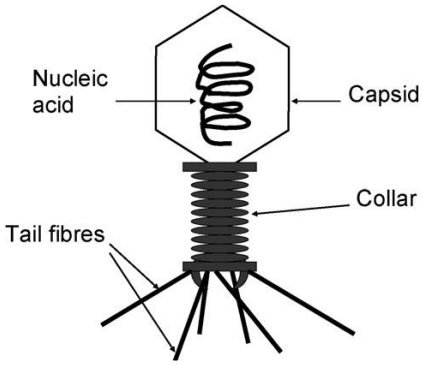


Figure 1.7 Schematic representation of a typical bacteriophage (adapted from: Hanlon, 2007).

1.7.3. Taxonomy of bacteriophages

The International Committee for Taxonomy of Viruses (ICTV) is presently responsible for the classification of the viruses. The taxonomy of these viruses is based on morphological (size and shape) and molecular characteristics (type of nucleic acid) (Table 1.5, Figure 1.8). Phages, of which there are currently 13 families and 30 genera, belong to the *Caudovirales* order, based on the phage tail structure, are divided into three families: (1) *Myoviridae*, in which viruses have a contractile tail constituted by hem, a central tube and a big capsid head (~150 nm), (2) *Siphoviridae*, in which viruses have long not contractible tails, and a relatively small capsid head (~50-60nm) (3) *Podoviridae*, in which viruses have no contractile tail and have short tails and a small capsid head (~50-60 nm) (Ackermann, 2007, 2003; Drulis-Kawa et al., 2012). The other non-tailed phages (represent less than 4%), are classified into ten families, and are cubic, filamentous, or pleomorphic and contain double-stranded or single-stranded DNA or RNA as the genome.

Table 1.5 Basic properties of phage families (adapted from: Ackermann, 2003; Hanlon, 2007).

Shape	Nucleic acid	Family name	Characteristics
Tailed	dsDNA, Linear	*Myoviridae*	Contractile tail
	dsDNA, linear	*Siphoviridae*	Long, non-contractile tail
	dsDNA, linear	*Podoviridae*	Short, non-contractile tail
Polyhedral	ssDNA, circular	*Microviridae*	Icosahedral capsid
	dsDNA, circular, superhelical	*Corticoviridae*	Icosahedral capsid with lipid layer
	dsDNA, linear	*Tectiviridae*	Icosahedral capsid with inner lipoprotein vesicle
	ssRNA, linear	*Leviviridae*	Quasi-icosahedral capsid
	dsDNA, linear, segmented	*Cystoviridae*	Enveloped, icosahedral capsid, lipids
Filamentous	ssDNA, circular	*Inoviridae*	Rod-shaped with helical symmetry
	dsDNA, linear	*Lipothrixviridae*	Enveloped filaments, lipids
	dsDNA, linear	*Rudiviridae*	Helical rods
Pleomorphic	ssDNA, circular, superhelical	*Plasmaviridae*	Pleomorphic, envelope, lipids, no capsid
	dsDNA, circular,superhelical	*Fuselloviridae*	Envelope, lipids, no capsid

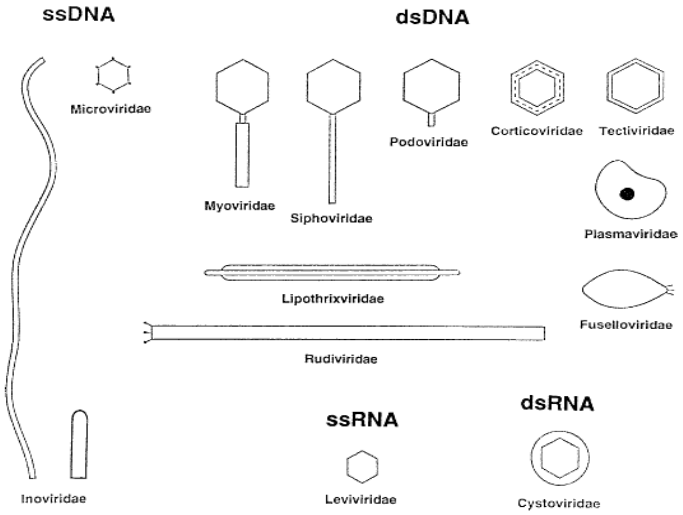


Figure 1.8 Schematic representation of the morphology of bacteriophages (adapted from: Ackermann, 2003).

1.7.4. The phage life cycle

The life cycles of phages, typically, can be classified broadly into categories, lytic (virulent) and lysogenic (temperate phages) cycles (Figure 1.7) (Hanlon, 2007; Weinbauer,

2004). There is a third way by which phages can also interact with their hosts, the pseudolysogenic cycle (Figure 1.9) (Weinbauer, 2004).

In the lytic cycle, the phage injects its genome into the host cell (Hogg, 2013). To multiply itself, the phage needs to cause lysis of the host cell to release the newly formed phages (Skurnik and Strauch, 2006). Phages adsorb to specific receptor sites on the surface of the host cell, which is followed by subsequent irreversible attachment. Ability to recognize and attach to receptor molecules on the cell surface largely dictates the host range of a bacteriophage. The penetration of tail through cell walls degraded enzymatically drives insertion of phage DNA into the cytoplasm of the host. Once inside the cell, the bases of the phage DNA are often modified to protect against the attack by restriction enzymes and cellular nucleases. The viral genome is transcribed by RNA polymerases of the host cell, producing premature mRNA. The premature mRNA function is to take over the metabolic machinery of the bacterium and redirect the metabolic processes to the manufacture of new viral components (Hanlon, 2007). After the replication and assembly of new phage particles within the host cell, the new phages are released to the environment (Hanlon, 2007). Almost all of the dsDNA phages develop enzymes that attack the bacterial peptidoglycan, like lysozymes that act at the sugar links, endopeptidases that break peptide bonds or amidases that act on amide links (Fischetti, 2005). These lytic enzymes (usually called muralytic enzymes or endolysins), coded by the phage genome, are produced within the cytoplasm but require another enzyme to allow them to cross the cytoplasmic membrane to reach its substrate. This enzyme is a holin that ruptures the membrane, allowing the lysin to degrade the peptidoglycan (Fischetti, 2005; Young et al., 2000). The holin controls the timing of the cell lysis and the release of the phage progeny. The filamentous phage can escape the host cell by extrusion through the cell wall without causing the destruction of the host, these

phages did not present relevance for phage therapy (Hanlon, 2007). The period of time between the attachment of a phage particle to the cell surface and the release of the newly synthesized phages is called the latent period, sometimes also known as the burst time (Hogg, 2013).

Temperate phages are viruses that do not enter automatically on a lytic cycle, the phage genome will integrate the genome of the host cell. A copy of the phage genome is stably maintained in a repressed state within the host genome and replicates in concert with the host chromosome (Hogg, 2013; Skurnik and Strauch, 2006). The temperate phages induce a state of lysogeny in the bacterial host (Hanlon, 2007). Cells can undergo multiple rounds of division but, occasionally, one will spontaneously lyse and release progeny phage. Alternatively, a population of lysogenic cells can be induced to lyses by submitting them to stress and treatment with mutagens or by exposure to ultraviolet light. The prophage directs the synthesis of a repressor protein which blocks the transcription of its own genes and also those of closely related bacteriophages. The presence of a prophage may therefore confer a certain type of bacterial cell immunity to other infections by phages. Lysogenic bacteria may have other advantages, in terms of acquisition of genes that confer increased virulence or pathogenicity. When one prophage escapes regulation by the repressor, its DNA is cut free inducing a lytic cycle. But, excision of the prophage DNA is often imprecise and bacterial genes adjacent to the prophage DNA may be incorporated into infectious phage DNA and then transferred into host cells. Temperate phages are generally avoided for direct use as therapeutics because they may mediate transduction of genetic material from one bacterial cell to another. Indeed, temperate phages may transmit genes that increase the virulence of the host in a process known as lysogenic conversion. As a result of their replication cycle, they do not kill all of the bacteria that they infect. Moreover, a bacterial cell harboring a

prophage within its genome becomes immune to infection by the same or closely related phages, a process known as superinfection immunity (Deresinski, 2009; Gill and Hyman, 2010; Wagner and Waldor, 2002). In contrast, virulent phages have the ability to replicate exponentially on a bacterial culture and can rapidly eliminate bacteria regardless of their antibiotic resistance profiles. This makes virulent phages very appealing candidates for use as biotherapeutic agents.

The pseudolysogen cycle (i.e., false lysogen) is described as a phenomenon where there is a constant production of phage in the presence of high host cell abundance (Ackermann and DuBow, 1987). The phage lysis results not in total host death and the abundance of phage coexists with exponential host growth. This might be the result of a mixture of resistant and sensitive host cells and/or a mixture of virulent and temperate phages Thus, in pseudolysogeny infection, the bacteriophage can either proceed with lytic infection or enter a dormant intracellular phase but in this case, the phage genome does not integrate into host cellular replicons (Wommack and Colwell, 2000). Pseudolysogeny is an environmental condition in which the bacterial cell coexists in an unstable relationship with infective viruses (Ripp and Miller, 1998, 1997). Under these conditions, host cells do not provide enough energy in order to phage entering into a true lysogenic or lytic condition.

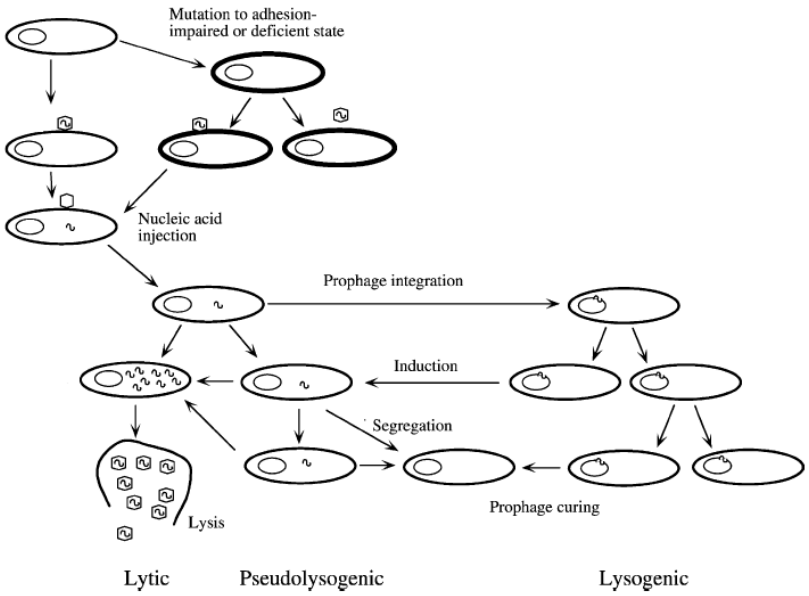


Figure 1.9 Schematic representation of the phage life cycle (adapted from: Weinbauer, 2004).

1.8. Phage therapy: a new technology in bivalve depuration

The use of phages as antibacterial agents, may offer an alternative treatment for bacterial infections. The use of phages as therapeutic agents in human medicine, veterinary science, agriculture and to control food-borne diseases is a great potential new tool (Loc-Carrillo and Abedon, 2011; Lu and Koeris, 2011; Maura and Debarbieux, 2011). However, for our knowledge, there is only one report on the combination of phage therapy and bivalve depuration. In this study, Rong et al. (2014) concluded that the application of bacteriophage (VPp1) could reduce the population of *V. parahaemolyticus* in infected oysters during depuration without water recirculation.

Their properties and behavior *in vitro* strongly support their use, including large-scale commercialization, however, still further testing *in vivo* is needed to complement the large information generated by *in vitro* studies. Among these factors is included the detailed understanding of the kinetic bacteria /phage. The selection of appropriate bacteriophages and the most adequate multiplicity of infection to be used in phage therapy

is a critical stage to achieve a successful phage-mediated control of pathogenic bacteria. Criteria such as the adsorption rate, latent period, burst size, lysogenic induction capacity, survival in the environment and emergency of phage-resistant mutants are determinant in the election of suitable phages to use in phage treatment. Another important aspect to consider is the narrow host range of phages. In order to apply phage therapy, use of bacteriophages with a broader spectrum of activity should be a priority in the investigation of these agents. Despite this, this apparent weakness of phage can be remedied through the use of viral mixtures, thus extending their range of action. Quality assurance in the massive use of this tool is, in their therapeutic role as well as their safety, is a very important aspect to consider. This issue can be a major obstacle for its development, due to the time and resources required to be invested in, among other issues, sequencing the entire genome and study of pharmacokinetic and pharmacodynamics properties (Loc-Carrillo and Abedon, 2011).

1.8.1. Variation of bacterial communities in the harvesting waters

In order to apply a successful phage therapy during depuration of bivalves it is essential to know the dynamics of the natural microbial community in the harvesting waters and in bivalves, with detail on the most important pathogenic bacteria of bivalves associated illness. The phage specificity can be a disadvantage when is not known the bacteria that cause the infection. However, this difficulty is overcomed when phage therapy is applied to cases when the main pathogenic bacteria are well known (Almeida et al., 2009). The success of phage therapy to inactivate shellfish pathogenic bacteria during the depuration process depends also of the comprehensive knowledge of the seasonal and spatial variation of the overall bacterial community, including the main pathogenic bacteria

of bivalves associated illness and the indicators of microbiological water quality, in the water of the harvesting areas and in bivalves. These studies may help define critical periods when phage therapy/depuration should be applied to reinforce the depuration process. Several studies observed that most outbreaks of *Salmonella* infection occurred during hot periods (summer and early autumn) (Checkley et al., 2000; Zhang et al., 2012), indicating that the summer season is a critical time period for the consumption of bivalve molluscs. Other studies also observed that the incidence of *V. parahaemolyticus* and *V. vulnificus* (Davis and Sizemore, 1982) and of *Aeromonas* (Maalej et al., 2004) increased in the summer months.

1.8.2. Selection of phages

The selection of appropriate phages becomes a critical factor to the success of the phage therapy as a successful phage-mediated control of pathogenic bacteria. Beside the efficiency on bacterial inactivation, among the main criteria required to select viruses for phage therapy are 1) host range, 2) adsorption rate, 3) survival in the environment; 4) the burst size and latent period; 5) no potential for lysogenic conversion and/or generalized transduction and, of course, 6) efficiency of bacterial inactivation must be evaluated and 7) safety are determinant in the election of suitable phages to use in phage treatment (Almeida et al., 2009; Mateus et al., 2014; Nakai, 2010;Pereira et al., 2011a; Skurnik and Strauch, 2006).

Therapeutic phages should have a very broad host range, which means that phages must present high virulence on a large number of bacterial strains (Koskella and Meaden, 2013; Mirzaei and Nilsson, 2015). The selected phages with a wide host range limits the number of phages in the library, and reduces the cost for clinical trials (Mirzaei and

Nilsson, 2015). Mirzaei and Nilsson (2015), observed that spot test cannot be used for identification and selection of phages to a phage library and should be replaced by EOP assays. These authors observed that high EOP was not correlated to the results from the spot tests (Mirzaei and Nilsson, 2015). Lysis is a plausible mechanism which happens when an overload of phages simultaneously infects a bacterium leading to lysis either from the action of lysins or from rapid depletion of the cells resources (Abedon, 2011).

An important factor in production is in the bacteriophage infective process: adsorption to the host cell. Studies on T-even and T-odd phages have revealed that a number of environmental factors, such as, ion concentrations, organic cofactors (i.e. L-tryptophan), pH and temperature, can have a significant impact on the adsorption of the virus to the host cell (Storms et al., 2010). The increased of the adsorption efficiency had an effect similar to increasing the initial multiplicity of infection; the number of phages produced during amplification decreased (Storms et al., 2010).

The success of phage therapy to control pathogenic bacteria also depends on viral survival and viability in the seawater, maintaining their lytic attributes. Although there are some data available about the study of the mechanisms and rates of mortality or loss of infectivity of phages in marine waters, but little is known about their time of survival in the marine environment. Suttle and coworkers have observed several processes implicated in the loss of infectivity of viruses in seawater. They used various indigenous marine phages (Suttle and Chen, 1992), cyanophage (Suttle and Chan, 1994), and a virus of *Micromonas pusilla*, a cosmopolitan marine phytoplankter (Cottrell and Suttle, 1995) and concluded that sunlight was the dominant factor controlling decay of viral infectivity in seawater. De Paepe and Taddei (2006) by comparing life history traits of 16 phages infecting *E. coli*, showed that their mortality rate is constant with time and negatively correlated to their

multiplication rate in the bacterial host. They showed that the capsid thickness and the density of the packaged genome account for 82% of the mortality rate (De Paepe and Taddei, 2006). In other studies, the authors observed that the phages of *Aeromonas salmonicida* (Pereira et al., 2011a) and *V. parahaemolyticus* (Mateus et al., 2014) survived several months in seawater (3 to 9 months).

The phage burst size (number of phages produce by each host cell) and the latent period (time elapsed from virus entry into the cell until the first progeny are released) are also important factors to consider when phages are selected. Selecting phages with high burst size (i.e. producing a large number of progenys phage), is very important. Phages cannot be administered in high doses per diffuse very poorly. A high burst size increases the probability that phages reach target bacteria, which is crucial for achieving an efficient infection. If phages can eliminate infecting bacteria faster than they can replicate, a high burst size also results in a lower risk of selection for phage resistant bacteria (Mirzaei and Nilsson, 2015). The phages that have a short latency period during isolation of new phages are more prevalent than other phages in the sample. This may be desirable for the phage therapy but a phage with long latency period and higher burst size can be never found, if a quick phage is present in sufficiently high titre in the sample from the beginning, even if this rapid phage has only a fraction of the slower phage's burst size (Mirzaei and Nilsson, 2015). The phages with high burst sizes and short latent periods are more effective to control bacteria; however, great burst sizes are associated with a long latent period (Abedon, 2011) which makes the selection for phage therapy difficult. Mateus et al. (2014) observed that the use of phages with high burst sizes and short lytic cycles clearly improves the efficiency of phage therapy. In this study, the authors used three phages of the *V. parahaemolyticus* (VP-1, VP-2 and VP-3) and observed that the VP-3 phage

presents the highest burst size (more than 3 times of those of VP-1 and VP-2 phages), the shortest lytic cycle (less than a half of those of VP-1 and VP-2 phages) and was more efficient to inactivate its host than VP-1 and VP-2 phages (more 2 log of inactivation, with the maximum of inactivation occurring 4–6 h before than those of VP-1 and VP-2 phages) (Mateus et al., 2014).

To select phages with therapeutic potential it is essential to assure that there is none potential for lysogenic conversion in order to avoid the expression of genes that encode toxins and to maintain their lytic characteristics, inactivating the pathogenic bacteria efficiently. The potential of lysogenic conversion can be evaluated through the detection of genes encoding integrase enzymes. The selection of phages must also have in consideration a potential ability to perform generalized transduction, i.e. the transmission of genes between bacteria (e.g. of virulent genes or other toxic factors on the bacteriophage) (Skurnik and Strauch, 2006). The sequencing of the phage genome for use with therapeutic agents should be made prior to further experiments (Holguín et al., 2015). The sequencing and annotation of the genome is important for the assessing of presence of potential toxins and genes associated with the lysogenic cycle, which is one concern when application of phage therapy is discussed (Skurnik and Strauch, 2006). Temperate phages should be avoided as therapeutic agents due to a variety of disadvantageous traits that could compromise the safety of the treatment. The ability to induce lysogeny rather than be obligatorily lytic potentially enhances the success of the bacterial host, making them resistant to related phages. Furthermore, they have the potential to introduce genes to the bacterial host in the process of lysogenic conversion (Abedon et al., 2011).

In the preparations of phage suspensions it is also necessary to remove the bacterial debris (such as lipopolysaccharides and endotoxins), since this components can be fatal for the treated organism (Carlton, 1999; Efrony et al., 2007; Inal, 2003).

1.8.3. Phage-resistant mutants

A major concern regarding the use of phages to control infections is the emergence of phage-resistant mutants. The development of phage-resistant bacteria can arise due to alteration or loss of the bacterial cell surface receptor, blocking the receptor by the bacterial extracellular matrix, inhibition of phage DNA penetration, production of modified restriction endonucleases degrading phage DNA, or inhibition of phage intracellular development (Labrie et al., 2010). The mutations affecting phage receptors represent the most frequent cause of phage resistance (Heller, 1992; Labrie et al., 2010). The receptors of the phages receptors are diverse bacterial surface-exposed molecules including many outer membrane proteins, sugar residues in the O antigen or lipopolysaccharide (LPS) core, teichoic acids, polysaccharides of the capsule or slime layer, or components of flagella and pili (Heller, 1992). Many phage receptors are essential virulence factors in pathogenic bacteria, some of which were identified through the selection of phage-resistant mutations. The receptors include capsular polysaccharides (Pickard et al., 2010; Smith et al., 1987; Smith and Huggins, 1982), adhesion and invasion factors(Begum et al., 2010; Pruzzo et al., 1983; Ricci and Piddock, 2010), a protein involved in intracellular growth (Spears et al., 2008), and, very commonly, different components of LPS (Petty et al., 2007, 2006; Zhang and Skurnik, 1994). If a phage receptor lies in a surface structure important for virulence, the phage-resistant mutants resulting from a loss or alteration of the receptor will be a virulent or attenuated. Such mutant bacterial clones will then be eliminated from the host

by the immune system and therefore should not present a problem if they arise during phage treatment (Levin and Bull, 2004; Ricci and Piddock, 2010; Smith et al., 1987; Smith and Huggins, 1982). Another way to overcome the problem of phage resistance is to use phage cocktails during treatment. The success of phage cocktails in the case of bacterial resistance requires that the selected phages do not possess overlapping cross resistance that is, bacterial mutants which are resistant to one phage are still sensitive to the other, and vice versa. Therefore, a cocktail consisting only of phages which use bacterial LPS as their receptors, for example, could be theoretically less successful than a cocktail made up of phages which utilized independent receptors. The ability of the phages in the cocktail to penetrate to bacteria sufficiently to achieve adsorption by multiple phages is also required. The possibility that phages could interfere with each other upon coinfection is a concern (Abedon and Thomas-Abedon, 2010). Pereira et al. (2016a) evaluated the efficacy of the E-2 and E-4 and a mixture of both phages (E-2/E-4) to control *Enterobacter cloacae*, and the results showed that the application of the phage cocktail limits the development of resistance by the bacteria. However, in several studies was observed that the colonies of resistant bacteria were smaller than that of the non-resistant ones and the former displayed slower growth (Pereira et al., 2016a; Silva et al., 2016). Colonies formed by resistant bacteria were visible only after 5 - 6 days of incubation but the non-resistant bacterial colonies were visible after 24 h of incubation. These results indicate that phage-resistant bacteria tend to be less fit and, consequently, it is expected that they will be eliminated from the environment faster than their wild-type relatives. Similar results were already registered for other bacterial hosts (Mateus et al., 2014; Silva et al., 2016).

1.8.4. Multiplicity of infection

The most adequate multiplicity of infection (MOI) to be used in phage therapy is yet a controversial aspect. It has been stated that, contrarily to the case of chemicals and other substances, precise initial doses may not be essential in treatment, because of the self-perpetuating nature of phages, revealed by an increasing of phage titers along with bacteria in infected fish or in contaminated waters (Mateus et al., 2014; Nakai, 2010; Silva et al., 2014b). Rong et al. (2014) concluded that the application of phage (VPp1) could reduce the population of *V. parahaemolyticus* in infected oysters (10^5 CFU/mL) during depuration, with decreases, after 36 h of depuration, of 1.24, 1.99, 1.74 and 2.35 log CFU/g, respectively, in the negative control (oysters infected *V. parahaemolyticus* and not treated with phage) and in oysters treated with phage VPp1 at MOI 10, 1 and 0.1. Martínez and Hipólito-Morales (2013) reported that the phage Vpms1 was effective in eliminating the adverse effects of *V. parahaemolyticus* in brine shrimp with a MOI of 45, 4.2, 2.25 and 0.45. These authors demonstrated that the reduction in the dosage of phages does not induce a significant reduction in the efficacy of Vpms1, not being detected significant differences between the doses of Vpms1. The MOI has been referred as an important factor influencing phage therapy efficiency, differing among various animals used in *in vivo* experiments namely due to the complex physicochemical environment and host defenses (Oliveira et al., 2010; Rong et al., 2014) but differing also from results obtained in *in vitro* assays (Kim et al., 2015; Tsonos et al., 2014). Tsonos et al. (2014) obtained similar results, as the efficiency of the phage cocktail used in treating CH2- infected chickens *in vivo* was negligible, even though, *in vitro*, the phages in the cocktail were able to efficiently lyse the APEC strain CH2. However, (Park and Nakai, 2003) obtained similar results *in vitro* and *in vivo*, and reported that the efficiency of the phage cocktail (PPpW-3 and PPpw-4) was

higher than that of the PPpW-3 and PPpW-4 phages when used individually in treating *Pseudomonas plecoglossicida* in both assays. Further studies are needed in order to understand the effect of low and high MOI *in vitro* and *in vivo.*

1.9. Major advantages of phage therapy

Phage therapy presents many advantages as biocontrol agents, including:

(1) High specificity to target their host, determined by bacterial cell wall receptors, leaving untouched the remaining microbiota, a property that favors phages over other antimicrobials that can cause microbiota collateral damage. Owing to their host specificity, which can range from an ability to infect only a few strains of a bacterial species to, more rarely, a capacity to infect more than one relatively closely related bacterial genus (Gill and Hyman, 2010) phages only minimally impact health-protecting normal flora bacteria (Gupta and Prasad, 2011; Skurnik et al., 2007).

(2) Self-replication and self-limiting, meaning that low or single dosages will multiply as long as there is still a host threshold present, multiplying their overall antimicrobial impact.

(3) As bacteria develop phage defense mechanisms for their survival, phages continuously adapt to these altered host systems.

(4) Low inherent toxicity, since they consist mostly of nucleic acids and proteins. However, phages can interact with immune systems, at least potentially resulting in harmful immune responses, though there is little evidence that this actually is a concern during phage treatment (Alisky et al., 1998; Carlton, 1999; Kutateladze and Adamia, 2010). Nonetheless, it can be imperative for certain phage therapy protocols to use highly purified phage preparations (Skurnik and Strauch, 2006) to prevent

anaphylactic responses to bacterial components, such as the endotoxins that can be found in crude phage lysates (Skurnik et al., 2007). Phages similarly can release bacterial components while killing bacteria in *situ*, a property associated with lysis that also can result from the application of cell-wall disrupting antibiotics.

(5) Limited impact of resistance development, bacteria will certainly develop resistance to phages, but the phages have a higher mutation and replication rate, as they can get round the adaptation of the bacteria and development of resistance is therefore limited. Moreover, it is comparably easier to find new phages because phages co-evolve with their host bacteria, outnumbering bacteria in the environment by tenfold, make possible the rapid isolation of new lytic phages from the environment for phage-resistant bacterial mutants. So, even if the bacteria acquire phage resistance, new mutant phage that acts lytically against these bacteria can be used against the targeted bacteria (Matsuzaki et al., 2003). Smith and Huggins (1983) demonstrated that infections produced by phage-resistant mutants of an enteropathogenic strain of *E. coli* and their parents could be successfully controlled with mutant phage derived from phage that had been active against the parent bacteria (Durán et al., 2002; Lucena et al., 2004). Another possibility to reduce the appearance of resistant strains during treatment with the phage preparation is to make a mixture of different phage strains (Biswas et al., 2002; Watanabe et al., 2007). Moreover, phage resistant bacteria are still not necessarily pathogenic because selection for resistance could select against virulence (Levin and Bull, 2004; Nakai, 2010; Sandeep, 2006). The relatively narrow host range exhibited by most phages (Hyman and Abedon, 2010) limits the number of bacterial types with which selection for specific phage-resistance mechanisms can occur. In addition, some mutations to resistance

negatively impact bacterial fitness or virulence due to loss of pathogenicity-related phage receptors (Capparelli et al., 2010; Skurnik et al., 2007).

(6) Phages are relatively cheap and easy to isolate and propagate but may become time consuming when considering the development of a highly virulent, broad-spectrum, and non-transducing phage.

Chapter 2. Seasonal variation of bacterial communities in shellfish harvesting waters: Preliminary study before applying phage therapy

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2.1. Abstract

The recurrent emergence of infections outbreaks associated with shellfish consumption is an important health problem, which results in substantial economic losses to the seafood industry. Even after depuration, shellfish is still involved in outbreaks caused by pathogenic bacteria, which increases the demand for new efficient strategies to control the shellfish infection transmission. Phage therapy during the shellfish depuration is a promising approach, but its success depends on a detailed understanding of the dynamics of bacterial communities in the harvesting waters. This study intends to evaluate the seasonal dynamics of the overall bacterial communities, disease-causing bacterial populations and bacterial sanitary quality indicators in two authorized harvesting-zones at Ria de Aveiro. During the hot season, the total bacterial community presented high complexity and new prevalent populations of the main shellfish pathogenic bacteria emerged. These results indicate that the spring/ summer season is a critical period during which phage therapy should be applied.

Keywords: shellfish harvesting waters, phage therapy, pathogenic bacteria, seasonal variation

2.2. Introduction

Bivalve shellfish is a nutritive food source whose consumption and commercial value have risen dramatically worldwide. Bivalves, such as mussels, clams and oysters, as filter feeding organisms, can concentrate contaminants from the surrounding water, including microorganisms that can cause several infectious diseases to humans (Brands et al., 2005; Butt et al., 2004; FAO, 2004; Huss et al., 2000; Muniain-Mujika et al., 2003; Robertson,

2007). Moreover, shellfish like oysters and sometimes clams or cockles, is often consumed raw or just lightly cooked (Bosch et al., 2009). This cooking habit, together with the fact that the whole animal, including viscera, is consumed, represents a major public health concern since shellfish act like passive carriers of human pathogenic microorganisms (Bosch et al., 2009; Lees, 2000; Murchie et al., 2005; Romalde et al., 2002).

Pathogenic bacteria associated with seafood such as bivalves can be categorized into three general groups: indigenous, non-indigenous and processing contamination (Feldhusen, 1999; Iwamoto et al., 2010; Reilly and Kaferstein, 1997). *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Clostridium botulinum* and *A. hydrophila* are indigenous bacteria of marine or estuarine environments. Non-indigenous enteric bacteria such as *Salmonella* spp., pathogenic *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica* result from faecal contamination. Some bacteria, such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium perfringens*, are introduced during processing (Feldhusen, 2000).

The microbiological control of shellfish products, as well as the classification of growing areas according to the sanitary quality, assumes particular importance (Despacho nº 14515, 2010). Adequate safeguards can be valuable in minimizing the probability of shellfish microbial contamination, from harvesting to consumption, and in the protection of public health. In Europe, the Directives 2006/ 113/CE (Directive 2006/113/CE, 2006) and 2004/41/CE (Directive 2004/41/CE, 2004) are guidelines to control the levels of microbiological indicators of shellfish. The classifications of bivalves harvesting areas in Europe are based on the *E. coli* concentration measured in 100 g of flesh bivalve and intra-valvular liquid (FIL). The harvesting zones are group in four classes, from A through D. The sequence corresponds to healthy, non-healthy, highly non-healthy and forbidden

harvesting when the most probable number (MPN) of *E. coli* per 100 g of FIL is ≤ 230, ≤ 4600, ≤ 46.000 and > 46.000, respectively. This classification defines whether shellfish can be sent directly for consumption or needs prior treatment before its commercialization (Lees, 2000). All shellfish sent for direct human consumption without any additional processing must comply with the standard of less than 230 *E. coli* in 100 g of FIL in more than 90% of samples. Harvesting from polluted (category B and C) areas is allowed but shellfish must undergo treatment, before being commercialized. In this case, shellfish can be placed on the market for human consumption following controlled self-purification in tanks of clean seawater (commercial depuration), prolonged relaying in clean seawater or commercial heat treatment or processing by any other acceptable method (Jones et al., 1991; Lees, 2000; Murchie et al., 2005). Where *E. coli* concentrations exceed 46.000 per 100 g of FIL (category D) harvesting is forbidden.

Depuration is a useful method to eliminate microorganisms from bivalves when conducted under conditions that maximize the natural filtering activity, which results in expulsion of intestinal contents. However, some pathogenic microorganisms are resistant to this process (FAO, 2008; Martínez et al., 2009; Rong et al., 2014). To reduce the risk of the development and transmission of infections caused by microbial pathogens, including multidrug-resistant bacteria, other technologies associated with depuration, such as phage therapy (application of lytic phages to prevent and/or to treat bacterial infections) must be developed. The association of the phage therapy with the depuration process would contribute to the improvement of the decontamination, reducing the time required, and consequently the production costs, increasing the bivalve safety and quality. As specific pathogen-killers, bacteriophages are effective agents for controlling bacterial infections, without affecting the normal flora (Hawkins et al., 2010; Park and Nakai, 2003). Moreover,

this is a relatively inexpensive method (Almeida et al., 2009). The potential of the use of bacteriophages to control bacterial diseases has been reported across diverse fields by numerous researchers (Almeida et al., 2009; Bueno et al., 2012; Hooton et al., 2011; Karunasagar et al., 2007; Lim et al., 2012; Mateus et al., 2014; Nakai and Park, 2002; Park and Nakai, 2003; Pereira et al., 2011a; Rong et al., 2014; Silva et al., 2014a; Viazis et al., 2011; Vinod et al., 2006). However, the inherent phage specificity, which is one of the major advantages of phage therapy, requires an extensive knowledge of phage bacterial hosts, including their spatial and temporal dynamics. Therefore, in order to apply a successful phage therapy during the depuration of bivalves it is essential to conduct a detailed study of the dynamics of the whole natural microbial community in the harvesting waters, focusing on the most important pathogenic bacteria (Pereira et al., 2011b).

The concentration of microorganisms within bivalves is higher than in the milieu where they growth, but the composition of their microbiota reflects the microbiological quality of harvesting waters (FAO, 1994). Numerous water characteristics, such as temperature, salinity, pH, concentration of nutrients and pollutants, as well as the season, influence the composition of microbial communities associated with bivalves (Richards, 2001). Consequently, the monitoring of the microbiological quality of the harvesting water is a crucial factor that must be taken into account to control outbreaks associated with shellfish consumption.

This work aimed to evaluate the seasonal dynamics of the composition of bacterial communities, including the disease-causing bacteria, and the sanitary quality bacterial indicators, in the water of two differently classified (statutes B and C) harvesting zones at the Ria de Aveiro (Portugal). The obtained results will be used to define critical periods when phage therapy/depuration should be applied to reinforce the depuration process.

2.3. Material and methods

2.3.1. Study area and sampling

Ria de Aveiro is an estuarine system located in the northwestern coast of Portugal (8''4'40W, 40'39'N) with an area of 47 km^2 (Almeida and Alcantara, 1992) connected to the Atlantic by a narrow opening (Figure 2.1), where the culture of bivalves is an activity of great socio-economic importance. The most exploited species are: grooved carpet shell clam (*Venerupis decussatus*), pullet carpet shell clam (*Venerupis pullastra*), cockle (*Cerastoderma edule*), blue mussel (*Mytilus edulis*) and grooved razor shell clam (*Solen marginatus*), reaching an annual production of about 5000 tons (Sobral et al., 2000). Samples were collected in two authorized harvesting zones. One located in Mira channel (40º36'30''N, 8º44'52''W), classified as a statute B (230–4600 MPN *E. coli* per 100 g of FIL) and the other in Ílhavo channel (40º37'50''N, 8º41'9''W) classified as a statute C (4600–46.000 MPN *E. coli* per 100 g of FIL) (Despacho n.º 15264, 2013). Ílhavo channel (CI) is close to port and industrial facilities and receives the effluent of the sewage treatment plant. Mira channel (CM) is a recreational area which has almost no industrial activity and it is subjected to some anthropogenic contamination. Sampling was performed on six dates in February, April, June, August, October and December 2012. In each sampling site, four water samples were collected at 20 cm below surface with sterile glass bottles. Samples were kept cold in refrigerated boxes and immediately transported to the laboratory, where they were processed within a period of 1–2 h.

2.3.2. Meteorological conditions and water properties

Precipitation and solar irradiance prior to sampling events were recorded at the meteorological station of the University of Aveiro, located on the vicinity of sampling

sites. 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 25 ºC, with a pH probe (Orion, Model 290 A).

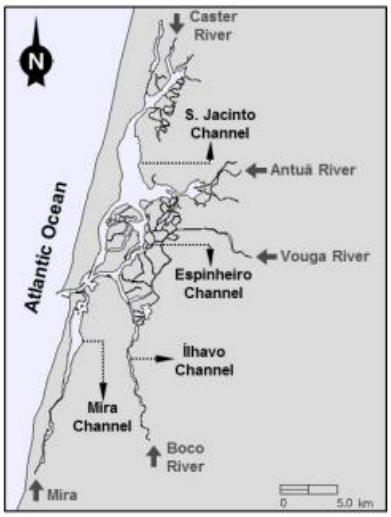

Figure 2.1. Ria de Aveiro lagoon (Portugal) with the main channels of the estuarine system, channels indicated with arrows.

2.3.3. Quantification of E. coli

E. coli was enumerated by the filter membrane method (adapted ISO 9308-1 and ISO16649-2) using the selective culture medium Triptone Bile X- glucuronide (Merck, Germany). Plates were incubated at 37 ± 1 ºC for 24 h and the number of colony forming units per one hundred milliliters (CFU/100 mL) was determined. Three independent samples were analyzed at each sampling date and for each sample, two replicated analyses were conducted.

2.3.4. Quantification of the main pathogenic bacterial groups

Samples were filtered through 0.22 µm polycarbonate filters (GE Osmonics), fixed with 4% paraformaldehyde for 30 min and rinsed twice with 1x Phosphate Saline Buffer (PBS) and Milli Q water. The filters were dried and stored at room temperature until hybridization. The relative abundances of specific groups of bacteria were determined by Fluorescent in situ hybridization (FISH), using 16S rRNA target probes (Amann et al., 1990) labelled with CY3. The probe mix Eub338-II-III (Daims et al., 1999) was used to quantify bacteria belonging to the Domain Bacteria. The bacteria belonging to the non-indigenous Enterobactereaceae family and to the indigenous *Vibrio*, *Aeromonas* and *Salmonella* genera were detected with the specific probes ENT183 (Friedrich et al., 2003), VIB572a (Huggett et al., 2008), AERO1244 (Böckelmann et al., 2000) and Salm-63 (Kutter et al., 2006), respectively. Three replicates were made for each probe. Filter sections were placed on a parafilm covered glass slide and overlaid with 30 µL hybridization solution containing 2.5 ng/mL of probe. The hybridization solution was composed by 0.9 M NaCl, 20 mM Tris–HCl (pH 7.4), 0.01% SDS, and the optimum concentration of formamide for each probe (Pernthaler et al., 2001). Filters were incubated in a hybridization oven at 46 ºC for 90 min. After hybridization, filters were washed for 20 min at 48 ºC in a washing 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 2mg/mL of 4, 6-diamidino-2-phenylindole (DAPI) and mounted on glass slides with Vectashield:Citifluor (1:4). The DAPI staining provides a measure of the abundance of total microorganisms. Samples were examined with a Leica DM microscope equipped with

the appropriate filter sets for DAPI and CY3 fluorescence. For each of the three replicates of each sample, 10 random optical fields were counted.

2.3.5. Evaluation of the structural diversity of bacterial communities

2.3.5.1. DNA extraction

For DNA extraction, four water samples of 300 mL were filtered through 0.22 lm pore-size filters (Poretics, USA). Collected cells were resuspended in 2 mL of TE buffer (pH 8.0) and centrifuged for 15 min at 13.000 *g*. After resuspension in 200 µL TE, 1 mg/mL lysozyme solution was added to induce cell lysis and incubated at 37 ºC for 1 h (Pereira et al., 2011b). DNA extraction was performed using the genomic DNA purification kit (MBI Fermentas, Lithuania). After extraction, DNA was resuspended in TE buffer and stored at - 80ºC until analysis. The yield and quality of DNA were checked after electrophoresis in a 0.8% (w/v) agarose gel.

2.3.5.2. Amplification of 16S ribosomal RNA gene sequences

The extracted DNA was used to amplify 16 rRNA gene fragments, using a nested PCR approach. In the first PCR, the universal bacterial primers 27F (5´-AGA GTT TGA TCC TGG CTC AG-3´) and 1494R (5´-TAC GGT TAC CTT GTT ACG AC-3´) were used to amplify ca. 1450 bp of the 16S rRNA gene (Weisburg et al., 1991). A reaction mixture of 25 µL was prepared containing 1 x PCR buffer (MBI Fermentas, Vilnius, Lithuania), 0.2 mM deoxynucleoside triphosphates (MBI Fermentas, Vilnius, Lithuania), 3.75 mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania), 4% (vol/vol) bovine serum albumin (BSA – Sigma–Aldrich, Co., St. Louis, MO), 0.1 µM primers, 1U Taq polymerase (MBI Fermentas, Vilnius, Lithuania) and template DNA (ca. 10 ng). Reaction was performed in

a MyCyclerTM termal cycler (Bio-Rad Laboratories, Hercules, CA, USA). Denaturation of the template for 5 min at 94 ºC was followed by 32 cycles of 45 s at 94 ºC, 45 s at 56 ºC, and 2 min at 72 ºC. The final extension lasted 10 min at 72 ºC. For DGGE analysis, a 410-bp rRNA gene fragment was amplified with primers F968-GC-clamp (5´-GC-clamp-AACGCGAAGAACCTTAC-3´) and R1401 (5´-GCGTGTGTACAAGACCC-3´) (Integrated DNA Technologies, BVBA, Munich, Germany) (Nübel et al., 1996) using as template 1 µL of the product obtained from the first PCR. Amplification was conducted in a MyCyclerTM thermal cycler (Hercules, CA, USA). Additionally, the reaction mixture contained 4% acetamide (Sigma–Aldrich, Co., St. Louis, MO), 0.2 mM deoxynucleoside triphosphates (MBI Fermentas, Vilnius, Lithuania), 1 x PCR buffer (MBI Fermentas, Vilnius, Lithuania), 0.1 µM of each primer, 3.75 mM $MgCl_2$ (MBI Fermentas, Vilnius, Lithuania), and 1 U Taq polymerase (MBI Fermentas, Vilnius, Lithuania). After 4 min of initial denaturation at 94 ºC, 34 cycles of 1 min at 95 ºC, 1 min at 53 ºC and 1.30 min at 72 ºC were performed. Finally, an extension step at 72 ºC for 10 min was carried Positive (DNA from a known bacterial species) and negative (water only) controls were included in every PCR reaction. PCR products were checked after electrophoresis in a 0.8% (w/v) agarose gel.

2.3.5.3. Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with the DCode System (Universal Mutation Detection System, Bio-Rad. PCR amplification products were loaded onto 6.5% acrylamide gels using a denaturing gradient ranging from 40% to 58%, where 100% denaturant corresponded to 7 M urea (VWR International, LLC, Radnor, PA) and 40% (v/v) formamide (Sigma–Aldrich, Co., St. Louis, MO). A marker standard composed by 11

bands halting at different denaturant concentrations was included in the extremities of each gel (Heuer et al., 2002). The electrophoresis was performed at 58 ºC for 160 min at 160 V in 1 x Tris–acetate-EDTA buffer (5 Prime, Hamburg, Deutschland). After electrophoresis, the gels were stained and fixed for 20 min with 0.3 g silver nitrate (Sigma–Aldrich, Co., St. Louis, MO) in 0.1% (v/v) etanol 96% (Aga S. A., Loures, Lisboa) and 0.005% acetic acid (Merck KGaA, Darmstadt, Germany), rinsed, and then submerged in a developing solution of 0.003% (v/v) formaldehyde 37% (Sigma–Aldrich, Co., St. Louis, MO) and 0.33% sodium hydroxide (9%) (Merck KGaA, Darmstadt, Germany). Finally, 0.75% Na_2CO_3 was added to stop the development, and the gel was scanned using a Molecular FX apparatus (Molecular Image FX apparatus, Bio-Rad Hercules).

2.3.5.4. Analysis of DGGE patterns

The gels were digitalized and analyzed with the software package Gelcompar 4.0 program (Applied Maths) as previously described by Smalla et al. (2001). After automatic band search, the bands detected were carefully checked and artefacts were removed. The band positions and their corresponding intensities (surface) from each treatment were exported to Excel (Microsoft). The band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane. The effect of each treatment was evaluated based on the calculated Bray–Curtis similarities, using analysis of similarities (ANOSIM) to assess the significance of separation between microbial communities from different treatments (Ramette, 2007). The R value in ANOSIM ranges from 0 to 1, where $R > 0.75$ indicates significant differences, $R > 0.5$ moderate separation, $R = 0.25$ to 0.5 indicates broadly overlapping but separable and $R < 0.25$ high similarity (Clarke and Gorley, 2001; Sheader and Van Dover, 2007).

2.3.6. Data analysis

The statistical analysis of data was performed with the IBM SPSS Statistics 22.0 software. Normal distribution was assessed by the Kolmogorov–Smirnov test and homogeneity of variances by the Levene test. The non-parametric test Mann–Whitney U was used to compare *E. coli* concentration and the abundance of possible pathogenic bacterial groups at the two studied sites. To test the significance of differences within sampling events was used the Friedman's ANOVA and the post hoc test described by Siegel and Castellan, (1988), using the critical difference of 3.74. The relations between the different parameters were examined using a Spearman correlation. The values were considered significantly different after applying the Bonferroni´s correction to a = 0.05.

