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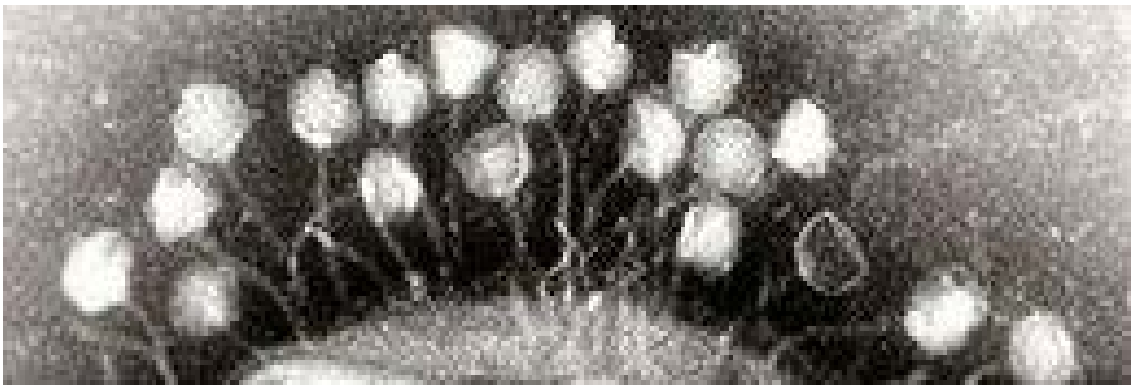

Novel surrogates for viral load: bacteriophages infecting *E. faecalis*. Presence and survival in different matrices

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Abstract

The maintenance of microbiological water quality is of special concern worldwide. There are a number of pathogens contained in polluted water, including faecal bacteria, enteric viruses and pathogenic protozoa. At the turn of years, the faecal water contamination has caused a series of outbreaks. There are various methods used to predict the presence of pathogens in waters. From among proposed faecal contamination indicators there are bacteriophages.

The aim of this work was to further characterize enterophages, a novel group of phages infecting *E. faecalis*, as faecal viral indicators, by means of their occurrence in untreated and treated wastewater, ability to replicate at different temperatures, stability in different types of water and genetic material composition. Moreover, the comparison of Puerto Rican *E. faecalis* host strain against Portuguese strains was performed, in order to test the potential universality of Porto Rican strain. The enterophages were detected and isolated using single layer and double layer methods. In order to analyse their genetic material, the gel electrophoresis was applied.

The method for enterophage recovering has been found to be simple and not time-consuming. The detection and enumeration of enterophages in untreated wastewaters revealed their prevalence in raw sewages. The comparison of enterophages' concentration between touristic and non-touristic sites proved a significant increase of enterophage number in touristic locations during warm-month period, due to the influx of tourists to holiday resorts. The test for ability of enterophages to replicate at different temperatures proved that they may replicate at 37 °C and 41 °C. The low detection of enterophages in treated wastewater might signify their substantial sensitivity to water treatment. The analysis on stability of enterophages in distilled, tap and wastewater revealed that enterophages have the highest survivability in wastewater. The nucleic acid analysis indicated that enterophages are composed of double-stranded DNA. Furthermore, the test for global applicability of Puerto Rican *E. faecalis* strain demonstrated the possible universality of this host strain for recovering of enterophages.

Keywords: Enterophages, bacteriophages, Microbial Source Tracking, water quality.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

AFLP- amplified fragment length polymorphism

ARA- antibiotic resistance analysis

$CaCl_2$- calcium chloride

cDNA- complementary DNA

CFU- colony forming unit

C. jejuni/coli- Campylobacter jejuni/coli

CNS- Central Nervous System

CUP- carbon-source utilization profiling

CVA- coxsackie A virus

CVB- coxsackie B virus

DNA- deoxyribonucleic acid

dsDNA- double-stranded deoxyribonucleic acid

DGGE- denaturing- gradient gel electrophoresis

DWD- Drinking Water Directive

E1- sample containing effluents

EEC- European Economic Community

EC- European Commission

E. coli- Escherichia coli

E. coli O157:H7- enterohemorrhagic strain of the bacterium Escherichia coli

EEC- European Economic Community

E. faecalis (E. faecium, E. durans, E. hirae, E. gallinarum)- Enterococcus faecalis (faecium, durans, hirae, gallinarum)

ELISA- enzyme-linked immunosorbent assay

EU- European Union

FAME- fatty acid methyl ester

Feb1- sample collected in February

FIO- faecal indicator organism

GI-GV- genogroups I-V

G. box- gel imaging for fluorescence applications

GI- gastrointestinal tract

h- hour

H. pylori- Helicobacter pylori

HAV- Hepatitis A virus

HEV- Hepatitis E virus

HUS- haemolytic ureamic syndrome

ISO- International Organization for Standardisation

Jul1- samle collected in July

Jun1- sample collected in June

Kb- kilobase

k_d- decay constant

LAIST_ENT_001- name of *E. faecalis* isolated from Portuguese wastewater

LEDC- Less Economically Developed Countries

LD- library-dependent

LI- library-independent

Mar1- sample collected in March

min- minutes

mm- millimetre (10^{-3} m)

mg- milligram (10^{-3} g)

mL- millilitre

MST- Microbial Source Tracking

N^o – number

NaN_3- sodium azide

nm- nanometre (10^{-9} m)