2.4. Results

2.4.1. Meteorological conditions

The values of solar irradiance and precipitation are shown in Table 2.1. The three-day average values of solar irradiance prior to sampling events varied between 6515.4Wm^{-2}, in December and 24496.4Wm^{-2} in June. Precipitation (cumulative 21 days before sampling date) oscillated from 0.0 mm in February and 103.8 mm in December.

2.4.2. Water properties

The values of salinity, temperature, oxygen concentration and pH in the water samples collected at the two studied sites are shown in Table 2.1. In the Mira channel, salinity varied between 13.9, in December and, 28.6 in June and August. Temperature oscillated from 14.6 ºC in October to 22.8 ºC in August and the dissolved oxygen ranged between 6.2 mg/L in October and 10.8 mg/L in April. pH varied between 7.8 in August and 8.1 in

December. In the Ílhavo channel, salinity varied between 17.1 in December and 28.4 in August. Temperature oscillated from 15.2 ºC in December and 23.0 ºC in August and the dissolved oxygen ranged between 5.8 mg/L in October and 10.1 mg/L in February and April. pH varied between 7.8 in June and August and 8.4 in December.

Table 2.1 Water properties in the Mira and Ílhavo channels during the study period.

Sampling dates	Precipitation (mm)	Solar irradiance (W m^{-2})	Mira Channel				Ílhavo Channel			
			Salinity	Temperature (ºC)	pH	Dissolved oxygen (mg/L)	Salinity	Temperature (ºC)	pH	Dissolved oxygen (mg L)
February	0.0	11820.0	26.4	15.0	7.9	9.7	26.1	15.5	8.0	10.1
April	62.6	22584.2	26.0	16.2	8.0	10.8	26.9	15.9	7.9	10.1
June	28.4	24496.4	28.6	20.5	7.9	7.5	28.2	20.8	7.8	6.2
August	18.4	16061.2	28.6	22.8	7.8	7.1	28.4	23.0	7.8	6.4
October	75.2	8617.6	26.5	14.6	7.9	6.2	27.8	15.7	7.9	5.8
December	103.8	6515.4	13.9	14.9	8.1	9.3	17.1	15.2	8.4	9.6

2.4.3. E. coli concentration

The variation of *E. coli* concentration (Figure 2.2) was distinct in the two sampling sites (Mann–Whitney U test, $p < 0.05$). In the Ílhavo channel, the concentration of *E. coli* was higher than in the Mira channel and varied between 21 CFU/mL (April 2012) and 208 CFU/mL (December 2012). A clear increase in the abundance of *E. coli* was noticed over the study period (June, August, October and December), reaching the highest values on June and December. The seasonal differences were statistical significant between April and, June, August and December (Friedman´ s test, $p < 0.05$). In the Mira channel, the highest value of *E. coli* was obtained in December 2012 (24.5 CFU/100 mL) and the lowest in April 2012 (5.75 CFU/100 mL). The differences between these two sampling moments were statistically significant (Friedman's test, $p < 0.05$).

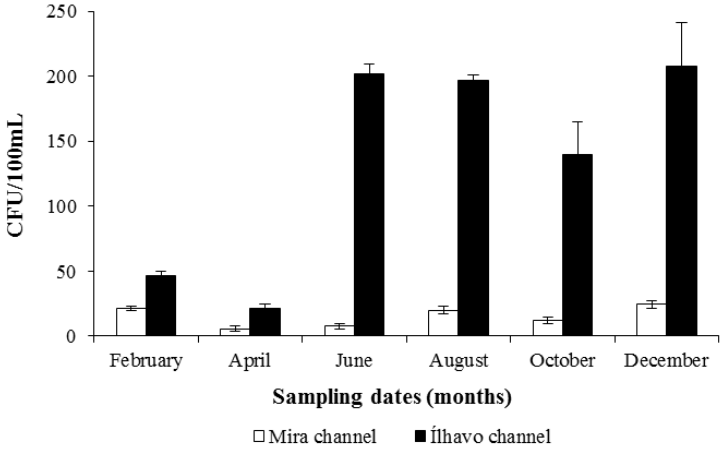

Figure 2.2 Seasonal variation of *E. coli* in Mira and Ílhavo channels

2.4.4. Abundance of total bacterial cells

Microbial abundance (DAPI counts) was significantly higher (Mann–Whitney U test, $p < 0.05$) in the Ílhavo Channel (1.9 – 4.2 x 10^9 cells/L) compared to the Mira channel (1.6 –3.6 x 10^9 cells/L) (Figure 2.3). The relative abundance of the Bacteria domain (Eub338-II-III), ranged from 81.7% to 96.1% and was similar (Mann–Whitney U test, $p > 0.05$) in the Mira and Ílhavo channels (data not shown).

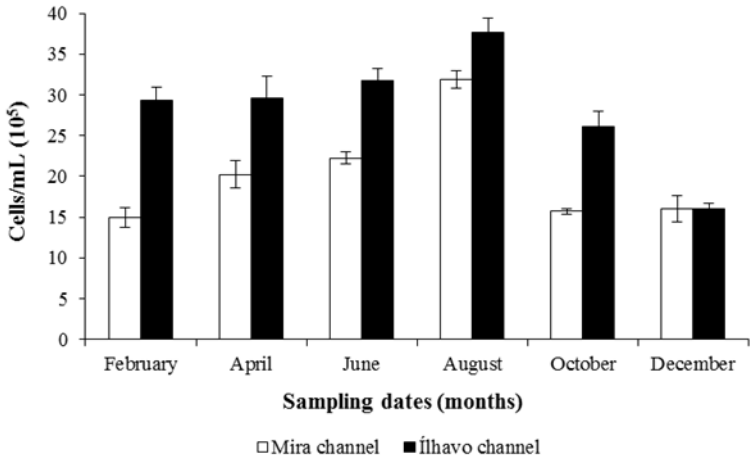

Figure 2.3 Total microbial abundance (DAPI counts) in Mira and Ílhavo channels.

2.4.5. Evaluation of the seasonal dynamics of bacterial community structure

Bacterial community structure in the water of Mira and Ílhavo channels was examined by comparing DGGE profiles of 16S-rDNA fragments corresponding to the different sampling moments (Figures 2.4 and 2.5). Reproducibility of PCR amplification and DGGE was confirmed by similar results obtained for the four samples analyzed at each date (Figures 2.4B and 2.5B). DGGE profiles of the Mira between the structural diversity of bacterial in water samples collected in the different months. The communities in the water samples collected in June and August 2012 showed the highest value of similarity (>75%) and the lowest similarity corresponded to the February samples (<50%). In October and December, the bacterial communities present in the water samples showed similarity index (~ 60%). Statistical analysis confirmed that the bacterial community in the Mira channel differed significantly among the different sampling moments (ANOSIM R = 1).

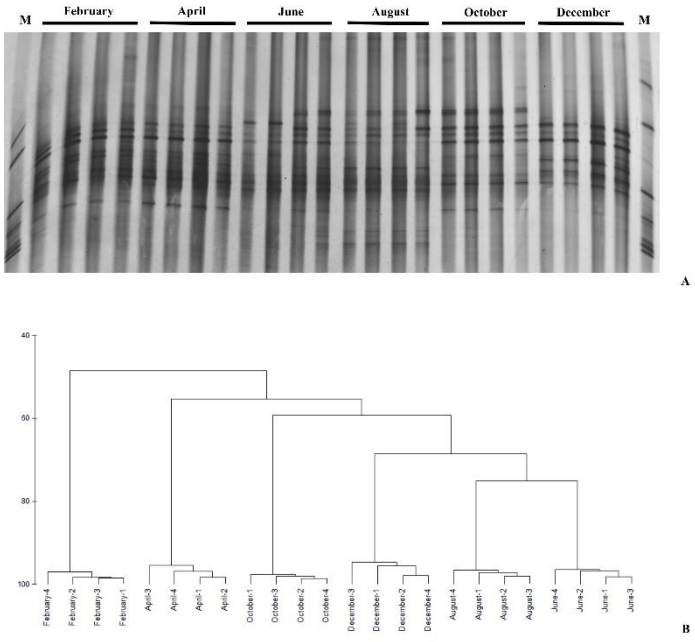

Figure 2.4 Seasonal DGGE profile of 16S rDNA of bacterial communities of the Mira channel (A) and dendrogram generated from the pattern of bands obtained by DGGE (B).

In the Ílhavo channel, the Bray–Curtis similarity index for bacterial population ranged from ~ 45% to ~80%, varying between sampling moments (Figure 2.5B). Statistical analysis confirmed that the bacterial community in the Ílhavo channel differed significantly between different periods (ANOSIM R = 1). Cluster analysis of the band patterns obtained from DGGE analysis (Figure 2.5B) of the water samples of the Ílhavo channel, showed the lowest similarity index between December (~ 45%) and the remaining samples (Figure 2.5B). The communities in the water samples collected in August showed the highest value of similarity (80%) with water samples collected in October 2012. In June, April and February, the bacterial communities present in the water samples showed similarity index (>60%).

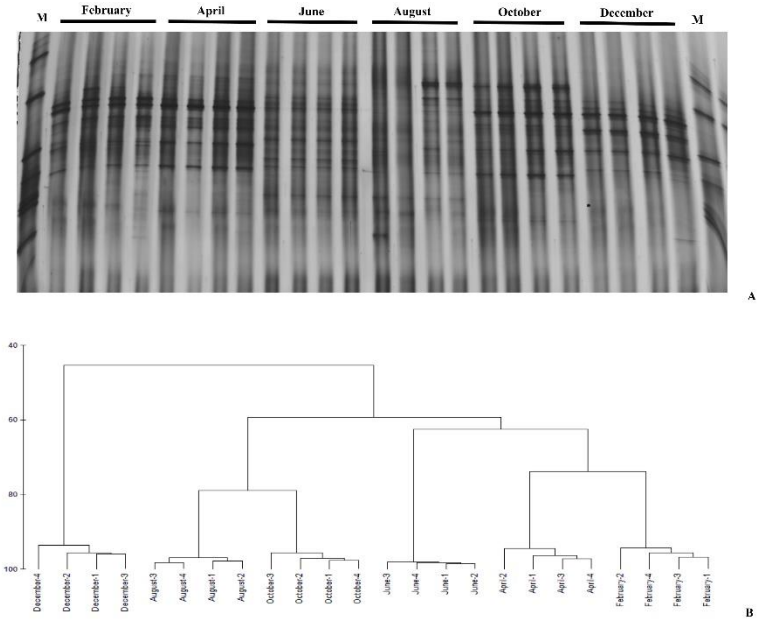

Figure 2.5 Seasonal DGGE profile of 16S rDNA of bacterial communities of the Ílhavo channel (A) and dendrogram generated from the pattern of bands obtained by DGGE (B).

DGGE profiles of 16S rDNA fragments of two sites (Figure 2.6) indicate that communities in the Mira and Ílhavo channel were statistically distinct in all sampling

moments (ANOSIM R = 0.408). A clear grouping by sampling site occurs for each sampling date (Figure 2.6B).

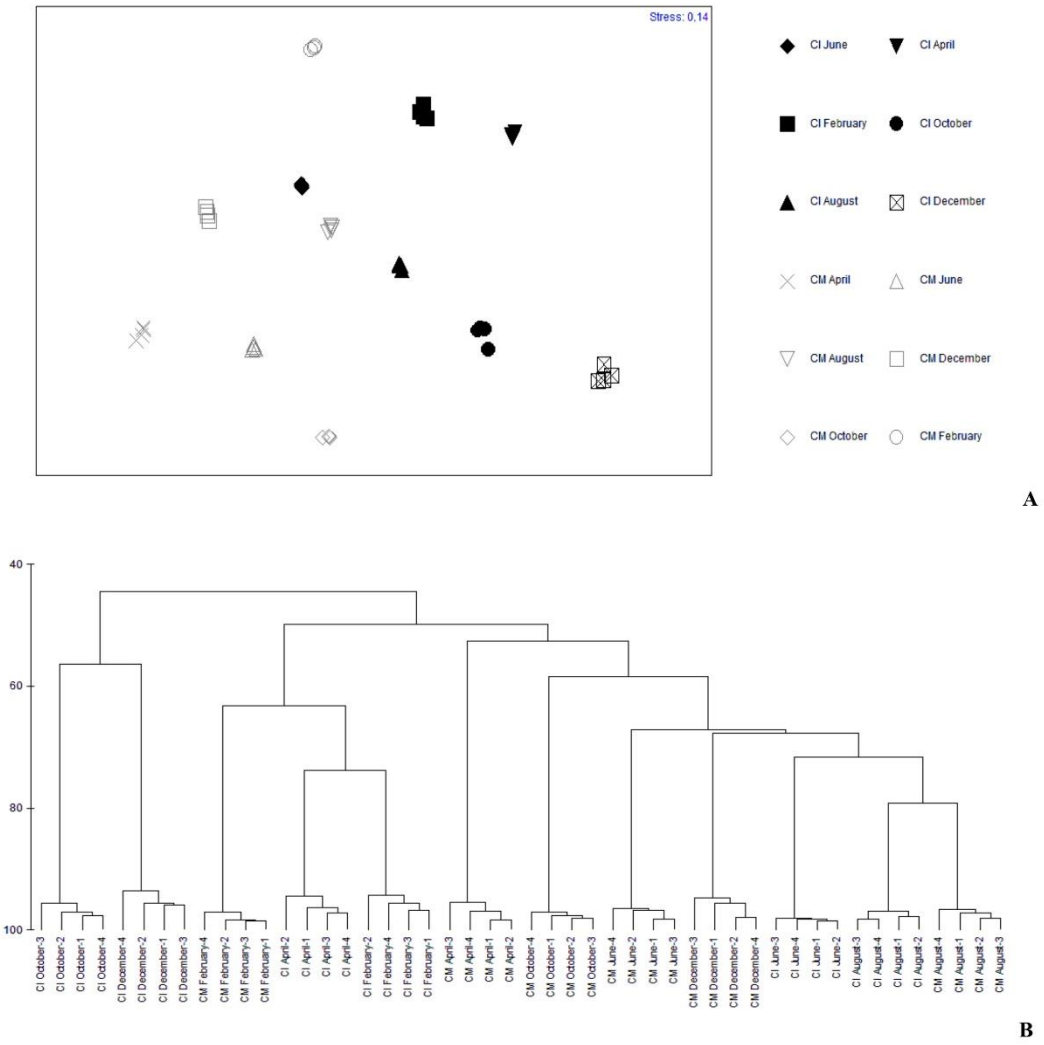

Figure 2.6 Comparison of DGGE patterns of 16S rDNA fragments amplified from water DNA of the Mira and Ílhavo channels at six sampling dates. (A) Multidimensional scaling (MDS) ordination and (B) Cluster analysis.

2.4.6. Abundance of potential pathogenic bacterial groups

In both sites and over the different sampling dates, Enterobactereaceae family was the most abundant bacterial group (Figure 2.7). The abundance of this family ranged from 0.29 to 1.1 x 10^9 cells/L and was different in the two sites (Mann–Whitney U test, $p < 0.05$). A clear and significant seasonal pattern of variation was observed in both channels, with highest and lowest values in the summer and winter, respectively (Friedman´ s test, $p < 0.05$).

Vibrio was the second most abundant group, varying between 1.2 and 5.0 x 10^8 cells/L. This group of bacteria showed a pattern of spatial variation that was different other groups, with higher relative values in the Mira channel. However, this difference was not statistically significant (Mann–Whitney U test, $p > 0.05$).

In the Mira channel, the least abundant group was *Aeromonas*, with exception of October samples (Figure 2.7A). *Aeromonas* showed a seasonal profile of variation (Friedman´ s test, $p < 0.05$), ranging from 4.0 x 10^7 cells/L to 1.8 x 10^8 cells/L in the Mira channel and from 1.2 to 2.4 x 10^8 cells/L in the Ílhavo channel. The abundance of *Aeromonas* was similar (Mann–Whitney U test, $p > 0.05$) in the two sites. In the Ílhavo channel, Salmonella corresponded to the least abundant group in February, April, October and December, whereas in June and August, *Aeromonas* was the least abundant pathogen (Figure 2.7B). The seasonal differences were statistically significant between hot and cold seasons (Friedman´ s test, $p < 0.05$). The relative abundance of *Salmonella* was similar (Mann–Whitney U test, $p > 0.05$) in the two sites, varying between 5.3 x 10^7 cells/L and 4.6 x 10^8 cells/L and showing a seasonal profile of variation with the highest values in summer and the lowest in winter.

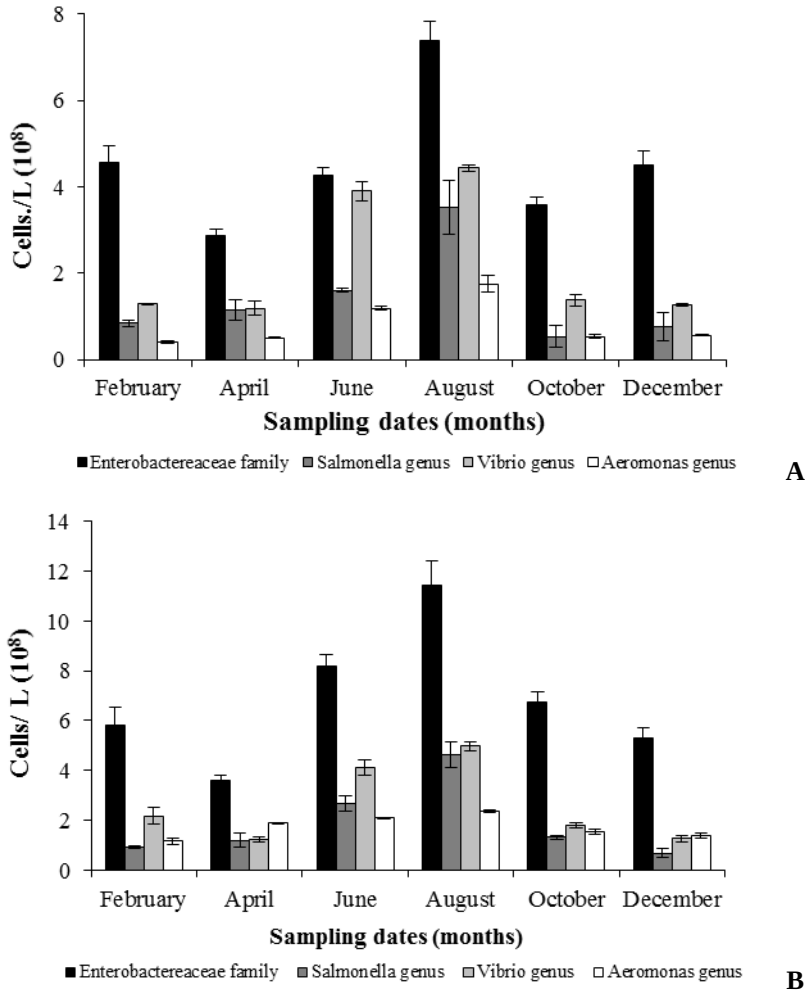

Figure 2.7 Abundance of Enterobactereaceae family, *Salmonella*, *Vibrio* and *Aeromonas* quantified by FISH in Mira (A) and Ílhavo (B) channels.

2.4.7. Correlations between bacteria and abiotic factors and between the different groups of bacteria

The values of the Spearman´ s rho correlation among the abundance of bacterial groups and abiotic factors at the two studied sites are shown in Table 2.2.

Table 2.2 Spearman's rho correlation between the abundance of bacterial groups and water proprieties at the Mira and Ílhavo channels.

Spearman Correlation (rs)	DAPI	Enterobactereaceae	*Aeromonas*	*Vibrio*	*Salmonella*	*E. coli*
Mira Channel						
Irradiation.	0.771 (n.s) N=6	-0.200 (n.s) N=6	0.200 (n.s) N=6	0.257 (n.s) N=6	0.771 (n.s) N=6	-0.257 (n.s) N=6
Precipitation.	-0.455(n.s) N=6	-0.698 (n.s) N=6	-0.334 (n.s) N=6	-0.820 (*) N=6	-0.455 (n.s) N=6	-0.213 (n.s) N=6
Temperature.	1.000 (**) N=6	0.371 (n.s) N=6	0.543 (n.s) N=6	0.486 (n.s) N=6	1.000 (**) N=6	0.029 (n.s) N=6
Salinity	0.580 (n.s) N=6	0.290 (n.s) N=6	0.580 (n.s) N=6	0.928 (**) N=6	0.580 (n.s) N=6	0.116 (n.s) N=6
Ílhavo Channel						
Irradiation	0.771 (n.s) N=6	0.257 (n.s) N=6	0.657 (n.s) N=6	0.314 (n.s) N=6	0.600 (n.s) N=6	-0.771(n.s) N=6
Precipitation	-0.395 (n.s) N=6	-0.820 (*) N=6	-0.213 (n.s) N=6	-0.941 (**) N=6	-0.516 (n.s) N=6	-0.213 (n.s) N=6
Temperature	1.000 (**) N=6	0.657 (n.s) N=6	0.943 (**) N=6	0.600 (n.s) N=6	0.943 (**) N=6	-0.600 (n.s) N=6
Salinity	0.943 (**) N=6	0.829 (*) N=6	0.886 (*) N=6	0.714 (n.s) N=6	1.000 (**) N=6	-0.486 (n.s) N=6

The abundance of total bacteria (DAPI counts) in the harvesting waters of both sites was correlated with temperature. At the Ílhavo channel, DAPI counts also correlated with salinity. The abundance of Enterobactereaceae was not correlated with any of the studied abiotic variables at the Mira channel, whereas at the Ílhavo channel correlated positively with salinity and negatively with precipitation. The abundance of *Aeromonas* also failed to show significant correlations at the Mira channel, whereas at the Ílhavo channel, there were positive correlations with salinity and temperature. At the Mira channel, the abundance of *Vibrio* was correlated with salinity and precipitation, whereas at the Ílhavo channel, was only correlated with precipitation. The abundance of *Salmonella* showed a strong correlation with temperature at both sites, and also with salinity at the Ílhavo channel. The abundance of *E. coli* was not correlated with any of the studied abiotic variables.

The values of the Spearman´ s rho correlation coefficient among the different bacterial groups at the two studied sites are shown in Table 2.3. At the Mira channel, the abundance of *E. coli* was correlated with the abundance of Enterobactereaceae and *Aeromonas*, whereas at the Ílhavo channel, it was not correlated with the abundance of other bacterial groups. The abundance of *Salmonella* was correlated with of the abundance of *Vibrio* and *Aeromonas* at the Mira channel, whereas at the Ílhavo channel was correlated with all of the other groups. The abundance of *Vibrio* was correlated with Enterobactereaceae and *Aeromonas* abundances at the Mira and Ílhavo channel. At the Ílhavo channel, the abundance of Enterobactereaceae was correlated with the *Aeromonas* abundance.

Table 2.3 Spearman´ s rho correlation between the concentration of *E. coli* and the abundance of Enterobactereaceae, *Vibrio*, *Aeromonas* and *Salmonella*, in Mira and Ílhavo channels.

Spearman Correlation (rs)	Enterobactereaceae	*Vibrio*	*Aeromonas*	*Salmonella*
		Mira Channel		
Vibrio	0.605 (**) N=18			
Aeromonas	0.453 (n.s) N=18	0.766 (**) N=18		
Salmonella	0.337(n.s) N=18	0.638 (**) N=18	0.596 (**)N=18	
E.coli	0.593 (**) N=18	0.405 (n.s) N=18	0.697 (**) N=18	0.177 (n.s) N=18
		Ílhavo Channel		
Vibrio	0.865 (**) N=18			
Aeromonas	0.662 (**) N=18	0.627 (**) N=18		
Salmonella	0.741 (**) N=18	0.709 (**) N=18	0.877 (**) N=18	
E. coli	0.091 (n.s) N=18	0.051 (n.s) N=18	-0.265 (n.s) N=18	-0.411 (n.s) N=18

2.5. Discussion

Depuration is a process by which shellfish release the intestinal content, including microorganisms. Its efficiency to remove bacteria depends on the bacterial group. For instance, presently practiced depuration is effective in the elimination of various faecal bacteria from shellfish but is not reliable in removing naturally occurring marine bacteria such as *V. parahaemolyticus* and *V. vulnificus* (FAO, 2008). Therefore, the association of

depuration with phage therapy, which can selectively inactivate the remaining pathogenic bacteria in the shellfish, would significantly improve the efficiency of the decontamination process. Moreover, the quality of the harvesting water from which shellfish concentrated bacteria highly influences the success of depuration. However, the classification of the harvesting waters is solely based on the concentration of one indicator of faecal pollution, *E. coli*, which is a non-indigenous indicator.

The present study showed that total bacterial number and the structure of the bacterial communities, which incorporates non and pathogenic bacteria, presented a broad temporal variation at both harvesting areas. The DGGE results showed that the similarity of the bacterial community structure between the sampling events was significantly different. At both areas, the bacterial community structure in the hot months (June and August) was more diverse and distinct from that in cold months (December and February). This temporal profile highlights the necessity of taking into consideration the dynamic of the whole community, including the seasonal variation, when choosing specific phages for the inactivation of shellfish pathogenic bacteria.

Compared to the total bacterial numbers, the variation of the relative abundance of particular bacterial groups was more pronounced during the sampling period. Among the potentially pathogenic bacteria, the family Enterobactereaceae, which comprises the indicator *E. coli* used to classify the harvesting waters, was the most abundant group, indicating that non-indigenous bacteria are prevalent and an important source of contamination in these shellfish growing areas. *Salmonella*, also a non-indigenous pathogenic bacterium, showed a seasonal pattern of variation, correlating strongly with temperature at both sites. Several studies also observed that most outbreaks of *Salmonella* infection occurred during hot periods (summer and early autumn) (Checkley et al., 2000;

Zhang et al., 2012), indicating that the summer season is a critical time period for the consumption of bivalve molluscs.

The indigenous *Vibrio* and *Aeromonas*, including *V. parahaemolyticus*, *V. vulfinicus*, *V. cholerae* and *A. hydrophila* (Colakoglu et al., 2006; Fukuda et al., 1996; Nakai and Park, 2002; Nakai et al., 1999), which are frequently associated with gastrointestinal outbreaks transmitted by the consumption of shellfish (Butt et al., 2004; FAO, 2004; Huss et al., 2000), were also present at high concentrations in the harvesting water. *Vibrio* and *Aeromonas* also presented a seasonal pattern of variation, with values up to 20 times higher in the hot season, when compared to the cold season. This observation reinforces the previous observation that the summer season is the critical period for gastrointestinal outbreaks associated with the consumption of bivalve molluscs. Other studies also observed that the incidence of *V. parahaemolyticus* and *V. vulnificus* (Davis and Sizemore, 1982) and of Aeromonads (Maalej et al., 2004) increased in the summer months. Both non- and indigenous bacterial groups, as the total bacterial number, registered the highest values in summer. During this season, the volume of effluents from sewage treatment plants increases due to the tourism pressure (Silva, 2013). The central region of Portugal is visited by a high number of tourists during this period (between July and September), with the lowest values of activity registered between November and March (Silva, 2013).

Fluorescence in situ hybridization, a culture-independent approach that uses specific probes for bacterial groups, provided a good overview of the real proportion of different cultivable and non-cultivable pathogenic bacteria and their dynamics in the harvesting waters. This information was crucial to the selection of phages to use in phage therapy during depuration, which must include specific phages of Enterobactereaceae, *Salmonella*, *Vibrio* and *Aeromonas* groups.

In general, the harvesting waters from the Ílhavo channel were more contaminated than from the Mira channel. This might be explained by the fact that Ílhavo channel is impacted by industrial facilities and receives the effluent of a sewage treatment plant. Moreover, this estuarine channel is classified as a C area due to a persisting natural occurring and anthropogenic microbial contamination. Contrastingly, the Mira channel has almost no influence of industrial activities, being only subjected to some contamination introduced by human and therefore classified as a B zone. The bacterial indicator used to classify the harvesting waters, *E. coli*, was present during the entire sampling period in the water of both harvesting areas, but, contrarily to Enterobactereaceae, *Salmonella*, *Vibrio* and *Aeromonas*, its seasonal variation was clearly stronger at the area located in the Ílhavo channel than in the Mira channel. Moreover, the seasonal profile of variation of *E. coli* was different of those observed for the other bacterial groups. In general, the abundance of the other bacterial groups studied in this work and the diversity of the bacterial community were higher during the hot season. However, for this bacterium, maximum values were observed in the cold months, namely in December in the Ílhavo channel. This might be due to the runoff from land and by resuspension of bottom sediments during the rainy period. In fact, the Enterobactereaceae family also showed lower decreases than those observe for *Vibrio* and *Aeromonas* groups in December and February. At the Mira channel, the abundance of *E. coli* was correlated with the abundance of Enterobactereaceae and *Aeromonas*, whereas at the Ílhavo channel, was not correlated with others bacterial groups. This spatial variability suggests that *E. coli* is not a good indicator of the presence of the other groups of bacteria, including pathogenic bacteria. Although a seasonal variation for the abundance of all bacterial groups and the bacterial community structure was observed at both harvesting zones, the seasonal profiles of variation were more evident in the

harvesting area of Mira channel. In the Ílhavo channel zone, a more land influenced area (Silva and Batista, 2008), the abundance and diversity of the bacteria might be influenced by other factors besides the seasonal variation. For harvesting areas where both seasonal and anthropogenic factors influence the abundance and structure of bacterial communities similar to this one, phage therapy during depuration must include phages against both indigenous and non-indigenous bacteria during the hot season and, during the cold season only against to non-indigenous bacteria. The overall bacterial community and the disease-causing bacteria, as well as the indicator of microbiological water quality of both shellfish harvesting areas showed clear but distinct patterns of variation. The highest concentration of the main fish pathogenic bacteria and the bacterial indicators of faecal contamination occurred during the summer season. However, the maximum value of non-indigenous bacteria was registered during the rainy season, which indicates that the risk of diseases outbreak to humans in the shellfish harvesting areas is present during the cold season as well. Despite of the permanent risk of outbreak throughout the year, the summer season, when the most common pathogenic bacteria responsible for the transmission of shellfish infections reach the highest density, is the critical period for shellfish disease transmission. It is easier, fast and more affordable to quantify bacterial indicators in harvesting waters than in bivalves. However, the guidelines to classify bivalve harvesting areas are based on the quantification of the levels of microbiological indicators in shellfish (in 100 g of FIL) and not in the harvesting waters. Further studies will be conducted in order to evaluate the concentration and seasonal variation of the different bacterial groups within shellfish. This knowledge will provide further information that will help in the choice of the more appropriate phages to use in phage therapy to apply during the depuration process.

2.6. Conclusion

In order to apply a successful phage therapy to inactivate shellfish pathogenic bacteria during the depuration process, is essential to have a comprehensive knowledge of the seasonal and spatial variability of the overall bacterial community, including the disease- causing bacteria and the indicators of microbiological water quality, in the water of the harvesting areas. A higher complexity of the whole community and an increase of abundance of the main pathogenic bacteria in the summer, indicate that this season is the critical time frame when phage therapy should be applied. In anthropogenic impacted and land influenced harvesting waters, phage therapy could be necessary also during the cold season.

Chapter 3. Characterization and *in vitro* evaluation of new bacteriophages for the biocontrol of *Escherichia coli*

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3.1. Abstract

In the present study two new phages (phT4A and ECA2) were characterized and their efficacy was evaluated separately and in cocktail (phT4A/ECA2) to control *Escherichia coli*. The isolated phages, phT4A and ECA2, belonged to the *Myoviridae* and *Podoviridae* family, respectively and both are safe (no integrase and toxin codifying genes) to be used in bacterial control. In general, the increase of multiplicity of infection (MOI) from 1 to 100 promoted a significant increase in the efficiency of phage phT4A and phage cocktail phT4A/ECA2. Both phages were effective against *E. coli*, but phage phT4A (reduction of 5.8 log CFU/mL after 8 h treatment) was more effective than phage ECA2 phage (reduction of 4.7 log CFU/mL after 8 h treatment). The use of a cocktail phT4A/ECA2 was significantly more effective (reductions of 6.2 log CFU/mL after 6 h treatment) than the use single phage suspensions of phT4A and ECA2 (reductions 5.3 log CFU/mL and 4.9 log CFU/mL, respectively, after 6 h treatment). The rate of emergence of phage-resistant mutants was lower for phage phT4A when compared with phage ECA2 and phage cocktail phT4A/ECA2.The results indicate that in addition to the efficacy, the potential development of phage-resistant mutants must also be considered in the design of phage cocktails.

***Keywords**:* Bacterial-phage inactivation, phage cocktail, multidrug resistance bacteria, phenotypic resistance

3.2. Introduction

Global bivalve production in aquaculture has consistently increased over the years from 7.1 million tonnes in 1995 to 14.2 million tonnes in 2010, and the consumer demand

is expected to further increase in the future (FAO, 2012). In Portugal, bivalve represent the most important seafood group, representing 48% of total aquaculture production (INE/DPGA, 2010), and assuming prominent role in sustainable seafood production.

Bivalve molluscan shellfish feed by filtering large volumes of seawater and accumulating food particles from their surrounding environment (Brands et al., 2005; Butt et al., 2004; FAO, 2004; Huss et al., 2000; Muniain-Mujika et al., 2003; Robertson, 2007). In order to protect public health and to provide safe products of high quality to consumers, several regulations have been imposed by the European Commission on shellfish production and trade chain in member states (FAO, 2008). These European regulations (EC Regulations, 2007, 2004b) establish limits for microorganism indicators (less than 300 faecal coliforms or less than 230 *E. coli* per 100 g of flesh and inter-valve liquid - FIL) and pathogens (absence of *Salmonella* spp. in 25 g of flesh shellfish) for a production area of bivalves suitable for direct human consumption. Pathogenic strains of *E. coli* are transferred to seafood through sewage pollution of the coastal environment or by contamination after harvest (Kanayama et al., 2015; Potasman et al., 2002). *E. coli* food infection causes abdominal cramping, water or bloody diarrhea, fever, nausea and vomiting (Kanayama et al., 2015; Macrae et al., 2005; Martins et al., 2004; Potasman et al., 2002; Ward et al., 1997).

Depuration conducted under conditions that maximize the natural filtering activity is a useful way of eliminating or reducing the concentration of microorganisms in bivalves by clearance of intestinal contents. However, some pathogenic microorganisms are resistant to this process (FAO, 2008; Martínez et al., 2009; Rong et al., 2014) . In order to reduce the risk of transmission of infections caused by microbial pathogens, including multidrug-resistant bacteria, it is essential to develop alternative approaches. One of the most

promising is the association of phage therapy (application of lytic phages to prevent and/or to treat bacterial infections) to the depuration process (Rong et al., 2014). This association will contribute to the improvement of the decontamination efficiency, likely reducing the time required for the depuration, and consequently, the production costs, with additional benefits in bivalve safety and quality. After depuration, bivalves may be intended for consumption if they have a level lower than 230 *E. coli* in 100 g of flesh and intra-valvular liquid (FIL), and absence of *Salmonella* spp. (FAO, 2008).

Phages, which are viruses that infect bacteria, have been used as therapeutic or prophylactic agents to control bacterial infections (Biswas et al., 2002; Bruttin and Brussow, 2005; Chhibber et al., 2008; Jun et al., 2014b, 2013; Kumari et al., 2009; Park et al., 2000). Phage-based applications have been demonstrated for food safety (Denes and Wiedmann, 2014; Endersen et al., 2014). As specific pathogen-killers, bacteriophages are effective agents for controlling bacterial infections, without affecting the normal microbiota (Hawkins et al., 2010; Park and Nakai, 2003; Pereira et al., 2011a). The association of depuration and phage therapy will contribute to the improvement of the decontamination efficiency, likely reducing the time required for the depuration, and consequently, the production costs, with additional benefits in bivalve safety and quality. To the best of our knowledge, there is only report on the combination of phage therapy and bivalve depuration. In this study, Rong et al. (2014) concluded that the application of bacteriophage (VPp1) could reduce the population of *Vibrio parahaemolyticus* in infected oysters during depuration without water recirculation (Rong et al., 2014).

The aim of this study was to evaluate the efficiency of two new phages of *E. coli* (phT4A and ECA2), individually or combined in cocktails, to control *E. coli* in the perspective of their potential use for phage-enhanced bivalve depuration.

3.3. Material and methods

3.3.1. Bacterial strains and growth conditions

The bacterial strain *E. coli* (ATCC 13706) was used in this study as phage host. The bacterial strains used in host range studies are listed in Table 3.1. *E. coli* (ATCC 25922), *Salmonella enterica* serovar Typhimurium (ATCC 13311 and ATCC14028), *Aeromonas hydrophila* (ATCC 7966), *Vibrio fischeri* (ATCC 49387), *Vibrio parahaemolyticus* (DSM 27657), *Vibrio anguillarum* (DSM 215 97), *Photobacterium damselae damselae* (DSM 7482), *Shigella flexneri* (DSM 4782), *Listeria innocua* (NCTC 11288), *Listeria monocytogenes* (NCTC1194) and *Aeromonas salmonicida* (CECT 894) were purchased from ATCC, DSM, NCTC and CECT collection, respectively. Five *Salmonella enterica* serovar Enteriditis strains were isolated from food samples and gently provided by Controlvet Laboratory. The other bacterial strains used in this study were isolated in previous works from water samples collected in Ria de Aveiro (Louvado et al., 2012; Pereira et al., 2016a). Fresh bacterial cultures were maintained in Tryptic Soy Agar (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth (TSB; Liofilchem, Italy) and grown overnight at 37 ºC. An aliquot of this culture (100 µL) was aseptically transferred to 10 mL of fresh TSB and grown overnight at 37 °C to reach an optical density (O.D. 600 nm) of 0.8, corresponding to about 10^9 cells per mL.

3.3.2. Phage isolation and purification

Two phages were isolated from sewage network of Aveiro (station EEIS9 of SIMRIA Multi Sanitation System of Ria de Aveiro) collected in different places at different times (December 2014 and March 2015). Sewage water was filtered through 0.45

µm pore size polycarbonate membranes (Millipore, Bedford, MA, USA). The filtrate was added to double-concentrated TSB medium with 1 mL of fresh culture of the host, *E. coli* (ATCC 13706). The mixtures were incubated at 37 ˚C for 18 h at 80 rpm, and then filtered through a 0.2 µm membrane (Millipore Bedford, MA, USA). Chloroform (final volume of 1%) was added to the supernatants and phage concentration was determined as described before. Plates were incubated at 37 ˚C and examined for the presence of lytic plaques after 12 h. One single plaque was removed from the agar, diluted in TSB, and then chloroform (final volume of 1%) was added to eliminate bacteria. The sample was centrifuged and the supernatant was used as a phage source for a second isolation procedure. Three successive single-plaque isolation cycles were performed to obtain pure phage stocks for both bacteria. All lysates were centrifuged at 10.000 *g* for 10 min at 4 ˚C, to remove intact bacteria or bacterial debris. The phage stock was stored at 4 ˚C and 1% chloroform (final volume) was added. The phage produced on *E. coli* was designated as phT4A and ECA2. The phage suspension titre was determined by the double-layer agar method using TSA as culture medium (Adams, 1959). The plates were incubated at 37 °C for 8 h and the number of lysis plaques was counted. The results were expressed as plaque forming units per millilitre (PFU/ mL).

3.3.3. Electron microscope examination

Phage particles of a highly concentrated suspension (10^9 PFU/mL) were negatively stained with 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, UK) and electron micrographs were taken using a JEOL 1011 transmission electron microscope (JEDL USA Inc, Peabody, MA, USA) operating at 100 kV. Images were acquired with a Gatan CCD-Erlangshen ES100W.

3.3.4. Phage DNA extraction

Bacteriophage suspensions (10^9 PFU/mL) of the two phages (phT4A and ECA2) were centrifuged 3 times at 13.000 *g* for 10 min. The phage lysates were ultracentrifuged (Beckman, Optima LE-80K) at 100.000 *g* for 2 h at 10 °C. The extraction of nucleic acid from phage particles was performed using the phage DNA isolation kit (Norgen Biotek Corp, Canadan), as indicated by the instructions provided by the manufacturer. Nucleic acid yield was quantified in the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The resulting product was electrophoresed through 0.8% agarose gel at 80 V for 40 min.

3.3.5. Whole genome sequencing and bioinformatics analyses

Phage DNA samples (176 and 200 ng of phage ECA2 and phage T4 DNA samples respectively) were firstly fragmented using NEB Next dsDNA Fragmentase (New England Biolabs) and digested DNA further processed for libraries construction, using reagents from KAPA Library Preparation Kit for Ion Torrent according to manufacturers' protocol. 470 bp long high quality libraries containing about 370 bp long insert were further used for template preparation using Ion PGM Hi-Q Template Kit and sequencing on Ion Torrent PGM machine using the chip316 v2 and Ion PGM HiQ Sequencing Kit according to manufacturers' protocol. The generated raw sequence data were analysed with CLC Genomics Workbench 8.5.1 (CLC Bio-Qiagen, Aarhus, Denmark). After quality trimming, the sequence reads were de novo assembled and the consensus sequence of the most represented contigs of both genomes, was generated. The annotation for both genomes was performed using the phage/plasmid pipeline from the RAST server Version 2.0 (Aziz et al., 2008). The nucleotide sequences were compared with those of other genes in GenBank using BLAST. Open reading frames (ORFs) were identified using the NCBI ORF Finder.

The genome sequences of both phages were deposited in GenBank under the accession numbers KX130727 (phage phT4A) and KX130726 (phage EC2A).

3.3.6. Phage host range determination and efficiency of plating (EOP) analysis

Bacterial strains used in the present work are listed in Table 3.1. As a first approach, phage host range was determined by spot testing according to (Adams, 1959). The plates were incubated at 37 °C and examined for plaques after 8 - 12 h. Bacterial sensitivity to a bacteriophage was established by a lysis cleared zone at the spot. According to the clarity of the spot, bacteria were differentiated into two categories: clear lysis zone (+), no lysis zone (-). The EOP was determined for bacteria with positive spot tests (occurrence of a clear lysis zone), using the double-layer agar method (Adams, 1959). For each phage, three independent experiments were performed. The EOP was calculated (average PFU on target bacteria / average PFU on host bacteria) (Kutter, 2009) along with the standard deviation for the three measurements.

3.3.7. Phage adsorption

Exponential host bacterial cultures of *E. coli* strain (ATCC 13706) were adjusted to a 0.8 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU/mL). Ten microliters of the phage suspensions were added to 10 mL of the *E. coli* strain (ATCC 13706) in order to have a MOI of 0.001 (Stuer-Lauridsen et al., 2003) and incubate at 25ºC. Aliquots of mixture were collected after 0, 5, 10, 15, 20, 25, 30, 40, 50, 60 and 70 min of incubation and chloroform (final volume 1%) was added. The mixture was centrifuged at 12.000 *g* for 5min and supernatants were immediately filtered by using 0.2 µm membrane (Millipore Bedford, MA, USA). The filtrates containing unadsorbed phages were diluted and titrated.

The plates were incubated at 37 °C and examined for plaques after 4-8 h. Adsorption was expressed as the percentage decrease of the phage titre in the supernatant, as compared to the time zero. Suspensions of phages without any cells were used as no-adsorption standard for calculations (Stuer-Lauridsen et al., 2003). Three independent assays were done.

3.3.8. One step growth assays

Exponential host bacterial cultures of *E. coli* strain (ATCC 13706) were adjusted to a 0.8 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU/mL). Ten microliters of the phage suspensions were added to 10 mL of the bacterial culture in order to have a MOI of 0.001 and incubated at 25 ºC (Mateus et al., 2014). The mixture was centrifuged at 12.000 *g* (ThermoHeraeus Pico, Hanau, Germany) for 5 min, the pellet was re-suspended in 10 mL of TSB at 25 ºC and then were diluted and titrated. The plates were incubated at 37 °C and examined for plaques after 4 - 8 h (Mateus et al., 2014). Three independent assays were done.

3.3.9. Bacterial kill curves

Bacterial inactivation was determined with the two single phage suspensions (phT4A and ECA2) and the phage cocktail (phT4A/ECA2, the two phages were mixed together and each phage at the same concentration) using the bacterium *E. coli* as host, at a MOI of 1 and 100. For each assay, two control samples were included: the bacterial control (BC) and the phage control (PC). The bacterial control was not inoculated with phages and the phage controls were inoculated with phages but not with bacteria. Controls and test samples were incubated exactly in the same conditions. Aliquots of test samples and of the bacterial and

phage controls were collected after 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation. In all assays, the phage titre was determined in duplicate through the double agar layer method after an incubation period of 4 – 8 h at 37 °C. Bacterial concentration was determined in duplicate in TSA after an incubation period of 24 h at 37 °C. Three independent experiments were performed for each condition.

3.3.10. Isolation of phage-resistant mutants

Sensitive *E. coli* was grown at 37 ºC for 16 h. A soft agar overlay seeded with 300 µL of fresh bacterial culture was then spotted with 10 µL of each of the two lytic phage suspensions. Using high phage titers were formed clear lysis plaque zones, where there was complete absence of visible bacterial growth after 6h of incubation. However, after 24 h of incubation, resistant colonies were observed in these zones. The phage resistant colonies were picked up and purified by successive sub-culturing in TSA, in order to remove attached phage particles, and were used in further experiments.

3.3.11. Determination of the rate of emergence of bacterial mutants

In order to determinate the mutation frequencies per cell, ten isolated colonies were picked and inoculated into ten tubes with TSB medium, grown at 37 °C for 24 h. Aliquots of 0.1 mL from the 10^{0} –10^{-3} dilutions were inoculated onto the double-layer plates and were incubated at 37 °C for five days (because some of the phage-resistant mutants grow very slowly). Simultaneously, 0.1 mL aliquots of 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated on TSA medium without phage, for determination of colony number. The averaged colony number of mutants (obtained from the ten isolated colonies) in 1 mL of culture (prepared from the culture with phages) was divided by the averaged colony number of the control

(prepared from the culture without phages) (Filippov et al., 2011). Three independent assays were performed.

3.3.12. Fitness of phage resistant mutants

The growth of the bacterial populations was quantified in the presence and in the absence of each of the phages and cocktail. Fitness of each bacterial population was analysed in two ways: (i) determining bacterial concentration after 6 and 12 h incubation and (ii) determining population growth rate using the optical density (OD).

Exponential host bacterial cultures of sensitive *E. coli* strain (ATCC 13706, without phage contact) and resistant mutants of phage phT4A, EC2A and cocktail phT4A/EC2A were adjusted to a 0.8 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU/mL). *E. coli* sensitive was added to 4 of the 10 samples to obtain a final concentration of 10^5 CFU/mL. Three of the inoculated samples with sensitive *E. coli* were inoculated with phages phT4A, EC2A and cocktail phT4A/EC2A, respectively (sensitive with phage phT4A, sensitive with phage EC2A and sensitive with cocktail phT4A/EC2A) to obtain a final concentration 10^7 PFU/mL and the remaining infected sample was not added of phages sensitive bacteria without phages). Resistant mutants to phage phT4A were added to 2 of 10 samples to obtain a final concentration of 10^5 CFU/mL. One of these samples was inoculated with phage phT4A (resistant with phage phT4A) to obtain a final concentration 10^7 PFU/mL and the remaining infected samples were not added of phages (resistant bacteria without phages). The same protocol was followed for the mutants resistant to phage ECA2 and to the cocktail phT4A/ECA2. Samples were incubated at 25 ºC and bacterial concentration was determined by the spread method in duplicate in TSA medium after an incubation period of 6 and 12 h post inoculation. The plates were

incubated at 37 °C and bacterial concentration (CFU/mL) was calculated after 24 h incubation. Three independent experiments were performed for each condition.

In parallel with this assay we determined 12 h growth curves using a spectrophotometer (Halo DB- 20, Dynamica Scientific) using culture turbidity as a proxy for bacterial density. OD_{600nm} was taken at 0, 2, 4, 6, 8, 10 and 12 post inoculation. Three independent experiments were performed for each condition.

3.3.13. Prophage detection in the host bacterium after phage addition

The phage resistant mutants isolated (section 3.3.11) were stress induced with mitomycin C (Sigma Chemical, St. Louis, MO, USA). An isolated colony was picked out from the lysis plaque, inoculated into tubes with TSB medium and stress-induced with mitomycin C (Sigma Chemical, St. Louis, MO, USA) at a final concentration of 1 μg/mL. Cells with temperate phages usually result in the release of the phage (after inducing it by mitomycin C). A negative control, without mitomycin C was prepared. The samples were incubated overnight at 37 °C and centrifuged (10.000 *g*, 10 min). The supernatant was filtered through 0.22 μm (GE Osmonics), and tested against each phage-sensitive host strain by applying the spot test. After an overnight incubation at 37 ºC, bacterial lawns were checked for clear zone. The presence of a clear zone after stress inducing indicates that bacteria have prophages in their genome. Three independent assays were performed.

3.3.14. Detection of host sensitivity to bacteriophages after one cycle of phage contact

The isolated phage resistant mutants (section 3.3.10) were used. Ten isolated colonies were picked out from the lysis plaque, inoculated in TSB medium and incubated

for 24 h. After incubation, the culture was tested against each phage- resistant host strain by applying the spot test and was also streak-plated on TSA plates. An isolated colony grown in TSA was selected and the procedure was repeated more 4 times. Overall, 5 streak-plating steps on solid medium were done (Pereira et al., 2016a). A control using phage sensitive strain was prepared. Controls and test samples were incubated exactly in the same conditions. After to be done 5 streak-plating steps on solid medium was determined efficacy of plating and adsorption of phages to resistant bacteria as described in Section 3.3.6 and 3.3.7, respectively. Three independent assays were done.

3.3.15. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. The geometric mean (GM) was calculated as the antilog of the mean of log10 transformed concentrations; the standard deviations (SD) of log10 transformed concentrations were also determined, as well as the 95% confidence interval for the mean and the range of values. Student's t-test was used to examine differences between the growth of resistant bacteria and sensitive bacteria in presence of the phage after 6 and 12h incubation, and the differences between the growth of resistant and sensitive bacteria in absence of the phage after 6 and 12h incubation. In order to determine whether or not to use a t-test, Levene's test for homogeneity of variances was used, assuming equal or unequal variances in the two groups compared, with the hypothesis of equal variance being rejected at $p > 0.05$.

Two-way ANOVA with repeated measures was used to analyze the statistical differences between growth curves of the sensitive and resistant bacteria in presence and absence of the phages during the sampling time. Normal distribution was assessed by the

Kolmogorov–Smirnov test and homogeneity of variances by the Levene´s test, and the significance was assumed when $p < 0.05$.

The existence of significant differences on bacterial concentration in killing curves assays was analyzed using a two-way ANOVA with repeated measures, with MOIs (1 and 100), phages (phT4A, EC2A and phT4A/EC2A) and treatment times being used as fixed factors. The significance of the differences was evaluated by comparing the results obtained in the test (BP phT4A, BP EC2A, BP phT4A/EC2A) and control samples (BC) for the different times among treatments of each of the three independent assays, and analyzing the results obtained in different treatments (BP phT4A MOI 1, BP EC2A MOI 1, BP phT4A/EC2A MOI 1, BP phT4A MOI 100, BP EC2A MOI 100, BP phT4A/EC2A MOI 100) and bacterial control (BC MOI 1 and BC MOI 100), along different times. The significance of the differences of viral concentration in killing curves assays was analyzed using a two-way ANOVA with repeated measures. The significance of differences was evaluated by analyzing the results obtained in the test (BP phT4A, BP EC2A, BP phT4A/EC2A) and phage control (PC) during the sampling time, and comparing the results obtained in the test (BP phT4A MOI 1, BP EC2A MOI 1, BP phT4A/EC2A MOI 1, BP phT4A MOI 100, BP EC2A MOI 100, BP phT4A/EC2A MOI 100) and phage control (PC phT4A MOI 1, PC EC2A MOI 1, PC phT4A/EC2A MOI 1, PC phT4A MOI 100, PC EC2A MOI 100, PC phT4A/EC2A MOI 100) for the different times, among treatments, of each of the three independent assays. Normal distribution was assessed by the Kolmogorov–Smirnov test and homogeneity of variances by the Levene´s test. Whenever significance was accepted, at $p < 0.05$, Tukey's multiple comparison test was used for a pairwise comparison of the means. To test the significance of the interactions, F test was applied.

3.4. Results

3.4.1. Phage isolation and purification

The phT4A and ECA2 phages, isolated from Aveiro municipal sewage using *E. coli* as host, formed clear plaques on the host strain with a diameter of 0.5 - 2 mm and 1 - 5 mm, respectively (Figure 3.1). High titre suspensions (10^9 PFU/mL) were obtained.

3.4.2. Virion morphology

Based on the morphological analysis by Transmission Electron Microscopy (TEM) (Figure 3.1), the two phages were identified as *Caudovirales*. Phage ECA2 has an icosahedral head with approximately 56 ± 2 nm of width and was identified as member of family *Podoviridae*. Phage phT4A has an elongated icosahedral head with approximately 80 ± 2 nm width and a contractile tail with a length approximately 107 ± 3 nm, being identified as a member of family *Myoviridae*.

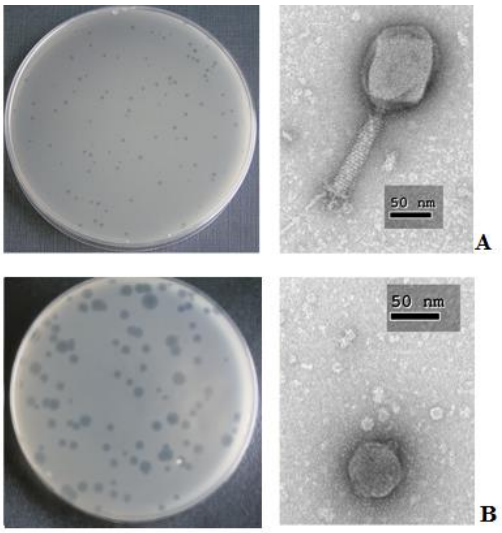

Figure 3.1 Examples of phage plaque morphologies and electron micrographs of phages *E. coli*. (A) Phage phT4A. (B) Phage ECA2. The bars represent 50 nm

3.4.3. Genome sequencing

The phage genomes were sequenced with an Ion Torrent PGM semiconductor approach. The sequencing run of phage genomes resulted in 246.183 reads of 358.2 bp mean length for phage ECA2, of which 206 413 were specific representing a fold coverage of approximately 1.354, and 52.403 reads of 380.5 bp mean read length for phage phT4A, of which 40.869 were specific, representing a fold coverage of approximately 59. Phage genomes were de novo assembled using CLC Genomics Workbench 8.5.1.

For phage phT4A 17.070 - fold coverage was obtained, 170.698 bp genome and a G+C content of 41.67%. For phage ECA2, 6.192- fold coverage, 38.890bp genome and 49.96% G+C content. The sequence analysis revealed the presence of 255 potential number of Coding Sequences (CDS) in phage phT4A and 47 potential CDS in phage ECA2 (Table 3.1S and 3.2S). In general, the genes of phages phT4A and ECA2can be roughly classified into three classes of clusters. The early (Class I), the class comprising gene clusters dealing with DNA metabolism (Class II) and finally the third group (Class III) with structural proteins of virion, helpers for assembly, and responsible for host lysis (Table 3.1S and 3.2S). None of the genomes featured any lysogenic related genes and it can be safety assumed that the two phages feature a lytic lifestyle. No genes encoding toxins, virulence factors or antibiotic resistance genes were identified based on amino acid sequence homology searches. Overall, the genome sequence of phage phT4A had the highest similarity with three of the *Myoviridae* phages: 99% with *Klebsiella* phage Matisse (accession number KT001918.1) and *Klebsiella* phage KP15 (accession number GU295964.1) and 98% with *Klebsiella* phage KP27 (accession number HQ918180.1). However, the genome sequence of phage ECA2 feature 98, 97 and 96% similarity with other three of the phages *Podoviridae Enterobacter* phage E-2 (accession number

KP791805.1), *Enterobacter* phage E-4 (accession number KP791807.1) and *Enterobacter* phage E-3 (accession number KP791806.1) respectively.

3.4.4. Analysis of phage host range and efficiency of plating (EOP)

Table 3.1 Lytic spectra of two phages *E. coli* isolates determined on 40 bacterial strains included in eight genera. Clear lysis zone (+), no lysis zone (-).

Species	Infectivity of phage	
	phT4A	ECA2
Salmonella Typhimurium ATCC 13311	+	+
Salmonella Typhimurium ATCC 14028	+	+
Salmonella Enteriditis CVA	+	+
Salmonella Enteriditis CVB	+	-
Salmonella Enteriditis CVC	+	+
Salmonella Enteriditis CVD	+	+
Salmonella. Enteriditis CVE	+	+
Escherichia coli ATCC 25922	-	-
Escherichia coli ATCC 13706	+	+
Escherichia coli BC30	-	-
Escherichia coli AE11	+	+
Escherichia coli AD6	+	+
Escherichia coli AF15	+	+
Escherichia coli AN19	-	-
Escherichia coli AC5	+	-
Escherichia coli AJ23	-	+
Escherichia coli BN65	+	+
Escherichia coli BM62	-	-
Shigella flexneri DSM 4782	-	-
Citrobacter freundii 6F	+	+
Citrobacter freundii 10I	+	+
Providencia vermicola	+	+
Providencia sp.	+	+
Proteus vulgaris	+	+
Proteus mirabilis	+	+
Klebsiella pneumoniae	-	-
Enterobacter cloacae	+	+
Listeria innocua NCTC 11288	-	-
Listeria monocytogenes NCTC 1194	-	-
Vibrio parahaemolyticus DSM 27657	-	-
Vibrio anguillarum DSM 21597	-	-
Vibrio fischeri ATCC 49387	-	-
Photobacterium damselae damselae DSM 7482	-	-
Aeromonas hydrophilla ATCC 7966	-	-
Aeromonas salmonicida CECT 894	-	-
Pseudomonas aeruginosa	-	-
Pseudomonas fluorescens	-	-
Pseudomonas putida	-	-
Pseudomonas segetis	-	-
Pseudomonas gingeri	-	-
Total number of strains lised	20	19

Spot test indicated that phage phT4A had the capacity to form completely cleared zones on 20 of the 40 strains and the phage ECA2 form cleared zones on 19 of the 40 strains (Table 3.1). However, EOP results indicate that the two phages formed phage lysis plates only in presence of the host.

3.4.5. Phage adsorption

Phage adsorption assays with phage phT4A showed that approximately 38% of the phage particles adsorbed to the host cell after 40 min and 97% adsorbed after 70 min (Figure 3.2). For phage ECA2, 46% of particles adsorbed to *E.coli* within 20 min and 98% after 40 min (Figure 3.2).

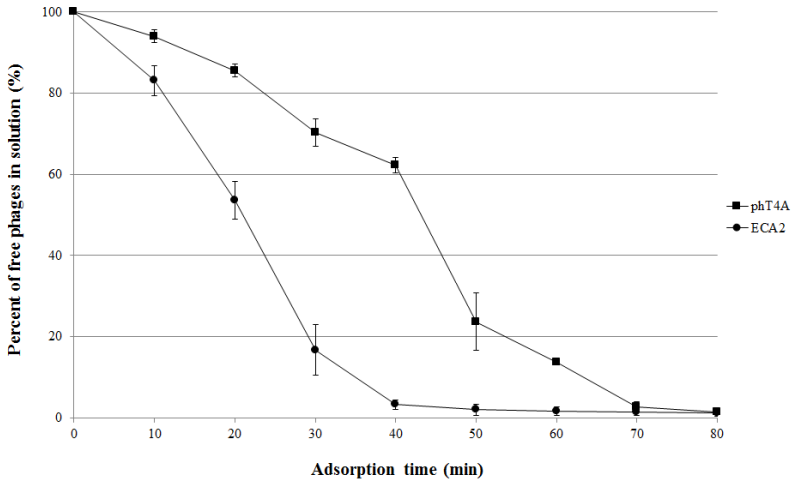

Figure 3.2 Adsorption of phages phT4A and ECA2 to *E. coli*. Percentage of unadsorbed phage is the ratio of PFU in the supernatant to the initial PFU and was determined by tittering an equivalent dilution of the phage in the absence of host cells. Values represent the mean of three experiments; error bars represent the standard deviation.

3.4.6. Burst size and explosion time

The one step growth curve for phages phT4A and ECA2 was determined in TSB at 25 ºC (Figure 3.3). From the triphasic curves obtained, an eclipse period of 30 min, a latent period of 50 min and a burst size of 17 ± 2 PFU/host cell were calculated for phage phT4A. The phage ECA2 phage is characterized by an eclipse period of 10 min, the latent time was at 30 min and each infected bacteria produced 117± 11 PFU/host cell.

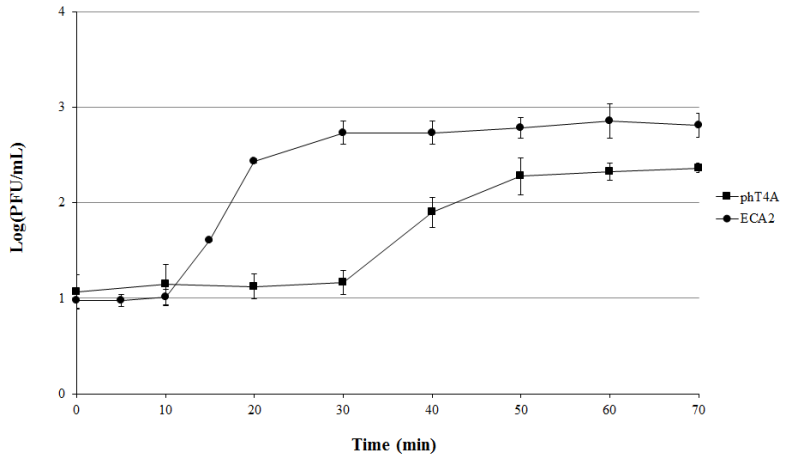

Figure 3.3 One-step growth curves of phages phT4A and ECA2 in the presence of *E. coli* as host. Values represent the mean of three experiments; error bars represent the standard deviation.

3.4.7. Killing curves

ANOVA revealed that the differences between sampling time among treatments ($F_{6,12}$ = 552.1, $p < 0.0001$) and density of the *E. coli* ($F_{7,14}$ = 575,7, $p < 0.0001$) were highly significant. There was also a significant interaction between treatments and density of the *E.coli* ($F_{42,84}$ = 118.7, $p < 0.0001$).

At a MOI of 1, the maximum of bacterium inactivation with phage phT4A phage was 5.8 log CFU/mL (Figure 3.4A), achieved after 8 h of phage therapy (ANOVA, $p < 0.05$). Increasing the MOI to a value of 100 did not significantly increase the inactivation factor (5.8 log CFU/mL) after the same period of time (ANOVA, $p > 0.05$).). However, after 4 h

of incubation, increasing the MOI from 1 to 100, significantly increases the inactivation (ANOVA, $p < 0.05$) (decreased of 1.5 log CFU/mL to MOI of 1 and of 4.0 log CFU/mL to MOI of 100) (Figure 3.4A). After 12 h, the inactivation factor was about 5.5 log CFU/mL for both MOI of 1 and 100 (ANOVA, $p > 0.05$) (Figure 3.4A).

For the phage ECA2, the maximum inactivation was about 4.9 log CFU/mL for both MOI, achieved after 6 h of applying phage therapy (ANOVA, $p < 0.05$) (Figure 3.4A). After 12 h, the rate of inactivation was still considerably high (3.9 log CFU/mL; ANOVA, $p < 0.05$) for both MOI (Figure 3.4A). At a MOI of 1, the inactivation of phages phT4A and ECA2 was statistically similar during the treatment (ANOVA, $p > 0.05$), however, at a MOI of 100, the inactivation of two phages was statistically different (ANOVA, $p < 0.05$). At a MOI of 100, the rate of bacterial inactivation with the phage phT4A (maximum reduction of 5.8 log CFU/mL after 8 h) was, in general, significantly higher (ANOVA, $p < 0.05$) than the one obtained with the phage ECA2 (maximum reduction of 4.9 log CFU/mL after 6 h). However, after 4 h of treatment, the bacterial inactivation of phage ECA2 was significantly higher (ANOVA, $p < 0.05$) than the obtained with the phage phT4A for the both MOIs (Figure 3.4A)

At MOI of 1, the maximum inactivation factor with phage cocktail phT4A/ECA2 was 5.3 log CFU/mL, achieved after 8 h of phage therapy (ANOVA, $p < 0.05$). Increasing MOI to a value of 100 did significantly increase the rate of inactivation (6.2 log CFU/mL) after the same period of time (ANOVA, $p > 0.05$) (Figure 3.4A). However, after 4 h, the inactivation factor was considerably higher for the MOI 100 (4.3 log CFU/mL for to MOI of 1 and 5.6 log CFU/mL for to MOI of 100; ANOVA, $p < 0.05$) and significantly higher (ANOVA, $p < 0.05$) than the one obtained with the phage phT4A for both MOI (1.5 log

CFU/mL for to MOI of 1 and 4.0 log CFU/mL for to MOI of 100; ANOVA, $p < 0.05$) and phage ECA2 for MOI of 100 (4.8 log CFU/mL; ANOVA, $p < 0.05$) (Figure 3.4A).

Bacterial density in the BC increased by 3.5 log CFU/mL (ANOVA, $p < 0.05$) during the first 12 h of incubation. The differences in bacterial concentration between different MOI values were not significant (ANOVA, $p > 0.05$) (Figure 3.4A).

ANOVA revealed that the differences between sampling time among treatments ($F_{6, 12} = 552.15$, $p < 0.0001$) and concentration of the phages ($F_{11, 22} = 341.3$, $p < 0.0001$) were highly significant. There was also a significant interaction between treatments and concentration of the phages ($F_{66, 132} = 113.2$, $p < 0.0001$).

No decrease on the phage survival was observed during the 12 h of the experiments for the phage alone and for the phage in the presence of the host (Figure 3.4B) in different treatments. While the phage control (PC) remained constant throughout the experiment (ANOVA, $p > 0.05$), when the phage phT4A was incubated in the presence of the host, a significant increase (1.5 log PFU/mL, ANOVA, $p < 0.05$) was observed for the MOI of 1 (ANOVA, $p < 0.05$) (Figure 3.4B). Increasing the MOI to 100 caused a smaller increase in phage survival (about 0.5 log PFU/mL). For phage ECA2, the survival factor increased up to 1.5 log PFU/mL for MOI of 1 and 0.35 log PFU/mL for MOI of 100. Similar survival factors were observed for phage cocktail phT4A/ECA2 (1.4 log PFU/mL for MOI of 1 and 0.3 log PFU/mL for MOI of 100) (Figure 3.4B).

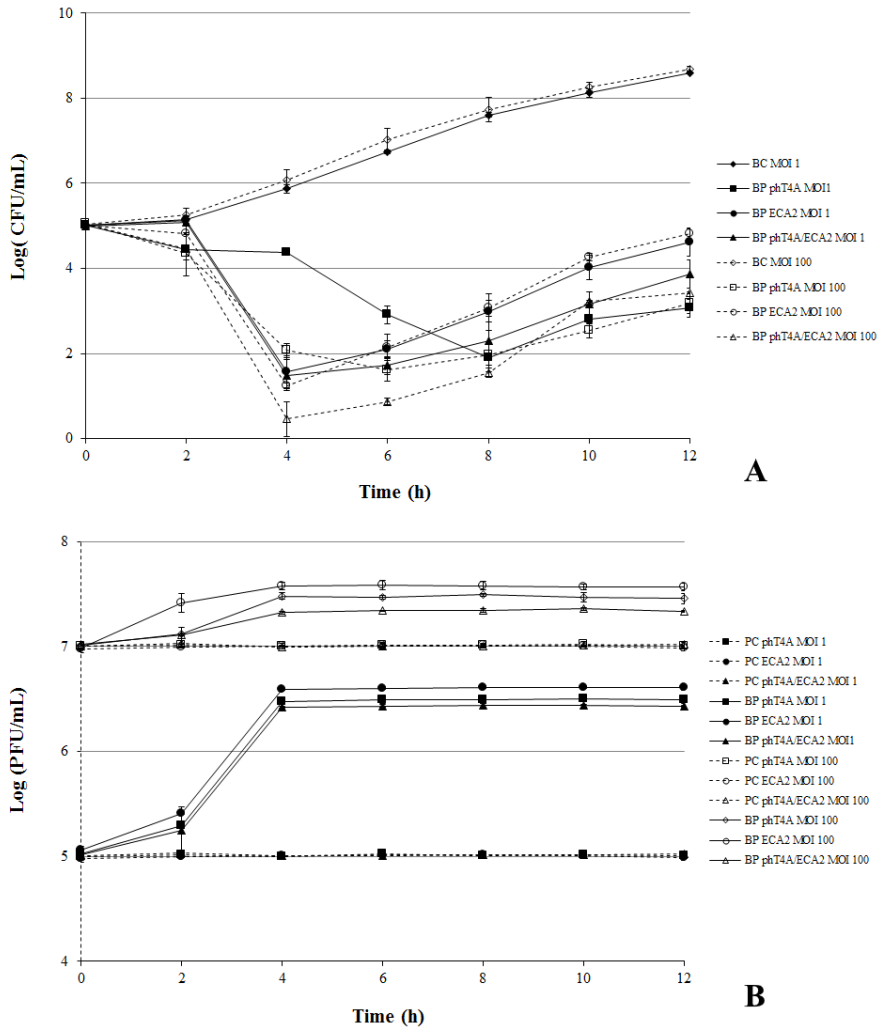

Figure 3.4 Inactivation of *E. coli* by the two phages alone (phT4A and ECA2) and phage cocktail (phT4A/ECA2) at a MOI of 1 and 100 during 12 h. A. Bacterial concentration: BC — bacteria control; BP — bacteria plus phage. B. Phage concentration: PC — phage control; BP — bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

3.4.8. Fitness of phage resistant mutants

In presence of phages differences between the growth of resistant bacteria and the growth of sensitive bacteria were observed, with resistant bacteria to phage phT4A (Figure 3.5A , t = 55.12, p = 0.0003 at 6 h and t = 18.07, p = 0.003 at 12 h), EC2A (Figure 3.5B, t

= 14.05, p = 0.005 at 6 h and t = 18.79, p = 0.0028) and cocktail phT4A/EC2A (Figure 3.5C, t = 14.33, p = 0.0048 at 6 h and t = 50.14, p = 0.0004) with higher concentration at 6 and 12 h than the sensitive bacteria. In absence of phages no differences were found between the concentration of resistant bacteria to the phage phT4A (Figure 3.5A , t = 0.3950, p = 0.7310 at 6 h and t = 0.6928, p = 0.5601), the phage EC2A (Figure 3.5B , t = 1.746, p = 0.2230 at 6 h and t = 0.6928, p = 0.5601 at 12 h) and to the cocktail phT4A/ECA2 (Figure 3.5C, t = 2.00, p = 0.1835 at 6 h and t = 2.097, p = 0.1710 at 12 h) and the concentration of sensitive bacteria.

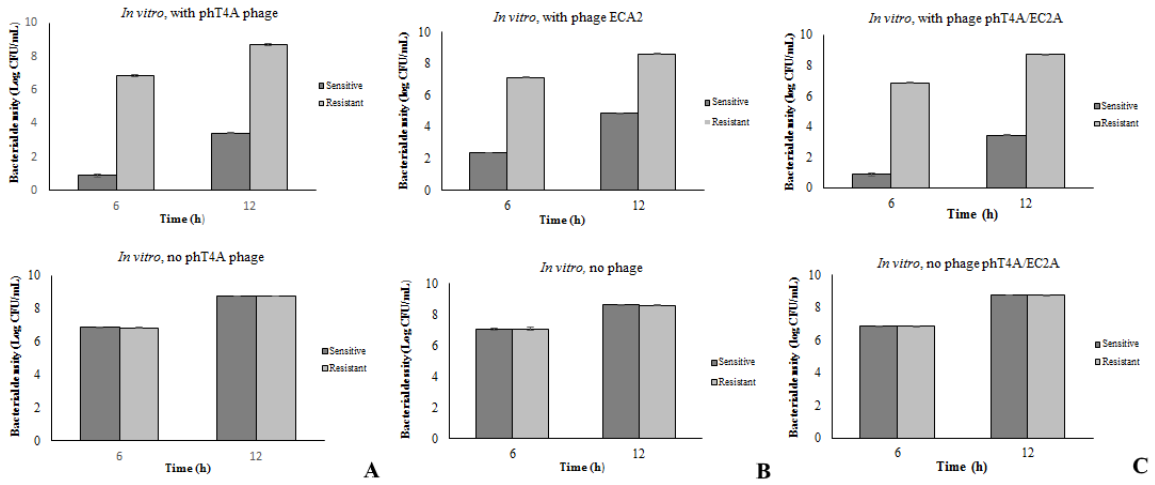

Figure 3.5 *In vitro* bacterial concentration of resistant mutants versus their sensitive bacteria in the presence or absence of phages after 6 and 12h. (A) phage phT4A, (B) phage EC2A and (C) cocktail phT4A/EC2A.

In parallel with this assay, we determined the growth curves using the sensitive bacteria and resistant bacteria for the phage phT4A, EC2A and phT4A/EC2A (Figure 3.6). For both phages and cocktail, ANOVA revealed significant differences (ANOVA, $p < 0.05$) between growth rate of the sensitive bacteria in presence the phage and, sensitive and resistant bacteria in absence of the phage and resistant bacteria with phage, after 8 h of

incubation. ANOVA did not reveal any difference in growth rates (ANOVA, $p > 0.05$) between the sensitive and resistant bacteria in the absence of phage and resistant bacteria with phage, during the 12 h incubation

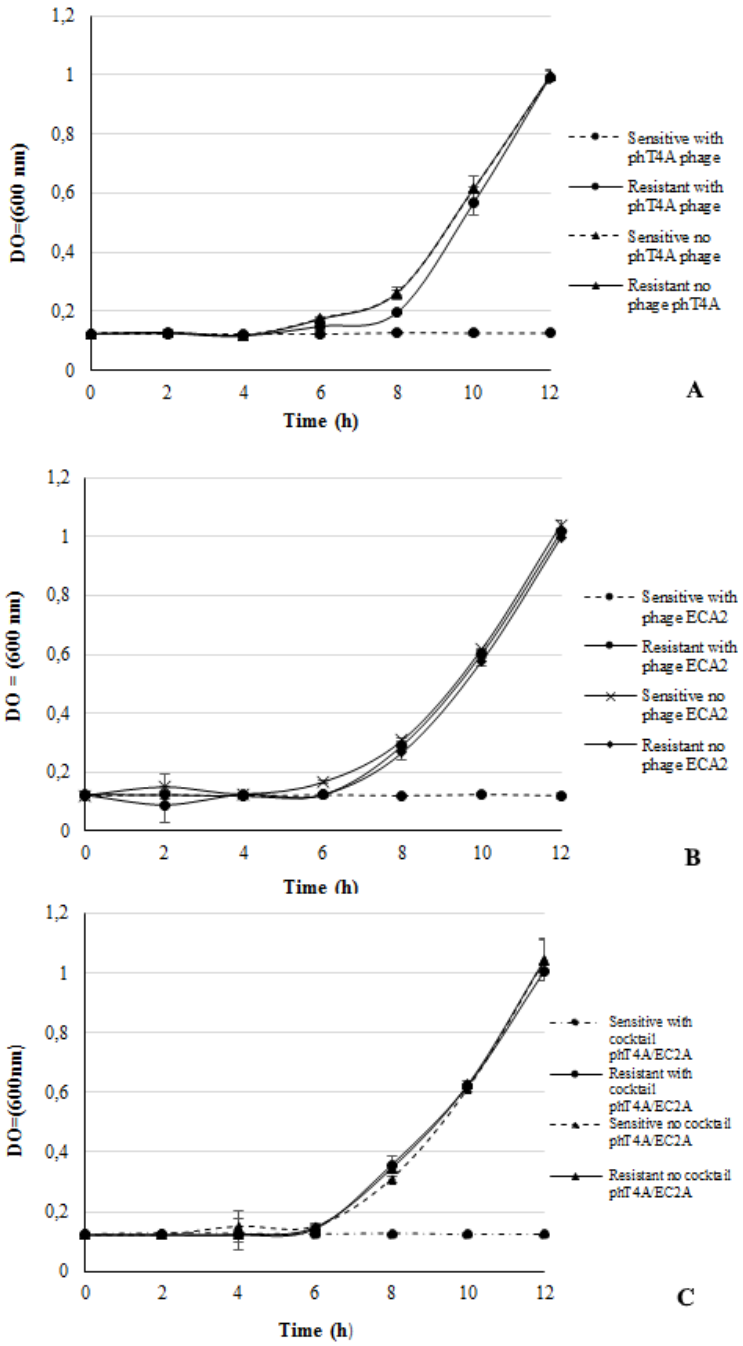

Figure 3.6 Growth curves of sensitive bacteria and resistant mutants in presence or absence of phages during 12h, with optical density readings at 600 nm. (A) phage phT4A, (B) phage EC2A and (C) cocktail phT4A/EC2A.

3.4.9. Rate of emergence of bacterial mutants resistant to bacteriophages

E. coli (ATCC 13706) showed different rates of phage-resistant mutants for single phage suspensions and for phage cocktails (Table 3.2). Mutants bacterial colonies were smaller than those of the control without added phage and were visible only after 6 days of incubation. In the control, colonies were visible after 24 h of incubation in similar conditions.

Table 3.2 Frequencies of *E. coli* spontaneous mutations to bacteriophage resistance

	Control sample (CFU mL^{-1})	Sample treated with phages	Frequency of mutants
phT4A	$1.07 \pm 0.64 \times 10^{9}$	$3.77 \pm 0.90 \times 10^{5}$	3.52×10^{-4}
ECA2	$5.70 \pm 0.18 \times 10^{8}$	$1.58 \pm 0.46 \times 10^{6}$	2.77×10^{-3}
phT4A/ECA2	$9.72 \pm 0.32 \times 10^{8}$	$1.17 \pm 0.51 \times 10^{6}$	1.20×10^{-3}

3.4.10. Prophage detection in the host after phage addition

No phages were detected in the supernatant of cultures of *E. coli* or in the mixture of bacteria and phages after treatment with mitomycin C (1µg/mL).

3.4.11. Host sensitivity to bacteriophages after one cycle of phage contact

After incubation in the presence of single phages and phage cocktail and five streak-plating steps on solid medium, host bacterium had the capacity to form completely cleared zones (positive spot test). However, EOP results indicated that the two phages and phage cocktail did not form phage lysis plates and cannot adsorb and replicate in the presence to the resistant strains bacteria after five streak-plating steps on solid medium.

3.5. Discussion

To our knowledge, there is not yet any report regarding the use of phages to control *E. coli* during depuration. In this study, the data provide proof of the principle that the application of phages (phT4A and ECA2) could reduce the population of *E. coli*. Elimination of *E. coli* in bivalves is important for public health because this pathogen can easily accumulate in raw bivalves (Kanayama et al., 2015; Potasman et al., 2002). Depuration is a process by which shellfish expulse their intestinal contents, however, some pathogenic microorganisms are resistant to this process (FAO, 2004; Martínez et al., 2009; Rong et al., 2014).

The selection of appropriate bacteriophages to be used in phage therapy is a critical stage to achieve a successful phage-mediated control of pathogenic bacteria. Criteria such as the host range, adsorption rate, latent period, burst size, lysogenic induction capacity, survival in the environment and safety are determinant in the election of suitable phages to be used in phage treatment (Mateus et al., 2014; Pereira et al., 2011a).

The genome sequence of the two phages isolated in this study was obtained, and no genes encoding products such as toxins, antibiotic resistance and integrase enzymes were detected by aminoacid sequence comparisons. Consequently, from our current state of knowledge, these phages can be considered safe to be used in phage therapy. Based on genomic analysis but also on the lack of lysogenic strains following the resistance development experiment, EC2A and phT4A phages can be considered as lytic bacteriophages which is a prerequisite for phage therapy. One of the major advantages of phage treatment is the phage specificity, since the non-target bacterial populations should remain relatively undisturbed (Koskella and Meaden, 2013). The two phages infected only their hosts, spot test indicated that phage phT4A had the capacity to form completely

cleared zones on 20 of the 40 strains, and the EC2A phage form cleared zones on 19 of the 40 strains. However, efficiency of plating (EOP) results indicated that the two phages did not form lysis plaques in none of the strains. Mirzaei and Nilsson (2015), obtained similar results, stating that spot test cannot be used for identification and selection of phages to a phage library and should be replaced by EOP assays. Lysis is a plausible mechanism which happens when an overload of phages simultaneously infects a bacterium leading to lysis either from the action of lysins or from rapid depletion of the cells resources (Abedon, 2011). In addition, as observed by Mirzaei and Nilsson (2015), high EOP was not correlated to the results from the spot tests. Further studies are needed to understand the different results obtained by both approaches.

Although, the genome sequence of phage phT4A has a similarity between 98-99% with three phages of the *Klebsiella pneumonie* (phage Matisse, phage KP15 and phage KP27), the phage phT4A not infect the strain of the *K. pneumonie* (negative spot test) used in this study. However, the genome sequence of the phage ECA2 has a similarity 96 - 98% with three phages of the *Enterobacter cloacae* (phage E-2, phage E-3 and phage E-4) and had the capacity to form completely cleared zones of the *E. cloacae*, but presented different host ranges. The host range is determined by the tail fibers on the phage and the cell receptors on the host cell (Beckendorf et al., 1973). Alterations of either of these structures alter the host range. Phages that differed in host range showed completely unrelated variable regions, while phages overlapping host ranges shared highly related variable regions (Brussow and Desiere, 2001).

Phage ECA2 presented the highest burst size (117±11 PFU/host cell), around 7 times higher than that of phage phT4A (17 ± 2 PFU/host cell) and the shortest latent period, 30 min (50 min for the phage phT4A). Similar higher burst size and small latent period were

also found in other bacteriophages such as *E. coli* O157:H7 phage CEV1 (150 PFU/cell, 26 min) (Raya et al., 2006). Other study shows for *E. coli* phage PPO1, as for the phage phT4A, a small burst size of 14 particles per cell with a latent period of 26 min (Fischer et al., 2004). The relatively short latent period and large burst size of phage ECA2 relatively to phage phT4A make the former more suitable to be used for the control of *E. coli* during bivalve depuration.

Before application of phages to inactivate pathogenic bacteria, like *E. coli*, during bivalves depuration, it is important to characterize *in vitro* the dynamics of phage-host replication. The kinetic theory of phage therapy predicts that the MOI could be critical to the bacterial inactivation efficiency. It has been shown, both *in vitro* and *in vivo*, that the reduction of pathogenic bacteria increases in parallel with MOI or that bacterial reduction occurs sooner at higher MOI values (ChiHsin et al., 2000; Pasharawipas et al., 2011; Prasad et al., 2011). However, Nakai (2010) related that the precise initial doses of phage may not be essential because of the self-perpetuating nature of phages, revealed by an increasing of phage titers along with bacteria (Nakai, 2010). In the present study, the increase in MOI from 1 to 100 promoted a significant increase in the efficiency of phage phT4A phage and of phage cocktail phT4A/ECA2. For the phage ECA2, the efficiency was similar for both MOI. However, the number of phages after 4 h of treatment in the presence of the host at MOI of 1 increased more (by 1.5 log PFU/mL) than at MOI 100 (by 0.5 log PFU/mL). This confirms the hypothesis that due to the self-perpetuating nature of phages precise initial doses of phage may not be essential.

Both single phage suspensions and phage cocktails of the phages isolated in this study can be used to inactivate *E. coli*. The phage cocktail was, however, more efficient than the single phage suspensions. Moreover, bacterial inactivation with the phage cocktail

occurred sooner than with phage phT4A suspension. These results are in accordance with other studies (Mateus et al., 2014; O'Flynn et al., 2006; Wagner and Waldor, 2002) that achieved a faster and higher inactivation by using phage cocktails, than that obtained with single-phage suspensions.

The emergency of phage-resistant mutants is currently a major concern related with the use of phages to control infections and it generally accepted that the selection of resistant strains can be prevented by the combined use of cocktails of two or more phages (Hooton et al., 2011; Sandeep, 2006; Scott et al., 2007). In this study, the use of phage cocktails, as well as the use of single-phage suspensions, did not prevent the occurrence of phage-resistant mutants. The rate of proportion of phage-resistant bacteria (determined using only resistant mutants selected from the surviving bacteria after treatment) to phage cocktail phT4A/ECA2 was, however, slightly lower than that observed when phage ECA2 was tested, but around 3 times higher than that of phage phT4A. Similar results have been observed for other phages (Filippov et al., 2011; Silva et al., 2014a).

Bacterial resistance to phages can evolve via a number of different mechanisms, which vary in their specificity. Many of these resistance mechanisms impose a significant fitness cost (Bohannan et al., 1999; Brockhurst et al., 2005), but these costs can vary across environments and the degree of competition for resources (Lennon et al., 2007; Quance and Travisano, 2009). Given the potential use of phages as biocontrol against pathogenic bacteria, a clear understanding of costs associated with evolved resistance could lead to more effective management strategies (Flynn et al., 2004; Heringa et al., 2010; Levin and Bull, 2004). In the experiments of cost of resistance no differences were observed between the concentration of sensitive bacteria and resistant mutants when grown in the absence of phages. Similar results have been observed by Meaden et al. (2015). Under standard

laboratory conditions, Meaden et al. (2015) found no differences in density or maximum growth rates of sensitive versus resistant mutants when grown, in the absence of phages, however, when grown in plant the resistant mutants exhibited reduced densities relative to the sensitive population. These authors demonstrate that phage-mutants that have evolved resistance via a single mutational step pay a substantial cost for this resistance when grown on their *in vivo*, but do not realize any measurable growth rate costs *in vitro*. In future studies, it is necessary to study the cost of resistance in bivalves since that resistance to phage can significantly alter bacterial growth, and therefore that phage-mediated selection in nature is likely to be an important component of bacterial pathogenicity.

After five streak-plating steps on solid medium, the resistant bacteria showed positive spot test, which was not observed for the first two streak-plating steps. In a first glance, it seems to be due to lysogenic induction. However, the experiments of lysogenic induction showed no evidence of lysogeny (results of the mitomycin test) was observed during the phage addition experiments. Moreover, genes codifying to integrase were not detected in the phages genomes. This behavior can be due some genomic or phenotypic modifications in the resistant cells after phage-stress stop. More studies of the research group are ongoing in order to clarify this comportment. Previous results of research group (Pereira et al., 2016b) for *S.* Typhimurium and three specific phages showed that the phage-resistant bacteria did not recover sensitivity, phages did not adsorbed to phage-resistant bacteria and after five streak-plating steps on solid medium these bacteria remained resistant. The authors compared the spectral changes of *S.* Typhimurium resistant and phage-sensitive cells, and observed relevant differences for peaks associated to amide I (1620 cm^{-1}) and amide II (1515 cm^{-1}) from proteins and from carbohydrates and phosphates region (1080-1000 cm^{-1}). This results suggested that the bacteria have evolved

a range of barriers apparently irreversible (associated to amide I and amide II form proteins, carbohydrates and phosphates) to prevent phage adsorption and, consequently, prevent infection.

3.6. Conclusion

The results of the present work suggest that phage therapy using phages individually or combined in cocktails, can be an effective alternative to reduce the concentration of *E. coli*, particularly if combined in a phage cocktail. Although phage ECA2 presents a higher burst size and a shorter latent period, inactivating *E. coli* sooner than phage phT4A, the latter is less inducer of resistance to phage infection. Consequently, to select phages to be used in cocktails, it is necessary to take into account not only phage efficacy but also its propensity to develop phage-resistant mutants.

3.7. Supplementary material

Table 3.1S Coding sequences identified in phT4A phage of *E. coli* strain.