NLV- Norwalk-like virus

NoV- Norovirus

PBS- Phosphorus Buffer Solution

PFGE- pulse-field gel electrophoresis

PFP- filamentous, polyhedral and pleomorphic shapes

PFU- plaque forming unit

PR- Porto Rican strain of *E. faecalis*

PV- polioviruses

QPCR- quantitative PCR

RAPD- Random Amplified Polymorphic DNA

Rep- PCR- repetitive polymerase chain reaction

RNA- Ribonucleic acid

rRNA- ribosomal RNA

rpm- rotations per minute

SARS- Severe Acute Respiratory Syndrome

Spp.- species

STIb, LTIIa, STII- *E. coli* toxin genes

T_{90}- time of 90% decrease in PFU concentrations

TSA- Trypticase Soy Agar

TAE buffer- Tris-acetate-EDTA buffer

T-RFLP- Terminal Restriction Fragment Length Polymorphisms analysis

TSB- Tryptic Soy Broth

URT- upper respiratory tract

USA- United States of America

UV- ultraviolet light

WHO- World Health Organisation

WFD- Water Framework Directive

WWTP- Wastewater Treatment Plant

V- Voltage

vs.- versus

µL- microliter (10^{-6} L)

µm- micrometre (10^{-6} m)

°C- Celsius degree

%- percentage

INTRODUCTION

In recent years, an expansive urbanization and increase in human population have resulted in gradual deterioration of water quality. The poor quality of water is a serious problem worldwide. More than a billion people has no access to safe drinking water and millions die each year, suffering numerous waterborne infections after bathing in contaminated recreational waters. The natural aquatic ecosystems become microbiologically polluted through discharges of effluents from wastewater treatment plants (WWTPs), agricultural soil leaching as well as surface runoff, containing pathogenic organisms especially of faecal origin [1, 2, 3, 4, 5, 6, 7, 8].

Water is a natural resource that functions as an excellent carrier of numerous pathogens, such as faecal bacteria, enteric human viruses and pathogenic protozoa [5, 9]. The faecal water contamination can cause a series of diseases, starting with eye and throat infections, through skin irritations, and finally it may be a reason of serious gastrointestinal (GI) illnesses [2, 10, 11, 12]. The number and type of pathogens in aquatic systems varies with distinct populations [9].

In order to maintain water quality, the numerous microbiological standards have been established. Throughout the world most of the countries have set up certain norms, concerning water treatment and its final quality, on the basis of World Health Organisation's standards *2, 8, 10, 12, 13]. Within European Union there are four principal directives, enacted to manage the water policy within Member States, namely: the Urban Waste Water Treatment Directive (91/271/EEC) of 21 May 1991 [14], the Drinking Water Directive (98/83/EC) of 3 November 1998 [15, 16], the New Bathing Water Directive (2006/7/EC) [17, 18, 19] and Water Framework Directive (2000/60/EC) of 23 October 2000 [20]. The microbiological monitoring of waters, destined to public use in EU countries, basically relies on the concept of microbiological indicators [13, 16, 17, 20].

Monitoring of traditional faecal indicators, such as total or faecal coliforms, enterococci and *E. coli* solely indicates whether the body of water is impacted by faecal contamination. It does not provide any information on the source of such pollution, whereas this knowledge may help local communities to restore water quality and reduce the risk of disease outbreaks. Therefore, Microbial Source Tracking (MST) approach has been spawned, which may not only assess water quality more accurately but also determines the source of contamination in water environment [21, 22, 23]. The approach is based on the assumption that there are certain characteristics unique to the faecal microorganisms from specific hosts that may help to identify the source of faecal contamination [23, 24, 25]. MST may discriminate the sources in broad fashion, like human vs. nonhuman sources; however group comparisons (humans vs. livestock vs. wildlife), species specific results (humans vs. cows vs. pigs etc.) as well as species individual hosts (cows from certain farm vs. other farms etc.) can

be also performed [23]. Currently, all of MST methods possess several drawbacks, and there is no ideal MST technique that may be suggested as a standard for source tracking [21, 22, 23]. Majority of traditional MST applications require cultivation of target organisms and/or reference library. The effectiveness of all library-based methods strictly depends on size and composition of library. The database needs to be geographically and temporally specific. The genotypic characterization, which employs PCR usually require training of personnel and are labour-intensive. Another important aspect may be unavailability of primers for all relevant hosts [23].

On the other hand, in order to exhibit the decay of enteric viruses after wastewater treatment bacteriophages have been studied. From among most studied there are somatic coliphages, F^{+}RNA coliphages, bacteriophages infecting *Bacteroides fragilis.* However, the results on their resistance to water treatment are not consensual, providing different information, which bacteriophage is superior to the others [31, 32, 33].