Location	Gene name	Organism evidence	Identity	Evalue	Function
202_969	hypothetical protein KP-KP15_gp117	*Citrobacter* phage IME-CF2	73%	6e-128	Thioredoxin, phage-associated
966_1211	hypothetical protein CPT_Matisse125	*Klebsiella* phage Matisse	99%	0.0	hypothetical protein
1211_1468	hypothetical protein KP27_138	*Klebsiella* phage KP27	100%	2-e54	Phage protein
1465_2388	cytosine-specific methyltransferase	*Klebsiella* phage Miro	99%	0.0	DNA-cytosine methyltransferase (EC 2.1.1.37)
2370_2903	putative homing endonuclease RB16 6	Enterobacteria phage RB16	45%	5e-59	Phage-associated homing endonuclease
2962_3240	hypothetical protein GAP161_115	*Cronobacter* phage vB_CsaM_GAP161	100%	4e-63	Phage protein
3969_4484	hypothetical protein KP-KP15_gp111	*Klebsiella* phage KP15	98%	8e-126	Phage protein
4484_4804	hypothetical protein CPT_Miro118	*Klebsiella* phage Miro	100%	2e-71	hypothetical protein
4812_7028	hypothetical protein KP-KP15_gp109	*Klebsiella* phage KP15	100%	0.0	Phage protein
7065_7715	hypothetical protein CPT_Miro116	*Klebsiella* phage Miro	100%	5e-158	Phage protein
7712_7870	hypothetical protein CPT_Miro115	*Klebsiella* phage Miro	100%	7e-28	hypothetical protein
7867_8337	hypothetical protein CPT_Miro114	*Klebsiella* phage Miro	100%	2e-109	Phage protein
8353_8637	hypothetical protein KP-KP15_gp105	*Klebsiella* phage KP15	100%	3e-62	Phage protein

8739_8993	hypothetical protein CPT_Matisse111	*Klebsiella* phage Matisse	100%	2e-54	hypothetical protein
8995_9219	hypothetical protein CPT_Matisse110	*Klebsiella* phage Matisse	100%	9e-48	Phage protein
9254_9682	hypothetical protein ADS69_00109	*Enterobacter* phage phiEap-3	100%	2e-101	Phage protein
10109_9717	baseplate wedge subunit	*Klebsiella* phage KP15	100%	5e-90	Phage baseplate wedge subunit (T4-like gp25)
10664_10110	baseplate hub subunit	*Klebsiella* phage KP27	100%	5e-135	Phage baseplate hub
11404_10661	gp51 baseplate hub assembly protein	*Klebsiella* phage KP15	100%	4e-180	Phage baseplate
12296_11433	gp54 baseplate subunit	*Klebsiella* phage KP15	100%	0.0	Phage baseplate tail tube initiator
13379_12306	tail-tube assembly protein	*Klebsiella* phage Matisse	100%	0.0	Phage baseplate tail tube cap (T4-like gp48)
14985_13390	hypothetical protein KP-KP15_gp096	*Klebsiella* phage KP15	99%	0.0	Phage protein
15742_15167	baseplate distal hub subunit	*Klebsiella* phage KP15	100%	2e-137	Phage baseplate distal hub subunit (T4-like gp28)
15812_16921	baseplate hub subunit	*Klebsiella* phage KP27	100%	0.0	Phage baseplate hub
17295_16918	hypothetical protein KP-KP15_gp093	*Klebsiella* phage KP15	100%	1e-87	Phage protein
17593_17282	hypothetical protein CPT_Matisse99	*Klebsiella* phage Matisse	99%	2e-68	Phage protein
17894_17604	hypothetical protein CPT_Matisse98	*Klebsiella* phage Matisse	100%	4e-64	Phage protein
18555_17986	hypothetical protein ADS69_00091	*Enterobacter* phage phiEap-3	99%	4e-138	hypothetical protein
20161_18632	gp30 DNA ligase	*Klebsiella* phage KP15	100%	0.0	DNA ligase, phage-associated
20756_20142	hypothetical protein KP27_104	*Klebsiella* phage KP27	100%	7e-152	Phage protein
20974_20753	hypothetical protein KP27_103	*Klebsiella* phage KP27	100%	9e-47	Phage protein
21212_20976	hypothetical protein KP-KP15_gp085	*Klebsiella* phage KP15	100%	2e-48	Phage protein
21565_21248	putative homing endonuclease	*Klebsiella* phage KP27	58%	7e-34	Phage-associated homing endonuclease
22068_21763	hypothetical protein CPT_Margaery94	*Citrobacter* phage Margaery	93%	6e-55	T4-like phage protein, T4 GC1630
22451_22299	hypothetical protein CPT_Margaery91	*Citrobacter* phage Margaery	96%	3e-26	Phage protein
22618_22448	hypothetical protein ADS69_00084	*Enterobacter* phage phiEap-3	98%	5e-34	hypothetical protein
22842_22603	hypothetical protein KP-KP15_gp081	*Klebsiella* phage KP15	100%	8e-53	hypothetical protein
23138_22845	hypothetical protein KP-KP15_gp080	*Klebsiella* phage KP15	100%	3e-64	hypothetical protein
23533_23264	hypothetical protein KP27_095	*Klebsiella* phage KP27	100%	6e-58	Phage protein
23895_23533	Vs.6	*Klebsiella* phage KP15	100%	3e-82	Pyruvate formate-lyase (EC 2.3.1.54)
24331_23906	hypothetical protein KP27_093	*Klebsiella* phage KP27	100%	4e-101	Phage protein
24933_24514	hypothetical protein CPT_Miro82	*Klebsiella* phage Miro	99%	1e-95	Phage protein
25224_24943	hypothetical protein CPT_Miro81	*Klebsiella* phage Miro	99%	1e-62	Phage protein
25532_25459	tRNA	--	--	--	tRNA-Met-CAT
25791_25540	hypothetical protein KP-KP15_gp074	*Klebsiella* phage KP15	100%	1e-53	Phage protein
26143_25802	head assembly chaperone with GroEL	*Klebsiella* phage Miro	100%	2e-76	Phage capsid assembly chaperone
26665_26159	cytidine deaminase-	*Klebsiella* phage Matisse	99%	6e-125	dCMP deaminase (EC 3.5.4.12)
26939_26655	hypothetical protein KP-KP15_gp071	*Klebsiella* phage KP15	100%	6e-64	hypothetical protein
27183_26932	hypothetical protein KP-KP15_gp070	*Klebsiella* phage KP15	100%	1e-52	hypothetical protein
27467_27180	hypothetical protein ADS69_00071	*Enterobacter* phage phiEap-3	100%	2e-64	hypothetical protein
27642_27520	PseT 3' phosphatase and 5' polynucleotide kinase	*Klebsiella* phage KP15	98%	7e-20	3'-phosphatase, 5'-polynucleotide kinase, phage-associated
486_85	hypothetical protein JD18_241	*Klebsiella* phage JD18	88%	1e-77	hypothetical protein
4314_529	L-shaped tail fiber protein	*Enterobacter* phage phiEap-3	87%	0.0	Phage tail fiber protein

78_2120	rIIA protector from prophage-induced early lysis	*Klebsiella* phage KP27	99%	0.0	Phage rIIA lysis inhibitor
2120_3019	host membrane ATPase affecting protein	*Klebsiella* phage KP15	100%	0.0	Phage lysis inhibitor # T4-like rIIA-rIIB membrane associated #T4 GC1698
3065_3439	hypothetical protein KP-KP15_gp257	*Klebsiella* phage KP15	100%	6e-85	hypothetical protein
3471_3851	nucleoid disruption protein	*Klebsiella* phage KP27	100%	2e-89	Phage protein
3888_4136	hypothetical protein CPT_Matisse278	*Klebsiella* phage Matisse	100%	1e-50	Phage protein
4181_4411	hypothetical protein GAP161_268	*Cronobacter* phage vB_CsaM_GAP161	99%	1e-46	Phage protein
4408_4545	hypothetical protein GAP161_267	*Cronobacter* phage vB_CsaM_GAP161	100%	1e-24	hypothetical protein
4520_4849	hypothetical protein KP27_293	*Klebsiella* phage KP27	100%	4e-75	Phage protein
4846_5073	hypothetical protein KP27_292	*Klebsiella* phage KP27	100%	3e-50	hypothetical protein
5154_5951	hypothetical protein KP27_291	*Klebsiella* phage KP27	100%	0.0	Phage protein
5951_6241	hypothetical protein KP27_290	*Klebsiella* phage KP27	99%	2e-65	Phage protein
6349_6507	hypothetical protein KP-KP15_gp251	*Klebsiella* phage KP15	94%	2e-29	hypothetical protein
7155_6508	T holin lysis mediator	*Klebsiella* phage KP27	98%	1e-156	Phage holin
880_353	distal long tail fiber assembly catalyst	*Klebsiella* phage KP27	91%	6e-117	Phage tail fibers
4801_920	L-shaped tail fiber protein	*Klebsiella* phage KP15	80%	0.0	Phage tail fiber protein
1495_32	NrdA ribonucleotide reductase A subunit	*Klebsiella* phage KP15	100%	0.0	Ribonucleotide reductase of class Ia (aerobic), alpha subunit (EC 1.17.4.1)
2410_1535	Td thymidylate synthetase	*Klebsiella* phage KP15	99%	0.0	Thymidylate synthase (EC 2.1.1.45)
2990_2412	dihydrofolate reductase	*Klebsiella* phage Matisse	99%	3e-139	Dihydrofolate reductase (EC 1.5.1.3)
3244_2987	hypothetical protein KP-KP15_gp058	*Klebsiella* phage KP15	100%	4e-56	Phage protein
3471_3241	hypothetical protein KP-KP15_gp057	*Klebsiella* phage KP15	100%	4e-48	Phage protein
3758_3468	hypothetical protein KP27_068	*Klebsiella* phage KP27	100%	3e-65	Phage protein
5527_3818	hypothetical protein ADS69_00056	*Enterobacter* phage phiEap-3	99%	0.0	Phage protein
5795_5601	hypothetical protein KP-KP15_gp054	*Klebsiella* phage KP15	95%	9e-36	Phage protein
5852_6571	putative Mob-like HNH homing endonuclease	*Cronobacter* phage vB_CsaM_GAP161	64%	2e-115	Phage-associated homing endonuclease
8249_6558	recombination endonuclease subunit	*Klebsiella* phage KP27	99%	0.0	Phage recombination-related endonuclease Gp46
8520_8233	hypothetical protein KP-KP15_gp052	*Klebsiella* phage KP15	100%	9e-63	hypothetical protein
9538_8507	gp47 recombination endonuclease subunit	*Klebsiella* phage KP15	99%	0.0	Phage recombination-related endonuclease Gp47
9916_9590	hypothetical protein KP-KP15_gp050	*Klebsiella* phage KP15	100%	8e-73	Phage protein
10124_9909	hypothetical protein KP-KP15_gp049	*Klebsiella* phage KP15	100%	6e-42	Phage protein
10648_10121	gp55 RNA polymerase sigma factor	*Klebsiella* phage KP15	100%	1e-128	T4-like phage RNA polymerase sigma factor for late transcription
10953_11888	RnaseH	*Klebsiella* phage KP15	100%	0.0	Phage ribonuclease H (EC 3.1.26.4)
11898_12161	DsbA dsDNA binding	*Klebsiella* phage KP15	100%	7e-56	Phage double-stranded DNA binding protein #T4-like dsbA, late transcriptional regulation #T4 GC1668
12165_12410	gp33 late promoter transcription	*Klebsiella* phage KP15	100%	9e-53	Phage transcriptional regulator
12407_13066	helicase assembly protein	*Klebsiella* phage Matisse	100%	2e-155	Phage DNA helicase loader
13082_14053	ssDNA binding protein	*Enterobacter* phage phiEap-3	100%	0.0	Single stranded DNA-binding protein, phage-associated
14109_14378	RpbA RNA polymerase binding protein	*Klebsiella* phage KP15	100%	9e-58	Phage RNA polymerase binding protein
14405_15070	gp45 sliding clamp DNA polymerase	*Klebsiella* phage KP15	100%	3e-156	Sliding clamp DNA polymerase accessory protein, phage associated
15129_16127	gp44 clamp-loader subunit	*Klebsiella* phage KP15	100%	0.0	Replication factor C small subunit / Phage DNA polymerase clamp loader subunit
16130_16693	gp62 clamp-loader subunit	*Klebsiella* phage KP15	99%	5e-133	Phage DNA polymerase clamp loader subunit Gp62

16697_17059	RegA	*Klebsiella* phage KP27	100%	4e-83	Phage endoribonulcease translational repressor of early genes, regA
17128_17415	hypothetical protein CPT_Matisse40	*Klebsiella* phage Matisse	100%	2e-64	Phage protein
17412_17741	hypothetical protein CPT_Miro40	*Klebsiella* phage Miro	100%	3e-73	hypothetical protein
329_12	gp49 recombination endonuclease VII	*Klebsiella* phage KP15	99%	7e-72	Phage endonuclease
801_526	NrdC thioredoxin	*Klebsiella* phage KP15	100%	8e-62	Thioredoxin, phage-associated
1661_810	DNA adenine methylase	*Enterobacter* phage phiEap-3	99%	0.0	DNA adenine methylase (EC 2.1.1.72)
1960_1658	hypothetical protein KP27_266	*Klebsiella* phage KP27	100%	9e-67	Phage protein
3380_1947	nicotinamide phosphoribosyl transferase	*Klebsiella* phage KP15	100%	0.0	Nicotinamide phosphoribosyltransferase (EC 2.4.2.12)
3857_3408	hypothetical protein KP27_264	*Klebsiella* phage KP27	100%	5e-106	Phage protein
4127_3867	hypothetical protein KP27_263	*Klebsiella* phage KP27	100%	2e-55	Phage protein
4648_4205	hypothetical protein KP-KP15_gp224	*Klebsiella* phage KP15	100%	4e-106	hypothetical protein
5298_4717	hypothetical protein ADS69_00241	*Enterobacter* phage phiEap-3	99%	1e-139	Phage protein
6307_5288	RNA ligase 2	*Enterobacter* phage phiEap-3	100%	0.0	RNA ligase, phage-associated
6835_6311	postulated decoy of host sigma70 or sigmaS	*Klebsiella* phage KP27	100%	2e-126	Srd postulated decoy of host sigma70 or sigmaS
7191_6835	hypothetical protein KP-KP15_gp220	*Klebsiella* phage KP15	100%	2e-80	Phage protein
7519_7193	hypothetical protein CPT_Miro237	*Klebsiella* phage Miro	100%	1e-72	Phage protein
7692_7516	hypothetical protein KP-KP15_gp218	*Klebsiella* phage KP15	100%	4e-33	Phage protein
8006_7689	hypothetical protein KP-KP15_gp217	*Klebsiella* phage KP15	99%	1e-72	Phage protein
8914_8039	hypothetical protein CPT_Miro234	*Klebsiella* phage Miro	99%	0.0	Phage protein
9029_9868	hypothetical protein KP-KP15_gp215	*Klebsiella* phage KP15	100%	0.0	Phage protein
9918_10382	hypothetical protein KP-KP15_gp214	*Klebsiella* phage KP15	100%	4e-108	Phage protein
10933_10415	hypothetical protein KP27_250	*Klebsiella* phage KP27	99%	1e-124	Phage protein
11222_10947	Hoc large outer capsid protein	*Klebsiella* phage KP15	100%	1e-60	Phage capsid and scaffold
11766_11233	Hoc head outer capsid protein	*Klebsiella* phage KP15	100%	5e-125	Phage capsid and scaffold
12064_11795	hypothetical protein KP27_247	*Klebsiella* phage KP27	100%	5e-59	Phage protein
12715_12404	hypothetical protein KP-KP15_gp209	*Klebsiella* phage KP15	100%	1e-71	Phage protein
13431_12808	inhibitor of prohead protease	*Klebsiella* phage Miro	97%	1e-147	Protein inhbitor
13502_15001	Helicase	*Klebsiella* phage KP27	100%	0.0	DNA helicase, phage-associated
15088_15243	UvsW.1 hypothetical protein	*Klebsiella* phage KP15	100%	6e-27	DNA helicase, phage-associated
15694_15518	UvsY.-2	*Klebsiella* phage KP15	100%	2e-34	Phage protein
16113_15694	UvsY	*Klebsiella* phage KP15	100%	9e-97	Single stranded DNA-binding protein, phage-associated
17707_16418	capsid vertex protein	*Klebsiella* phage KP27	100%	0.0	Phage capsid vertex
18375_17740	SegD homing endonuclease	*Klebsiella* phage KP15	99%	1e-152	Phage-associated homing endonuclease
20029_18461	major capsid protein	*Klebsiella* phage Miro	99%	0.0	Phage major capsid protein
20837_20049	gp22 prohead core protein	*Klebsiella* phage KP15	100%	0.0	Phage prohead assembly (scaffolding) protein
21517_20870	prohead core scaffold protein and protease	*Klebsiella* phage Matisse	100%	7e-153	Phage prohead assembly (scaffolding) protein
21924_21520	gp68 prohead core protein	*Klebsiella* phage KP15	100%	3e-89	Phage capsid and scaffold
22106_21933	prohead core protein	*Klebsiella* phage Matisse	98%	1e-27	Phage prohead core protein
23750_22176	gp20 portal vertex protein	*Klebsiella* phage KP15	100%	0.0	Phage portal vertex of the head
24291_23806	gp19 tail tube protein	*Klebsiella* phage KP15	100%	4e-117	Phage tail fibers

26331_24340	tail sheath protein	*Klebsiella* phage Miro	99%	0.0	Phage tail sheath monomer
28180_26351	large terminase protein	*Enterobacter* phage phiEap-3	100%	0.0	Phage terminase, large subunit
28691_28149	gp16 small terminase protein	*Klebsiella* phage KP15	100%	2e-128	Phage terminase, small subunit
29512_28688	gp15 tail sheath stabilizer and completion protein	*Klebsiella* phage KP15	100%	0.0	Proximal tail sheath stabilization protein
30339_29593	gp14 neck protein	*Klebsiella* phage KP15	96%	7e-175	T4-like phage head completion, neck hetero-dimeric protein (T4-like gp14)
30946_30332	putative endonuclease	*Escherichia* phage MX01	57%	8e-68	Phage-associated homing endonuclease
31882_30956	gp13 neck protein	*Klebsiella* phage KP15	99%	0.0	T4-like phage head completion, neck hetero-dimeric protein (T4-like gp13)
33363_31921	whisker protein	*Klebsiella* phage KP15	99%	0.0	Phage neck whiskers
33673_33368	whisker protein	*Klebsiella* phage Matisse	100%	2e-60	Phage neck whiskers
35072_33687	short tail fiber protein	*Klebsiella* phage Matisse	99%	0.0	Phage tail fibers
35750_35082	gp11 baseplate wedge subunit and tail pin	*Klebsiella* phage KP15	100%	2e-162	Phage baseplate wedge subunit and tail pin (T4-like gp11) # T4 GC 1597
37567_35750	baseplate wedge subunit and tail pin	*Klebsiella* phage KP27	99%	0.0	Phage baseplate wedge subunit and tail pin (T4-like gp10)
38427_37564	baseplate wedge tail fiber connector	*Klebsiella* phage KP27	99%	0.0	Phage baseplate wedge tail fiber connector (T4-like gp9)
39429_38437	baseplate wedge subunit	*Klebsiella* phage Matisse	100%	0.0	Phage baseplate wedge subunit (T4-like gp8)
42516_39430	baseplate wedge initiator	*Klebsiella* phage Matisse	99%	0.0	Phage baseplate wedge subunit #T4-like gp7
44522_42597	baseplate wedge subunit	*Klebsiella* phage Miro	99%	0.0	Phage baseplate wedge subunit (T4-like gp6)
46011_44554	hypothetical protein CPT_Miro198	*Klebsiella* phage Miro	99%	0.0	Phage protein
1824_55	baseplate hub subunit and tail lysozyme	*Enterobacter* phage phiEap-3	100%	0.0	Phage baseplate hub
2375_1821	baseplate wedge subunit	*Klebsiella* phage KP27	100%	2e-134	Phage baseplate wedge subunit #T4-like gp53
2426_2893	gp4 head completion protein	*Klebsiella* phage KP15	100%	2e-112	Phage head completion protein
2909_3763	DNA end protector protein	*Klebsiella* phage KP27	100%	0.0	Phage DNA end protector during packaging
4522_3800	hypothetical protein KP27_206	*Klebsiella* phage KP27	100%	1e-170	Phage protein
4610_4494	hypothetical protein KP27_206	*Klebsiella* phage KP27	100%	2e-09	hypothetical protein
4959_5489	gp3 tail completion and sheath stabilizer protein	*Klebsiella* phage KP15	100%	4e-129	Phage tail completion protein
5489_6184	deoxynucleoside monophosphate kinase	*Klebsiella* phage KP27	98%	3e-168	Deoxynucleotide monophosphate kinase (EC 2.7.4.13) #T4-like phage gp1 #T4 GC1586
6199_6459	chaperone for tail fiber formation	*Klebsiella* phage KP27	99%	3e-52	Phage protein
6511_7134	hypothetical protein KP27_202	*Klebsiella* phage KP27	99%	1e-150	T4-like phage protein, T4 GC1584
7142_7681	hypothetical protein KP27_201	*Klebsiella* phage KP27	100%	4e-131	Phage protein
7747_7887	hypothetical protein ADS69_00187	*Enterobacter* phage phiEap-3	100%	3e-27	Phage protein
7989_8117	hypothetical protein KP-KP15_gp168	*Klebsiella* phage KP15	100%	4e-20	hypothetical protein
8128_8463	hypothetical protein CPT_Matisse187	*Klebsiella* phage Matisse	100%	9e-78	Phage protein
8460_8753	hypothetical protein KP-KP15_gp167	*Klebsiella* phage KP15	100%	2e-67	Phage protein
8746_8997	hypothetical protein CPT_Miro182	*Klebsiella* phage Miro	99%	6e-56	hypothetical protein
8994_9239	hypothetical protein KP27_195	*Klebsiella* phage KP27	96%	7e-50	hypothetical protein
9240_9647	nudix hydrolase	*Enterobacter* phage phiEap-3	100%	7e-97	Nudix hydrolase, phage-associated
9658_10041	hypothetical protein KP27_193	*Klebsiella* phage KP27	98%	9e-89	Phage protein
10043_10300	hypothetical protein KP-KP15_gp164	*Klebsiella* phage KP15	99%	2e-55	Phage protein
10329_10652	hypothetical protein KP-KP15_gp163	*Klebsiella* phage KP15	100%	4e-74	Phage protein
10652_11179	hypothetical protein KP-KP15_gp162	*Klebsiella* phage KP15	100%	1e-129	Phage protein
11176_12090	hypothetical protein KP27_188	*Klebsiella* phage KP27	99%	0.0	hypothetical protein

12167_12370	hypothetical protein KP27_187	*Klebsiella* phage KP27	100%	1e-40	Phage protein
12380_12514	hypothetical protein M975_1922	*Buttiauxella brennerae* ATCC 51605	81%	5e-17	hypothetical protein
12689_13087	hypothetical protein ADS69_00174	*Enterobacter* phage phiEap-3	99%	4e-94	hypothetical protein
13095_13670	hypothetical protein ADS69_00173	*Enterobacter* phage phiEap-3	98%	1e-139	Phage protein
14014_14292	hypothetical protein CPT_Miro170	*Klebsiella* phage Miro	99%	5e-61	Phage protein
14364_14615	hypothetical protein RB16p161	Enterobacteria phage RB16	100%	3e-54	hypothetical protein
14612_14758	hypothetical protein RB16p160	Enterobacteria phage RB16	100%	2e-26	hypothetical protein
14755_14955	hypothetical protein GAP161_158	*Cronobacter* phage vB_CsaM_GAP161	95%	2e-37	hypothetical protein
15022_15276	hypothetical protein Lw1_gp163	*Escherichia* phage Lw1	96%	2e-53	Phage protein
15997_15269	hypothetical protein CPT_Miro165	*Klebsiella* phage Miro	95%	1e-172	hypothetical protein
16010_17164	radical SAM superfamily protein	*Klebsiella* phage Miro	96%	0.0	Phage protein
17161_17457	hypothetical protein ADS69_00160	*Enterobacter* phage phiEap-3	98%	4e-64	hypothetical protein
17454_17795	hypothetical protein ADS69_00159	*Enterobacter* phage phiEap-3	98%	1e-77	Phage protein
17776_18123	hypothetical protein KP27_173	*Klebsiella* phage KP27	100%	1e-78	Phage protein
18120_18293	hypothetical protein KP27_172	*Klebsiella* phage KP27	100%	3e-34	hypothetical protein
18304_18735	hypothetical protein KP27_171	*Klebsiella* phage KP27	100%	1e-103	Phage protein
18737_18997	hypothetical protein KP27_170	*Klebsiella* phage KP27	100%	1e-56	Phage protein
19067_19330	hypothetical protein KP27_169	*Klebsiella* phage KP27	99%	3e-57	Phage protein
19370_19693	hypothetical protein CPT_Matisse156	*Klebsiella* phage Matisse	99%	2e-72	Phage protein
19748_20158	hypothetical protein KP27_167	*Klebsiella* phage KP27	94%	6e-90	Phage protein
20226_20621	hypothetical protein KP27_166	*Klebsiella* phage KP27	100%	1e-90	Phage endolysin
21111_21635	homing endonuclease	*Klebsiella* phage Miro	99%	4e-126	Phage-associated homing endonuclease
21645_22991	PhoH domain protein	*Klebsiella* phage Matisse	100%	0.0	Predicted ATPase related to phosphate starvation-inducible protein PhoH
23234_23416	hypothetical protein	*Citrobacter* phage IME-CF2	27%	2e-26	hypothetical protein
23418_23972	hypothetical protein KP-KP15_gp137	*Klebsiella* phage KP15	100%	8e-134	Phage protein
24037_24678	hypothetical protein ADS69_00146	*Enterobacter* phage phiEap-3	100%	2e-156	T4-like phage protein, T4 GC1559
24746_24892	hypothetical protein KP-KP15_gp134	*Klebsiella* phage KP15	98%	2e-27	hypothetical protein
24910_25479	hypothetical protein KP-KP15_gp133	*Klebsiella* phage KP15	99%	8e-140	Phage protein
25489_25812	hypothetical protein KP-KP15_gp132	*Klebsiella* phage KP15	100%	1e-73	Phage protein
25812_25928	hypothetical protein CPT_Matisse143	*Klebsiella* phage Matisse	100%	9e-20	hypothetical protein
25879_26034	hypothetical protein KP27_156	*Klebsiella* phage KP27	100%	2e-28	hypothetical protein
26119_26592	hypothetical protein KP27_155	*Klebsiella* phage KP27	100%	8e-112	Phage protein
26595_26882	hypothetical protein KP27_154	*Klebsiella* phage KP27	99%	8e-62	hypothetical protein
26875_27090	hypothetical protein KP27_153	*Klebsiella* phage KP27	100%	2e-46	hypothetical protein
27273_27881	Tk thymidine kinase	*Enterobacter* phage phiEap-3	99%	4e-148	Thymidine kinase (EC 2.7.1.21)
27898_28290	hypothetical protein KP27_150	*Klebsiella* phage KP27	100%	3e-92	Phage protein
28844_29071	hypothetical protein KP27_148	*Klebsiella* phage KP27	100%	5e-47	Phage protein
29143_30057	hypothetical protein KP-KP15_gp124	*Klebsiella* phage KP15	100%	0.0	Phage protein
30303_30722	hypothetical protein KP27_146	*Klebsiella* phage KP27	99%	8e-91	hypothetical protein
30716_31294	bifunctional protein NMN adenylyltransferase/NU	*Klebsiella* phage Matisse	99%	4e-141	Nicotinamide-nucleotide adenylyltransferase, NadM family (EC 2.7.7.1) / ADP-ribose pyrophosphatase (EC 3.6.1.13)

	DIX hydrolase				
31468_31662	hypothetical protein RB16p128	Enterobacteria phage RB16	100%	4e-38	hypothetical protein
31659_31931	hypothetical protein KP-KP15_gp121	*Klebsiella* phage KP15	99%	5e-61	Phage protein
24_1181	UvsX RecA-like recombination protein	*Klebsiella* phage KP15	100%	0.0	Phage recombination protein
1219_1539	membrane-associated initiation of head vertex	*Klebsiella* phage KP15	100%	8e-71	Phage membrane-associated initiation of head vertex
1549_2985	gp41 replication and recombination DNA helicase	*Klebsiella* phage KP15	100%	0.0	DNA primase/helicase, phage-associated
3029_3298	hypothetical protein KP27_040	*Klebsiella* phage KP27	100%	4e-55	hypothetical protein
3378_3632	hypothetical protein KP-KP15_gp030	*Klebsiella* phage KP15	100%	2e-53	Phage protein
3725_3961	hypothetical protein KP-KP15_gp029	*Klebsiella* phage KP15	100%	3e-46	hypothetical protein
3958_4350	hypothetical protein KP27_036	*Klebsiella* phage KP27	100%	3e-92	Phage protein
4331_5062	hypothetical protein KP-KP15_gp027	*Klebsiella* phage KP15	100%	0.0	Phage protein
5068_5556	gp61.1 hypothetical protein	*Klebsiella* phage KP15	100%	1e-116	T4-like phage protein, T4 GC1491
5553_5771	hypothetical protein KP-KP15_gp025	*Klebsiella* phage KP15	100%	5e-47	hypothetical protein
5827_6087	hypothetical protein KP27_032	*Klebsiella* phage KP27	100%	9e-58	Hypothetical Zinc-finger containing protein
6091_7116	DNA primase subunit	*Klebsiella* phage KP27	100%	0.0	DNA primase (EC 2.7.7.-) / DNA helicase (EC 3.6.1.-), phage-associated
7124_7660	dCTP pyrophosphatase	*Klebsiella* phage KP15	96%	3e-127	dCTP pyrophosphatase (EC 3.6.1.12), phage-associated
8313_7654	Homing endonuclease	Enterobacteria phage JSE	45%	2e-72	Phage-associated homing endonuclease
8714_9004	hypothetical protein KP-KP15_gp021	*Klebsiella* phage KP15	100%	1e-63	Phage protein
9001_10329	DNA helicase	*Klebsiella* phage KP27	100%	0.0	DNA helicase (EC 3.6.1.-), phage-associated
10354_10662	hypothetical protein KP-KP15_gp019	*Klebsiella* phage KP15	100%	7e-68	Phage protein
10663_10947	hypothetical protein CPT_Matisse20	*Klebsiella* phage Matisse	100%	3e-64	Phage protein
10944_11615	DexA exonuclease A	*Klebsiella* phage KP15	100%	3e-165	T4-like phage DexA exonuclease A #T4 GC1472
11612_11893	hypothetical protein KP27_024	*Klebsiella* phage KP27	100%	6e-61	Phage protein
12334_12633	hypothetical protein KP-KP15_gp013	*Klebsiella* phage KP15	100%	6e-66	hypothetical protein
12722_12985	hypothetical protein KP-KP15_gp012	*Klebsiella* phage KP15	100%	5e-57	hypothetical protein
13059_13283	hypothetical protein KP27_016	*Klebsiella* phage KP27	100%	9e-47	Phage protein
13343_13672	hypothetical protein KP27_014	*Klebsiella* phage KP27	100%	1e-75	Phage protein
13712_14308	hypothetical protein CPT_Matisse11	*Klebsiella* phage Matisse]	100%	3e-147	Phage protein
14272_14583	hypothetical protein KP-KP15_gp008	*Klebsiella* phage KP15	98%	1e-71	Phage protein
14570_15163	hypothetical protein ADS69_00008	*Enterobacter* phage phiEap-3	97%	2e-140	Phage protein
15186_15626	hypothetical protein KP-KP15_gp006	*Klebsiella* phage KP15	99%	2e-105	Transcriptional regulator
15677_15790	hypothetical protein KP27_008	*Klebsiella* phage KP27	100%	6e-19	Phage protein
15858_17756	DNA topoisomerase large subunit	*Klebsiella* phage KP27	99%	0.0	Phage DNA topoisomerase large subunit (EC 5.99.1.3) #T4-like gp60 #T4 GC1464
4710_937	long tail fiber proximal subunit	*Enterobacter* phage phiEap-3	96%	0.0	Phage long tail fiber proximal subunit
5089_4790	hypothetical protein KP-KP15_gp244	*Klebsiella* phage KP15	100%	5e-67	Phage protein
5367_5086	Glutaredoxin	*Klebsiella* phage KP27	100%	2e-62	Glutaredoxin
5995_5327	hypothetical protein KP-KP15_gp242	*Klebsiella* phage KP15	100%	3e-160	Phage protein
7006_6038	hypothetical protein CPT_Matisse262	*Klebsiella* phage Matisse	97%	0.0	Phage protein
297_437	DenA endonuclease II	*Klebsiella* phage KP15	100%	3e-25	Phage endonuclease
418_1575	RNA ligase	*Klebsiella* phage Miro	99%	0.0	RNA ligase, phage-associated #T4-like RnlA #T4 GC1653

1908_2108	homing endonuclease	*Klebsiella* phage Miro	100%	9e-38	Phage endonuclease
2221_2412	i-spanin	*Klebsiella* phage Matisse	98%	2e-36	Phage protein
2409_2726	o-spanin	*Klebsiella* phage Matisse	99%	9e-70	Phage outer membrane lipoprotein Rz1
2711_2884	hypothetical protein KP27_081	*Klebsiella* phage KP27	100%	3e-32	hypothetical protein

Table 3.2S Coding sequences identified in ECA2 phage of *E. coli* strain.

Location	**Gene name**	**Organism evidence**	**Ident**	**E-value**	**Function**
120_353	tail protein	*Citrobacter* phage SH2	97%	3e-42	Phage major tail protein
467_1057	tail fiber protein	*Enterobacter* phage E-3	100%	3e-143	Phage tail fiber protein / T7-like tail tubular protein A
1073_3478	tail tubular protein B	*Citrobacter* phage SH1	99%	0.0	Phage tail fiber protein / T7-like tail tubular protein B
3551_3961	gp13	*Salmonella* phage phiSG-JL2	100%	1e-97	Phage protein inside capsid A
3964_4557	protein inside capsid B	*Enterobacter* phage E-3	98%	5e-137	Phage protein inside capsid B
4560_6803	internal (core) protein	*Enterobacter* phage E-3	98%	0.0	Phage internal (core) protein
6822_10784	internal (core) protein	*Enterobacter* phage E-2	99%	0.0	Phage internal (core) protein
10856_12544	tail fibers protein	*Enterobacter* phage E-2	67%	7e-123	Phage tail fibers
12585_12788	holin class II	*Enterobacter* phage E-3	100%	9e-41	Phage holin, class II
12792_13058	gp18	*Salmonella* phage phiSG-JL2	99%	3e-56	DNA packaging protein A, T7-like gp18
13148_13621	endopeptidase	*Citrobacter* phage SH2	59%	9e-55	Phage endopeptidase (EC 3.4.-.-) Rz
13621_14046	gp13.5	*Enterobacteria* phage EcoDS1	49%	1e-32	Phage endonuclease VII
14039_15802	DNA packaging protein B	*Yersinia* phage phiYeO3-12	99%	0.0	Phage DNA packaging
16047_16196	hypothetical protein vBYenPAB5_0045	*Yersinia* phage vB_YenP_AP5	100%	7e-26	Phage protein
17482_17847	hypothetical protein RU52_00001	*Citrobacter* phage phiCFP-1	98%	2e-80	Phage protein kinase
17847_17999	hypothetical protein RU52_00002	*Citrobacter* phage phiCFP-1	96%	2e-28	Phage protein
18068_18352	hypothetical protein	*Enterobacter* phage E-4	88%	2e-55	hypothetical protein
18374_18496	hypothetical protein phiYe-F10_00004	*Yersinia* phage phiYe-F10	95%	1e-16	hypothetical protein
18493_18690	hypothetical protein phiYe-F10_00005	*Yersinia* phage phiYe-F10	95%	7e-39	Phage protein
18712_19821	protein kinase	*Enterobacteria* phage T3	92%	0.0	Phage protein kinase (EC 2.7.11.1)
19892_22546	DNA-directed RNA polymerase	*Enterobacter* phage E-2	99%	0.0	DNA-directed RNA polymerase (EC 2.7.7.6)
22633_22905	gene 1.05 protein [	*Enterobacteria* phage T3	98%	2e-59	Phage protein
22998_23138	1.1 protein	Yersinia phage phiYeO3-12	100%	3e-24	Phage protein
23138_23416	gp1.2	*Salmonella* phage phiSG-JL2	98%	1e-61	dGTP triphosphohydrolase inhibitor
23443_23859	hypothetical protein	*Enterobacter* phage E-2	100%	4e-100	Phage protein
23856_24872	DNA ligase	*Enterobacter* phage E-2	100%	0.0	DNA ligase, phage-associated
25042_25299	1.6 protein	*Yersinia* phage phiYeO3-12	100%	1e-54	Phage protein
25299_25778	hypothetical protein	*Enterobacter* phage E-2	100%	2e-116	Phage protein
25765_25902	1.8 protein	*Yersinia* phage phiYeO3-12	100%	4e-24	Phage protein
25899_26135	host RNA polymerase inhibitor	*Yersinia* phage vB_YenP_AP5	100%	3e-52	Host RNA polymerase inhibitor, T7-like gp2
26188_26886	ssDNA-binding protein	*Enterobacter* phage E-2	99%	2e-169	T7-like phage ssDNA-binding protein
26886_27347	endonuclease	*Enterobacter* phage E-3	100%	1e-107	T7-like phage endonuclease (EC 3.1.21.2)

27340_27795	N-acetylmuramoyl-L-alanine amidase	*Yersinia* phage phiYeO3-12	99%	6e-109	Phage lysin, N-acetylmuramoyl-L-alanine amidase (EC 3.5.1.28)
28162_29676	helicase	*Yersinia* phage phiYeO3-12	99%	0.0	T7-like phage primase/helicase protein
29776_29988	4.3 protein	*Yersinia* phage phiYeO3-12	100%	8e-40	Phage protein
30001_30285	4.5 protein	*Yersinia* phage phiYeO3-12	100%	9e-64	Phage protein
30353_32467	DNA polymerase	*Enterobacter* phage E-3	99%	0.0	DNA polymerase (EC 2.7.7.7), phage-associated
32487_32786	HNS binding protein	*Enterobacter* phage E-3	97%	1e-63	Phage HNS binding protein
32786_32995	5.7 protein	*Yersinia* phage phiYeO3-12	100%	6e-43	Phage HNS binding protein
32992_33174	host recBCD nuclease inhibitor [	*Yersinia* phage vB_YenP_AP5	97%	2e-35	Phage inihibitor of recBCD nuclease
33171_34082	exonuclease	*Yersinia* phage phiYeO3-12	99%	0.0	T7-like phage exonuclease (EC 3.1.11.3)
34064_34177	gene 6.3 protein	*Enterobacteria* phage T3	92%	2e-14	Phage protein
34270_34515	gene 6.5 protein	*Enterobacteria* phage T3	100%	3e-53	Phage protein
34520_34771	hypothetical protein vBYenPAB5_0027	*Yersinia* phage vB_YenP_AP5	100%	8e-51	Phage protein
34799_35119	host specificity protein B	*Yersinia* phage phiYeO3-12	100%	4e-64	Phage protein
35130_36737	collar / head-to-tail joining protein	*Enterobacter* phage E-3	99%	0.0	Phage collar / T7-like phage head-to-tail joining protein
36839_37771	capsid assembly protein	*Yersinia* phage phiYe-F10	99%	0.0	Phage capsid and scaffold

Chapter 4. Application of phage therapy during bivalve depuration improves *Escherichia coli* decontamination

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4.1. Abstract

The present study investigated the potential application of the bacteriophage (or phage) phT4A, ECA2 and the phage cocktail phT4A/ECA2 to decrease the concentration of *Escherichia coli* during the depuration of natural and artificially contaminated cockles. Depuration in static seawater at MOI 1 with single phage suspensions of phT4A and ECA2 was the best condition, as it decreased by ~2.0 log CFU/g the concentration of *E. coli* in artificially contaminated cockles after a 4 h of treatment. When naturally contaminated cockles were treated in static seawater with single phage suspensions and the phage cocktail, similar decreases in the concentration of *E. coli* (~ 0.7 log CFU/g) were achieved. However, when employing the phage cocktail, a longer treatment time was required to obtain comparable results to those achieved when using single phage suspensions. When naturally contaminated cockles were depurated with phage phT4A in a recirculated seawater system (mimicking industrial depuration conditions), a 0.6 log CFU/g reduction of *E. coli* was achieved after a 2 h of treatment. When the depuration process was performed without phage addition, a 4 h treatment was necessary to obtain a similar decrease. By combining phage therapy and depuration procedures, a reduction in the depuration period can be achieved for marine bivalves, thus decreasing the potential costs associated with depuration and, possibly, even enhance the quality and safety of depurated bivalves destined for human consumption.

Keywords: phages, cockles, *Cerastoderma edule*, food safety

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4.2. Introduction

Enteric pathogen strains of *Escherichia coli* are widely distributed in coastal areas and are a causative agent of gastroenteritis in humans after consumption of contaminated seafood (Kanayama et al., 2015; Potasman et al., 2002). Cockles (*Cerastoderma edule*) are filter feeder bivalve molluscs that accumulate food particles and small organisms by circulating large volumes of seawater; consequently, microorganisms, including human pathogens, are retained and accumulate in their tissues (Lees, 2000). Moreover, as bivalves are often consumed raw or lightly cooked, they are potential vectors for pathogenic *E. coli*.

Depuration is a method applied to reduce and eliminate human pathogens from bivalves. Briefly it consists of a flow-through or recirculation system operating with chemically (chlorine, ozone, iodophores or activated oxygen) or physically (UV-C light) disinfected water that allow bivalve purification under controlled conditions (Croci et al., 2002; Wang et al., 2010). After depuration, bivalves may be destined for human consumption if they display less than 230 most probable number (MPN) *E. coli* per 100 g of flesh and intra-valvular liquid (FIL), as well as no detectable levels of *Salmonella* spp. (FAO, 2008). However, some pathogenic microorganisms are known to be resistant to this process and remain in bivalves after depuration (FAO, 2008; Martínez et al., 2009; Rong et al., 2014).

In order to reduce the risk of infections by microbial pathogens through the consumption of bivalves, it is essential to develop alternative approaches to conventional depuration practices. One of the most promising approaches is to combine bivalve depuration with phage therapy (the application of lytic phages to prevent and/or to treat bacterial infections). The use of lytic phages to reduce food-borne pathogens has emerged as a promising tool for food safety (Denes and Wiedmann, 2014; Endersen et al., 2014).

These viruses are target-specific, self-replicating, rapid bactericides and do not modify normal food properties. A few publications have demonstrated that phages can be used to successfully decrease the load of *E. coli* in food, especially in meat and poultry products (Atterbury et al., 2007; Goodridge and Bisha, 2011; Raya et al., 2006; Sillankorva et al., 2012). However, the combination of depuration and phage therapy to eliminate pathogenic bacteria in bivalves is an innovative approach which is still being investigated. A previous study by Rong et al. (2014) already reported the combined use of these two processes to eliminate *Vibrio parahaemolyticus* from artificially contaminated oysters. However, this study did not replicate industrial depuration procedures, neither tested the suitability of this approach on naturally contaminated bivalves.

The aim of the present study was to evaluate, for the first time, the efficiency of two new phages of *E. coli* (phT4A and ECA2), individually or combined in a cocktail, to control *E. coli* in natural and artificially contaminated cockles in static water and during depuration mimicking industrial procedures currently employed.

4.3. Material and Methods

4.3.1. Bacterial strains

E. coli (ATCC 13706), a microbiological indicator of shellfish depuration efficiency, was used to contaminate cockles. Fresh plate bacterial cultures were maintained in solid Tryptic Soy Agar (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth (TSB; Liofilchem, Italy) and was grown overnight at 37 ºC. An aliquot of this culture (100 µL) was aseptically transferred to 10 mL of fresh TSB and grown overnight at 37 °C to reach an optical density (O.D600 = 0.8), corresponding to about 10^9 cells per mL.

4.3.2. Phage preparation

The phages phT4A and ECA2, two lytic phages previously isolated from sewage samples, evidenced to be effective in lysing *E. coli* even at low multiplicity of infection (MOI) (tested range: 1 / 100) in studies *in vitro*. Two phages were prepared in SM buffer (0.1 M NaCl, 8 mM MgSO4, 20 mM Tris-HCl, 2% (w/v) gelatin, pH 7.5) at 37 °C during 6 h, using *E. coli* as host (unpublished data). Double-plaque assays were performed as described by to determine the phage titer, with the phages being stored at 4 °C. The titer of both phages was approximately 10^9 plaque-forming units (PFU)/mL.

Phage phT4A and ECA2 contained a linear double-stranded DNA with a size of 170.698 bp and 61.915 bp, respectively (unpublished data). Morphologically, phage phT4A belongs to the family *Myoviridae* and is characterised by an icosahedral head with a diameter of 80 ± 2 nm 75 nm width and a long contractile tail of 107 ± 3 nm. ECA2 phage has an icosahedral head with approximately 56 ± 2 nm of width and was identified as member of family *Podoviridae*. None of the genomes featured any lysogeny related genes and it can be securely assumed that the two phages feature a virulent lifestyle. No genes encoding toxins, virulence factors or antibiotic resistance were identified based on aminoacid sequence homology searches. The genome sequences were deposited in GenBank under the accession numbers KX130727 (phage phT4A) and KX130726 (phage EC2A).

4.3.3. Collection of cockle samples

Cockles were selected as biological models to test the efficacy of phage therapy against *E. coli* infections and were purchased from Falcamar Lda. (Vila do Conde, Portugal), a bivalve wholesaler, after being depurated according to industrial processing

protocols (48 h at 15-16 °C in seawater irradiated with UV-C). These specimens were later artificially contaminated with *E. coli* in the laboratory (see below). Naturally contaminated cockles were collected in Mira Channel (Ria de Aveiro, Portugal; 40º36'30"N, 8º44'52"W), a bivalve production area ranked as B (230 – 4600 MPN *E. coli* per 100 g of FIL) (Despacho n.º 15264, 2013). Mira channel is a recreational area subjected to anthropogenic contamination (Pereira et al., 2015). Cockles were collected in June, July, September and October 2015.

Live cockles were dry transported to the laboratory by the research team in a controlled temperature container, under an oxygen saturated atmosphere.

4.3.4. Phage application during cockles depuration in static seawater

4.3.4.1. Accumulation of *E. coli* in cockles

Non contaminated cockles were maintained in independent tanks (10 cm length x 9 cm width x 15 cm height), acting as static systems, filled with sterile synthetic seawater (0.6 L) and equipped with its own aerator, during 12h before being infected with *E. coli*. Temperature was maintained at 16 ± 1 °C, pH 8.0 ± 0.2, salinity 35 and dissolved oxygen above 5.5 mg/L during the whole trial. Synthetic seawater was prepared by mixing a synthetic salt brand (Tropic Marin Pro Reef salt – Tropic Marine, Germany) with water purified by a reverse osmosis system (Aqua-win RO-6080, Thailand). After the immersion period cockles were washed with sterile synthetic seawater and placed in independent tanks with sterile synthetic seawater.

A total of 5 groups of cockles were randomly formed, each group with three replicates of 30 cockles (a total of 5 groups x 3 replicates x 30 cockles = 450 cockles). Four groups were infected with *E. coli* (test tanks), with the fifth group being used as the

uninfected group (control cockles - CC). The 3 replicates from each of the 5 groups were maintained in independent tanks as described above. A fresh culture of *E. coli* was added to the four test groups to obtain final concentrations of 10^5, 10^6, 10^7 and 10^8 colonies-forming units (CFU)/mL. Cockles were sampled at 0, 6, 12 and 24 h during the accumulation process to determine cultivable bacteria concentrations. *E. coli* concentration was determined in chromocult coliform agar (Merck, Germany) at the start of the assays (time 0). At each sampling time, four cockles were randomly selected from each tank and their FIL was homogenized with a Bag Mixer 400 (Interscience, France). Ten grams were blended in 90 mL of alkaline peptone water (Liofilchem, Italy). The homogenized samples were then ten-fold serially diluted and 1 mL from each dilution was spread on the non-specific TSA plates. All plates were incubated at 37 °C for 24 h. The counts in TSA medium allows to detect added *E. coli*, as well as other cultivable bacteria already present in cockles (including other *E. coli* strains). This assay was repeated three times in different periods to secure independent replication.

4.3.4.2. Depuration of artificially contaminated cockles in the presence of phages

Cockles were maintained in independent tanks as described above. After the immersion period cockles were washed with sterile synthetic seawater and placed in independent tanks with sterile synthetic seawater (Figure 4.1). In these experiments 14 groups of cockles were used. Each group included 3 replicates of 30 cockles each (for a total of 14 groups x 3 replicates x 30 specimens = 1260 cockles). Each replicate was stocked in an independent tank as described above. *E. coli* was added to 7 of the 14 groups to obtain a final concentration of 10^5 CFU/mL. In the other 7 groups no *E. coli* was added. Cockles from the 14 groups remained for 12 h in the tanks under the same conditions.

Following 12 h of incubation cockles were washed with sterilized artificial seawater and placed in clean tanks with no *E. coli* contamination. From the 7 groups of cockles infected with *E. coli*, 3 were inoculated with phages (single phage suspensions of phT4A and ECA2 phage and of phT4A/ECA2 cocktail at MOI 1 (BP MOI 1 phT4A, BP MOI 1 ECA2 and BP MOI 1 phT4A/EC2), another three with the same phage suspensions at a MOI 100 (BP MOI 100 phT4A, BP MOI 100 ECA2 and BP MOI 100 phT4A/ECA2) and to the remaining infected group no phage was added (bacteria control - BC). In order to obtain a MOI of 1 and 100, 10^5 PFU/mL for MOI 1 and 10^7 PFU/mL for MOI 100 of the single phage suspensions (phT4A or ECA2) or phage cocktail (phT4/ECA2) were inoculated. Concerning the 7 groups of cockles not infected with *E. coli*, 3 were inoculated with phages (single phage suspensions of phT4A and ECA2 phage and of phT4A/ECA2 cocktail at MOI 1 (PC MOI 1 phT4A, PC MOI 1 ECA2 and PC MOI 1 phT4A/ECA2), three groups with the same phage suspensions at a MOI 100 (PC MOI 100 phT4A, PC MOI 100 ECA2 and PC MOI 100 phT4A/ECA2) and the remaining group was not inoculated with phages. All 14 groups were incubated exactly under the same conditions. Cockles of test tanks and controls were sampled at 0, 2, 4, 6, 8, 10 and 12 h of incubation. Four cockles were randomly selected from each tank to determine bacterial and phage concentration, homogenized as described above and 10 g were blended in 90 mL alkaline peptone water (Liofilchem, Italy). The phage titer was determined in duplicate for all assays through the double agar layer method after an incubation period of 8 - 12 h at 37 °C. Cultivable bacterial concentration was determined by the spread method in duplicate in TSA medium after an incubation period of 24 h at 37 °C. *E. coli* concentration was determined in chromocult coliform agar (Merck, Germany) at the start of the assays (time

0). All plates were incubated at 37 ºC for 24 h. This assay was repeated three times in different periods to secure independent replication.

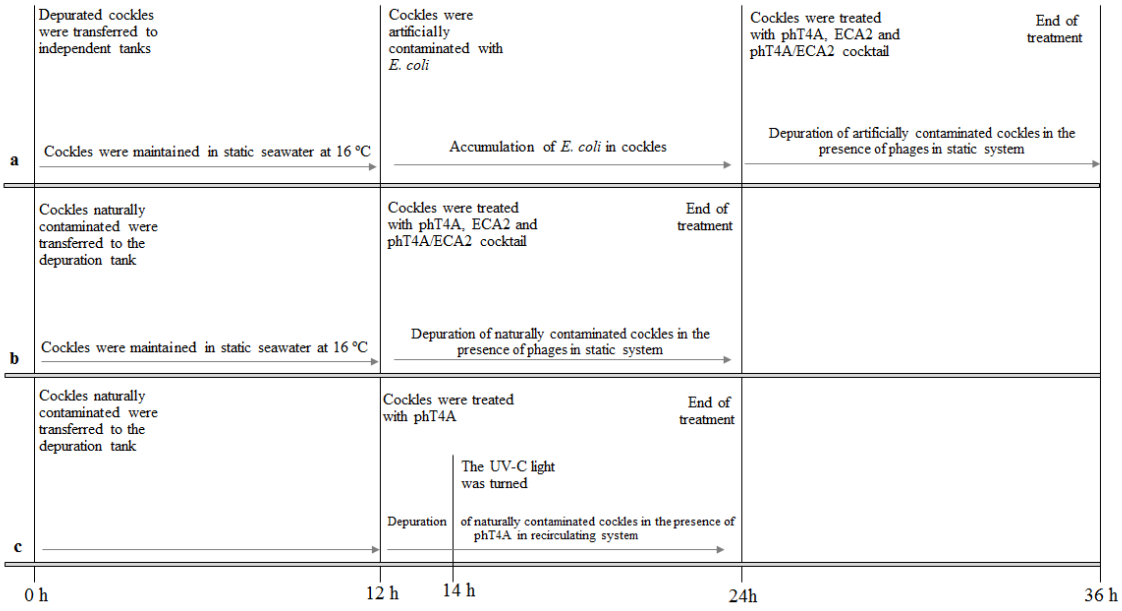

Figure 4.1 Schematic representation of the depuration experiments of cockles employing phages. (a) Depuration of artificially contaminated cockles with *E. coli* with phT4A, ECA2 phages and phT4A/ECA2 cocktail in static system (see section 4.3.4.2), (b) Depuration of naturally contaminated cockles with phT4A, ECA2 phages and phT4A/ECA2 cocktail in static system (see section 4.3.5), (c) Depuration of naturally contaminated cockles with phT4A phage in a recirculating system (see section 4.3.7).

4.3.5. Depuration of naturally contaminated cockles in the presence of phages

Naturally contaminated cockles were washed with sterile synthetic seawater and placed in clean water tanks with sterile synthetic seawater as described above (see also Figure 4.1). In these experiments 4 groups of cockles were used. Each group included 3 replicates of 30 cockles each (a total of 4 groups x 3 replicates x 30 specimens = 360 cockles). All replicates were maintained in independent tanks as described above. Three of the groups were inoculated with phages (single phage suspensions of phT4A and ECA2

phage and of phT4A/ECA2 cocktail), while the remaining group was not inoculated with any phages. The phage suspensions were inoculated as described above, with an initial concentration of 10^5 PFU/mL (selected according to the results of previous tests in artificially contaminated cockles). All replicates were incubated exactly under the same conditions. Cockles were collected after 0, 2, 4, 6, 8, 10 and 12 h of incubation. Four cockles were randomly selected from each tank to determine bacterial and phage concentration, homogenized as described above and 10 g were blended in 90 mL alkaline peptone water. The phage titer, cultivable bacterial concentration and *E. coli* concentration was determined as described above. This assay was repeated three times in different periods to secure independent replication.

4.3.6. Effect of ultraviolet (UV) irradiation on phages

In these experiments the effect of UV irradiation on phages was investigated by inoculating 10^5 PFU/mL of phT4A phage on 14 L of aerated synthetic seawater and irradiating it with UV light during 12 h (phT4A UV). A control treatment was prepared by replicating this procedure, with the exception of UV irradiation (CphT4A). Each group included 3 replicates, with temperature being maintained at 16 ± 1 °C, pH 8.0 ± 0.2, salinity 35, and dissolved oxygen above 5.5 mg/L, during the whole trial. Aliquots of each group were collected after 0, 2, 4, 6, 8, 10 and 12 h of incubation. In each group, the phage titre was determined in duplicate through the double agar layer method after an incubation period of 4 – 8 h at 37 °C. This assay was repeated three times in different periods to secure independent replication.

4.3.7. Depuration of naturally contaminated cockles with phT4A phage in a recirculating system

The efficacy of the phT4A phage (selected according to the results of the tests described above) was evaluated during depuration in a recirculating system mimicking an industrial depuration process (Figure 4.1 and 4.2). The rationale for this trial was to evaluate if this approach could be successfully employed in industrial depuration facilities to inactivate *E. coli*. Cockles were washed with sterile synthetic seawater and placed in clean water tanks with sterile synthetic seawater as described above. In this experiment 2 groups of cockles were formed, each with 3 replicates of 30 cockles each (for a total of 2 groups x 3 replicates x 30 cockles = 180 cockles). All replicates were maintained in independent tanks with a volume of 0.6 L. Only one of the groups was inoculated with 10^5 PFU/mL of phT4A phage. Tanks from each group were connected in parallel to twin recirculated water systems mimicking an industrial depuration facility. Each recirculated system was equipped with a 14 L tank holding a submerged water pump (Eheim Compact+ 3000, Germany), a protein skimmer (RedSea Berlin Air-Lift 60, Israel) equipped with air pump (API Rena Air 200, USA) and UV filter (TMC V2 Vecton 120 Nano, UK) with a recirculating submerged water pump (Eheim Compact 1000, Germany) (Figure 4.2). Temperature was controlled by using a common 120 L water bath connected to a cooling unit (Hailea HC 500-A, China) through a submerged water pump (Eheim Compact+ 3000, Germany) (Figure 4.2). To avoid the effect of UV irradiation on the phages, the UV light was only switched on 2 h after the beginning of the depuration process (the time required for cockles to filtrate and accumulate the phages). All replicates were incubated exactly under the same conditions. Aliquots of test samples and controls were collected after 0, 2, 4, 6, 8, 10 and 12 h of incubation. Four cockles were randomly selected from each tank to

determine bacterial and phage concentration, homogenized as described above and 10 g were blended in 90 mL alkaline peptone water. The phage titer, cultivable bacterial concentration and *E. coli* concentration was determined as described above. This assay was repeated three times in different periods to secure independent replication.

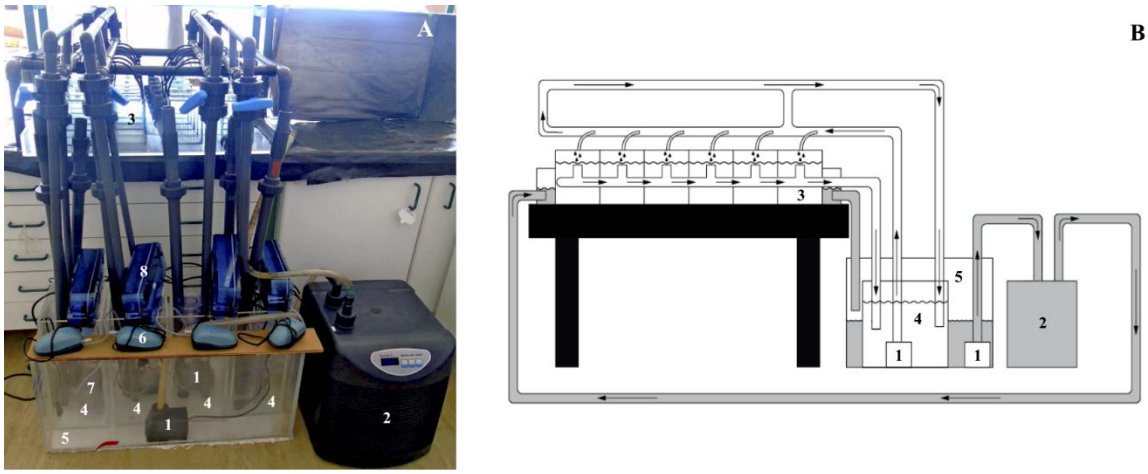

Figure 4.2 Front view (A) and schematic representation of the lateral view (B) of the depuration system. 1 - Submerged water pump (Eheim Compact+ 3000), 2 - cooling unit (Hailea HC 500-A), 3 – independent tanks, 4 – water reservoir, 5 – water bath for temperature control, 6 - air pump (API Rena Air 200), 7 - protein skimmer (RedSea Berlin Air-Lift 60), 8- UV filter (TMC V2 Vecton 120 Nano) with a recirculating submerged water pump, ⟶ water circulation system, ▭ cooling system, ▭ depuration system.

4.3.8. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Normal distributions were assessed by Kolmogorov–Smirnov test and homogeneity of variances was assessed by Levene's test. Two-way analysis of variance (ANOVA) was used to compare significant differences among the accumulation different concentrations of E. coli in cockles and control at different times. The significance differences on bacterial and viral concentration in cockles artificially contaminated was analysed using a three-way ANOVA

with MOIs (1 and 100), phages used in treatment (phT4A, ECA2 and phT4A and ECA2) and treatment times being used as fixed factors. The existence of significant differences of the phage treatment in cockles naturally contaminated and treatment time on bacterial and viral concentration was analyzed using a two-way ANOVA. For each situation, the significance of differences was evaluated by comparing the results obtained in the test samples and control samples for the different times of each of the three independent assays. The significance of the effect of UV-C irradiation on the phT4A phage and irradiation time on phage inactivation was assessed by one-way analysis of variance (ANOVA) model. A value of $p < 0.05$ was considered statistically significant and in these cases. Tukey's multiple comparison test was used for a pairwise comparison of the means.

4.4. Results

4.4.1. Phage application during cockles depuration in a static system

4.4.1.1. Accumulation of *E. coli* in cockles

The total number of cultivable bacteria and *E. coli* concentration present in the cockles at the start of the assay was 3.1 log CFU/g and 1.5 log CFU/g, respectively. Live cockles were assessed after 24 h of incubation in the presence of *E. coli* at 16 ± 1 °C (Figure 4.3). Accumulation of *E. coli* in cockles increased rapidly during the first 6 h, with counts decreasing in all four infected groups until they remained comparatively steady after 12 h and onward. Following 12 h of incubation, the mean values of bacteria in cockles were 5.0, 5.9, 7.1 and 8.0 log CFU/g in infected groups with 10^5, 10^6,10^7 and 10^8 CFU/mL of *E. coli*, respectively (Figure 4.3). The abundance of cultivable bacteria in contaminated groups (10^5, 10^6,10^7 and 10^8 CFU/mL) after 6, 12 and 24 h, was statistically

different (ANOVA, $p < 0.05$; Figure 4.3). Incubation by immersion during 12 h in an *E. coli* culture with 10^5 CFU/mL was the selected procedure for follow-up experiments.

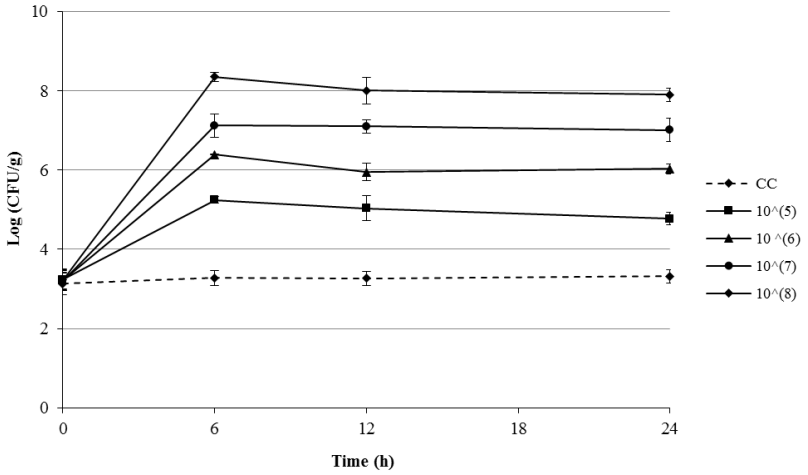

Figure 4.3 Accumulation of *E. coli* in cockles in different infected groups after 24 h. Final concentrations of *E. coli* (log CFU/g) in groups of cockles initially contaminated with 10^5 CFU/mL; 10^6 CFU/mL, 10^7 CFU/mL and 10^8 CFU/mL, as well as the uninfected group (control cockles - CC).

4.4.1.2. Depuration of artificially contaminated cockles in the presence of phages

The total number of cultivable bacteria and *E. coli* concentration present in the cockles at the start of the assay was 2.6 log CFU/g and 1.2 log CFU/g, respectively Bacterial density in the BC and CC remained constant (ANOVA, $p > 0.05$) during the 12 h of treatment.

At a MOI of 1, the maximum of cultivable bacterial inactivation in cockles treated with phT4A phage relatively to the bacterial control was 2.0 log CFU/g, which was achieved after 4 h of phage therapy, and significantly higher than the one obtained with the phT4A/ECA2 (0.4 log CFU/g) (Figure 4.4A). Phage inactivation with phT4A phage at MOI 1 was significantly higher from that MOI 100 (ANOVA, $p < 0.05$) after 4 h, 6 h, 8 h

and 10 h of treatment. However, after 2 and 12 h of treatment, the rate of inactivation for both MOI was similar (ANOVA, $p > 0.05$). Increasing the MOI to a value of 100 enhanced the highest inactivation rate (1.0 log CFU/g) was achieved after 6 h of treatment, and after 4 h it was 0.6 log CFU/g after 4 h of treatment (Figure 4.4A).

The maximum rate of bacterial inactivation in cockles treated with ECA2 phage relatively to the bacterial control was 1.9 log CFU/g achieved after 4 h of treatment at a MOI of 1 (ANOVA, $p < 0.05$) and significantly higher than the one obtained with the phT4A/ECA2 (0.4 log CFU/g) (Figure 4.4A). However, after 2 h, the inactivation rate was considerably higher for the MOI 1 (1.2 log CFU/g) and significantly higher (ANOVA, $p < 0.05$) than the one obtained with the phT4A phage (0.6 log CFU/g; ANOVA, $p < 0.05$) and PhT4A/ECA2 cocktail phage (0.5 log CFU/g; ANOVA, $p < 0.05$) for MOI 1. At 12 h of treatment the inactivation rate remained quite high (1.6 log CFU/g) (ANOVA, $p < 0.05$) (Figure 4.4A). Phage inactivation with EC2 phage at MOI 1 was significantly higher from that MOI 100 (ANOVA, $p < 0.05$) at the different times. At the highest MOI value (100), the maximum value of inactivation was 1.1 log CFU/g after 6 h of treatment.

The maximum of cultivable bacteria inactivation in cockles treated with phT4A/EC2 cocktail phage was 1.8 log CFU/g achieved after 8 h treatment (ANOVA, $p < 0.05$; Figure 4.4A). Phage inactivation at MOI 1 was only significantly different from that of MOI 100 after 6 h and 8 h of treatment (ANOVA, $p < 0.05$). Increasing the MOI to a value of 100 enhanced the highest inactivation rate (1.0 log CFU/g), which was achieved after 10 h of treatment, and after 6 h it was 0.8 log CFU/g (Figure 4.4A). At a MOI of 100, the rate of inactivation with the single suspensions of phT4A and ECA2 phage was similar (ANOVA, $p > 0.05$) than the obtained with the phT4A/ECA2 cocktail phage.

The abundance of phT4A and ECA2 single phage suspensions and of phT4A/ECA2 phage cocktail in cockles, both in the absence (PC) and in the presence of the host *E. coli* (BP, Figure 4.4 B) increased during the first 2 h treatment, and then remained constant until the end of the treatment (ANOVA, $p > 0.05$).

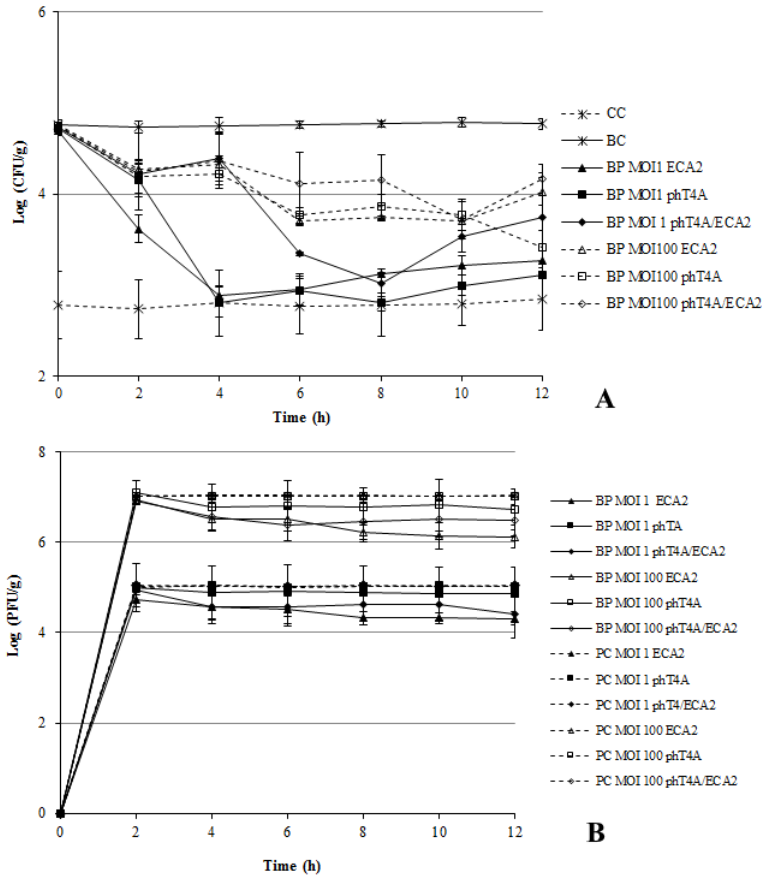

Figure 4.4 Inactivation of *E. coli* of artificially contaminated cockles by the two single phage suspensions (phT4A and ECA2) and the phage cocktail (phT4A/ECA2) at a MOI of 1 and 100 during 12 h. A. Bacterial concentration: CC - uninfected group (control cockles); BC — bacteria control; BP — bacteria plus phage. B. Phage concentration: PC — phage control; BP — bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

4.4.1.3. Depuration of naturally contaminated cockles in the presence of phages

The total number of cultivable bacteria and *E. coli* concentration present initially in naturally contaminated cockles was 3.4 log CFU/g (CC) and 1.5 log CFU/g, respectively. Bacterial density in the CC remained constant (ANOVA, $p > 0.05$) during the 12 h of treatment (Figure 4.5A).

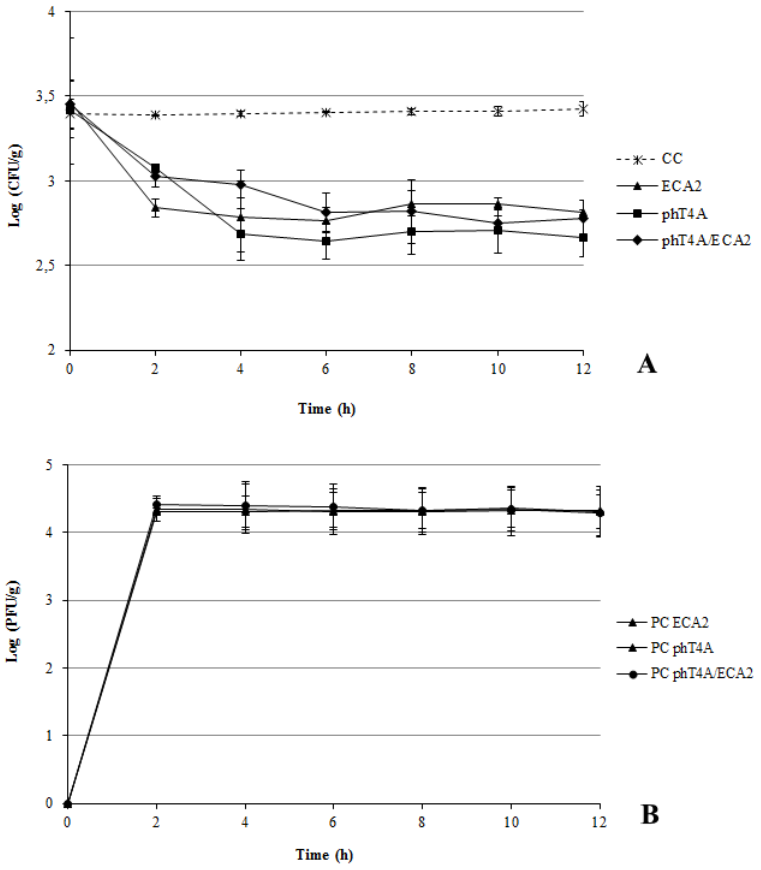

Figure 4.5 Inactivation of *E. coli* of naturally contaminated cockles by the two single phage suspensions (phT4A and ECA2) and the phage cocktail (phT4A/ECA2) during 12 h. A. Bacterial concentration: CC - uninfected group (control cockles), ECA2— cockles treated with ECA2 phage, phT4A – cockles treated with phT4A phage, phT4A/ECA2 – cockles treated with phT4A/ECA2 cocktail. B. Phage concentration: PC- phage control. Values represent the mean of three experiments; error bars represent the standard deviation.

The maximum reduction of cultivable bacteria in cockles treated with phT4A and ECA2 phage was 0.8 and 0.7 log CFU/g, respectively, after 6 h of treatment, being statistically different from the untreated group (ANOVA, $p < 0.05$; Figure 4.5A). The highest inactivation rate (0.7 log CFU/g) was achieved after 10 h of treatment with phT4A/ECA2, and after 6 h it was 0.6 log CFU/g after 6 h of treatment (ANOVA, $p < 0.05$; Figure 4.5A). Phage inactivation with phT4A, ECA2 phage and phT4A/ECA2 cocktail phage was similar (ANOVA, $p > 0.05$) at the different times.

The abundance of phT4A and ECA2 phage and phT4A/ECA2 phage cocktail in cockles increased during the first 2 h of treatment, and then remained constant until the end of the treatment (ANOVA, $p > 0.05$; Figure 4.5B).

4.4.2. Effect of ultraviolet (UV-C) irradiation on phages

Significant reductions (ANOVA, $p < 0.05$) in phT4A phage counts exposed to UV-C irradiation (phT4A UV) were observed after 2 h of incubation with a reduction of approximately 4.5 log PFU/mL, which was statistically different from the untreated group (C phT4A) (Figure 4.6). The phT4A phage was inactivated to the limit of detection (5.0 log PFU/mL of reduction) by UV-C irradiation after 4 h of treatment (Figure 4.6). The abundance of phT4A in untreated group (C phT4A) remained constant until the end of the treatment (ANOVA, $p > 0.05$).

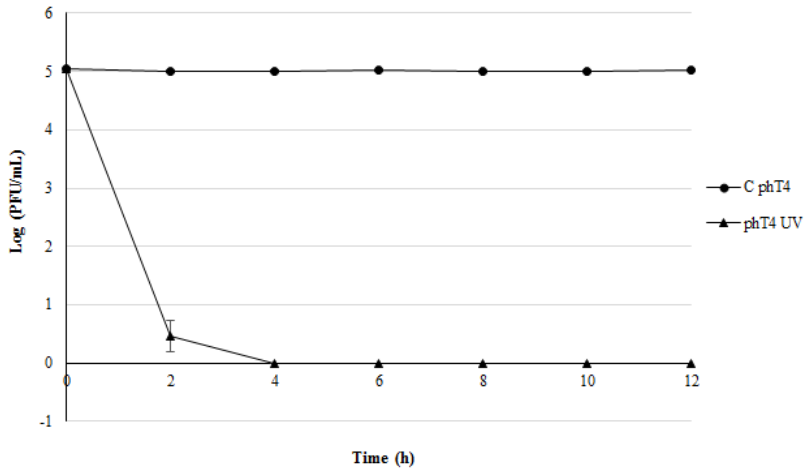

Figure 4.6 Effect of UV-C irradiation on the phT4A phage and synthetic seawater recirculation during 12 h. (C phT4) synthetic water with phT4A phage but not purified by UV-C irradiation (phT4A UV) synthetic seawater with phT4A phage and purified by UV-C light.

4.4.3. Depuration of naturally contaminated cockles with phT4 phage in a recirculating system

Total cultivable bacteria and *E. coli* density present initially in naturally contaminated cockles depurated was 4.6 log CFU/g (CC, Figure 4.7A) and 1.5 log CFU/g, respectively. Significant reductions (ANOVA, $p < 0.05$) in cultivable bacteria counts were also observed with the phT4A phage after 2 h of treatment with a reduction of approximately 0.6 log CFU/g, which was statistically different from the untreated group (Figure 4.7A). However, after 4 h of depuration, the concentration of the bacteria was similar (ANOVA, $p > 0.05$; Figure 4.7A) in the treatment with phT4A phage and in the cockles control.

The abundance of phT4A phage in cockles increased during the first 2 h treatment, then decreased by one log of magnitude between 2 and 6 h, and then remained constant until the end of the treatment (ANOVA, $p > 0.05$; Figure 4.7B).

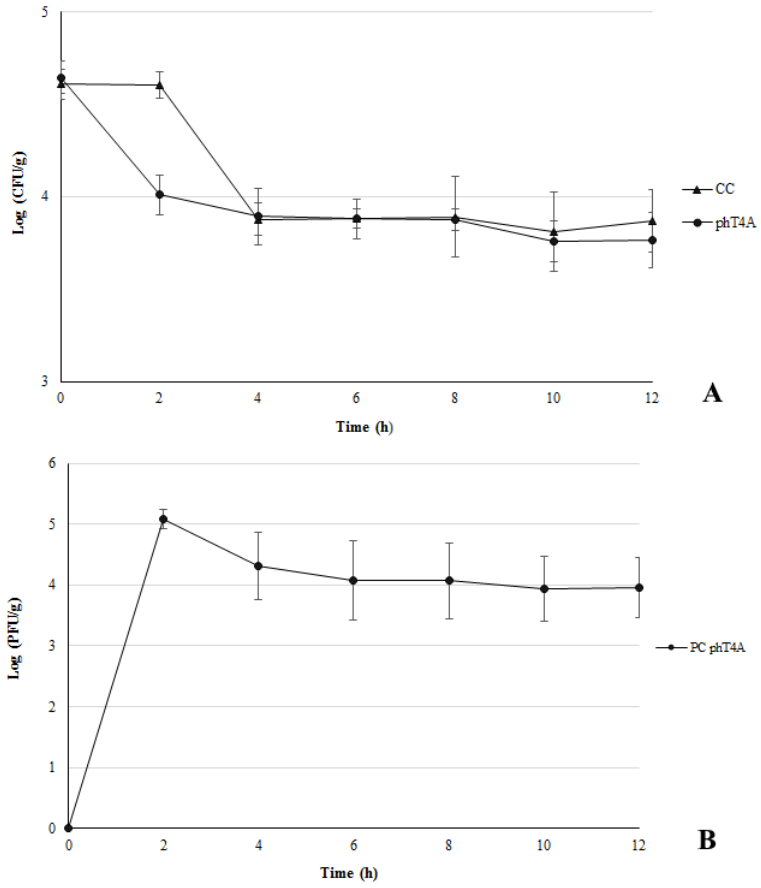

Figure 4.7 Inactivation of *E. coli* during depuration with recirculating water of natural contaminated cockles with and without phT4A phage suspension during 12 h. A. Bacterial concentration: CC - uninfected group (control cockles), phT4A— cockles treated with phT4A phage, B. Phage concentration: PC phT4A — Phage control Values represent the mean of three experiments; error bars represent the standard deviation.

4.5. Discussion

The elimination of *E. coli* in cockles is of extreme importance for public health, as some pathogenic strains of this species, used also as indicator of faecal pollution to classify bivalve production zones and to evaluate the depuration efficiency, can easily be accumulated in raw bivalves and pose a serious threat to human health (Kanayama et al., 2015; Potasman et al., 2002). Although, bacterial elimination mostly depends on the

depuration process that is achieved by filtering cockles (Croci et al., 2002; Wang et al., 2010), this process is not always efficient to eliminate pathogenic microorganisms from their tissues (FAO, 2008; Martínez et al., 2009; Rong et al., 2014). Although many studies have demonstrated that phages can be used to successfully decrease the concentration of *E. coli* in food, especially in meat and poultry products (Atterbury et al., 2007; Goodridge and Bisha, 2011; Raya et al., 2006; Sillankorva et al., 2012), to date, no research on inactivation of *E.coli* with phages during depuration has been reported in the literature. Rong et al. (2014) reported the effectiveness of phage application to reduce the population of *V. parahaemolyticus* in the oyster depuration. However, these studies focused only on the depuration application of a phage in artificially contaminated oysters without water circulation. The experiments did not replicate industrial depuration procedures and neither tested the suitability of this approach on naturally contaminated bivalves. In the present study, we evaluated the inactivation of *E. coli* in artificially and naturally contaminated cockles by applying phage therapy during the depuration process, both with and without water circulation.

One of the current challenges to phage therapy studies is to demonstrate its feasibility *in vivo*, according to several authors (S. Pereira et al., 2016; Silva et al., 2016, 2014b; Vieira et al., 2012), the assays *in vitro* are not sufficient to understand the interactions phage–bacteria *in vivo*. Tsonos et al. (2014) obtained similar results, as the efficiency of the phage cocktail used in treating CH2- infected chickens *in vivo* was negligible, even though, *in vitro*, the phages in the cocktail were able to efficiently lyse the APEC strain CH2. However, Park and Nakai (2003), which obtained similar results *in vitro* and *in vivo*, reported that the efficiency of the phage cocktail (PPpW-3 and PPpw-4) was higher than

that of the phages PPpW-3 and PPpW-4 when used individually in treating *Pseudomonas plecoglossicida* in both assays.

E. coli concentrations in cockles increased rapidly during the first 6 h and then the concentration remained stable. Similar results have been observed by Rong et al. (2014) during accumulation of *V. parahemolyticus* in oysters. Although there is no consensus in the literature on the process of filtration in bivalves, many studies have shown that it is a physiological process regulated by many ecological factors, primarily by temperature, but also by salinity, particle size and concentration (Shumway and Rodrick, 2009; WHO, 2010).

Seafood is considered as the most difficult food to treat with phages since it has an uneven and large surface area, which physically limits the distribution of phage particles to reach bacterial targets (Guenther et al., 2009). However, some studies report the efficiency of phages for bacterial inactivation in bivalves (Jun et al., 2014; Rong et al., 2014). Also, Pelon et al. (2005) tested, *in vitro*, the use of *Vibrio vulnificus* specific bacteriophage and a bactericidal (antimicrobial component existing in oysters and designed by anti–*V. vulnificus* factor) for *V. vulnificus*, and observed that although each treatment alone can reduce *V. vulnificus* numbers, the simultaneous use of both treatments, was more efficient (decrease more 2 log CFU/mL when using only bactericidal 1%) (Pelon et al., 2005). In the current study, both single phage suspensions and the phage cocktail tested can be used to inactivate *E. coli* (inactivation of 2.0 log CFU/g, 1.9 log CFU/g and 1.8 log CFU/g, respectively, for single phage suspensions of phages phT4A and ECA2 and for phage cocktail phT4A/ECA2 in artificially contaminated cockles during the depuration process). For the two single phage suspensions (ECA2 and phT4A), after 4 h of treatment, all added bacteria were inactivated by the phages. The bacterial concentration in the contaminated

cockle groups reached values similar to those observed for the control group that was neither added of *E. coli* nor treated with phages. The phage cocktail was, however, less efficient than the single phage suspensions and needs a longer time (8 h) to attain a similar level of *E. coli* reduction.

According to Rong et al. (2014), the successful application of phages for the treatment of artificial *V. parahaemolyticus* infections in oyster depend on many factors, such as time of incubation with phages during depuration without water circulation; temperature of depuration; bacterial concentration used to infect target bivalves and concentration of phage administered to inactivate the bacteria in the bivalves, that is, the multiplicity of infection (MOI) (Rong et al., 2014). The MOI has been referred as an important factor influencing phage therapy efficiency, differing among various animals used *in vivo* experiments namely due to the complex physicochemical environment and host defences (Rong et al., 2014) but differing also from results obtained in *in vitro* assays (Kim et al., 2015; Tsonos et al., 2014). As stated before and contrarily to the case of chemicals and other substances, precise initial doses of phages may not be essential to inactivate *in vitro* important aquaculture pathogenic bacteria due to their self-perpetuating nature (Nakai, 2010). Rong et al. (2014) concluded that the application of phage (VPp1) could reduce the population of *V. parahaemolyticus* in infected oysters (10^5 CFU/mL) during depuration, with decreases, after 36 h of depuration, of 1.24, 1.99, 1.74 and 2.35 log CFU/g, respectively, in the negative control (oysters infected *V. parahaemolyticus* and not treated with phage) and in oysters treated with phage VPp1 at MOI 10, 1 and 0.1. Martínez and Hipólito-Morales (2013) reported that the phage Vpms1 was effective in eliminating the adverse effects of *V. parahaemolyticus* in brine shrimp with a MOI of 45, 4.2, 2.25 and 0.45. These authors demonstrated that the reduction in the dosage of phages does not

induce a significant reduction in the efficacy of Vpms1, not being detected significant differences between the doses of Vpms1. In our study, the low dose of phage (MOI 1, 10^5 PFU/mL) had a significantly higher effect on bacterial reduction in cockles than the high dose (MOI 100, 10^7 PFU/mL). All treated cockle groups (treatment with phage phT4A, ECA2 and the phage cocktail phT4A/ECA2) revealed a significantly decrease in the rate of *E.coli* inactivation in cockles (0.8 - 1.0 log CFU/g) during treatment when MOI was increased from 1 to 100. However, the application of a low phage titer is cost-effective, as theoretically, a low MOI ratio is advantageous for large scale production and commercialisation of phage products, since it would reduce the cost of preparation, purification and application of phage products.

Studies on the removal of bacteria during "traditional" depuration (without phage addition) using bivalves artificially challenged with bacterial cultures show a greater degree of removal than studies using naturally contaminated shellfish (FAO, 2008). The type and quantity of initial contamination is also related with depuration efficiency, as more contaminated bivalves require longer depuration times and different microorganisms respond differently to the purification process (Croci et al., 2002; Jones et al., 1991; Richards, 1988). In this study, when naturally contaminated cockles (using 0.6 L of seawater added with phages, but without water recirculation) were depurated with the single suspensions of the two phages tested and the phage cocktail, the maximum reduction of cultivable bacteria (0.7 - 0.8 log CFU/g) was less than that observed in artificially contaminated cockles (reductions 1.8 - 2.0 log CFU/g). However, *E. coli* concentration used in contaminated cockles experiments was significantly higher (about 3.5 log CFU/g) than that observed in naturally contaminated cockles (about 1.5 log CFU/g). This indicates that naturally occurring *E. coli* in cockles can be effectively removed using depuration in

the presence of phages. Moreover, the two phages tested in this study (produced on a specific strain of *E. coli*) can inactivate the natural bacteria belonging to the *E. coli* species which are present in cockles with a similar efficiency than that observed for the *E. coli* specific host used to produce both phage suspensions.

In the current study it was also tested the depuration in the presence of phages in conditions similar to those used in industrial depuration facilities, and phage phT4A was selected to be used for depuration of natural contaminated cockles in a circulating system. A 0.6 log CFU/g reduction of cultivable bacteria in cockles was achieved after a 2 h treatment (using 14 L seawater treated with UV-C light and supplemented with phages). However, when depuration occurred without the supply of phages (control group, using 14 L seawater treated with UV-C light and without addition of phages) 4 h of treatment were necessary to obtain the same level of bacterial reduction. In the control group, the reduction occurred only during the 2 - 4 h time frame of treatment. After 4 h, no reduction of bacterial concentration was observed, which indicated that no *E. coli*, or other bacteria, were removed from the cockles after this period.

Using the specific medium to detect *E. coli* initial concentration in the cockles it was obtained a value of 1.5 CFU/mL. Taking this into account, some bacteria detected with this medium (chromocult coliform agar) are not inactivated by the phage during the depuration process. Further studies, using a higher concentration of *E. coli*, using cockles naturally contaminated with a higher content of *E. coli* or artificially contaminated cockles with *E. coli*, in conditions similar to those used in industrial depuration facilities, that is in the recirculating system, are needed. In the control samples, without phage addition, after 4 h, the concentration of bacteria was constant, which means that a high concentration of bacteria, including *E coli* and other than bacteria, were not removed by depuration. Further

studies in the recirculating system, using cocktails of phages targeted for different genera of the main pathogenic bacteria transmitted by bivalve consumption, such as *V. parahaemolyticus*, *Salmonella enterica* serovar Enteritidis and serovar Typhimurium (Butt et al., 2004; Feldhusen, 2000), are also needed to evaluate the efficacy of phage therapy during depuration to inactivate the main pathogenic bacteria not removed by simple depuration.

The success of phage therapy to control pathogenic bacteria in bivalves depends on viral survival and viability in seawater during depuration. After the accumulation period of phages in the cockles, their concentration remained relatively stable during the treatment period in the artificially and naturally contaminated cockles, which indicate that the tested phages are adequate to be used during the depuration. Whenever seawater is disinfected with UV-C light, the UV-C light application must be done before phage application, this because the phage concentration was affected by UV-C irradiation. However, the phages and bacteria accumulated inside the cockles were not affected by UV-C irradiation, because there was no decrease in the concentration of the phage and bacteria after 4 h of treatment. UV radiation kills viruses by chemically modifying their genetic material, DNA and RNA. The nucleic acid within the virus particle plays a crucial role in the absorption of UV radiation and in virus inactivation (Lytle and Sagripanti, 2005).

In 2006, the Food and Drug Administration (FDA) approved the use of a commercial phage cocktail (such as List-Shield and Intralytix.) as suitable to be included on both raw and ready-to-eat food products. Having this approval into account and bearing in mind that phages are naturally found in all food products (Soni et al., 2010), we do not foresee major regulatory difficulties preventing the use of phages phT4A and EC2A during cockles depuration process.

4.6. Conclusion

Overall, application of single phage suspensions and phage cocktails was found to be effective for the control of *E. coli* in artificial/natural contaminated cockles during depuration. Among all of the treatment groups, artificially contaminated cockles depurated with the phT4A and ECA2 phage at a MOI of 0.1 was the most efficient condition. This approach holds the potential to reduce the depuration time. We can also envisage that an increase in the efficiency of this process can be achieved, through the use of phage cocktails treating multiple pathogens transmitted to humans through bivalve consumption, broadening the spectrum of action of phage therapy. However, further studies on this topic are required to confirm this potential break through.

By combining phage therapy and depuration a reduction in the depuration period can be achieved for marine bivalves, thus decreasing the potential costs associated with depuration and, possibly, even enhance the quality and safety of depurated bivalves destined for human consumption.

Chapter 5. Bacteriophages with potential to inactivate *Salmonella* Typhimurium: use of single phage suspensions and phage cocktails

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5.1. Abstract

The aim of this study was to compare the dynamics of three previously isolated bacteriophages (or phages) individually (phSE-1, phSE-2 and phSE-5) or combined in cocktails of two or three phages (phSE-1/phSE-2, phSE-1/phSE-5, phSE-2/phSE-5 and phSE-1/phSE-2/phSE-5) to control *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) in order to evaluate their potential application during depuration. Phages were assigned to the family *Siphoviridae* and revealed identical restriction digest profiles, although they showed a different phage adsorption, host range, burst size, explosion time and survival in seawater. The three phages were effective against *S.* Typhimurium (reduction of ~ 2.0 log CFU/mL after 4 h treatment). The use of cocktails was not significantly more effective than the use of single phages. A big fraction of the remained bacteria are phage-resistant mutants (frequency of phage-resistant mutants 9.19 $x10^{-5}$ - 5.11 $x10^{-4}$) but phage-resistant bacterial mutants was lower for the cocktail phages than for the single phage suspensions and the phage phSE-1 presented the highest rate of resistance and phage phSE-5 the lowest one. The spectral changes of *S.* Typhimurium resistant and phage-sensitive cells were compared and revealed relevant differences for peaks associated to amide I (1620 cm^{-1}) and amide II (1515 cm^{-1}) from proteins and from carbohydrates and phosphates region (1080-1000 cm^{-1}). Despite the similar efficiency of individual phages, the development of lower resistance indicates that phage cocktails might be the most promising choice to be used during the bivalve depuration to control the transmission of salmonellosis

Keywords: Bacterial-phage inactivation, phage cocktails, *Salmonella* Typhimurium, salmonellosis, phenotypic resistance

5.2. Introduction

Enteric pathogen strains of *Salmonella* spp. are widely distributed in coastal areas and are a causative agent of gastroenteritis in humans after consumption of contaminated seafood (Kanayama et al., 2015; Potasman et al., 2002). *Salmonell*a introduced into marine environments readily contaminates the fauna, especially bivalves, which concentrate the marine microbiota via filter feeding, being even used as an indicator of the depuration efficiency of bivalves (FAO, 2008). Bivalve molluscs that accumulate food particles and small organisms by circulating large volumes of seawater; consequently, microorganisms, including human pathogens, are retained and accumulate in their tissues (Brands et al., 2005; Butt et al., 2004; FAO, 2004; Huss et al., 2000; Muniain-Mujika et al., 2003; Robertson, 2007). Moreover, as bivalves are often consumed raw or lightly cooked, they are potential vectors for pathogenic *Salmonella* sp.. Although more than 2500 serovars of *Salmonella enterica* have been identified, the human infections are caused by a limited number of serovars (Grimont and Weill, 1997). *Salmonella enterica* serovar Enteritidis and serovar Typhimurium belong to the most common serovars isolated during outbreaks of foodborne salmonellosis in the United States and European Union (Finstad et al., 2012). *Salmonella* most commonly causes acute gastroenteritis, with symptoms including diarrhea, abdominal cramps, and fever. Other clinical manifestations can include enteric fever, urinary tract infections, bacteremia, and severe fecal infections.