Recently, the enterophages have been detected and proposed as markers of human faecal contamination. They are novel group of phages that specifically infect *Enterococcus spp.* Enterophages may be present in untreated and treated wastewaters and possess relatively narrow range of hosts [5, 27, 28+. There are found to replicate at 22°C, 37°C and 41°C found; however, there is still unknown how many groups of these phages exist [5, 28]. The majority of phages infecting *E. faecalis* are double- stranded DNA tailed phages, corresponding to *Siphoviridae* family [5, 27]. The preliminary studies on enterophages have revealed, that their occurrence does not restrict to particular geographical areas, thus they can be tested as faecal indicators in different types of water [5]. The absence of enterophages in cattle faeces may suggest that they are specific to human faecal contamination and could be used for indication of human enteric viruses. The enterophage resistance to removal treatment proved to be higher than resistance of other bacteriophages, such as coliphages. Moreover, it has reported been reported in literature that enterophages have similar die-off rates in fresh and marine waters to enteric viruses [5]. The latter characteristics may imply the potential usefulness of enterophages as surrogates for enteric viruses.

The aim of this study was to further characterize enterophages as faecal viral indicators by means of their prevalence in different water matrices, ability to replicate at specific temperatures, stability in different types of water as well as their genetic material composition. Moreover, the comparison between different strains of *E. faecalis* was performed, in order to assess the potential universality of Porto Rican for recovery of enterophages.

The prevalence of enterophages was determined in untreated and treated wastewaters received from wastewater treatment plants (WWTPs) from locations, receiving high numbers of tourists during warm months and from locations with low input from tourists in Portugal. The comparison of enterophages' concentrations in samples belonging to touristic and non-touristic

sites in accordance to cold- and warm-month periods was performed. In order to analyse the enterophage ability to grow at different temperatures, the microorganisms were replicated at 37°C and 41°C. The stability of enterophages was tested in distilled, tap and sewage water and the time of 90% reduction in their concentration was determined. The analysis of enterophage genetic material composition was done, in order to define if they are composed of DNA or RNA. Finally, the applicability of different *E. Faecalis* host strains for enterophage recovery was examined through comparison of Porto Rican host strain and six *E. Faecalis* isolates received from Portuguese wastewaters.

2. Microbiological water quality

2.1. Importance and control of water quality

The maintenance of microbiological water quality is of special concern. This include waters used for various purposes, namely water intended for drinking and used in food preparation, treated recreational waters as swimming pools, as well as untreated waters used for recreation like sea, river and lake water [1, 2]. In natural aquatic systems the microbial quality of water is affected by various pathogens, including faecal bacteria, viruses and pathogenic protozoa [1, 3, 4, 5]. The sources of faecal contamination could be a point discharge of effluents from wastewater treatment plants, as well as non-point sources such as leaching of soil and, particularly in rural areas, the manure runoff [3, 4, 6, 29, 30].

In the course of years, the epidemiologists and microbiologists struggle with the faecal pollution problem, in order to protect public from number of outbreaks due to consumption of infected water and bathing in contaminated recreational waters. The problem afflicts especially Less Economically Developed Countries (LEDC) [3, 7]. The World Health Organisation (WHO) estimates that more than a billion people have no access to safe drinking water, and more than two million, principally children, die each year suffering infectious waterborne diseases. The acute diarrhoea is one of the most frequent causes of morbidity and mortality in Third World [3, 7, 10, 30].

The problem of faecal water contamination could be eliminated or at least reduced through the adoption of appropriate water quality practises, in particular, disinfection practises during potable water production and treatment of sewages [3, 9, 31, 32, 33], but also through intensified control of surface water quality [9]. However, especially in developing countries, the wastewater before discharged to the aquatic system still only goes through partial or even no disinfection [9]. Traditionally, the assessment of water quality is being performed through the analyses of faecal indicator organisms (FIOs), which are supposed to reveal the potential presence of pathogens in waters [2, 3, 34, 35]. Nevertheless, the scientists frequently have faced cases, in which water outbreaks occurred due to the presence of pathogens regardless of presence or absence of faecal indicators [1, 3].

The number of outbreaks has been reported worldwide. The epidemics had different courses, from mild, through moderate to substantial [2]. The most recent serious outbreak, reported in the literature, took place between October and November 2010 in South China. An acute gastroenteritis infection occurred due to the consumption of water contaminated with Norovirus, from both the delivery point and the terminal point of the pipe network of local waterworks [36]. The Norovirus was also a contributor to the large outbreak of acute gastroenteritis among Dutch

scouts in Belgium in 2007. The water for drinking, washing and cleaning purposes came from nearby contaminated farmer's well *37].

In developing countries, where populations live under poor sanitary conditions, Hepatitis E virus (HEV) caused serious outbreaks. The endemics have been reported Central, South and Southeast Asian countries, in Africa, Middle East, Mexico. Moreover, for a number of years, India suffered with infection of this pathogen as well. The most recent outbreak in India took place in 2004 in Baripada [38].