Depuration is a useful method to reduce microorganisms from bivalves when conducted under conditions that maximize the natural filtering activity, which results in expulsion of intestinal contents. However, some pathogenic microorganisms are resistant to this process (FAO, 2008; Martínez et al., 2009; Rong et al., 2014). In order to reduce the

risk of transmission of infections caused by microbial pathogens, including multidrug-resistant bacteria, it is essential to develop alternative approaches. One of the most promising is the association of phage therapy (application of lytic phages to prevent and/or to treat bacterial infections) to the depuration process. This association will contribute to the improvement of the decontamination efficiency, likely reducing the time required for the depuration, and consequently, the production costs, with additional benefits in bivalve safety and quality. After depuration, the bivalves may be intended for consumption if they have a level of less than 230 *Escherichia coli* in 100 g of flesh bivalve and intra-valvular liquid (FIL), and absence of *Salmonella* sp. (FAO, 2008).

The use of phages to control bacterial infections has been reported across numerous fields by many researchers (Bueno et al., 2012; Hooton et al., 2011; Karunasagar et al., 2007; Lim et al., 2012; Mateus et al., 2014; Park and Nakai, 2003; Pereira et al., 2011a; Rong et al., 2014; Silva et al., 2016, 2014a; Viazis et al., 2011; Vinod et al., 2006). As specific pathogen-killers, phages are effective agents for controlling bacterial infections, without affecting the normal microbiota (Hawkins et al., 2010; Park and Nakai, 2003a; Pereira et al., 2011a). Despite of phage therapy can be an eco-friendly alternative to prevent and control pathogenic bacteria, there is a major concern regarding the use of phages in the treatment of infectious diseases due to the emergence of phage-resistant mutants (Gill and Hyman, 2010; Silva et al., 2014a). Resistance may arise due to the alteration or loss of the bacterial cell surface receptors, blocking of the receptors by the bacterial extracellular matrix, inhibition of phage DNA penetration, production of modified restriction endonucleases that degrade the phage DNA, or due to the inhibition of the phage intracellular development (Labrie et al., 2010). Genetic mutations affecting phage receptors represent the most frequent cause of bacterial phage resistance (Heller, 1992;

Labrie et al., 2010). However, nowadays, there is a growing recognition that the emergence of phage-resistant mutants can be due to tolerance to phage exposure. In this situation bacterial populations may maintain their viability in the presence of phages, remaining genetically sensitive to them (Bull et al., 2014; Vieira et al., 2012). Phenotypic resistance may be i) induced – the products of phage-lysed bacteria result in a change in uninfected bacterial gene expression, reducing adsorption; ii) intrinsic - reduced adsorption is due to a physiological or gene expression state that exists prior to the phage introduction; and iii) dynamic - degradation or blocking of bacterial receptors by phage proteins released during cell lysis (Bull et al., 2014).

Although the development of phage-resistance, when only one phage is used, has already been reported (Levin and Bull, 2004; Nakai, 2010; Sandeep, 2006; Scott et al., 2007; Silva-aciares et al., 2013; Skurnik and Strauch, 2006; Tanji et al., 2005; Vieira et al., 2012), this limitation can be overcame by the combined use of more than one phage at the same time, that can be achieved, by the use of phage cocktails (Chan et al., 2013; Crothers-Stomps et al., 2010; Mateus et al., 2014). Phage cocktails not only potentially provide means to circumvent resistance to the presence of a single phage, but they also allow the treatment of multiple pathogens simultaneously (Cairns et al., 2009; Kunisaki and Tanji, 2010; Merabishvili et al., 2009). Therefore, the high specificity of phages, that sometimes can be considered to be a disadvantage of phage therapy, namely when the pathogenic bacteria are not known, may be circumvented by the use of phage cocktails, which broaden the spectrum of action (Chan et al., 2013; Sulakvelidze et al., 2001).

The aim of this study was to evaluate the efficiency of three new phages of *S.* Typhimurium (phSE-1, phSE-2 and phSE-5), individually or combined in cocktails of two or three phages, to control *S.* Typhimurium growth. As the selection of appropriate phages

is a key factor in the success of phage mediated control of bivalves infections, the three phages were characterized in terms of survival in the marine environment, host range, latent period, burst size and adsorption to the host. Nowadays, a major concern regarding the use of phages to control infections is the emergency of phage-resistant mutants and it has been stated that resistance can be overcome by the combined use of two or more phages. In this sense, phage cocktails were tested, and the development of phage-resistant mutants was evaluated after exposition to single phage suspensions and to phage cocktails.

5.3. Material and Methods

5.3.1. Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 5.1. *S.* Typhimurium (ATCC 13311 and ATCC14028), *Escherichia coli* (ATCC 25922 and 13706), *Aeromonas hydrophila* (ATCC 7966), *Vibrio fischeri* (ATCC 49387), *Vibrio parahaemolyticus* (DSM 27657), *Vibrio anguillarum* (DSM 21597), *Photobacterium damselae damselae* (DSM 7482), *Shigella flexneri Listeria innocua* (NCTC 11288), *Listeria monocytogenes* (NCTC1194) and *Aeromonas salmonicida* (CECT 894) were purchased from ATCC, DSM, NCTC and CECT collection, respectively. Five strains *S.* Enteriditis were isolated from food and gently provided by Controlvet Laboratory. The other bacterial strains used in this study were isolated in previous works from water samples collected in Ria de Aveiro (Alves et al., 2008; Louvado et al., 2012; Pereira et al., 2016a). Fresh plate bacterial cultures were maintained in solid Tryptic Soy Agar medium (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth medium (TSB; Liofilchem, Italy) and was grown overnight at 37 ºC. An aliquot of this culture (100 μL) was aseptically transferred to 10 mL of fresh TSB medium and

grown overnight at 37 °C to reach an optical density (O.D. 600) of 0.8, corresponding to about 10^9 cells per mL.

5.3.2. Phage isolation and purification

Three phages (phSE-1, phSE-2 and phSE-5) were isolated from sewage network of Aveiro (station EEIS9 of SIMRIA Multi Sanitation System of Ria de Aveiro) collected in different places at different times (January 2014, April 2014 and June 2014). Sewage water was filtered through 0.45 µm pore size polycarbonate membranes (Millipore, Bedford, MA, USA). The filtrate was added to double-concentrated TSB medium with 1 mL of fresh culture of the host, *S.* Typhimurium (ATCC 13311). The mixtures were incubated at 37 ˚C for 18 h at 80 rpm, and then filtered through a 0.2 µm membrane (Millipore Bedford, MA, USA). Chloroform (final volume of 1%) was added to the supernatants and phage concentration was determined as described before. Plates were incubated at 37 ˚C and examined for the presence of lytic plaques after 12 h. One single plaque was removed from the agar, diluted in TSB, and then chloroform (final volume of 1%) was added to eliminate bacteria. The sample was centrifuged and the supernatant was used as a phage source for a second isolation procedure. Three successive single-plaque isolation cycles were performed to obtain pure phage stocks for both bacteria. All lysates were centrifuged at 10.000 *g* for 10 min at 4 ˚C, to remove intact bacteria or bacterial debris. The phage stocks were stored at 4 °C and 1% chloroform (final volume) (Scharlau, Spain) was added. The phages were designated as phSE-1, phSE-2 and phSE-5. The phage suspension titers were determined by the double-layer agar method using TSA as culture medium (Adams, 1959). The plates were incubated at 37 °C for 4 – 8 h and the number of lysis plaques was counted. The results were expressed as plaque forming units per millilitre (PFU/mL).

5.3.3. Electron microscope examination

Phage particles of a highly concentrated suspension (10^9 PFU/mL) were negatively stained with 2% uranyl acetate (Electron Microscopy Sciences, Hatfield, UK) and electron micrographs were taken using a JEOL 1011 transmission electron microscope (JEDL USA Inc, Peabody, MA, USA) operating at 100 kV and images were acquired with a Gatan CCD-Erlangshen ES100W.

5.3.4. DNA extraction and restriction enzyme digestion

Phage suspensions (10^9 PFU/mL) of the three phages (phSE-1, phSE-2 and phSE-5) were centrifuged 3 times at 13.000 *g* for 10 min. The phage lysates were ultracentrifuged (Beckman, Optima LE-80K) at 100.000 *g* for 2 h at 10 °C. The extraction of nucleic acid from phage particles was performed using the phage DNA isolation kit (Norgen Biotek Corp, Canadan), as indicated by the instructions provided by the manufacturer. Nucleic acid yield was quantified in the Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The resulting product was electrophoresed through 0.8% agarose gel at 80 V for 40 min. The extracted phage nucleic acid was analysed on a standard 0.8% (w/v) 1X Tris–acetate–EDTA (TAE) agarose gel. It was then digested with DNase I (Thermo Scientific, USA), RNase A (Thermo Scientific, USA) and *Eco*RI (Thermo Scientific, USA) following the manufacturer´s recommendations.

5.3.5. Whole genome sequencing and bioinformatics analyses

The genome of the two most effective (individually and in cocktail) phage suspensions (phSE-2 and phSE-5) were sequenced. Phage DNA samples were firstly fragmented using NEB Next dsDNA Fragmentase (New England Biolabs) and digested

DNA further processed for libraries construction, using reagents from KAPA Library Preparation Kit for Ion Torrent according to manufacturers' protocol. High quality libraries were further used for template preparation using Ion PGM Hi-Q Template Kit and sequencing on Ion Torrent PGM machine using the chip316 v2 and Ion PGM HiQ Sequencing Kit according to manufacturers' protocol.

The generated raw sequence data were analyzed with CLC Genomics Workbench 8.5.1 (CLC Bio-Qiagen, Aarhus, Denmark). After quality trimming, the sequence reads were *de novo* assembled and the consensus sequence of the most represented contigs of both genomes, was generated. The annotation for both genomes was performed using the phage/plasmid pipeline from the RAST server Version 2.0 (Aziz et al., 2008). The nucleotide sequences were compared with those of other genes in GenBank using BLAST. Open reading frames (ORFs) were identified using the NCBI ORF Finder. A conserved protein domain analysis was performed using BLASTP and the NCBI Conserved Domains Database. The homology level between the two phages was determined taking into account the values correspond to percent nucleotide identity, using blast (bl2seq) on NCBI home-page. The genome sequences were deposited in GenBank under the accession numbers KX015770 (phage phSE-2) and KX015771 (phage phSE-5).

5.3.6. Phage host range and efficiency of plating (EOP) analysis

As a first approach, phage host range was determined by spot testing according to Adams (1959). The plates were incubated at 37 °C and examined for plaques after 8 - 12 h. Bacterial sensitivity to a phage was established by a lysis cleared zone at the spot. According to the clarity of the spot, bacteria were differentiated into three categories: clear lysis zone (+), no lysis zone (-). The efficiency of plating (EOP) was determined for

bacteria with positive spot tests (occurrence of a clear lysis zone), using the double-layer agar method (Adams, 1959). The EOP was calculated (average PFU on target bacteria / average PFU on host bacteria) (Kutter, 2009) along with the standard deviation for the three measurements. For each phage, three independent experiments were done.

5.3.7. Phage adsorption

Ten microliters of the phage suspensions (final concentration 10^6 PFU/mL) were added to 10 mL of the bacterial culture [cell density of 10^9 colonies forming units per millilitre (CFU/mL), corresponding to a O.D600=0.8] to obtain a multiplicity of infection (MOI) of 0.001 (Stuer-Lauridsen et al., 2003) and incubate at 25ºC. Aliquots of mixture were collected after 0, 5, 10, 15, 20, 25, 30, 40, 50, 60 and 70 min of incubation and chloroform (final volume 1%) was added. The mixture was centrifuged at 12.000 *g* for 5min and supernatants were immediately filtered by using 0.2 µm membrane (Millipore Bedford, MA, USA). The filtrates containing unadsorbed phages were diluted and titrated. The plates were incubated at 37 °C and examined for plaques after 4-8 h. Adsorption was expressed as the percentage decrease of the phage titre in the supernatant, as compared to the time zero. Suspensions of phages without any cells were used as no-adsorption standard for calculations (Stuer-Lauridsen et al., 2003). Three independent assays were done.

5.3.8. One step growth assays

Exponential host bacterial cultures of *S.* Typhimurium were adjusted to a 0.8 O.D. at 600 nm (corresponding to a cell density of 10^9 CFU/mL). Ten microliters of the phage suspensions (final concentration: 10^6 PFU/mL) were added to 10 mL of the bacterial

culture in order to have a MOI of 0.001 and incubated at 25 °C (Mateus et al., 2014).The mixture was centrifuged at 12.000 *g* for 5 min, the pellet was re-suspended in 10 mL of TSB at 37 °C and then were diluted and titrated. The plates were incubated at 37 °C and examined for plaques after 4-8 h (Mateus et al., 2014). Three independent assays were done.

5.3.9. Phage survival determination

The survival of *S.* Typhimurium phages was tested in seawater. Water samples were collected in August 2014 at the Mira Channel in the estuarine system Ria de Aveiro (latitude: 40°37′51.44″N, longitude 8°40′31.75″W), which is located on the northwestern coast of Portugal (Almeida and Alcantara, 1992; Almeida et al., 2002). Mira Channel (40°36′30″N, 8°44′52″W) is one of the authorized harvesting area classified as a statute B [230 – 4600 most probable number (MPN) *E. coli* per 100 g of flesh bivalve and intra-valvular liquid (FIL)] (Pereira et al., 2015). Fifty millilitres of water was filtered through 0.45 μm and then by 0.22 μm pore-size membranes (Poretics, USA), which was followed by the addition of phage suspensions of about 10^7 PFU/mL. The samples were then incubated at 25 °C without shaking, in the dark. Phage titre was determined at time zero and at intervals of 14 days by the double-layer agar method. The plates were incubated at 37 °C and examined for lysis plaques after 4 - 8 h. For each phage, three independent experiments were done.

5.3.10. Bacterial kill curves

Bacterial inactivation was determined using one phage alone (phSE-1, phSE-2 or phSE-5) and with phage cocktails (two or all the three phages mixed together, each phage

at the same concentration) using the bacterium *S.* Typhimurium as host, at a MOI of 100. The tested phage cocktails were: phSE-1/phSE-2, phSE-1/phSE-5, phSE-2/phSE-5 and phSE-1/phSE-2/phSE-5 phages. For each assay, two control samples were included: the bacterial control (BC) and the phage control (PC). The bacterial control was inoculated with *S.* Typhimurium but not with phages and the phage controls were inoculated with phages but not with bacteria. Controls and test samples were incubated exactly in the same conditions. Aliquots of test samples and of the bacterial and phage controls were collected after 0, 2, 4, 6, 8, 10, 12 and 24 h of incubation. In all assays, the phage titre was determined in duplicate through the double agar layer method after an incubation period of 4 – 8 h at 37 °C. Bacterial concentration was determined in duplicate in TSA medium after an incubation period of 24 h at 37 °C. Three independent experiments were performed for each condition.

5.3.11. Statistical analysis

The statistical analysis of data was performed using the IBM SPSS Statistics 22.0 software. Normal distributions were checked by Kolmorov-Smirnov test and homogeneity of variances by Levene test. The significance of bacterial and viral concentrations between treatments and along the experiments was tested using two-way ANOVA and Bonferrori post-hoc test. For different treatments, the significance of differences was evaluated by comparing the result obtained in the test samples with the results obtained for the correspondent control samples, for the different times. A value of $p<0.05$ was considered to be statistically significant.

5.3.12. Determination of the rate of emergence of bacterial mutants

In order to determinate the mutation frequencies per cell, ten isolated colonies were picked and inoculated into ten tubes with TSB medium, grown at 37 °C for 24 h. Aliquots of 0.1 mL from the 10^{0} –10^{-3} dilutions were inoculated on TSA plates which were incubated at 37 °C for five days (because some of the phage-resistant mutants grow very slowly). Simultaneously, 0.1 mL aliquots of 10^{-5}, 10^{-6} and 10^{-7} dilutions were plated on TSA medium without phage, for determination of CFU numbers. The averaged colony number of mutants (obtained from the ten isolated colonies) in 1 mL of culture (prepared from the culture with phages) was divided by the averaged colony number of the control (prepared from the culture without phages (Filippov et al., 2011). Three independent assays were performed.

5.3.13. Prophage detection in the host bacterium after phage addition

In order to evaluate if the phage was capable of lysogenic induction (i.e. has the ability to incorporate its own genome in the bacterial genome), a test using mitomycin C was applied (Mendes, 2009; Pereira et al., 2016a) The petri plates used to determine the spontaneous *S.* Typhimurium mutants resistant to phages were used. An isolated colony was picked out from the lysis plaque, inoculated into tubes with TSB medium and stress-induced with mitomycin C (Sigma Chemical, St. Louis, MO, USA) at a final concentration of 1 μg/mL. Cells with temperate phages usually result in the release of the phage (after inducing it by mitomycin C). The samples were incubated overnight at 37 °C and centrifuged (10.000 *g*, 10 min). The supernatant was checked for the presence of phages by applying the spot test. The presence of a clear zone after stress inducing indicates that bacteria have prophages in their genome. Three independent assays were performed.

5.3.14. Detection of host sensitivity to phages after one cycle of phage contact

The petri plates used to determine the spontaneous *S.* Typhimurium mutants resistant to phages were used. Ten isolated colony was picked out from the lysis plaque, inoculated in TSB medium and incubated for 24 h. After incubation the culture was used to do the spot test and was also streak-plated on TSA solid medium. An isolated colony in TSA was selected and the procedure was repeated more 4 times. Overall 5 streak-plating steps on solid medium were done (Pereira et al., 2016a). After to be done 5 streak-plating steps on solid medium was determined efficacy of plating and adsorption of phages to resistant bacteria as described in Section 5.3.6 and 5.3.7, respectively. Three independent assays were done.

5.3.15. Spectral characterization of S. Typhimurium phage resistant and susceptible colonies

In order to assess the spectral differences of spontaneous *S.* Typhimurium mutants resistant to phages, mid-infrared spectroscopy was performed, as previously described (Moreirinha et al., 2015). The petri plates used to determine the spontaneous *S.* Typhimurium mutants resistant to phages were used. Ten isolated colonies was picked out from the lysis plaque inoculated on SS medium (Liofilchem, Italy). After 24 h of incubation at 37 °C, the colonies were examined by infrared spectroscopy. Colonies were collected with a loop and placed directly on the crystal of a horizontal single reflection platinum ATR accessory. The colonies were air-dried and then measured. Control *S.* Typhimurium and mutants resistant to individual and cocktail phages were analysed. Spectroscopic acquisition was carried out in a MIR (Bruker ALPHA FTIR Spectrometer)

with a resolution of 4 cm^{-1} and 32 scans, in the mid-infrared (region between 4000 and 600 cm^{-1}). At least three replicate spectra were obtained for each sample.

Mid-infrared spectra, obtained in OPUS format (OPUS version 6.5 software, Bruker, Germany), were transferred via JCAMP.DX format to an in-house developed data analysis package (CATS build 97) (Barros, 1999). Principal component analysis (PCA) was used to find the major sources of variability in data, detect outliers and detect the probable presence of clusters. Previous to PCA, the spectra were standard normal deviate (SNV) corrected.

5.4. Results

5.4.1. Phage isolation and enrichment

Phages phSE-1, phSE-2 and phSE-5, isolated from Aveiro municipal sewage using *S.* Typhimurium (ATCC 13311) as host, formed clear plaques on the host strain with a diameter of 0.5 - 2 mm, of 1 - 3 mm and of 2 - 5 mm, respectively (Figure 5.1). High titre suspensions (2-4 x 10^9 PFU/mL) were obtained for the three phages.

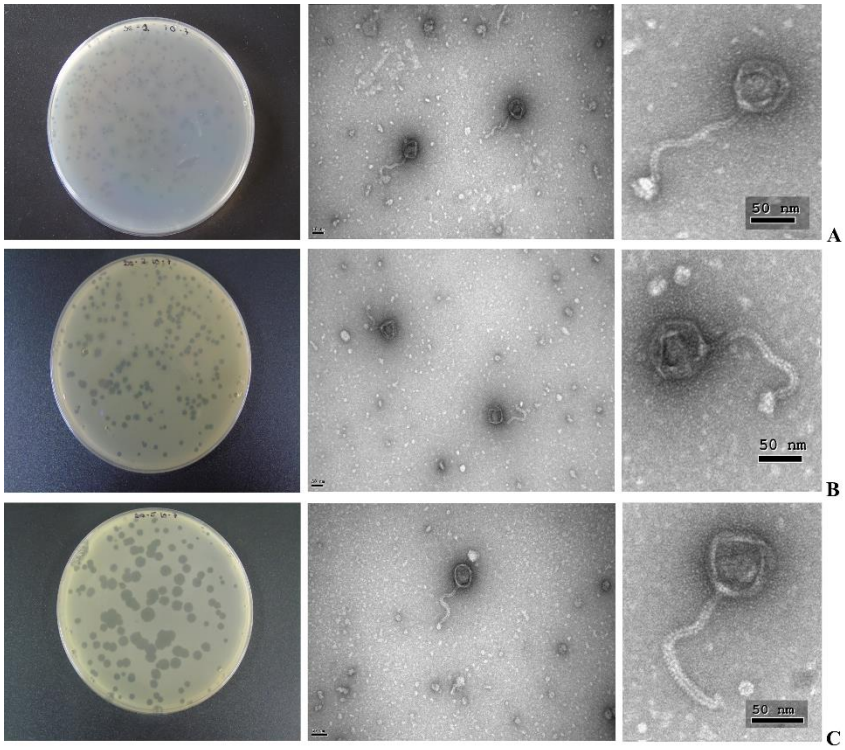

Figure 5.1 Examples of phage plaque morphologies and electron micrographs of *Salmonella* phages. (A) Phage phSE-1. (B) Phage phSE-2. (C) Phage phSE-5. The bars represent 50 nm.

5.4.2. Virion morphology

Based on the morphological analysis by TEM (Figure 5.1), all three phages were identified as order Caudovirales and family *Siphoviridae* of double-stranded DNA phages. The three phages have an icosahedric head with approximately 67 ± 3nm. Phages phSE-1, phSE-2 and phSE-5 displayed binary symmetry and a long and flexible tail with a length of approximately 152 ± 2 nm, 177 ± 2nm and 160 ± 2 nm, respectively (Figure 5.1)

5.4.3. Phage genomic characterisation

The analysis of nucleic acid type suggested that phSE-1, phSE-2 and phSE-5 are DNA phages as the genome was completely digested by DNase I, but refractory to the

activities of RNase A. The three phages presented indistinguishable restriction digest profiles and estimated genome size was approximately 50 kbp.

5.4.4. Genome Analysis

The phage genomes were sequenced with an Ion Torrent PGM semiconductor sequencing approach. A total of 331.839 reads were produced, with 376 bp average read length, resulting in a total of 121.880.159 bases. Specifically, 38.487 sequence reads were obtained for phSE-2 and 293. 352 sequence reads for phSE-5 phage. Phage genomes were *de novo* assembled using CLC Genomics Workbench 8.5.1. For phSE-2 phage 49.167-fold coverage was obtained, 49.167 bp genome and a G+C content of 42.89%. For phSE-5 phage, 9.490- fold coverage, 49.178 bp genome and 45.18% G+C content. The sequence analysis revealed the presence of 83 potential number of Coding Sequences (CDS) in both phages (Table 5.1S and 5.2S). In general, the genes of phSE-2 and phSE-5 can be roughly classified into three classes of clusters. The early (Class I), the class comprising gene clusters dealing with DNA metabolism (Class II) and finally the third group (Class III) with structural proteins of virion, helpers for assembly, and responsible for host lysis (Table 5.1S and 5.2S). None of the genomes featured any lysogenic related genes and it can be safety assumed that the two phages feature a lytic lifestyle. No genes encoding toxins, virulence factors or antibiotic resistance genes were identified based on amino acid sequence homology searches. All two genomes (phSE-2 and phSE-5) were very similar on nucleotide level (99% similarity). Overall, the genome sequence of phSE-2 phage had the highest similarity with two of the *Siphoviridae* phages: 95% with *Citrobacter* phage Stevie (accession number KM236241.1) and 94% with *Salmonella* phage 36 (accession number KR296690.1). However, the genome sequence of phSE-5 phage feature 94% similarity

with other two of the *Siphoviridae* phages, Phage TLS (accession number AY308796.1) and *Salmonella* phage FSL SP-126 (accession number KC139513.1).

5.4.5. Phage host range and efficiency of plating analysis (EOP)

Spot test indicated that phage phSE-1 and phSE-2 had the capacity to form completely cleared zones on 27 of the 42 strains, and the phSE-5 phage form cleared zones on 25 of the 42 strains (Table 5.1). However, efficiency of plating (EOP) results indicated that the three phages formed phage lysis plates only in 3 strains (*E. coli* AE11, Bioluminescent *E. coli* and *Citrobacter freundii* 10I) of the 42 strains (14% strains) tested (Table 5.1). Phage phSE-1 infected *E. coli* AE11, Bioluminescent *E. coli* and *C. freundii* 10I, presenting an efficacy of $1.70x10^{-6}$, 22.25 and $1.74x10^{-3}$%, respectively. The phSE-2 phage infected *E. coli* AE11, Bioluminescent *E. coli* and *C. freundii* 10I with an efficacy of $4.73x10^{-6}$, 34.50 and $2.28x10^{-3}$ %. The phSE-5 phage also infected *E. coli* AE11, Bioluminescent *E. coli* and *C. freundii* 10I, presenting an efficacy of $1.41x10^{-5}$, 68.22 and $5.89x10^{-4}$ % respectively (Table 5.1).

Table 5.1 Host range and efficiency of plating of three *Salmonella* phages isolates determined on 47 bacterial strains included in eight genera. Clear lysis zone (+), not lysis zone (-)

Species	Infectivity of phage			Efficacy of plating (%)		
	phSE-1	phSE-2	phSE-5	phSE-1	phSE-2	phSE-5
Salmonella. Typhimurium ATCC 13311	+	+	+	100	100	100
Salmonella Typhimurium ATCC 14028	+	+	+	0	0	0
Salmonella Typhimurium WG49	+	+	+	0	0	0
Salmonella Enteriditis CVA	+	+	+	0	0	0
Salmonella Enteriditis CVB	+	+	+	0	0	0
Salmonella Enteriditis CVC	+	+	+	0	0	0
Salmonella Enteriditis CVD	+	+	+	0	0	0
Salmonella Enteriditis CVE	+	+	+	0	0	0
Escherichia coli ATCC 25922	+	+	+	0	0	0
Escherichia coli ATCC 13706	+	+	+	0	0	0
Escherichia coli BC30	+	+	+	0	0	0
Escherichia coli AE11	+	+	+	$1.70x10^{-6}$	$4.73x10^{-6}$	$1.41x10^{-5}$
Escherichia coli AD6	+	+	+	0	0	0
Escherichia coli AF15	-	-	-	0	0	0
Escherichia coli AN19	+	+	-	0	0	0
Escherichia coli AC5	+	+	+	0	0	0
Escherichia coli AJ23	+	+	+	0	0	0
Escherichia coli BN65	+	+	+	0	0	0
Escherichia coli BM62	+	+	+	0	0	0
Bioluminescent *Escherichia coli*	+	+	+	22.3	34.5	68.2
Shigella flexneri DSM 4782	-	-	-	0	0	0
Citrobacter freundii 6F	+	+	+	0	0	0
Citrobacter freundii 10I	+	+	+	$1.74x10^{-3}$	$2.28x10^{-3}$	$5.89x10^{-4}$
Providencia sp.	+	+	+	0	0	0
Providencia vermicola	+	+	+	0	0	0
Providencia vulgaris	+	+	+	0	0	0
Providencia mirabilis	+	+	+	0	0	0
Klebsiella pneumonia	+	+	+	0	0	0
Enterobacter cloacae	+	+	-	0	0	0
Listeria innocua NCTC 11288	-	-	-	0	0	0
Listeria monocytogenes NCTC 1194	-	-	-	0	0	0
Vibrio parahaemolyticus DSM 27657	-	-	-	0	0	0
Vibrio anguillarum DSM 21597	-	-	-	0	0	0
Vibrio fischeri ATCC 49387	-	-	-	0	0	0
Photobacterium damselae damselae DSM 7482	-	-	-	0	0	0
Aeromonas hydrophilla ATCC 7966	-	-	-	0	0	0
Aeromonas salmonicida CECT 894	-	-	-	0	0	0
Pseudomonas aeruginosa	-	-	-	0	0	0
Pseudomonas fluorescens	-	-	-	0	0	0
Pseudomonas putida	-	-	-	0	0	0
Pseudomonas segetis	-	-	-	0	0	0
Pseudomonas gingeri	-	-	-	0	0	0
Total number of strains infected	27	27	25			

5.4.6. Phage adsorption

Phage adsorption assays with phSE-1 showed that approximately 55% of the phage particles adsorb to *S.* Typhimurium after 40 min and 85% adsorbed after 50 min (Figure 5.2). For phage phSE-2, 55% of particles adsorbs to *S.* Typhimurium within 50 min and

65% after 60 min (Figure 5.2). Phage adsorption of phSE-5 was approximately 55% of particles adsorbs to *S.* Typhimurium within 40 min and about 90% after 60 min (Figure 5.2).

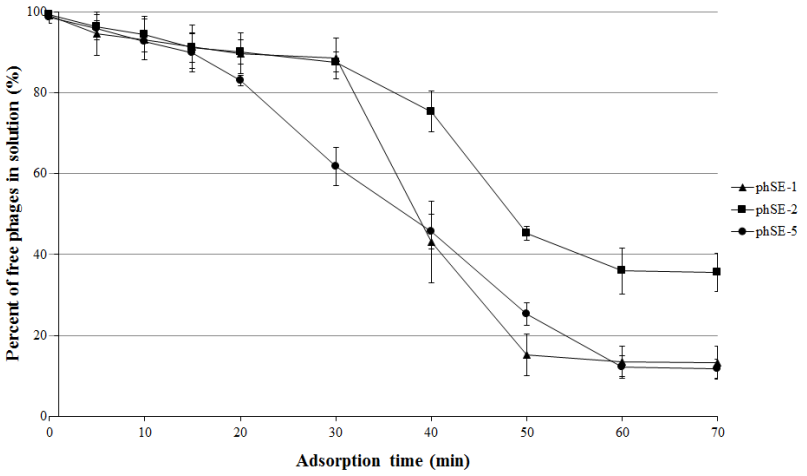

Figure 5.2 Adsorption of bacteriophages phSE-1, phSE-2 and phSE-5 to *S.* Typhimurium. The percent of unadsorbed phage is the ratio between the PFU in the supernatant to the initial sample and in an equivalent dilution of the phage in the absence of host cells. Values represent the mean of three experiments; error bars represent the standard.

5.4.7. Burst size and explosion time

Growth curve for phages phSE-1, phSE-2 and phSE-5 was determined in TSB at 25 ºC (Figure 5.3). From the triphasic curves obtained, an eclipse period of 20 min, a latent period of 30 min and a burst size of 28±3 PFU/host cell were calculated for phSE-1. The phSE-2 phage is characterized by an eclipse time of 20 min, a latent period of 40 min and each infected bacteria produced 53±5 PFU/host cell. phSE-5 is characterized by an eclipse period of 15min, a short latency of 30 min and burst size of 149±14 PFU/host cell.

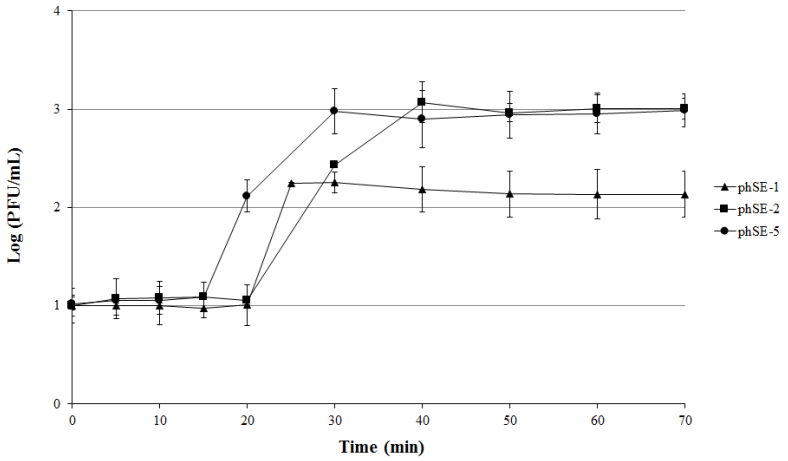

Figure 5.3 One-step growth curves of phSE-1, phSE-2 and phSE-5 phages in the presence of *S.* Typhimurium as host. Values represent the mean of three experiments; error bars represent the standard deviation.

5.4.8. Phage survival in seawater

The experiments of phage survival in the seawater samples revealed that all the three phages maintained their viability during long periods in seawater (Figure 5.4). All of the studied phages persisted viable for at least 8 months at 25 °C. The abundance of phSE-1 phage remained constant during 28th days, then decreased by three orders of magnitude between 42 and 126th day (ANOVA, $p < 0.05$), and reached a plateau until the 168th day. After 168th days, the abundance of phSE-1 phage decreased by two orders of magnitude. The phSE-2 phage abundance remained constant during 28th days, then decreased by two orders of magnitude between 42 and 112th day (ANOVA, $p < 0.05$) and reached a plateau until the 140th day. Afterwards, the phage titer decreased by two orders of magnitude until the 252th day. The phSE-5 phage abundance remained constant during 70th days, then decreased by 2.5 log between 84th and 126th day and reached a plateau until the 168th day. Afterwards, the phage titer decreased by one order of magnitude until the 252th day.

Between 126 and 168th days, the abundance of phSE-1 phage was similar (ANOVA, $p > 0.05$) to the obtained by phSE-2 and phSE-5 phages. The abundance of phSE-1, phSE-2 and phSE-5 phages at the end of the experiment was statistically different.

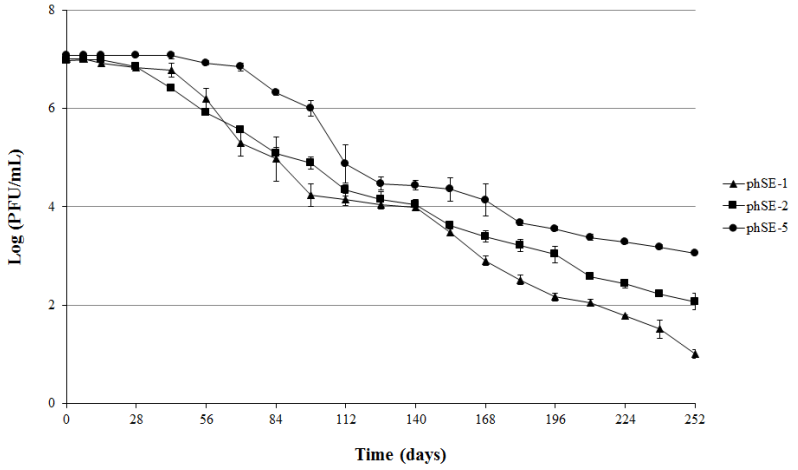

Figure 5.4 Survival of phSE-1, phSE-2 and phSE-5 phages in seawater. Values represent the mean of three experiments; error bars represent the standard deviation.

5.4.9. Bacterial killing curves

5.4.9.1. Bacterial killing curves using single-phage suspensions

According to Figure 5.5, after 4 h of treatment there was a significant reduction in *S.* Typhimurium counts with phSE-1, phSE-2 and phSE-5 phage of 1.8, 1.7 and 1.9 log CFU/mL, respectively (ANOVA, $p < 0.05$) (Figure 5.5A) when compared with those of the bacterial control (BC). The rates of inactivation after 4 h treatment, were statistically similar (ANOVA, $p > 0.05$) for the three phages. However, after 12 h, the rate of bacterial inactivation with the phSE-5 and phSE-2 phages (2.5 and 2.4 log, respectively) was similar (ANOVA, $p > 0.05$) and, twice higher (ANOVA, $p < 0.05$) than those obtained with the phSE-1 (Figure 5.5A).

Bacterial density in the BC increased by 3.5 log CFU/mL (ANOVA, $p < 0.05$) during the 12 h of incubation (Figure 5.5A).

No decrease of the phage survival was observed during the 12 h of the experiments for the phage alone and for the phage in the presence of its host in different treatments (Figure 5.5B). The phage alone (PC) was constant during all time (ANOVA, $p > 0.05$) and when they were incubated in the presence of its host a significant increase (ANOVA, $p < 0.05$) of 0.4, 0.5 and 0.35 log PFU/mL was observed for phSE-1, phSE-2 and phSE-5, respectively, after 2 h of incubation (Figure 5.5B).

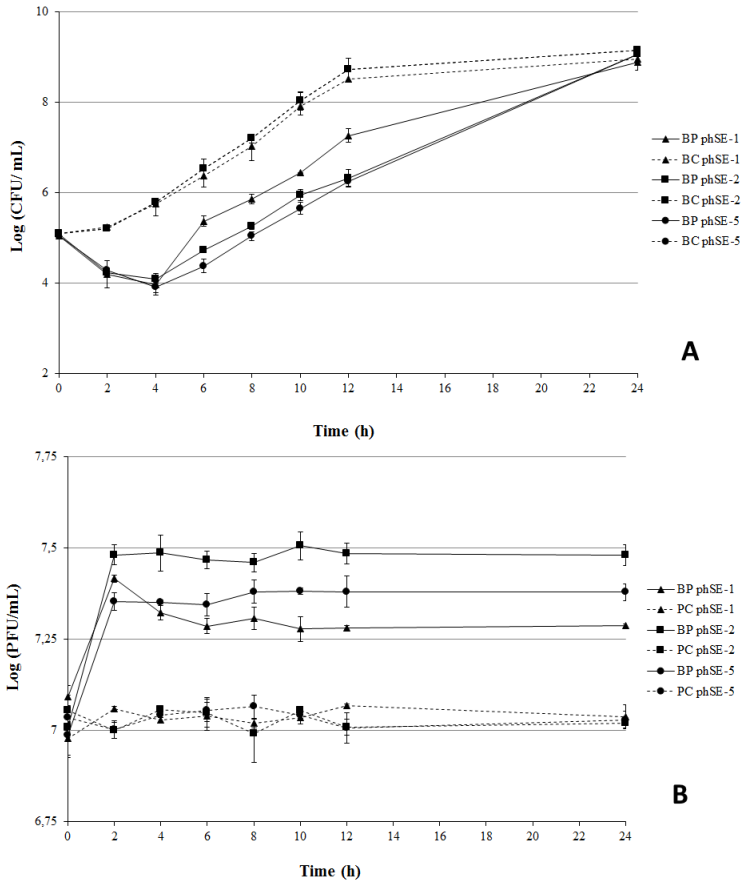

Figure 5.5 Inactivation of *S.* Typhimurium by the three phages (phSE-1, phSE-2 and phSE-5) at a MOI of 100 during 24 h. A. Bacterial concentration: BC — bacteria control; BP —bacteria plus phage. B. Phage concentration: PC — phage control; BP — bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

5.4.9.2. Bacterial killing curves using phage cocktails

The rates of inactivation during the treatment, were statistically similar (ANOVA, $p > 0.05$) for the four phage cocktails (Figure 5.6A).

The bacterial inactivation with the phSE-1/phSE-2 phage cocktail was already 1.7 log CFU/mL (ANOVA, $p < 0.05$) after 4 h incubation, being statistically similar to those with single phage suspensions of phSE-1 and phSE-2 (Figure 5.6A). However, after 12 h incubation, the rate of bacterial inactivation with phSE-1/phSE-2 phage cocktail was statistically different from that obtained with the phSE-1 phage (ANOVA, $p < 0.05$) and similar to that obtained for the phage phSE-2 (ANOVA, $p>0.05$) (Figure 5.6A). The bacterial inactivation with the phSE-1/phSE-5 after 4 h incubation was also 1.7 log CFU/mL (ANOVA, $p < 0.05$) (Figure 5.6A). This value was not statistically different (ANOVA, $p > 0.05$) from the results of the phage therapy with the phSE-1 and phSE-5 phages. However, after 12 h incubation, the rate of bacterial inactivation of the phSE-1/phSE-5 were significantly different from those obtained in the assays of phage therapy with the phSE-1 phage alone (ANOVA, $p < 0.05$), but similar to those obtained with the phSE-5 phage alone (ANOVA, $p > 0.05$).

In the phage treatment with the phage cocktail phSE-2/phSE-5, after 4 h of treatment, the rate of bacterial inactivation was 2.0 log CFU/mL (ANOVA, $p < 0.05$) (Figure 5.6A). The inactivation was not statistically different (ANOVA, $p > 0.05$) from those obtained with single phage suspension of phSE-2 and phSE-5.

The bacterial inactivation with the phSE-1/phSE-2/phSE-5 phage cocktail was 1.8 log CFU/mL (ANOVA, $p < 0.05$) (Figure 5.6A), which was not statistically different from the values obtained for the phage treatment with the three phages individually (ANOVA, $p > 0.05$). However, after 12 h incubation, the rate of bacterial inactivation with this cocktail

was statistically different (ANOVA, $p < 0.05$) from the experiments with the phSE-1 phage, but was similar (ANOVA, $p > 0.05$) to those obtained in the assays with phages phSE-2 and phSE-5.

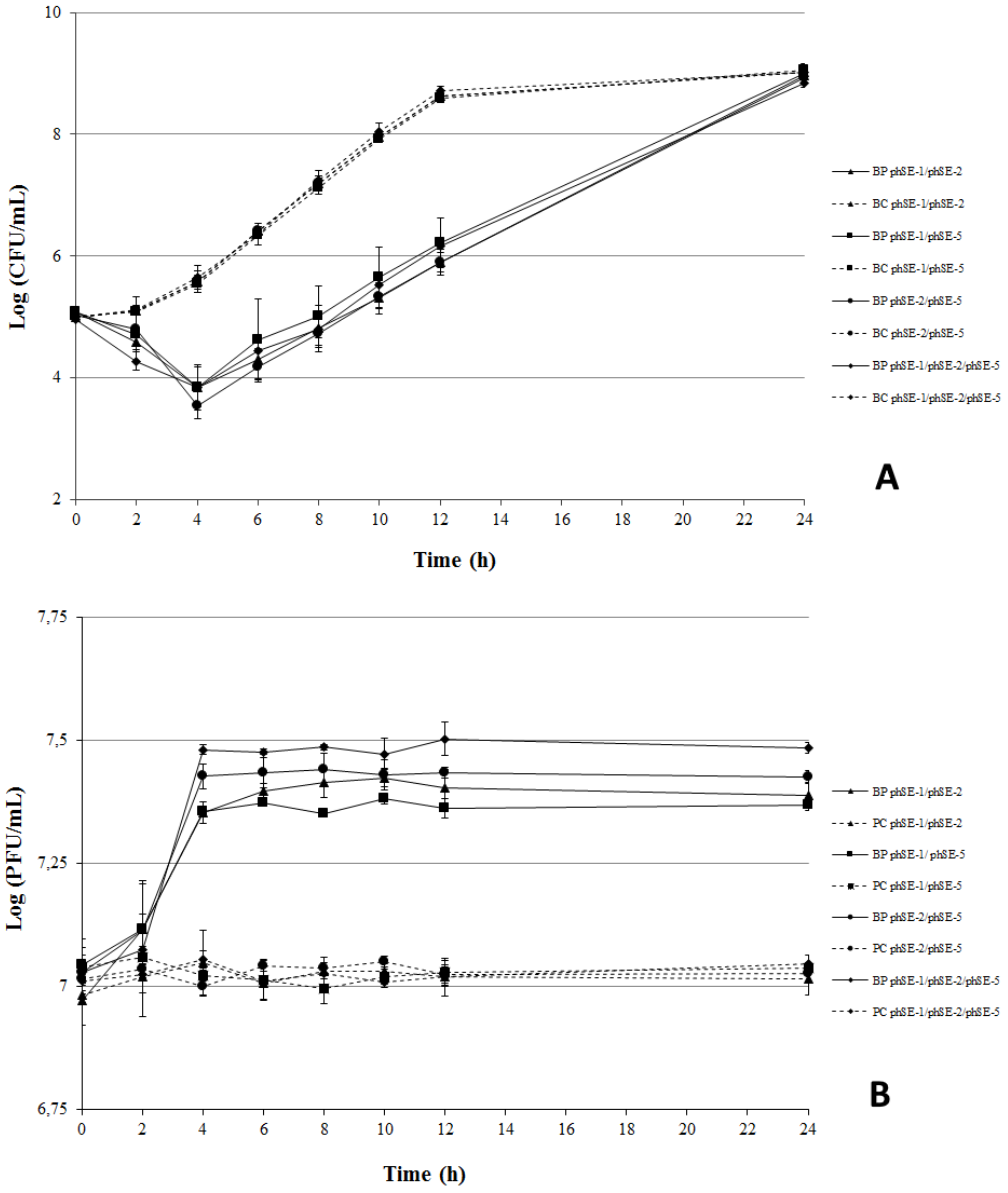

Figure 5.6 Inactivation of S. Typhimurium by phage cocktails at a MOI of 100 during 24 h. A. Bacterial concentration: BC—bacteria control; BP—bacteria plus phage. B. Phage concentration: PC — phage control; BP — bacteria plus phage. phSE-1- phage phSE-1; phSE-2 - phage phSE-2; phSE-5 - phage phSE-5. Values represent the mean of three experiments; error bars represent the standard deviation.