However, not only LEDCs have being afflicted with waterborne outbreaks. The other large outbreak of gastroenteritis occurred in Switzerland in August 1998. More than 50% of the 3500 inhabitants in Swiss village suffered the infection with the strains of Norwalk-like viruses (NLVs). The drinking water was highly contaminated with enteric viruses due to the failure of pumping system in local wastewater treatment plant [39]. In the period 1999-2006, 413 outbreaks were recorded in Spain. They took place irrespective of compliance with legislation and conducted microbiological controls. However, in USA there have being 19 million diseases, related to consumption of contaminated water, estimated each year [2].

The viral outbreaks are not the sole that affect the populations. Numerous bacterial and parasitic protozoa epidemics have been reported as well. In 1993 in Milwaukee (USA) the *Cryptosporidium* parasitic protozoan outbreak affected more than 400,000 people. On the other hand, in Europe there were 106 outbreaks documented, in particular of *Giardia duodenalis* and *Cryptosporidium parvum protozoa* origin [3].

Certain epidemiological evaluations also revealed the outbreaks of a mixed aetiology. In 2000 in Walkerton, Ontario (Canada) two pathogens, namely *Cryptosporidium* and *E.coli O157:H7* affected over 2,300 persons [3, 10]. Moreover, in South Bass Island, Ohio (USA) in 2004 a massive microbiological contamination occurred. The ground water used for drinking purposed was faecally polluted with total coliforms, *E. coli*, enterococci, *Campylobacter*, *Arcobacter*, coliphages and adenoviruses, resulting in 1,450 cases of gastroenteritis. In Europe in 2007 eight countries reported 17 waterborne outbreaks that involved 10,912 cases, where the leading microorganisms were *Campylobacter*, norovirus, *Giardia* and *Cyptosporidium.* The biggest epidemics of multiple aetiologies occurred in Denmark (453 cases), Finland (8,000 cases) and Slovenia [3].

The numerous outbreaks taking place each year and the demand for safe drinking water generated high societal and epidemiological alarm. The WHO has being highly engaged in this field. The preventive approach with important guidelines of universal application has been developed in order to monitor the quality of all water types [2, 3, 10]. Recently, bacteriophages have been studied to exhibit the decay of enteric viruses after wastewater treatment. From among most studied there are somatic coliphages, F^{+}RNA coliphages, bacteriophages infecting *Bacteroides fragilis.*

However, the results on their resistance to water treatment are not consensual, providing different information, which bacteriophage is superior to the others [31, 32, 33].

2.2. Pathogens and diseases

Most of the microorganisms present in fresh and marine water are of no concern for human health; however, some of them contribute to hazardous health outcomes. Depending on their characteristics, pathogens may cause asymptomatic or mild poisoning, and those transmitted through faecal-oral route, such as enteric viruses, cause serious diseases, such as hepatitis and meningitis [2, 40]. However, water is not a natural habitat of pathogens. It is solely a carrier of morbific bacteria and viruses, which are introduced into aquatic system directly from infected humans or animals as well as indirectly through discharges of raw or insufficiently treated sewage and surface runoff of animal manure [2, 9, 10, 11, 12].

The epidemiological studies have revealed that contaminated water can be a reason of serious gastrointestinal (GI) diseases, eye, ear, nose and throat infections, skin irritations, as well as respiratory system illnesses [2, 10, 11, 12]. These morbidities can be orally transmitted through drinking untreated or contaminated water, but also due to bathing and other recreational activities in waters containing excrements [2, 9, 10, 12]. The adverse health outcomes have been found to be higher in swimmers comparing to non-swimmers [11, 12]. The microbial generic spectrum in contaminated water depends on state of health of specific watershed's inhabitants, whereas the number of pathogens mostly varies with the population [9]. Additionally, the relative risks from exposure to waters contaminated by waterfowl, poultry or livestock excretes may be different from those associated with human sewage-impacted waters [11].

Pathogenic bacteria and protozoa

Despite the fact, that majority of illnesses caused by bacteria contained in polluted water are relatively mild, there are certain bacterial and protozoa pathogens, which cause severe health risks to humans, among others *Escherichia spp.*, *Campylobacter spp., Mycobacterium spp., Salmonella spp.*, *Leptospira interrogans, Shigella spp., Helicobacter pylori, Vibrio Cholerae;* and pathogenic protozoa such as *Entamoeba histolytica, Giardia Lamblia, Cryptosporidium parvum* [9, 12, 28, 30]. Most of them have being known for many years; however others, such as *Helicobacter pylori*, are emerging as new pathogens. The poisoning caused by *H. pylori* leads to gastroenteritis, anaemia as well as more serious stomach ulcers and gastric cancer [9, 10, 12, 41]. *Escherichia spp*., although belong to alimentary canals of humans and are not considered to be pathogenic, contain several

strains like *E. coli O157:H7* bacillus, which when introduced to human body evokes diarrhoea [9, 12, 42]. Bacteria that belong to *Campylobacter spp*., in particular *C. jejuni* and *C. coli*, are the major factor of gastroenteritis diseases and sequelae. On the other hand, *Salmonella spp.* cause water outbreaks of epidemics as acute gastro-intestinal disorders, wheras *Shigella spp.* induce shigellosis, which is also acute infectious disease [9, 42].

Viruses

From among viruses found in wastewaters there are human Enteroviruses (Polioviruses [PV], Coxsackieviruses A and B [CVA, CVB] and Echoviruses), human Adenoviruses, Caliciviruses (Norovirus, human Calicivirus, Rhinoviruses and Hepatitis E [HEV]), human Astrovirus, human Parvovirus, human Coronaviruses, Hepatitis A [HAV] and Rotaviruses [10, 12, 30, 43, 43]. The enterovirus infection starts in mucosal tissue in gastrointestinal tract and usually remains in this site. Nevertheless, the replication of viruses leads to viraemia, causing dissemination of infection to other organs, such as heart, Central Nervous System (CNS) and skin [43, 44]. Adenoviruses are the group of DNA viruses, highly resistant to chemical and physical agents, including tertiary or UV radiation wastewater treatment. The course of diseases is usually mild; however, some fatal cases have been reported in the literature. The transmission of adenovirus may occur through swimming in faecally- polluted recreational waters but also through droplets [3, 12]. Noroviruses (NoVs) are predominant infectious agents of non-bacterial acute gastroenteritis worldwide [46, 47, 48]. There are five genogroups of NoVs discovered (GI- GV) and those, which infect humans belong to GI, GII and GIV genogroups [47, 48]. NoVs have been found to be highly resistant to inactivation processes; therefore they may be detected in treated wastewaters and surface waters [23]. Besides the above mentioned viruses, the polyomaviruses and novel emerging enteric viruses such SARS coronavirus, human parechovirus and zoonotic influenza viruses deserve special considerations [3, 42, 40].

The review of the most prevalent pathogens with related diseases and main symptoms are presented in the Table 1 [2, 12, 13, 30, 41].

Table 1. Prevalent pathogens with related diseases and main symptoms [2, 12, 13, 30, 41]

Pathogen	Primary symptoms	Disease
Bacterial		
Campylobacter spp.	Diarrhoea, abdominal pain, fever, malaise	Enteritis, meningitis, carditis
Escherichia spp.	Severe bloody diarrhoea, abdominal cramps	Gastroenteritis, haemolytic-uremic syndrome (HUS)
Helicobacter pylori	Nausea, abdominal pain, hypochlorhydria, gastritis	Gastroenteritis, ulcers, anaemia, gastric cancer
Leptospira Interrogans	High fever, severe headache, chills, muscle aches, vomiting, diarrhoea, abdominal pain	Leptospirosis
Mycobacterium spp.	Cough, fatigue, sweats, weight loss, haemoptysis, sputum production	Respiratory and gastrointestinal tract infections
Salmonella spp.	Fever, diarrhoea, malaise, abdominal pain, delirium	Enteric gastroenteritis, typhoid
Shigella spp.	Severe abdominal pain, diarrhoea, bloody stools	Bacillary dysentery
Viral		
Enteroviruses	Fever, headache, myalgia, nausea, abdominal pain	Acute gastroenteritis, herpangina, acute haemorrhagic conjunctivitis, paralysis, aseptic meningitis, CNS disease
Adenoviruses	Fever, upper respiratory tract (URT) symptoms, conjunctivitis	Cystitis, nephritis, pneumonia, inflammation of URT
Noroviruses	Fever, diarrhoea, explosive vomiting	Acute gastroenteritis

2.3. European Union's standards

The European Union has enacted four principal directives in order to manage the water policy within member countries, namely:

- The Urban Waste Water Treatment Directive (91/271/EEC) of 21 May 1991 concerning the discharges of municipal and some industrial wastewaters [14];
- The Drinking Water Directive (98/83/EC) of 3 November 1998 relating to potable water quality [15, 16];
- The New Bathing Water Directive (2006/7/EC) concerning the healthiness of recreational waters [17, 18, 19];
- Water Framework Directive (2000/60/EC) of 23 October 2000 regarding water resources management [20].

The Urban Waste Water Treatment Directive regulates the collection, treatment and discharge of wastewater from both domestic sources and certain industrial sectors. Its main objective is to protect the environment and humans from any adverse effects caused by discharge of urban wastewaters [14]. According to the Directive, Member States ensure that collected urban wastewater is appropriately treated, as follows:

- for discharges to fresh-water and estuaries from agglomerations populated less than 2000 inhabitants;
- for discharges to coastal waters from agglomerations populated less than 10000 inhabitants [14].