Bacterial density in the BC increased by 3.6 log CFU/mL (ANOVA, $p < 0.05$) during the 12 h of incubation (Figure 5.6A).

Phage concentration did not decrease significantly (ANOVA, $p > 0.05$) during the 12 h of experiment for the phage cocktail controls (PC) (Figure 5.6B). The suspensions with the phage cocktails, when incubated in the presence of the host, presented a significant increase (ANOVA, $p < 0.05$). An increase of 0.4 log was observed after 4 h of incubation (ANOVA, $p < 0.05$) for the phage cocktails phSE-1/phSE-2, phSE-2/phSE-5 and phSE-1/phSE-2/phSE-5 and after the concentration remained constant until 12 h (Figure 5.6B). When the phSE-1/phSE-2 was incubated in the presence of the host, phage started to increase sooner (0.4 log PFU/mL; ANOVA, $p < 0.05$), after only 2 h (Figure 5.6B).

5.4.10. Determination of the rate of emergence of bacterial mutants

S. Typhimurium showed different rates of phage-resistant mutants frequencies for single phage suspensions and for phage cocktails (Table 5.2). The bacterial colonies of the mutants were smaller than those of the non-phage added control and were visible only after 6 days of incubation. In control, colonies were visible after 24 h of incubation in the same conditions.

Table 5.2 Frequency of *S.* Typhimurium spontaneous phage-resistant mutants.

	Control sample (CFU mL^{-1})	Sample treated with phages	Frequency of mutants
phSE-1	$1.19 \pm 0.26 \times 10^9$	$6.06 \pm 0.51 \times 10^5$	5.11×10^{-4}
phSE-2	$1.45 \pm 0.34 \times 10^9$	$3.53 \pm 0.84 \times 10^5$	2.43×10^{-4}
phSE-5	$1.84 \pm 0.55 \times 10^9$	$2.34 \pm 0.53 \times 10^5$	1.27×10^{-4}
phSE-1/phSE-2	$1.68 \pm 0.56 \times 10^9$	$1.54 \pm 0.40 \times 10^5$	9.19×10^{-5}
phSE-1/phSE-5	$1.58 \pm 0.25 \times 10^9$	$1.75 \pm 0.32 \times 10^5$	1.10×10^{-4}
phSE-2/phSE-5	$1.76 \pm 0.33 \times 10^9$	$1.80 \pm 0.39 \times 10^5$	1.02×10^{-4}
phSE-1/phSE-2/phSE-5	$1.61 \pm 0.45 \times 10^9$	$1.99 \pm 0.56 \times 10^5$	1.24×10^{-4}

5.4.11. Prophage detection in the host bacterium after phage addition

No phages were detected in the supernatant of cultures of *S.* Typhimurium or in the mixture of bacteria and phages after treatment with mitomycin C (1µg/mL), demonstrating the absence of inducible prophages.

5.4.12. Detection of host sensitivity to phages after one cycle of phage contact

After incubation in the presence of single phages and phage cocktails and five streak-plating steps on solid medium, host bacterium had the capacity to form completely cleared zones (positive spot test). However, EOP results indicated that the three phages and phage cocktails did not form phage lysis plates and cannot adsorb and replicate in the presence to the resistant strains bacteria after five streak-plating steps on solid medium.

5.4.13. Spectral characterization of S. Typhimurium phage resistant and susceptible colonies

Principal component analysis of the spectra showed that mutants resistant to single phage suspensions, as well as mutants resistant to phage cocktails (BP), were well discriminated from non-phage added control (BC) (Figure 5.7A). It was also observable that three distinct groups are formed: bacteria resistant to individual phages; bacteria resistant to phage cocktails and bacteria control. Figure 5.7B shows that the peaks that contribute to the discrimination of the sensitive bacteria (BC) from the resistant ones (BP) are: 2917, 1072, 1052 and 1020 cm^{-1} for the sensitive bacteria (positive PC1) and 1620 and 1515 cm^{-1} for the resistant bacteria (negative PC1). It was performed a second PCA (Figure 5.8) with the spectra correspondent to bacteria sensitive to individual phages and bacteria resistant to phage cocktails, in order to observe whether these two groups remained discriminated and if the peaks that contributed to their discrimination were the

same that contributed for the discrimination in Figure 5.7. The bacteria resistant to individual phages were well discriminated from bacteria resistant to cocktails (Figure 5.8A) and the peaks that characterized the bacteria resistant to individual phages were 1693, 1620 and 1615 cm^{-1}, while the peaks that characterized the bacteria resistant to phage cocktails were characterized by peaks at 2917, 1079 and 1020 cm^{-1}.

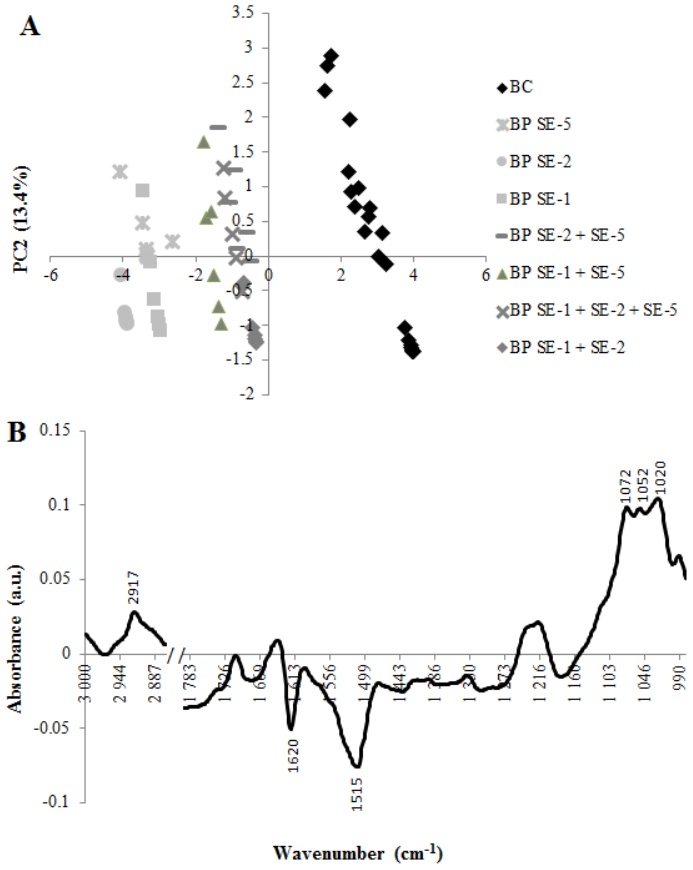

Figure 5.7 A - Scores scatter plot of the mid-infrared spectra of the bacteria in the spectral region between 3000-2800 cm^{-1} and 1800-980 cm^{-1}. BC—bacteria control; BP—bacteria plus phage. phSE-1- phage phSE-1; phSE-2 - phage phSE-2; phSE-5 - phage phSE-5. B - Loadings plot profile of the mid-infrared spectra of the bacteria in the spectral region between 3000-2800 cm-1 and 1800-980 cm-1. BC—bacteria control; BP—bacteria plus phage. phSE-1- phage phSE-1; phSE-2 - phage phSE-2; phSE-5 - phage phSE-5.

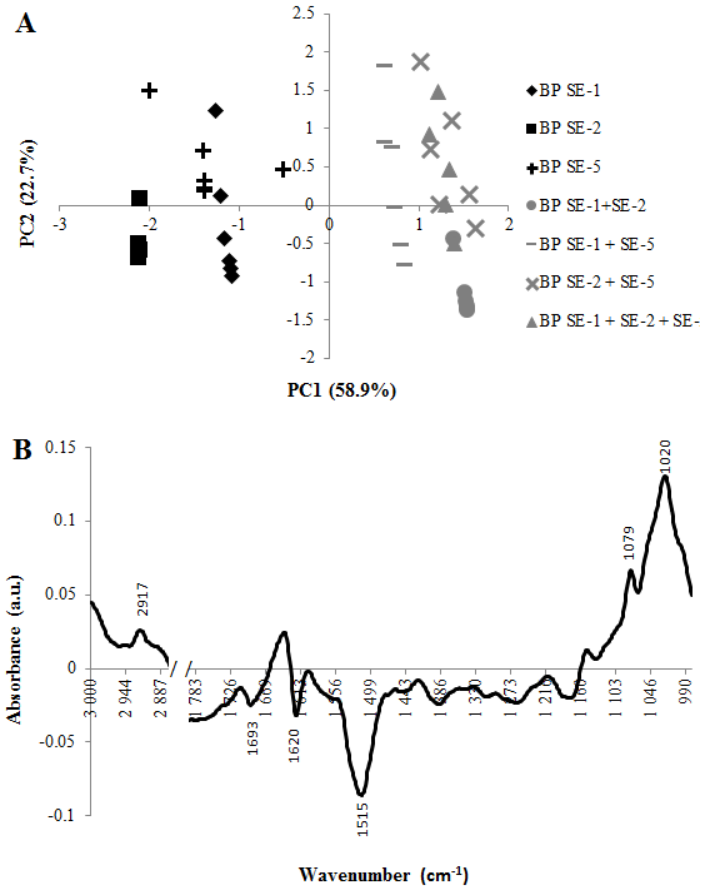

Figure 5.8 A - Scores scatter plot of the mid-infrared spectra of the bacteria in the spectral region between 3000-2800 cm^{-1} and 1800-980 cm^{-1}. BP—bacteria plus phage. phSE-1- phage phSE-1; phSE-2 - phage phSE-2; phSE-5 - phage phSE-5. B - Loadings plot profile of the mid-infrared spectra of the bacteria in the spectral region between 3000-2800 cm^{-1} and 1800-980 cm^{-1}. BC—bacteria control; BP—bacteria plus phage. phSE-1- phage phSE-1; phSE-2 - phage phSE-2; phSE-5 - phage phSE-5.

5.5 Discussion

Depuration is the unique treatment applied in order to reduce microbial contamination in bivalves, but some pathogenic microorganisms are resistant to this process. Therefore, the association of phage treatment during depuration can be an innovative and promising approach that is still in the initial stage, needing more work and

experiments. Just one study has reported the simultaneous use of the two processes in the elimination of *V. parahaemolyticus* (Rong et al., 2014), but, as far as we know, there are not any studies focused in the inactivation of the indicator of the depuration efficiency of bivalves and causal agent of a high number of enteric infections in the world, *S.* Typhimurium.

The selection of appropriate phages to be used in phage therapy is a critical stage to achieve a successful phage-mediated control of pathogenic bacteria. Criteria such as the host range, adsorption rate, latent period, burst size, survival in the environment and efficiency of bacterial inactivation must be evaluated (Mateus et al., 2014).

The three dsDNA phages tested in this study (isolated on *S.* Typhimurium) 1) infected the same hosts (*E. coli* AE 11, bioluminescent *E. coli* and *C. freundii*), 2) presented high efficiency to inactivate the *S.* Typhimurium, 3) presented long periods of survival in marine water but the concentration of phSE-5 phage decrease slowly in seawater, however, 4) the phSE -5 phage had a short latent period than the phSE-2 phage and higher burst size than the other two phages, adsorbing faster to its host.

The three phages presented similar morphology and its restriction digest profiles were indistinguishable from each other. However, there were evident differences between them, namely in their phage adsorption, host range, burst size, explosion time and survival in seawater are enough to consider all of them to evaluate their potential to inactivate *S.* Typhimurium as single suspensions or as cocktails.

The phage burst size and latent period are important factors to consider when phages are selected. Phages with high burst sizes and short latent periods are more effective to inactivate bacteria (Abedon and Culler, 2007; Mateus et al., 2014). Phage phSE-5 presented the highest burst size (149 ± 14 PFU/host cell) which was around 3 to 5 times

higher than those presented by phSE-2 and phSE-1 phages (53 ± 5 and 28 ± 3 PFU/host cell, respectively). The latent period was short, 30 min (30 and 40 min for phSE-2 and phSE-1, respectively). Having into account the number of phages produced by host cell (burst size) and the necessary time to replicate them (time of explosion), phage phSE-5 should be the most adequate to be used during bivalve depuration.

One of the major advantages of phage treatment is the phage specificity, although a therapeutic phage should be able to lyse the majority of the strains of a given bacterial species (Almeida et al., 2009). The three phages infect a similar host range, which can be explained by the fact that all of the phages were isolated using the same strain of *S.* Typhimurium as a host. Spot test indicated that phage phSE-1 and phSE-2 had the capacity to form completely cleared zones on 27 of the 42 strains, and the phSE-5 phage form cleared zones on 25 of the 42 strains. However, efficiency of plating (EOP) results indicated that the three phages formed phage lysis plates only in 3 strains (*E. coli* AE11, Bioluminescent *E. coli* and *C. freundii* 10I) of the 42 strains tested. Mirzaei and Nilsson (2015), obtained similar results, stating that spot test cannot be used for identification and selection of phages to a phage library and should be replaced by EOP assays. Lysis is a plausible mechanism which happens when an overload of phages simultaneously infects a bacterium leading to lysis either from the action of lysins or from rapid depletion of the cells resources (Abedon, 2011). In addition, as observed by Mirzaei and Nilsson (2015), high EOP was not correlated to the results from the spot tests. Further studies are needed to understand the different results obtained by both approaches.

Before the application of phages to inactivate pathogenic bacteria, like *S.* Typhimurium, during bivalves depuration, it is important to characterize the *in vitro* the dynamics of phage-host replication. Both single phage suspensions and phage cocktails

isolated in this study can be used to inactivate *Salmonella* (reduction of bacterial concentration of almost 3 log CFU/mL). Unlike other studies (Mateus et al., 2014; O'Flynn et al., 2006; Wagner and Waldor, 2002), the efficiency of all phage cocktails tested (two or three phages mixed together) to inactivate *S.* Typhimurium was similar to the single phage suspensions. For instance, Mateus et al. (2014) showed that there is a faster and higher rate of bacterial inactivation by using phage cocktails, relatively to the use of single-phage suspensions. In this study, it was not detected an increase in efficiency due to the use of phage cocktails, even when the phage phSE-5, which present the more suitable characteristics to be used in phage treatment (a shorter latent period, bigger burst size and slower decrease in seawater) is in the mixture, comparatively to the use of the single phage suspensions.

The emergence of phage-resistant mutants during phage infection has been reported in many other studies (Kudva et al., 1999; O'Flynn et al., 2006; Park and Nakai, 2003; Silva et al., 2016, 2014a; Tomat et al., 2013; Vieira et al., 2012) Other major challenge of bacterial inactivation by phages is the selection of phages that are unable to induce lysogeny. The experiments of lysogenic induction with mitomycin C (1 µg/mL) showed that no phages were detected in the supernatant of *S.* Typhimurium cultures or in the mixture of bacteria and phages after treatment with mitomycin C, demonstrating the absence of inducible prophages. Phages presented lytic cycles with no evidence of lysogeny induction. Moreover, genes codifying to integrase were not detected in the phSE-2 and phSE-5 genomes.

Phage resistance may result from mutations that alters cell surface receptors, restriction modification, or abortive infection associated with the presence of clustered regularly interspaced short palindromic repeats (CRISPRs) in the bacterial genome

(Allison and Klaenhammer, 1998; Barrangou et al., 2007). In this study, the frequency of phage-resistant mutation was 10^{-4}-10^{-5} CFU/mL. With such a low mutation frequency, phage resistance should not hinder the use of phages as biocontrol agents against the pathogenic bacteria, as stated before by other authors (Flynn et al., 2004; Tanji et al., 2004). In this study, the potential of using phage cocktails to minimize the development of phage resistant mutants was tested. Using a phage cocktail containing different phages against the same bacterial species can decrease the likelihood of selecting phage-resistant mutants (Carter et al., 2012; Flynn et al., 2004; Tanji et al., 2004; Tomat et al., 2013). Because different phages may attach to different receptors on the host, mutations in one phage receptor gene may not alter the mutant susceptibility to another phage that attaches to a different receptor on the bacterial cells (Tanji et al., 2004). However, the use of phage cocktails, as well as the use of single-phage suspensions, did not prevent the occurrence of phage-resistant mutants. The emergence of phage resistant mutants was slightly lower for phage cocktails than for single phage suspensions. Nevertheless, colonies of phage-resistant mutants were smaller than colonies formed by the non-phage added control and were visible only after 5 days of incubation. In the non-phage added control, bacterial colonies were visible after 24 hour of incubation. These results suggest that the remaining bacterial mutants (forming small size colonies and showing slow growth) maintained their viability in the presence of phages but their phenotypes were affected. Similar results were already observed in other studies (Flynn et al., 2004; Mizoguchi et al., 2003; Pereira et al., 2016a). These decrease in the bacterial growth after phage exposure could be fitness cost which can contribute to their elimination from the environment faster than their wild-type parents. Further studies are needed to confirm this hypothesis.

Phage-resistant bacteria did not recover sensitivity, phages did not adsorbed to phage-resistant bacteria and after five streak-plating steps on solid medium these bacteria remained resistant. In order to understand why the surviving viable bacteria did not recuperate their sensitivity to the phages, infrared spectroscopy was used to study the surface of the bacterial cells. The spectral changes of *S.* Typhimurium resistant and phage-sensitive cells were compared. Spectral differences between resistant mutants and the controls were clear, being the differences more relevant for peaks associated to amide I (1620 cm^{-1}) and amide II (1515 cm^{-1}) from proteins and from carbohydrates and phosphates region (1080-1000 cm^{-1}). Taking this into account, it is a possibility that the proteins present in the bacteria cellular envelope of the resistant cells, which can be used as receptors for phages, are someway modified, thus leading to the observed resistance. The peak at 2917 cm^{-1}, which appears in the controls but not in the resistant cells, has been previously associated with methylene, which is associated with proteins and amino acids, evidencing that phages could have induced changes in protein composition. However, shall be taking in consideration that the cellular envelope contains also lipids which can arise signals in the same region (Santos et al., 2013). The alterations in the spectral region of 1080-1000 cm^{-1} (Barth, 2007; Movasaghi et al., 2008) are more difficult to understand, as this region comprises not only vibrations of amino acids, but also signals due to sugars and phosphates, originating very complex bands. Comparing the group of resistant bacteria corresponding to exposition to single suspensions with that corresponding to resistant bacteria treated with phage cocktail, it is noticed that the peaks that separated these two groups were almost the same that separated resistant and controls for this spectral region. The more noticeable alteration is a peak at 1693 cm^{-1} (Barth, 2007; Movasaghi et al., 2008) observed in bacterial resistant cells treated with single phages, which seems to be related to

amide I β-linked to protein conformation. The peaks 2917 and 1020 cm^{-1}, present in the controls, re-appear in the cells treated with the cocktails after five streak-plating steps on solid medium but not in the cells treated with the single suspensions. Overall, the results suggests that the bacteria have evolved a range of barriers apparently irreversible (associated to amide I and amide II form proteins, carbohydrates and phosphates) to prevent phage adsorption and, consequently, prevent infection. As infrared spectroscopy only allows to detect alterations on cell surface, external proteins seem to be greatly implied in this process. The bacterial resistance to the phages maybe mainly due to phenotypic alterations on bacterial surface. In order to better understand the spectral changes observed when the whole cells spectra is used and, it is important to analyze bacteria protein, DNA and lipids cellular fractions in separate with infrared spectroscopy (work in progress). Although all the phages, individually or combined in cocktails, inactivated the pathogenic bacterium *S.* Typhimurium with the same efficiency, the use of phage cocktails induce lower rates of resistance development. Therefore, phage cocktails might be the most promising choice to be used during the bivalve depuration to control the transmission of salmonellosis. However, it is essential to previously select the phages for the cocktails, having into account not only its efficacy and its propensity to develop phage-resistant mutants, but also the growth characteristics. As phage phSE-5 present the shortest latent period, the largest burst size, the slowest decrease in seawater, and the highest sensitivity on surviving bacteria, this phage should be one to be used in the cocktails. Contrarily, as the phage phSE-1 increases the frequency of resistance of the cocktails, this must be avoided. Taking into account this information, the genome of the phSE-5 and phSE-2 were sequenced in order to use them in posterior *in vivo* tests during the depuration process. The genome sequence of phSE-2 and phSE-5 confirmed that they had similar

genome sequences (99% similarity) and no genes encoding products such as toxins, antibiotic resistance and integrase enzyme were detected by aminoacid sequence comparisons. Consequently, from our current state of knowledge, these phages can be considered safe to be used in phage therapy.

5.6 Conclusion

Bacterial inactivation efficiency with phSE-5 and phSE-2 phage suspension alone and combined in phage cocktails, associated with the safety of the phages and their long periods of survival, paves the way for depth studies, especially *in vivo* studies, concerning the control of *Salmonella* in bivalves. The results of this study highlight the importance of previously test the efficacy of phages to inactivate bacteria *in vitro* before moving on to *in vivo* experiments.

5.7 Supplementary material

Table 5.1S Coding sequences identified in phSE-2 phage of *S.* Typhimurium strain.

ID	Location	Start	Stop	Strand	Function
1	1_576	1	576	+	JK_59P
2	657_854	657	854	+	Phage protein
3	856_1020	856	1020	+	Phage protein
4	1013_1195	1013	1195	+	hypothetical protein
5	1192_1437	1192	1437	+	Phage protein
6	1450_1962	1450	1962	+	Phage protein
7	2037_2246	2037	2246	+	Phage protein
8	2243_2500	2243	2500	+	Phage protein
9	2497_2823	2497	2823	+	Phage protein
10	2883_3011	2883	3011	+	Phage protein
11	3013_3240	3013	3240	+	Phage protein
12	3423_3584	3423	3584	+	Phage protein
13	3568_3750	3568	3750	+	Phage protein
14	3750_3980	3750	3980	+	Phage protein

15	4070_4594	4070	4594	+	Phage terminase, small subunit
16	4606_6177	4606	6177	+	Phage terminase, large subunit
17	6231_7526	6231	7526	+	Phage portal protein
18	7523_8194	7523	8194	+	Phage protein
19	8191_9300	8191	9300	+	Phage capsid and scaffold
20	9313_9801	9313	9801	+	Phage protein
21	9845_10285	9845	10285	+	Phage protein
22	10375_11352	10375	11352	+	hypothetical protein
23	11414_11686	11414	11686	+	JK_24P
24	11732_12136	11732	12136	+	JK_23P
25	12133_12504	12133	12504	+	JK_22P
26	12497_12937	12497	12937	+	Phage protein
27	12927_13319	12927	13319	+	Phage protein
28	13334_13996	13334	13996	+	Phage tail fibers
29	14074_14388	14074	14388	+	Phage protein
30	14436_14705	14436	14705	+	Phage protein
31	14744_17653	14744	17653	+	Phage tail length tape-measure protein 1
32	17653_18000	17653	18000	+	Phage minor tail protein
33	18067_18825	18067	18825	+	Phage minor tail protein
34	18822_19544	18822	19544	+	Phage tail assembly protein
35	19537_20136	19537	20136	+	Phage tail assembly protein
36	20218_23994	20218	23994	+	Phage tail fiber protein
37	24455_25510	24455	25510	+	Exodeoxyribonuclease VIII
38	25586_25876	25586	25876	+	Phage tail length tape-measure protein
39	25923_26585	25923	26585	+	Phage-associated recombinase
40	26624_27049	26624	27049	+	Single-stranded DNA-binding protein
41	29494_27083	29494	27083	-	Phage tail fibers
42	30581_29655	30581	29655	-	DNA primase/helicase, phage-associated
43	31248_30640	31248	30640	-	Phage protein
44	31337_33310	31337	33310	+	DNA helicase, phage-associated
45	33313_33720	33313	33720	+	Phage protein
46	33792_34070	33792	34070	+	Phage protein
47	34072_34812	34072	34812	+	DNA adenine methyltransferase, phage-associated
48	34814_35044	34814	35044	+	Phage protein
49	35116_35280	35116	35280	+	Phage protein
50	35261_35503	35261	35503	+	JK_65P
51	35587_36702	35587	36702	+	Phage protein
52	36814_37038	36814	37038	+	Phage holin
53	37038_37529	37038	37529	+	Phage lysin (EC 3.2.1.17) # Phage lysozyme or muramidase (EC 3.2.1.17)
54	37526_37915	37526	37915	+	Phage protein
55	38150_37929	38150	37929	-	hypothetical protein
56	38364_38227	38364	38227	-	hypothetical protein

57	38740_38348	38740	38348	-	Phage protein
58	40323_38743	40323	38743	-	Phage protein
59	40639_40391	40639	40391	-	Phage protein
60	40998_40636	40998	40636	-	Phage protein
61	41766_41071	41766	41071	-	Phage protein
62	42189_41965	42189	41965	-	Phage protein
63	42412_42191	42412	42191	-	Phage protein
64	42638_42471	42638	42471	-	Phage protein
65	42868_42635	42868	42635	-	Phage protein
66	43149_42868	43149	42868	-	Phage protein
67	43521_43327	43521	43327	-	Phage protein
68	43754_43518	43754	43518	-	Phage protein
69	43969_43751	43969	43751	-	Phage protein
70	44328_44044	44328	44044	-	Phage protein
71	45091_44975	45091	44975	-	hypothetical protein
72	45054_45341	45054	45341	+	hypothetical protein
73	45353_45796	45353	45796	+	JK_47P
74	46035_45913	46035	45913	-	hypothetical protein
75	46104_46262	46104	46262	+	Phage protein
76	46246_46527	46246	46527	+	Phage protein
77	46533_46790	46533	46790	+	Phage protein
78	46940_47116	46940	47116	+	hypothetical protein
79	47190_47495	47190	47495	+	hypothetical protein
80	47679_48242	47679	48242	+	3'-phosphatase, 5'-polynucleotide kinase, phage-associated
81	48223_48411	48223	48411	+	Phage protein
82	48422_48607	48422	48607	+	Phage protein
83	48685_48996	48685	48996	+	hypothetical protein

Table 5.2S Coding sequences identified in phSE-5 phage of *S.* Typhimurium strain

CDS	Location	Start	Stop	Strand	Function
1	4318_542	4318	542	-	Phage tail fiber protein
2	4999_4400	4999	4400	-	Phage tail assembly protein
3	5714_4992	5714	4992	-	Phage tail assembly protein
4	6469_5711	6469	5711	-	Phage minor tail protein
5	6883_6536	6883	6536	-	Phage minor tail protein
6	9792_6883	9792	6883	-	Phage tail length tape-measure protein 1
7	10100_9831	10100	9831	-	Phage protein
8	10462_10148	10462	10148	-	Phage protein
9	11202_10540	11202	10540	-	Phage tail fibers
10	11609_11217	11609	11217	-	Phage protein
11	12039_11599	12039	11599	-	Phage protein

12	12403_12032	12403	12032	-	JK_22P
13	12804_12400	12804	12400	-	JK_23P
14	13122_12850	13122	12850	-	JK_24P
15	14161_13184	14161	13184	-	hypothetical protein
16	14604_14251	14604	14251	-	Phage protein
17	15223_14735	15223	14735	-	Phage protein
18	16345_15236	16345	15236	-	Phage capsid and scaffold
19	17013_16342	17013	16342	-	Phage protein
20	18305_17010	18305	17010	-	Phage portal protein
21	19930_18359	19930	18359	-	Phage terminase, large subunit
22	20466_19942	20466	19942	-	Phage terminase, small subunit
23	20807_20556	20807	20556	-	Phage protein
24	20989_20807	20989	20807	-	Phage protein
25	21134_20973	21134	20973	-	Phage protein
26	21544_21317	21544	21317	-	Phage protein
27	21674_21546	21674	21546	-	Phage protein
28	22060_21734	22060	21734	-	Phage protein
29	22314_22057	22314	22057	-	Phage protein
30	22520_22311	22520	22311	-	Phage protein
31	23107_22595	23107	22595	-	Phage protein
32	23365_23120	23365	23120	-	Phage protein
33	23544_23362	23544	23362	-	hypothetical protein
34	23701_23537	23701	23537	-	Phage protein
35	23900_23703	23900	23703	-	Phage protein
36	24556_23981	24556	23981	-	JK_59P
37	24731_24549	24731	24549	-	Phage protein
38	25039_24728	25039	24728	-	hypothetical protein
39	25302_25117	25302	25117	-	Phage protein
40	25501_25313	25501	25313	-	Phage protein
41	26045_25482	26045	25482	-	3'-phosphatase, 5'-polynucleotide kinase, phage-associated
42	26534_26229	26534	26229	-	hypothetical protein
43	26784_26608	26784	26608	-	hypothetical protein
44	27191_26934	27191	26934	-	Phage protein
45	S27478_27197	27478	27197	-	Phage protein
46	27620_27462	27620	27462	-	Phage protein
47	27689_27811	27689	27811	+	hypothetical protein
48	28371_27928	28371	27928	-	JK_47P
49	28670_28383	28670	28383	-	hypothetical protein
50	29396_29680	29396	29680	+	Phage protein
51	29755_29973	29755	29973	+	Phage protein
52	29970_30206	29970	30206	+	Phage protein
53	30203_30397	30203	30397	+	Phage protein
54	30575_30856	30575	30856	+	Phage protein

55	30856_31089	30856	31089	+	Phage protein
56	31086_31253	31086	31253	+	Phage protein
57	31312_31533	31312	31533	+	Phage protein
58	31535_31759	31535	31759	+	Phage protein
59	31958_32653	31958	32653	+	Phage protein
60	32726_33088	32726	33088	+	Phage protein
61	33085_33333	33085	33333	+	Phage protein
62	33401_34981	33401	34981	+	Phage protein
63	34984_35376	34984	35376	+	Phage protein
64	35360_35497	35360	35497	+	hypothetical protein
65	35574_35795	35574	35795	+	hypothetical protein
66	36198_35809	36198	35809	-	Phage protein
67	36686_36195	36686	36195	-	Phage lysin (EC 3.2.1.17) # Phage lysozyme or muramidase (EC 3.2.1.17)
68	36910_36686	36910	36686	-	Phage holin
69	38137_37022	38137	37022	-	Phage protein
70	38463_38221	38463	38221	-	JK_65P
71	38608_38444	38608	38444	-	Phage protein
72	38910_38680	38910	38680	-	Phage protein
73	39652_38912	39652	38912	-	DNA adenine methyltransferase, phage-associated
74	39932_39654	39932	39654	-	Phage protein
75	40411_40004	40411	40004	-	Phage protein
76	42387_40414	42387	40414	-	DNA helicase, phage-associated
77	42476_43084	42476	43084	+	Phage protein
78	43143_44069	43143	44069	+	DNA primase/helicase, phage-associated
79	44230_46641	44230	46641	+	Phage tail fibers
80	47100_46675	47100	46675	-	Single-stranded DNA-binding protein
81	47801_47139	47801	47139	-	Phage-associated recombinase
82	48138_47848	48138	47848	-	Phage tail length tape-measure protein
83	48990_48214	48990	48214	-	Phage exonuclease

Chapter 6. Application of bacteriophages during depuration reduces the load of *Salmonella* Typhimurium in cockles

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6.1. Abstract

As bivalve molluscs are filter feeder, often consumed raw or lightly cooked and are frequently cultivated in contaminated waters, they are implicated in food-borne disease transmission to human. The present study investigated the potential application of the bacteriophage (or phage) phSE-5 and phSE-2/phSE-5 cocktail to decrease the load of *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) during the depuration of natural and artificially contaminated cockles (*Cerastoderma edule*). Although a previous study reported the combined use of these two processes to eliminate *Vibrio parahaemolyticus* from artificially contaminated oysters, this study did not replicate industrial depuration procedures and did not test the suitability of this approach on naturally contaminated bivalves. Cockles were artificially infected with 10^5 and 10^6 colony-forming units (CFU)/mL of *S.* Typhimurium in static seawater and each infected group was treated with four different MOI values: 0.1, 1, 10 and 100. Depuration in static seawater at MOI of 0.1 with single phage suspension of phSE-5 provided the best results, as it decreased by ~ 1.7 log CFU/g the load of *S.* Typhimurium after a 4 h treatment. At a MOI of 0.1, the rate of inactivation with phSE-5 phage was higher when compared with the results obtained using phSE-2/phSE-5 cocktail. However, in naturally contaminated cockles treated in static seawater with single phage suspensions and phage cocktail phSE-2/phSE-5, similar decreases in *S.* Typhimurium concentration (~ 0.9 log CFU/g) were achieved. When artificially contaminated cockles were depurated with phSE-5 phage in a recirculated seawater system (mimicking industrial depuration conditions), a 0.9 and 2.0 log CFU/g reduction of *S.* Typhimurium was reached after 4 and 6 h treatment, respectively, relatively to the bacterial control (BC). Once the depuration process was performed without phage, a 6 h treatment was needed to obtain a 1.1 log CFU/g reduction

of *S.* Typhimurium. Results indicated that combining phage therapy with depuration procedures enhance bivalve microbial safety for human consumption by improving decontamination efficiency, proving that this technology can be transposed to the bivalves industry. Moreover, this approach also displays the advantage of reducing the time required for depuration and consequently its associated costs.

Keywords: bacteriophage, cockles, *Cerastoderma edule*, food safety

6.2. Introduction

Seafood, particularly filter feeder bivalve molluscs, are well represented in food-borne disease statistics (Potasman et al., 2002). As the majority of bivalves used for human consumption, cockles (*Cerastoderma edule*) accumulate food particles and small organisms by circulating large volumes of seawater; consequently, microorganisms, including human pathogens, are retained and can accumulate in their tissues (Brands et al., 2005; Butt et al., 2004; FAO, 2004; Huss et al., 2000; Muniain-Mujika et al., 2003; Robertson, 2007). Moreover, as bivalves are often consumed raw or lightly cooked, they are potential vectors of pathogenic *Salmonella enterica* serovar Typhimurium (*Salmonella* Typhimurium) (Lees, 2000). *Salmonella* has long been recognized as an importance foodborne pathogen and a major public health burden worldwide (Hungaro et al., 2013). *Salmonella enterica*, namely *Salmonella enterica* serovar Enteritidis and serovar Typhimurium, has been considered the causal agent of a high number of enteric infections in the world, transmitted mainly by raw foods and cross-contamination of ready-to-eat products (Bell and Kyriakides, 2002). As *Salmonella* is introduced through a number of pathways into marine environments and readily contaminate existing fauna, especially

bivalves which concentrate marine microbiota via filter feeding, this microorganism is commonly used as a proxy to infer depuration efficiency of bivalves destined for human consumption (FAO, 2008).

Depuration is a method applied to reduce and eliminate human pathogens from bivalves. Briefly, it consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores and activated oxygen) or physically (UV-C irradiation) disinfected seawater to allow purification under controlled conditions (Croci et al., 2002; Wang et al., 2010). This process of depuration usually occurs in 2 days (Lees, 2000). After depuration, bivalves may be destined for consumption if they have a level of less than 230 *Escherichia coli* in 100 g of flesh bivalve and intra-valvular liquid (FIL), and absence of *Salmonella* sp. (FAO, 2008). However, it is already well documented that a number of pathogenic microorganisms still persist in depurated bivalves (FAO, 2008; Martínez et al., 2009; Rong et al., 2014). In order to reduce the risk of transmission of infections caused by microbial pathogens, including multidrug-resistant bacteria, it is essential to develop alternative and/or complementary approaches to "traditional" bivalve depuration practices. One of the most promising is the association of phage therapy (application of lytic phages to prevent and/or to treat bacterial infections) to the depuration process (Pereira et al., 2016a; Rong et al., 2014).

The use of lytic phages to reduce food-borne pathogens has emerged as a promising tool for food safety. These viruses are target-specific, self-replicating, rapidly bactericidal, and do not alter normal food properties (Galarce et al., 2014). A few publications have demonstrated that phages can be used to successfully reduce *Salmonella* spp. in foods, especially meat and poultry products (Bielke et al., 2007; Bigwood et al., 2008; Goode et al., 2003; Goodridge and Bisha, 2011; Guenther et al., 2009; Hooton et al., 2011).

However, the combination of depuration and phage therapy to eliminate pathogenic bacteria in bivalves is an innovative approach which is still being investigated. A previous study by Rong et al. (2014) already reported the combined use of these two processes to eliminate *V. parahaemolyticus* from artificially contaminated oysters. However, this study did not replicate industrial depuration procedures and did not test the suitability of this approach on naturally contaminated bivalves.

The aim of the present study was to evaluate, for the first time, the efficiency of phSE-5 individually or combined in a cocktail (phSE-2/phSE-5), to control *S.* Typhimurium in natural and artificially contaminated cockles in static water and during depuration mimicking industrial procedures currently employed using artificially contaminated bivalves.

6.3. Material and Methods

6.3.1. Bacterial strains

S. Typhimurium (ATCC 13311), a microbiological indicator of shellfish depuration efficiency (FAO, 2008), was used to infect cockles. Fresh plate bacterial cultures were maintained in solid Tryptic Soy Agar (TSA; Liofilchem, Italy) at 4 °C. Before each assay, one isolated colony was aseptically transferred to 10 mL of Tryptic Soy Broth (TSB; Liofilchem, Italy) and was grown overnight at 37 ºC. An aliquot of this culture (100 µL) was aseptically transferred to 10 mL of fresh TSB and grown overnight at 37 °C to reach an optical density (O.D. 600 nm = 0.8), corresponding to about 10^9 cells per mL. Counts of *S.* Typhimurium was performed using Brilliant green agar (BGE, Liofilchem, Italy) in artificially contaminated cockles , while TSA medium was used for naturally contaminated cockles.

6.3.2 Phage preparation

The phages phSE-2 and phSE-5, two lytic phages previously isolated from sewage samples, evidenced to be effective in lysing *S.* Typhimurium even at multiplicity of infection (MOI) of 100 in studies *in vitro* (Pereira et al., 2016b). The two phage suspensions were prepared in SM buffer (0.1 M NaCl, 8 mM MgSO4, 20 mM Tris-HCl, 2% (w/v) gelatin, pH 7.5) at 37 °C during 4-6 h, using *S.* Typhimurium as host. Double-plaque assays were performed as described by Pereira et al. (2016b) to determine the phage titer, with the phages being stored at 4 °C. The titer of both phages was approximately 10^9 plaque-forming units (PFU)/mL.

Phage phSE-2 and phSE-5 belong to the family *Siphoviridae* and both are safe (no integrase and toxin codifying genes) to be used in bacterial control (Pereira et al., 2016b). The genome sequences were deposited in GenBank under the accession number KX015770 for phSE-2 and KX015771 for phSE-5.

6.3.3. Collection of cockle samples

Cockles were selected as biological models to test the efficacy of phage therapy against *S.* Typhimurium infections and purchased from Falcamar Lda. (Vila do Conde, Portugal), a bivalve wholesaler, after being depurated according to industrial processing protocols (48 h at 15-16 °C in seawater radiated with UV-C). These specimens were later artificially contaminated with *S.* Typhimurium in the laboratory (see below). Naturally contaminated cockles were collected in Mira Channel (Ria de Aveiro, Portugal; 40º36'30"N, 8º44'52"W), a bivalve production area ranked as B (230 – 4600 MPN *E. coli* per 100 g of FIL) (Despacho n.º 15264, 2013). Mira channel is a recreational area subjected to anthropogenic contamination (Pereira et al., 2015). Cockles were collected in

February, March, April, July 2015 and January 2016.Live cockles were dry transported to the laboratory by the research team in a controlled temperature container, under an oxygen saturated atmosphere.

6.3.4. Phage application during cockles depuration in static seawater

6.3.4.1. Accumulation of *S.* Typhimurium in cockles

Non contaminated cockles were maintained in independent tanks (10 cm length x 9 cm width x 15 cm height), acting as static systems, filled with sterile synthetic seawater (0.6 L) and equipped with its own aerator, during 12 h before being infected with *S.* Typhimurium. Temperature was maintained at 16 ± 1 °C, pH 8.0 ± 0.2, salinity 35, and dissolved oxygen above 5.5 mg/L during the whole trial. Synthetic seawater was prepared by mixing a synthetic salt brand (Tropic Marin Pro Reef salt – Tropic Marine, Germany) with water purified by a reverse osmosis system (Aqua-win RO-6080, Thailand). After the immersion period cockles were washed with sterile synthetic seawater and placed in independent tanks filled with sterile synthetic seawater.

A total of 5 groups of cockles were randomly formed, each group with three replicates of 30 cockles (a total of 5 groups x 3 replicates x 30 cockles = 450 cockles). Four groups were infected with *S.* Typhimurium (test tanks), with the fifth group being used as the cockles control. The 3 replicates from each of the 5 groups were maintained in independent tanks as described above. A fresh culture of *S.* Typhimurium was added to the four test groups obtain to obtain final concentrations of 10^5, 10^6, 10^7 and 10^8 CFU/mL. Cockles were sampled at 0, 6, 12 and 24 h during the accumulation process. This assay was repeated three times in different periods to secure independent replication.

6.3.4.2. Depuration of artificially contaminated cockles in the presence of phSE-5 phage

Cockles were maintained in independent tanks as described above. After the immersion period, cockles were washed with sterile synthetic seawater and placed in tanks independents with sterile synthetic seawater (Figure 6.1). In these experiments, 21 groups of cockles were used. Each group included 3 replicates of 30 cockles each (19 groups x 3 replicates x 30 specimens =1890). Each replicate was stocked in an independent tank as described above. *S.* Typhimurium was added to 6 of the 21 groups to obtain a final concentration of 10^5 CFU/mL, and was added to the other 5 groups to obtain a final concentration of 10^6 CFU/mL. In the other 9 groups no *S.* Typhimurium was added. Cockles from the 21 groups remained for 12 h in the tanks under the same conditions. Following 12 h of incubation, cockles were washed with sterilized artificial seawater and placed in clean tanks with no *S.* Typhimurium contamination. From the 11 groups of cockles infected with *S.* Typhimurium, 8 were inoculated with phSE-5 phage at a MOI of 0.1, 1, 10 and 100 (BP MOI 0.1 phSE-5, BP MOI 1 phSE-5, BP MOI 10 phSE-5 and BP MOI 100 phSE-5), 1 was inoculated with cocktail phSE-2/phSE-2 at a MOI of 0.1 (BP phSE-2/ BPphSE-5) and no phage was added to the two remaining infected groups (bacteria control - BC). In groups infected with *S.* Typhimurium (final concentration of 10^5 CFU/mL), the single phage suspension (phSE-5) was inoculated in order to obtain a MOI of 0.1, MOI of 1, MOI of 10 and 100, 10^4 PFU/mL for MOI of 0.1, 10^5 PFU/mL for MOI of 1, 10^6 PFU/mL for MOI of 10 and 10^7 PFU/mL for MOI of 100 and the cocktail suspension (phSE-2/phSE-5) was inoculated in order to obtain a MOI of 0.1. In groups infected with *S.* Typhimurium (final concentration of 10^6 CFU/mL), final concentrations of 10^5 PFU/mL, 10^6 PFU/mL and 10^7 PFU/mL and 10^8 PFU/mL were inoculated in order to obtain a MOI of 0.1, 1, 10 and 100, respectively. Concerning the 10 groups of cockles not

infected with *S.* Typhimurium, 8 were inoculated with phSE-5 phage at a MOI of 0.1, 1, 10 and 100 (PC MOI 0.1 phSE-5, PC MOI 1 phSE-5, PC MOI 10 phSE-5 and PC MOI 100 phSE-5), 1 was inoculated with cocktail phSE-2/phSE-5 and the remaining group was not inoculated with phages (CC). All 21 groups were incubated exactly under the same conditions. Aliquots of test samples and controls were collected after 0, 2, 4, 6, 8, 10 and 12 h of incubation. This assay was repeated three times in different dates to secure independent experiments.

6.3.5. Depuration of naturally contaminated cockles in the presence of phages

Naturally contaminated cockles were washed with sterile synthetic seawater and placed in clean water tanks with sterile synthetic seawater as described above (Figure 6.1). For each contaminated group, cockles were divided into four treatments groups (4 groups x 3 samples x 30 specimens = 360 cockles): phage treatment with phSE-5 or cocktail phSE-2/phSE-5 and no phage or cocktail treatment. The phage suspensions were inoculated as described above, with an initial concentration of 10^4 PFU/ mL (selected according to the results of previous tests in artificially contaminated cockles). After that, the procedure was similar to that described in section 6.3.4.2.Three independent experiments were done in different dates.

6.3.6. Effect of ultraviolet (UV) irradiation on phages

The effect of UV irradiation on phages was investigated by inoculating 10^5 PFU/ mL of phSE-5 phage on 14 L of aerated synthetic seawater and irradiating it with UV irradiation during 12 h (phSE-5 UV). A control treatment was prepared by replicating this procedure, with the exception of UV irradiation (CphSE-5). Each group included 3

replicates, with temperature being maintained at 16 ± 1 °C, pH 8.0 ± 0.2, salinity 35, and dissolved oxygen above 5.5 mg/L, during the whole trial. Aliquots of each group were collected after 0, 2, 4, 6, 8, 10 and 12 h of incubation. This assay was repeated three times in different periods to secure independent replication.