The Drinking Water Directive (DWD) assures that Member States provide their customers with clean and tasty water, which does not contain any concentration of microorganisms, parasites and any other substances that may constitute the potential risk for human health. The tap water has to meet the minimum microbiological requirements established by the Directive, while the Member States are obliged to guarantee the healthiness and purity of water that is intended for consumption, by regular monitoring of the water quality using the methods specified in the Directive. The microbiological monitoring basically relies on indicator concept that is the absence of *E.coli,* enterococci and *Pseudomonas aeruginosa* in tap water [16]. Currently, the DWD provides high level of drinking water safety throughout its Member States [15, 16]. However, in several Member States the outbreaks through drinking of the water that fulfilled *E. coli* standards has revealed certain shortcomings for this bacterial indicator as traditional approach for microbiological security. For this reason, the future development project of EU Drinking Water Directive assumes the establishment of new microbiological parameters [16].

The New Bathing Water Directive (2006/7/EC) was adopted in 2006 to protect public health from the risk of accidental and chronic diseases caused by human pathogens present in contaminated water. The procedure for monitoring of recreational waters established by new directive gives more reliable results than the assessment under the first European Bathing Water Directive from 1976. It induces the Member States to minimise the health risks to bathers, basing on appropriate determination of contamination origin. Regarding the viruses, the directive induces scientists to search for reference parameters and appropriate detection methods [13, 18, 19]. The new directive reduces the list of 19 pollutants that need to be monitored to just two microbial indicators of faecal contamination with *E. coli* and intestinal enterococci [13, 17, 20]. The new EU standards for those indicators are presented in Table 2.

Table 2. European Union's microbiological criteria for recreational waters [13, 17, 20]

Inland waters					
	Parameter	Excellent quality	Good quality	Sufficient quality	Reference methods of analysis
1	Intestinal enterococci (CFU/100ml)	200*	400*	330**	ISO 7899-1 or ISO 7899-2
2	Escherichia coli (CFU/100ml)	500*	1000*	900**	ISO 9308-3 or ISO 9308-1
Coastal and transitional waters					
1	Intestinal enterococci (CFU/100ml)	100*	200*	185**	ISO 7899-1 or ISO 7899-2
2	Escherichia coli (CFU/100ml)	250*	500*	500**	ISO 9308-3 or ISO 9308-1

(*) Based on 95-percentile estimation.
(**) Based on 90-percentile estimation.

The EU Water Framework Directive (WFD) was established in response to the increasing pollution and increasing demand for clean rivers, lakes and beaches throughout Member States. It provides the framework for protection and improvement of a quality of all types of waters, among others rivers, lakes, estuaries, coastal waters, groundwater as well as their natural habitat. It assumes that good status for all waters should be achieved by December 2015 [20].

Throughout the European Union, all Member States are legally obliged to conform to the standards established in several Directives. They have to ensure the minimum quality standards of various types of water, starting with drinking water purity, through recreational water healthiness, inclusive of urban waste water treatment standards. Each country has to monitor the quality of waters using standardized methods, reduce the pollution of water bodies and protect against further deterioration. The important aim is to prevent morbidity from enteric [14, 16, 17, 18, 19, 20].

3. Microbial Source Tracking

Traditionally, the quality of water has been estimated by cultivation and enumeration of faecal indicator bacteria, such as total or faecal coliforms, enterococci and *E. coli*. Monitoring of these microorganisms could indicate whether the body of water is impacted by faecal contamination or not, and aid to protect public health. However, it is known that traditional approaches do not identify the source of faecal pollution, whereas this knowledge may help local communities to restore water quality and reduce the risk of disease outbreaks.

Therefore, a Microbial Source Tracking (MST) approach has been spawned, which may not only assess water quality more accurately but also determines the source of contamination in water environment [21, 22, 23]. MST is based on the assumption that there are characteristics unique to the faecal microorganisms from specific hosts and, with the help of these characteristics, the source of faecal microbial contamination can be identified [23, 24, 25]. The possible source discriminations are:

- Broad fashion discrimination (human vs. nonhuman sources),
- Species specific results (humans vs. cows vs. pigs etc.),
- Group comparisons (humans vs. livestock vs. wildlife),
- Species individual hosts (cows from certain farm vs. other farms etc.) [23].

There are various methods that can be used to seek for the origin of contamination in water. The typical MST applications employ genotypic (molecular) or phenotypic (non-molecular) characterization of microorganisms from water bodies. Genotypic methods rely on certain aspects of organism DNA sequence, while phenotypic analyses measures specific feature that is expressed [23, 24, 25]. The methods are further divided into library-dependent (LI) and library-independent (LI), from which some require cultivation of target organisms and the others are culture-independent [23, 24, 49]. Despite the fact, that no single method that is undoubtedly superior to another has been found yet, new MST methods are still being developed [23, 24].

3.1. Library-dependent, culture-based methods

The LD methods are based on a host-origin database of isolates from known faecal sources, so called library. The microorganisms isolated from unknown sources are analysed in order to provide a set of 'fingerprint' patterns that are further compared with the fingerprints of the library. The effectiveness of all library-based methods strictly depends on the size and composition of library.

The database needs to be representative of most of the faecal sources in water bodies without distinction of geographic areas. Moreover, it should be stable over time so that there is no need to continually create new libraries [23, 49, 50]. The library-dependent methods include both phenotypic and genotypic tests and are culture-based [23, 49].

Phenotypic characterization

☐ Antibiotic resistance analysis (ARA)

It is one of the most common tools applied in MST field. It is well recognized that bacteria from different origins (human or animal) are exposed to different antibiotics, therefore presenting distinct susceptibilities. The method is based on patterns of susceptibility of bacteria to a range of therapeutic agents. The environmental isolates are grown in variety of antibiotics with different concentrations and consequently compared to the established library [23, 24, 45]. The size and composition of the library is an important criterion. The library should represent the diversity of patterns of antibiotic susceptibility found in water environment [23, 45, 50]. Human faecal bacteria are supposed to be more susceptible to antibiotics other than those from livestock or wildlife sources [23, 24]. ARA employs two types of analysis, the sample-level and isolate-level analyses. The sample-level analysis is used when single major source is taken into account; however the isolate-level one is more appropriate to examine the samples contaminated by more sources [24].

☐ Carbon-source utilization profiling (CUP)

The CUP technique, also called biochemical or phenotypic fingerprinting, relies on the utilization of different carbon and nitrogen substrates by bacteria, conducting to a high range of phenotypic diversity. The consumption of substrates can be followed using a colorimetric reaction and measured by an ELISA [21, 22, 23].

☐ Fatty acid methyl ester (FAME) profiling

The FAME profiling relies on testing of bacterial membrane fatty acids. The technique is still at the proof-of-concept and biological likelihood level of testing, and more research is needed to detect the specific mechanisms that cause the host specificity. However, latter studies have found that FAME profiles may show both qualitative (presence of FAME distinctive feature) and quantitative (variation in mass of FAMEs) host-specific differences [21, 22, 49].

Genotypic characterization

☐ Repetitive DNA sequences (rep-PCR)

The rep-PCR technique differentiates between sources of pollution using repetitive intergenic DNA sequences. In order to produce DNA fragments of various sizes, the DNA between adjoining

repetitive extragenic elements is amplified using polymerase chain reaction (PCR) and primers. The resulting mixture of amplified DNA fragments is subsequently size-fractioned by agarose-gel electrophoresis. The fingerprints obtained are analysed through recognition of patterns and their comparison with the library [21, 22, 23, 24, 51]. Bacteria, which have identical fingerprints, are considered as being the same strain, while those possessing similar patterns are considered as being genetically related [23, 45].

☐ Random Amplified Polymorphic DNA (RAPD)

The RAPD technique, unlike the traditional PCR analysis, does not require any knowledge of specific DNA sequence of target organism, since the segments of DNA that are amplified are random. The assumption is that PCR performed with non-selective primers at high stringency produce a series of strain specific PCR products, depending on primer and template used. After separation on agarose gels and stained with ethidium bromide, these PCR products produce a series of species- or strain-specific bands that are used as fingerprint of the bacterial genome [23].

☐ Amplified Fragment Length Polymorphism (AFLP)

The AFLP analysis is sensitive fingerprinting technique, which applies combination of DNA digestion with restriction enzymes and PCR amplification. In the first place, the restriction enzymes digest genomic DNA. Afterwards, the short specific adaptors are ligated to digested fragment ends to provide sufficient length of known sequence for primers to be used for PCR. In order to prevent amplification of all the digested fragments, additional PCR primers are employed for the second round of PCR. They differ from the initial primers in supplementary 1-3 nucleotide bases, thus amplification occurs just in a subset of initial fragments. The supplementary nucleotides increase specificity and decreases the number of PCR final products. The accurate sampling of entire genome depends on sufficient number of primers used. Currently, there is no standard set of primers to be used for MST for any bacterial species [23].

☐ Pulse-field gel electrophoresis (PFGE)

The PFGE method is most common genotyping method in epidemiological investigations. It applies direct analysis of microbial genome and PCR is not performed. The digestion of total DNA genome by rare-cutting restriction enzyme results in production of large fragments, which cannot be separated in standard agarose gel electrophoresis unit, since the gel pore size restricts their migration. Therefore, the large fragments of DNA genome are divided through exposure to alternatively pulsed, perpendicularly oriented electrical fields. The determination of fragment sizes is performed by comparison to molecules of known size [21, 22, 23, 24].

☐ Ribotyping

Ribotyping, also called 'molecular fingerprinting', is based on identification of microorganisms through genetic differences in genomic sequences the analysis of DNA fragments created from restriction enzyme digestion. The genes coding for the ribosomal ribonucleic acid (rRNA) tend to be highly conserved in microorganisms. The DNA of cultured faecal isolate is extracted and digested with restriction enzymes. Afterwards, it is subjected to gel electrophoresis, transferred to a membrane and hybridized with labelled rRNA probes. The ribotyping analysis uses oligonucleotide probes for detection of rRNA sequences, generating fingerprints for microbial isolates [21, 22, 23, 24, 45, 51].