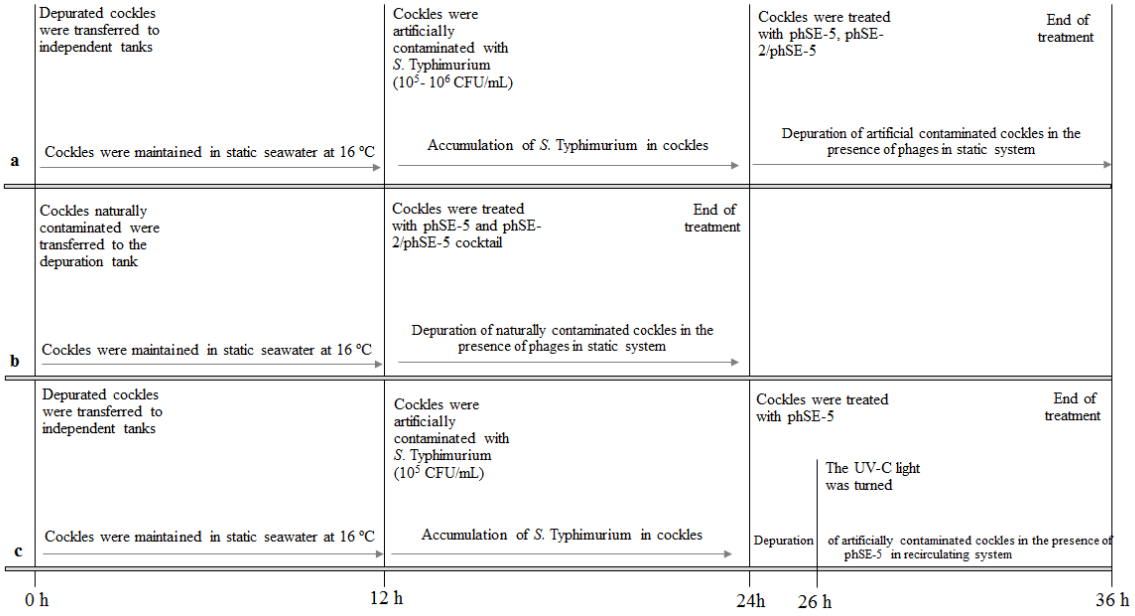

Figure 6.1 Schematic representation of the depuration experiments of cockles employing phages. (a) Depuration of artificially contaminated cockles with *S.* Typhimurium with phSE-5 and phSE-2/phSE-5 cocktail in static system (see sections 6.3.4.2), (b) Depuration of naturally contaminated cockles with phSE-5 and phSE-2/phSE-5 cocktail in static system (see section 6.3.5), (c) Depuration of artificially contaminated cockles with phSE-5 phage in a recirculating system (see section 6.3.7).

6.3.7. Depuration of artificially contaminated cockles with phSE-5 phage in a recirculating system

The efficacy of the phSE-5 phage (selected according to the results of the tests described above) was evaluated during depuration in a recirculating system mimicking the industrial depuration process. The rationale for this trial was to evaluate if this approach

could be successfully employed in industrial depuration facilities to inactivate *S.* Typhimurium. Tanks from each group were connected in parallel to twin recirculated water systems mimicking an industrial depuration facility. Each recirculated system was equipped with a 14 L tank holding a submerged water pump (Eheim Compact+ 3000, Germany), a protein skimmer (RedSea Berlin Air-Lift 60, Israel) equipped with air pump (API Rena Air 200, USA) and UV filter (TMC V2 Vecton 120 Nano, UK) with a recirculating submerged water pump (Eheim Compact 1000, Germany). Temperature was controlled by using a common 120 L water bath connected to a cooling unit (Hailea HC 500-A, China) through a submerged water pump (Eheim Compact + 3000, Germany). Cockles were washed with sterile synthetic seawater and placed in clean water tanks with sterile synthetic seawater as described above (Figure 6.1). For each contaminated group, cockles were divided into four treatments groups (4 groups x 3 samples x 30 specimens = 360 cockles): cockles treated with phage phSE-5 with 10^4 PFU/mL (BP), cockles inoculated with *S.* Typhimurium with 10^5 CFU/mL and no treated with phage, cockles not inoculated with bacteria and not treated with phage (CC), cockles no inoculated with bacteria but treated with phage (PC). To avoid the effect of UV irradiation on the phages, the UV irradiation was only switched on 2 h after the beginning of the depuration process (the time required for cockles to filtrate and accumulate the phages. After that, the procedure was similar to that described in section 6.3.4.2. This assay was repeated three times in different dates.

6.3.8. Determination the concentration of bacteria and phage

To determine the *S.* Typhimurium counts, oysters were sampled from treatment groups at different intervals during the accumulation and depuration process. At each

sampling time, four cockles were randomly selected from each tank and were homogenized with Bag Mixer 400 (Interscience, France). Ten grams were blended in 90 mL of alkaline peptone water (Liofilchem, Italy). The homogenized samples were then ten-fold serially diluted and 1 mL from each dilution was spread on the specific BGE plates (for cockles during accumulation of *S.* Typhimurium and for artificially contaminated cockles) or TSA medium (for naturally contaminated cockles). All plates were incubated at 37 °C for 24 h. The counts in BGE medium allows to detect added *S.* Typhimurium, as well as other *Salmonella* spp. already present in cockles. The phage titer was determined in duplicate for all assays through the double agar layer method after an incubation period of 8 - 12h at 37 ºC.

6.3.9. Statistical analysis

Statistical analysis was performed using GraphPad Prism 6. Normal distributions were assessed by Kolmogorov–Smirnov test and homogeneity of variances was assessed by Levene's test. Two-way analysis of variance (ANOVA) was used to test for significant differences among the accumulation of different concentrations of *S.* Typhimurium in cockles at different times. The existence of significant differences on bacterial and viral concentration in cockles artificially contaminated with 10^5 CFU/mL of *S.* Typhimurium was analysed using a three-way ANOVA with MOIs (0.1, 1, 10 and 100), phages (phSE-5, phSE-2/phSE-5) and treatment times being used as fixed factors. The significance of differences on bacterial and viral concentration in cockles artificially contaminated with 10^6 CFU/mL of *S.* Typhimurium was analysed using a two-way ANOVA. The existence of significant differences between phage treatment in cockles naturally contaminated and treatment time on bacterial and viral concentration was analysed using a two-way

ANOVA. For each experiment, the significance of differences was evaluated by comparing the results obtained in the test and control samples for the different times of each of the three independent assays. The significance of the effect of UV-C irradiation on the phT4A phage and irradiation time on phage inactivation was assessed by one-way ANOVA. Whenever significance was accepted, at $p < 0.05$, Tukey's multiple comparison test was used for a pairwise comparison of the means.

6.4. Results

6.4.1. Phage application during cockles depuration in static seawater

6.4.1.1. Accumulation of *S.* Typhimurium in cockles

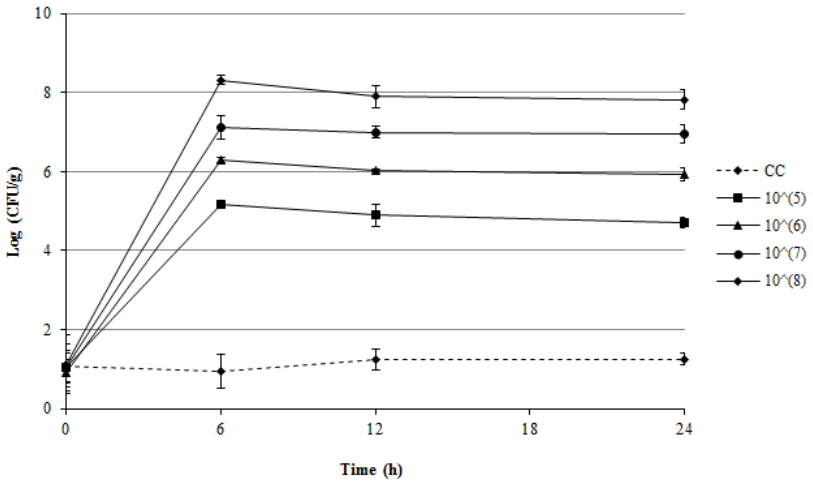

Figure 6.2 Accumulation of *S.* Typhimurium in cockles in different infected groups after 24 h. Final concentrations of *S.* Typhimurium (log CFU/g) in groups of cockles initially contaminated with 10^5 CFU/mL; 10^6 CFU/mL, 10^7 CFU/mL and 10^8 CFU/mL, as well as the cockles control (CC).

Live cockles were assessed after 24 h of incubation in the presence of *S.* Typhimurium at 16 ± 1 °C (Figure 6.2). Accumulation of *S.* Typhimurium in cockles increased rapidly during the first 6 h, with counts decreasing after this time in all four

infected groups until they remained comparatively steady after 12 h and onward. Following 12 h of incubation, the mean values of bacteria in cockles were 4.9, 6.0, 7.0 and 7.9 log CFU/g in infected groups with 10^5, 10^6,10^7 and 10^8 CFU/mL of *S.* Typhimurium, respectively (Figure 6.2). The abundance of *S.* Typhimurium in contaminated groups (10^5, 10^6,10^7 and 10^8 CFU/mL) after 6, 12 and 24 h, was statistically different (ANOVA, $p < 0.05$; Figure 6.2). Incubation by immersion during 12 h in an *S.* Typhimurium culture with 10^5 CFU/mL was the selected procedure for follow-up experiments.

6.4.1.2. Depuration of artificially contaminated cockles in the presence of phSE-5 phage and phSE-2/phSE-5 cocktail phage

At a MOI of 0.1 (the infected concentration of *S.* Typhimurium was 10^5 CFU/mL), the maximum of bacterial inactivation in cockles treated with phSE-5 phage relatively to the bacterial control was 1.7 log CFU/g (ANOVA, $p < 0.05$), which was achieved after 4 h of phage therapy (Figure 6.3A). Increasing the MOI to a value of 1 did not significantly increase the rate of inactivation (1.0 log CFU/g) after the same period of time (ANOVA, $p > 0.05$). At higher values of MOI (10 and 100), it was shown that the rate of inactivation slightly decreases with the MOI, with maximum values of 1.0 log CFU/g for a MOI of 10 and 0.6 log CFU/g of reduction at a MOI of 100, respectively, after 4 and 8 h of treatment (Figure 6.3A). However, this decrease in the rate of inactivation at MOI of 10 was not statistically significant when compared with the MOI of 1 and 10 (ANOVA, $p > 0.05$) after 6, 8, 10 and 12 h treatment. The results showed that phage treatment with phSE-5 phage in cockles infected with *S.* Typhimurium (10^5 CFU/mL at MOI of 0.1) could achieve significant reduction in less time of depuration.

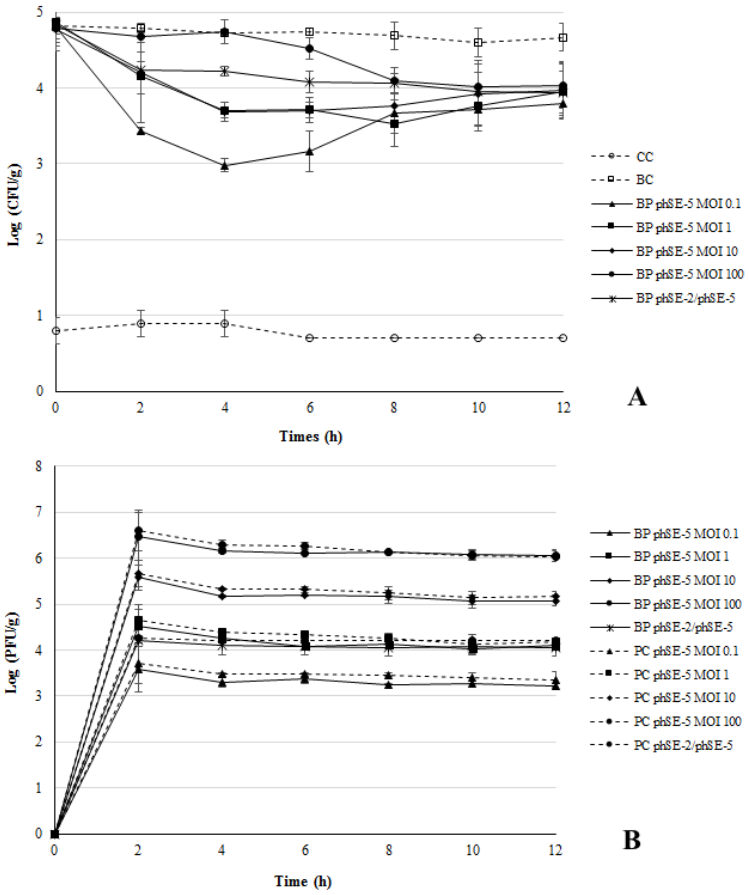

Figure 6.3 Inactivation of *S.* Typhimurium in artificially contaminated cockles with concentration of *S.* Typhimurium was 10^5 CFU/mL, by phSE-5 phage at a MOI of 0.1, 1, 10 and 100 during 12 h. A. Bacterial concentration: CC – cockles control; BC — bacteria control; BP — bacteria plus phage. B. Phage concentration: PC — phage control; BP — bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

The maximum of bacteria inactivation in cockles treated with phSE-2/SE-5 cocktail phage (MOI of 0.1) was 0.7 log CFU/g achieved after 6 h treatment (ANOVA, $p < 0.05$; Figure 6.3A). At a MOI of 0.1, the rate of inactivation with the single suspension of phSE-5 phage was significantly higher (ANOVA, $p > 0.05$) than the obtained with the phSE-2/phSE-5 cocktail phage after 2, 4 and 6 h of treatment. After 8 h of treatment, the rate of inactivation for phSE-5 phage and phSE-2/phSE-5 cocktail was similar (ANOVA, p >

0.05). The rate of inactivation with the phage cocktail was similar to the obtained with the phSE-5 phage at MOI of 1, 10 and 100 during 12 h of treatment (ANOVA, $p > 0.05$). Bacterial density in the bacterial control and cockles control remained constant (ANOVA, $p > 0.05$) during the 12 h of treatment (Figure 6.3A). The abundance of phSE-5 phage and phSE-2/phSE-5 in cockles, in the absence (PC) and in the presence of the host *S.* Typhimurium (BP, Figure 6.3B) increased during the first 2 h treatment, and then remained constant until the end of the treatment (ANOVA, $p > 0.05$).

At a MOI of 0.1 (the infected concentration of *S.* Typhimurium was 10^6 CFU/mL) in cockles treated with phSE-5 phage was 1.4 log CFU/g, achieved after 4 h of treatment (Figure 6.4A). However, after 2 h, the inactivation rate was considerably higher for the MOI of 0.1 (1.3 log CFU/g) and significantly higher (ANOVA, $p < 0.05$) than the one obtained for the MOI of 1 (0.2 log CFU/g; ANOVA, $p < 0.05$) and for the MOI of 100 (0.1 log CFU/g; ANOVA, $p < 0.05$). Increasing the MOI of 0.1 to 10 did not significantly increase the rate of inactivation during 12 h of treatment (ANOVA, $p > 0.05$). After 6 h of treatment, the rate of inactivation for both MOIs was similar (ANOVA, $p > 0.05$). The rate of inactivation decreased with the MOI, with maximum values of 1.0 log CFU/g for a MOI of 1 and 10 and 1.2 log CFU/g of reduction at a MOI of 100, respectively, after 6 and 12 h of treatment (Figure 6.4A). Bacterial density in the bacterial control and cockles control remained constant (ANOVA, $p > 0.05$) during the 12 h of treatment (Figure 6.4A). Phage density in the PCs and in the presence of the host *S.* Typhimurium (BPs) increased during the first 2 h treatment, and then remained constant until the end of the treatment (ANOVA, $p > 0.05$; Figure 6.4B).

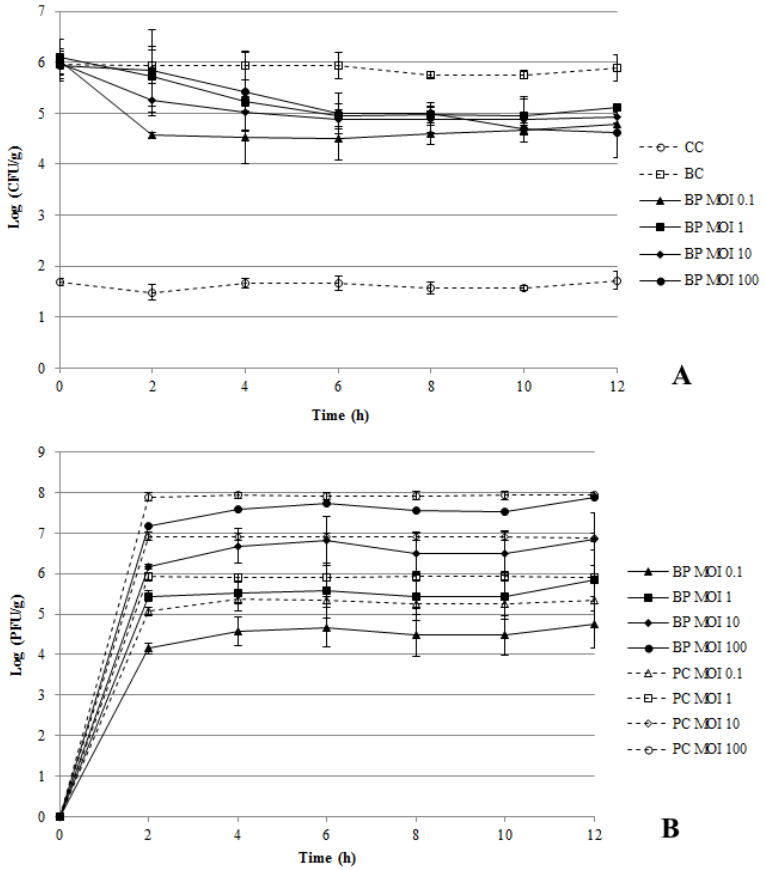

Figure 6.4. Inactivation of *S.* Typhimurium in artificially contaminated cockles with concentration of *S.* Typhimurium was 10^6 CFU/mL, by phSE-5 phage at a MOI of 0.1, 1, 10 and 100 during 12 h. A. Bacterial concentration: CC - cockles control; BC — bacteria control; BP — bacteria plus phage. B. Phage concentration: PC — phage control; BP — bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

6.4.2. Depuration of naturally contaminated cockles in the presence of phages

The maximum reduction of bacteria in cockles treated with phSE-5 was 0.9 log CFU/g after 6 h of treatment, being statistically different from the untreated group (ANOVA, $p < 0.05$), and similar than the one obtained with the phSE-2/phSE-5 (0.7 log CFU/g) (ANOVA, $p > 0.05$; Figure 6.5A). However after 2 h, the inactivation rate was considerably higher with phSE-5 phage (0.5 log CFU/g) and significantly higher than the

one obtained with the phSE-2/phSE-5. The highest inactivation rate (0.9 log CFU/g) was achieved after 4 h of treatment with phSE-2/SE-5, and after 6 h it was 0.8 log CFU/g (ANOVA, $p < 0.05$; Figure 6.5A). Phage inactivation with phSE-5 phage and phSE-2/phSE-5 cocktail phage was similar (ANOVA, $p > 0.05$) after 2 h treatment.

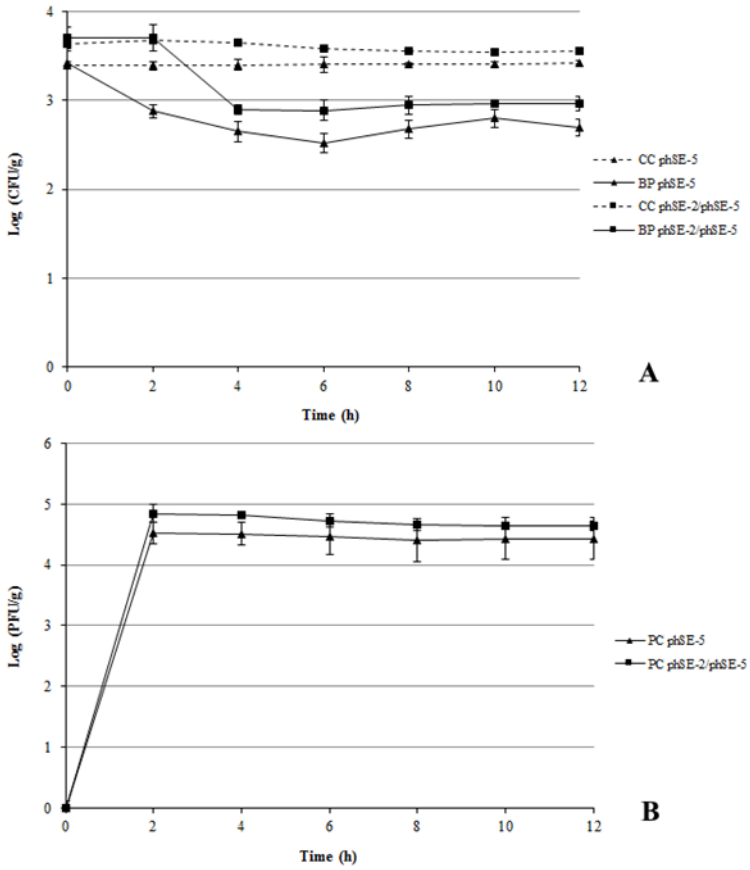

Figure 6.5 Inactivation of *S.* Typhimurium in natural contaminated cockles by phSE-5 phage and phSE-2/phSE-5 cocktail phage during 12 h. A. Bacterial concentration: CC - cockles control, phSE-5 — cockles treated with phSE-5 phage. B. Phage concentration: PCphSE-5- phage control. Values represent the mean of three experiments; error bars represent the standard deviation.

The abundance of phSE-5 phage and phSE-2/phSE-5 cocktail in cockles increased during the first 2 h of treatment, and then remained constant until the end of the treatment (ANOVA, $p > 0.05$; Figure 6.5B).

6.4.3. Effect of ultraviolet (UV) irradiation on phages

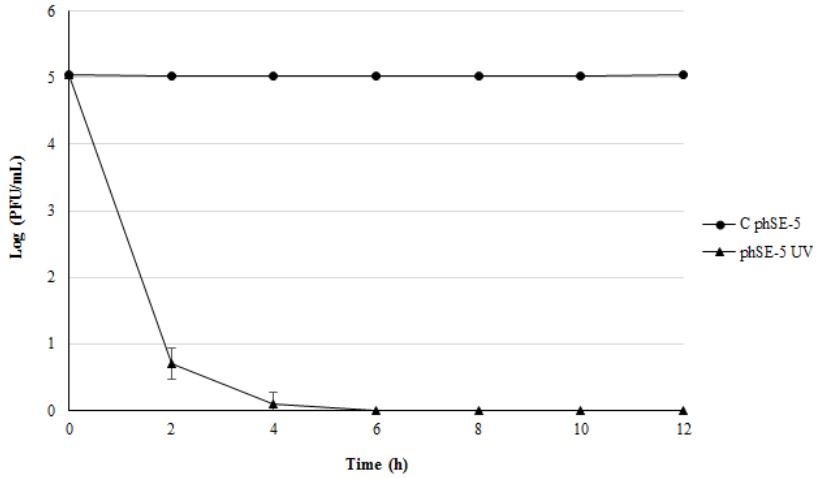

Figure 6.6 Effect of UV-C irradiation on the phSE-5 phage and synthetic seawater recirculation during 12 h. (C phSE-5) synthetic water with phSE-5 phage but not purified by UV-C irradiation (phSE-5 UV) synthetic seawater with phT4A phage and purified by UV-C irradiation.

Significant reductions (ANOVA, $p< 0.05$) in phSE-5 phage counts exposed to UV-C irradiation (phSE-5 UV) were observed after 2 h of incubation with a reduction of approximately 4.3 log PFU/mL, which was statistically different from the untreated group (C phSE-5) (Figure 6.6). The phSE-5 phage was inactivated to the limit of detection (5.0 log PFU/mL of reduction) by UV-C irradiation after 4 h of treatment (Figure 6.6). The abundance of phSE-5 in untreated group (C phSE-5) remained constant until the end of the treatment (ANOVA, $p > 0.05$).

6.4.4. Depuration of artificially contaminated cockles with phSE-5 phage in a recirculating system

The maximum of bacteria inactivation in cockles with phSE-5 was 2.0 log CFU/g after a 6 h treatment (Figure 6.7A). After 2 and 4h of treatment, the rate of inactivation was 0.7 log CFU/g and 0.9 log CFU/g, respectively, which was statistically different from the bacterial control (ANOVA, $p < 0.05$). Bacterial density in the bacterial control (BC) remained constant (ANOVA, $p > 0.05$) during the first 4 h of treatment, then decreased by 1.1 log CFU/g after 6 h of treatment, compared with initial bacterial density (5.0 log CFU/g) (Figure 6.7A). After 6 h of treatment, the bacterial density remained constant until the end of the treatment. Bacterial density in cockles control (CC) remained constant (ANOVA, $p > 0.05$; Figure 6.7A) during the first 4 h of treatment, then decreased to the limit of detection (~ 0.6 log CFU/g).

The abundance of phSE-5 in cockles, both in the absence (PC) and in the presence of the host *S.* Typhimurium (BP, Figure 6.7 B) increased during the first 2 h of treatment, then decreased by 0.6 log PFU/g between 2 and 4 h treatment. After a 4 h treatment, the abundance of phSE-5 remained constant until the end of the treatment (ANOVA, $p > 0.05$).

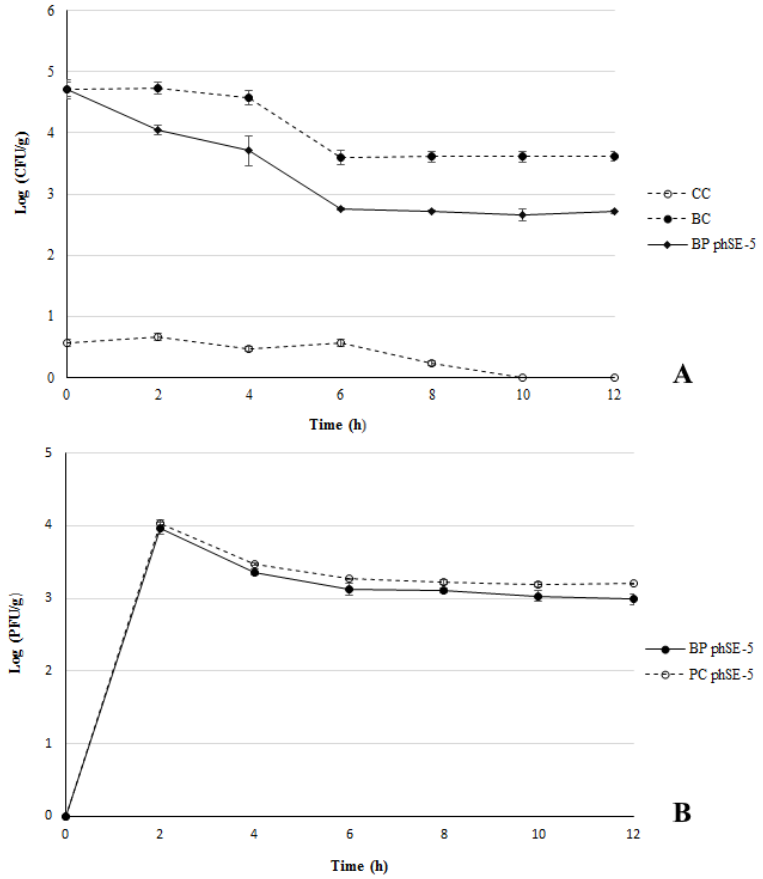

Figure 6.7 Inactivation of *S.* Typhimurium during depuration of artificial contaminated cockles with phSE-5 phage and water recirculation during 12 h. A. Bacterial concentration: CC - cockles control, BPphSE-5 - cockles treated with phSE-5 phage, B. Phage concentration: PC - phage control; BP - bacteria plus phage. Values represent the mean of three experiments; error bars represent the standard deviation.

6.5. Discussion

Salmonella Typhimurium is the most frequent causative agent of human gastroenteritis (Butt et al., 2004; Oliveira et al., 2011a). Taking this into account, the elimination of *S.* Typhimurium in cockles is paramount for public health, as pathogenic strains of this species can easily accumulate in raw bivalves (Hungaro et al., 2013; WHO, 2010). One of the current challenges faced when performing phage therapy studies is to

demonstrate its feasibility *in vivo*. According to several authors (Pereira et al., 2016a; Silva et al., 2014a, 2016; Vieira et al., 2012), *in vitro* tests are not sufficient to understand phage–bacteria interactions *in vivo*. While the use of lytic phages to reduce food-borne pathogens has already been suggested as a promising tool for food safety (Hungaro et al., 2013; Sillankorva et al., 2012), to date, only a single study has addressed the potential use of phage therapy (during depuration in a system without water circulation) to inactivate pathogenic *V. parahaemolyticus* in artificially contaminated oyster (Rong et al., 2014). In the present study, we evaluated the inactivation of *S.* Typhimurium in artificially and naturally contaminated cockles by applying phage therapy during the depuration process, both with and without water circulation.

An artificially infected cockles, using different concentrations of *S.* Typhimurium, was established to evaluate the effect of the single phage suspensions and phage cocktail on cockles depuration. *S.* Typhimurium counts in cockles decreased from 6 to 12 h of accumulation, which may have been due to the self-cleaning of cockles, only a certain level of bacteria could colonize in the body after accumulation. Therefore, the accumulation of *S.* Typhimurium was performed for 12 h, when accumulated bacterial counts were comparatively steady. Similar results have been observed during the accumulation of *V. parahemolyticus* in oysters (Rong et al., 2014).

Single phage suspension (phSE-5) tested in this study can be used to inactivate *S.* Typhimurium in artificially contaminated cockles contaminated with 10^5 CFU/mL and 10^6CFU/mL. In general, increasing the concentration of *S.* Typhimurium in cockles (of 10^5 CFU/mL for 10^6 CFU/mL), did not shift the rate of inactivation during a 12 h treatment using the same MOI. Rong et al. (2014)obtained similar results, since these authors observed that the application of phage (VPp1) could reduce the population of *V.*

parahaemolyticus in infected oysters (10^5-10^7 CFU/mL) during depuration; indeed similar decreases after 36 h of depuration were recorded in oysters treated with VPp1 at MOI of 0.1,1 and 10. The phage cocktail (phSE-2/phSE-5) at MOI of 0.1 was, however, less efficient than the single phage suspensions and needed a longer time (6 h) to attain the maximum of inactivation for *S.* Typhimurium. Previous results using *in vitro* assays, in liquid medium at a MOI of 100, showed that the inactivation of *S.* Typhimurium with single suspensions of phSE-5 and with the phSE-2/phSE-5 phage cocktail was also effective Pereira et al., 2016b). In the *in vitro* assays performed, the rate of inactivation for phSE-5 phage and phSE-2/phSE-5 phage cocktail were 1.9 and 2.0 CFU/mL, respectively, achieved after 4 h of phage therapy (Pereira et al., 2016b).

Unlike in *in vivo* assays, previous trials *in vitro* displayed similar rates of bacterial inactivation with the phSE-5 phage and phSE-2/phSE-5 cocktail (reductions of 1.9 and 2.0 CFU/mL, respectively, after a 4 h treatment) (Pereira et al., 2016b). Park and Nakai (2003) recorded similar results using *in vitro* and *in vivo* assays, and reported that the efficiency of the phage cocktail (PPpW-3/PPpw-4) was higher than that of the PPpW-3 and PPpW-4 phages when used individually to treat *Pseudomonas plecoglossicida*.

The phage concentration applied is another factor influencing the elimination of the target microorganism during depuration (Rong et al., 2014). The dynamics of phage-bacterium interactions differ among various animal samples due to the complexity of the physicochemical environment, phage lysis activity, and host defenses (Clokie et al., 2011). Hence, the phage concentration used in a particular scenario can show dramatically different effects in other cases. In this study, the authors demonstrated that the application of phSE-5 phage at MOI of 0.1, 1, 10 and 100 in artificial contaminated cockles (10^5 CFU/mL) could reduce the population of *S.* Typhimurium during depuration in s static

system, with decreases of 1.7 log CFU/g after 4 h, 1.0 log CFU/g after 4 h, 1.0 log CFU/g after 4 h and 0.6 log CFU/g after 8 h, respectively. In this study the low dose of phSE-5 phage (MOI of 0.1, 10^4 PFU/mL) had a significantly higher effect on bacterial reduction in cockles than the highest dosage tested (MOI of 100, 10^7 PFU/mL). Rong et al.(2014) concluded that the application of the phage (VPp1) could reduce the population of *V. parahaemolyticus* in infected oysters (10^5 CFU/mL) during depuration, with decreases, after 36 h of depuration, of 1.24 log, 1.99, 1.74 and 2.35 log CFU/g, respectively, in the negative control (oysters infected *V. parahaemolyticus* and not treated with phage) and in oysters treated with phage VPp1 at MOI of 10, 1 and 0.1. Nakai et al. (1999) examined the protective effects of an anti-*Lactococcus garvieae* phage with a MOI < 1 by intraperitoneal injection or oral administration of phage against experimentally infected young yellowtails. Hooton et al. (2011) found that a high concentration (MOI > 10) of *S.* Typhimurium U288 phage cocktail could achieve successful results on artificially contaminated pig skins. The application of a low phage titer is cost-effective, as theoretically, a low MOI ratio is advantageous for large scale production and commercialization of phage products, as it would reduce their cost of preparation, purification and application.

Studies on the removal of bacteria during "traditional" depuration (without phage addition) using bivalves artificially challenged with bacterial cultures show a greater degree of removal than studies using naturally contaminated shellfish (FAO, 2008). In this study, when naturally contaminated cockles (using 0.6 L of seawater added with phages, but without water recirculation) were depurated with the phSE-5 phage, the maximum reduction of cultivable bacteria (reductions of 0.9 log CFU/g) was less than that observed in artificially contaminated cockles (reductions of 1.9 log CFU/g). However, when naturally contaminated cockles were depurated with the phSE-2/phSE-5 phage, the

maximum reduction of cultivable bacteria (0.9 log CFU/g) was similar to that observed in artificially contaminated cockles (0.7 log CFU/g). However, *S.* Typhimurium concentration used in contaminated cockles experiments was significantly higher (about 3.2 log CFU/g) than that observed in naturally contaminated specimens (about 1.0 log CFU/g). This indicates that naturally occurring *S.* Typhimurium in cockles can be effectively removed if the bivalves are depurated in the presence of phages.

While a maximum of 2.0 log CFU/g reduction of *S.* Typhimurium in cockles was achieved after a 6 h treatment (using 14 L of recirculated seawater treated with UV-C irradiation and supplemented with phages), after 2 h of treatment a bacterial reduction of 0.7 log CFU/g log was already perceptible. However, when depuration occurred without the supply of phages (control group, using 14 L of recirculated seawater treated with UV-C irradiation and without addition of phages) a small reduction of 1.1 log CFU/g of *S.* Typhimurium was obtained only after 4 h of treatment. Using conventional depuration practices, bacterial concentration is reduced only after 4 h of treatment, while in the presence of phages the bacterial reduction is already significant after 2 h; this result indicates that cockles decontamination in the presence of phages can shorten the duration of the depuration period. It is worth stating that after 6 h of treatment the number of *Salmonella,* even in the presence of the phages, remained stable. This can be due to the development of resistance by bacteria. Further studies are needed in order to test this hypothesis. *In vitro* treatments revealed the frequency of phage-resistant mutation to be low (10^{-4}-10^{-5} CFU/mL) and that these phage-resistant bacteria grow slowly. These findings will be also important to evaluate the growth of resistant bacteria isolated from phage treated cockles, as these bacteria frequently display altered surfaces and cell shapes, which reduce their fitness and attenuate their virulence (Carrillo et al., 2005; Flynn et al.,

2004). The modification of *Salmonella* LPS structure is believed to be an important phage adsorption ligand and is likely to severely affect its pathogenicity (Kong et al., 2011). The development of resistance in the host could reduce the efficacy of phage treatment and, consequently, appropriate measures to decrease the likelihood of resistance development are needed. Among these measures should be considered the use of phage cocktails including phages with broad host range or the use of rotating treatment schemes employing different phages (Guenther et al., 2009; Kunisaki and Tanji, 2010).

After the accumulation period of phages in cockles, their concentration remained relatively stable during the treatment period in artificially and naturally contaminated specimens, thus indicating that tested phages can be suitably employed during bivalve depuration. Whenever seawater is disinfected with UV-C irradiation, its application must be done before phage application, because the phage concentration is affected by UV-C irradiation. However, phages and bacteria that were accumulated inside the cockles were not affected by UV-C irradiation, because there was no decrease in the concentration of phage and bacteria after 4 h of treatment.

6.6. Conclusion

The application of phSE-5 phage and phSE-2/phSE-5 phage cocktails can be successfully employed to inactivate *S.* Typhimurium in cockles during depuration, especially if phSE-5 phage is used individually and a low MOI is employed. Overall, this approach holds the potential to reduce depuration time and increase the efficiency of this process, consequently holding the potential to reduce production costs and improve bivalve safety and quality for human consumption

Chapter 7. General conclusions and future perspectives

7.1 Conclusions

In this work a series of experiments were conducted to assess the sustainability of phage therapy to control infections by microbial pathogens through the consumption of bivalves, during depuration.

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

For apply a successful phage therapy during depuration of bivalves it is essential to know the dynamics of the natural microbial community in the harvesting waters, with detail on the most important pathogenic bacteria of bivalves associated illness.

- Enterobactereaceae family was the most abundant bacterial group.
- The indigenous *Vibrio* and *Aeromonas* were also present at high concentrations
- The harvesting waters from the Ílhavo channel (classified as a C area) were more contaminated than those from the Mira channel (classified as a B area).
- The overall bacterial community and the disease-causing bacteria, as well as the indicator of microbiological water quality of two harvesting areas showed clear but distinct patterns of variation.
- The seasonal variation of the bacterial community and specific groups that are responsible for the contamination of the bivalves, indicate that monitoring the seasonal variation should be done in order to select the more appropriate phages to inactive microbial pathogenic of the bivalves.
- A higher complexity of the whole community and an increase of abundance of the main pathogenic bacteria in hot months, indicate that this season is the critical time

frame when phage therapy should be applied. However, could be necessary the application of phage therapy during the cold season.

- The bacterial community structure in the hot months (June and August) was more diverse and distinct from that in cold months (December and February).
- Non- and indigenous bacterial groups, as the total bacterial number, registered the highest values in summer.
- *Vibrio* and *Aeromonas* genus also presented a seasonal pattern of variation, with values up to 20 times higher in the hot season, when compared to the cold season.

The new phages of *S.* Typhimurium and of *E. coli* tested in this study are potential candidates to be used during depuration. Phage characterization is an important aspect to select the most adequate phages.

- *S.* Typhimurium phages showed good survival time in seawater
- Phages with high burst sizes and short lytic cycles increase the efficiency of phage therapy.
    - The *E. coli* phage EC2A presents the high burst size and the short lytic cycle and inactivate *E. coli* sooner than phage phT4A.
- Efficiency of plating was not correlated to the results from the spot tests
    - Spot test indicated that the three phages of *S.* Typhimurium have capacity to form completely cleared zones on 25-27 of the 42 strains. However, EOP results indicated that the three phages form lysis plates only on 3 strains.
    - Spot test indicated that *E. coli* phages have the capacity to form completely cleared zones on 19-20 of the 40 strains, but EOP indicated that the two phages do not form phage lysis plates in none strain.

Phage-resistant bacteria tend to be less fit, so they can be expected to be eliminated from the environment fast than their wild-type relatives. Therefore, the evolution of resistance against phage might in this case promote bacterial mutants with reduced ability to cause shellfish disease. These results suggest that the emergence of phage-resistant mutants should not be a major problem to the application of phages to control bacterial infections in aquaculture

- The frequency of phage-resistant mutation was low (10^{-3}-10^{-5} CFU/mL).
- The use of phage cocktails, as well as the use of single-phage suspensions, did not prevent the occurrence of phage-resistant mutants.
- The use of the phage cocktail containing different phages against the same bacterial species can decrease the likelihood of selecting phage-resistant mutants
- The colonies of phage -resistant mutants were smaller than colonies formed by the non-phage added control and were visible only after 5 days of incubation.
- Phage-resistant bacteria did not recover sensitivity, phages did not adsorb to phage-resistant bacteria and after five streak-plating steps on solid medium these bacteria remained resistant by the spot test but not by the EOP technique.
- The spectral changes of *S.* Typhimurium resistant and phage-sensitive cells were compared and revealed relevant differences for peaks associated to amide I (1620 cm^{-1}) and amide II (1515 cm^{-1}) from proteins and from carbohydrates and phosphates region (1080-1000 cm^{-1}).

Precise initial doses may not be essential, due to the self-perpetuating nature of phages.

- The efficiency of phages phT4A and phage cocktail phT4A/EC2A *in vitro* increased in MOI from 1 to 100, but for phage EC2A the efficiency was similar for both MOI.
- *In vivo,* low MOI were efficient to inactivate *E. coli* (MOI of 1) and *S.* Typhimurium (MOI of 0.1).The efficiency of inactivation with phages *E. coli* at a low MOI (MOI of 1, reduction ~2.0 CFU/g) was twice more higher using a high MOI (MOI of 100, reduction ~1.0 CFU/g). The rate inactivation using phSE-2/phSE-5 was 1.7 and 0.6 log CFU/g at MOI 0.1 and 100, respectively, after 4 and 8h treatment.

***In vivo* studies are needed to transpose phage therapy for industry. Results *in vivo* were different from the results obtained *in vitro*.**

- The rate of *E. coli* inactivation, *in vitro,* using phages phT4A, ECA2 and phage cocktail phT4A/ECA2 was higher than the results recorded *in vivo*.
- Unlike *in vivo* assays, in previous *in vitro* experiments, the use of the cocktail phT4A/ECA2 was significantly more effective than the use of single phage suspensions of phT4A and ECA2
- The use of the cocktail phSE-2/phSE-5 *in vitro* assays was more effective than the when used *in vivo* assays.

The use of phage cocktail does not always increase the efficiency of treatment

- The phage cocktail phT4A/EC2A *in vitro* assays was more efficient than the single phage suspensions.

- *In vivo* assays, the phage cocktail was less efficient than the single phage suspensions and needs a longer time to attain a similar level of *E. coli* reduction.
- *In vitro*, the inactivation rate of phage cocktails to control *S.* Typhimurium was similar to that obtained using single phage suspensions
- The inactivation rate of artificially contaminated cockles depurated with the phSE-2/phSE-5 phage cocktail was lower than when phage phSE-5 was used individually. However, when naturally contaminated cockles were depurated with the phSE-2/phSE-5 phage cocktail, the maximum reduction of cultivable bacteria was similar than when the single phage phSE-5 suspension was used.

The efficiency of bacterial inactivation in artificially contaminated cockles was higher than that observed in naturally contaminated cockles

- In general, the rate of inactivation when artificially contaminated cockles were depurated with the phage/ phage cocktail *E. coli* and *S.* Typhimurium was higher than when naturally contaminated cockles were used

The application *E. coli* and *S.* Typhimurium phages can be successfully employed during depuration. Overall, this approach holds the potential to reduce the depuration time and increase the efficiency of this process, consequently holding the potential to reduce production costs and improve bivalve safety and quality for human consumption.

- During depuration mimicking industrial procedures using naturally contaminated cockles, the maximum reduction of cultivable bacteria in cockles was 0.6 log CFU/g after a 2 h of treatment with phage phT4A. However, when depuration occurred without the supply of phages, 4 h of treatment were necessary to obtain the same level of bacterial reduction.

- During depuration mimicking industrial procedures using artificially contaminated cockles, the maximum reduction of *S.* Typhimurium in cockles was achieved after a 6 h treatment (2.0 log CFU/g), however, after 4 h of treatment a bacterial reduction of 0.7 log CFU/g log was already perceptible. When depuration occurred without the supply of phages the reduction was 1.1 log CFU/g after 6 h of treatment.

- Whenever seawater is disinfected with UV-C light, the UV-C light application must be done before phage application because concentration phage was reduced after UV-C irradiation.

7.2 Future work

While successfully used *in vitro* and at laboratory scale, the feasibility of phage therapy at industrial large scale remains to be validated. This aspect will be of paramount importance for its rapid inclusion in depuration systems. The next step will be the evaluation of phage therapy at market scale, by testing it in industrial depuration systems with larger water volumes and higher bivalves stocking densities, employing phage concentrations suitable to control target bacteria, in real-case scenarios. Also, it is necessary to evaluate the efficiency of the isolated phages to inactivate bacteria in other species of bivalves. It is also important to isolate and characterize new phages to control other pathogenic bacteria transmitted to humans by bivalves (e.g. *Vibrio parahaemolyticus*, *Aeromonas hydrophilla*, *Staphylococcus aureus*, *Shigella flexneri* and *Listeria monocytogenes*).

The results of this and previous studies suggest that phage-resistant bacteria tend to be less fit or even lose their virulence properties, so they can be expected to be eliminated from the environment faster than their wild-type relatives and they can have a reduced ability of causing disease in shellfish. Further studies are needed in order to evaluate new phage-bacteria interactions and confirm these findings. It has been stated that phage-resistance development can be overcome by the combined use of two or more phages (phage cocktails) and that the use of phage cocktails has the ability to treat multiple pathogens. This feature broadens the action spectrum of phage therapy. Further studies using phage cocktails, specific for different bacteria, are needed in order to evaluate and optimize their application during bivalve depuration.

Chapter 8. References

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