3.2. Library-independent, culture-based methods

The LI culture-based methods rely on presence or absence of target organism or gene in the sample, thus they do not require source library. When the target for MST analysis is in low number, it is necessary to primarily enrich the sample or obtain isolates.

☐ F^+RNA coliphage typing

F^+ RNA coliphages distinguishes human and animal faecal contamination by typing isolates into one of four subgroups [23, 26, 45]. Groups II and III are highly associated with human faecal contamination, group IV coliphages are generally detected in faeces of animal and livestock origin, whereas group I coliphages were found to be present in both human and animal faeces in wastewater [23, 45, 46, 51].

The method may apply sero- or genotyping for typing of F^+ RNA coliphages [42, 51]. Serotyping uses group-specific antisera to test virus infectivity, while genotyping is based on hybridization of group specific oligonucleotides. The coliphage typing is library independent method, but can only broadly distinguish human and nonhuman faecal contamination [42].

Genotypic characterization

☐ Gene specific PCR

The gene specific PCR is based on knowledge that certain enterotoxin genes are belonged solely to *E. coli,* which infects species of warm-blooded mammals. It has been discovered that STIb gene, LTIIa gene and STII gene are carried out only by *E. coli* of human, bovine and swine origin, respectively. Enterococci virulence genes have been also used as targets for host specific markers. This method is still in developmental stage; however it indicates potential for future [21, 22, 23, 45].

3.1.3. Library-independent, culture-independent methods

Cultivation-independent methods are principally based on nucleic acid analyses. They employ a genetic marker from DNA extracted from water sample, without any culturing procedure. The specific markers are assayed with or without PCR amplification. The great advantage of this approach is the quickness of the process and possibility to determine novel markers, which would be difficult or even impossible with use of growth assays [21, 23].

Genotypic characterization

- ☐ Community fingerprinting

The fingerprinting is often used to monitor changes in community or to compare different communities. The main principle for fingerprinting analyses is that differences in banding patterns result from differences in microbial species comprising certain community [23]. The methods examine DNA size or conformation profiles obtained from microbial community. After amplification of rRNA genes or randomly separated DNA fragments, the amplicons can be separated by denaturing-gradient gel electrophoresis (DGGE). It separates genes of similar size but different nucleotide composition [21, 22, 24]. The DNA denaturant 'melts' a double-stranded DNA fragment moving across the gel. The differences in base sequences are controlled by differences in melting properties. Additionally, amplicons can be separated by size before restriction enzyme digestion (LH-RFLP) or after restriction enzyme digestion (T-RFLP). The method gives a number of phylotypes (different 16S rRNA genes) that are present in water sample [22, 24]. Another method for DNA separation by size is Terminal Restriction Fragment Length Polymorphisms analysis (T-RFLP) [21, 22, 23].

- ☐ Host- specific markers

The specific bacteria identification and quantification in samples by cultivation independent methods is based on information obtained from clone libraries or sequencing genes from cultivated organisms. The methods can be divided into PCR-based or direct probing, while the latter does not require PCR [23]. From among direct probing methods one can distinguish hybridization using oligonucleotide sequences for hybridizing with target DNA sequences. The PCR-based methods use traditional PCR with some variations. One of those can be simultaneous detection of several DNA targets, so called multiplex PCR, which increases sensitivity of overall detection by application of two amplification steps and quantifying the initial template by quantitative PCR (QPCR) [23]. From among bacterial markers there are *Bacteroides, Bifidobacterium, Rhodococcus coprophilus* and *Streptococcus* Lancefield Group D (*Streptococcus* and *Enterococcus*) [23].

☐ Virus specific markers

The viral techniques assume identification of enteric viruses with limited host ranges, such as human-specific adenoviruses and enteroviruses for human faecal contamination, bovine enteroviruses and porcine adenoviruses for animal- source faecal pollution, and teschoviruses for porcine faecal contamination. The viral methods performed for investigation of human sewages have given proper results. The direct detection of viral pathogens may give more information on pathogens presence than that provided by bacterial indicators. Nevertheless, the detection of human viruses requires large water samples and the concentration procedure [40, 41, 42].

The Table 3 presents the review of advantages and disadvantages of methods applied in Microbial Source Tracking [23, 51].

Table 3. Review of advantages and disadvantages of MST methods [23, 51]

Method	Advantages	Disadvantages
ARA	- Rapid and easy to perform - Requires limited training - May be useful to differentiate host source	- Requires reference library - Requires cultivation of target organism - Libraries geographically and/or temporally specific - Variations in methods used in different studies
CUP	- Rapid and easy to perform - Requires limited training	- Requires reference library - Requires cultivation of target organism - Libraries geographically and/or temporally specific - Variations in methods used in different studies - Results often inconsistent
rep-PCR	- Highly reproducible - Rapid and easy to perform - Requires limited training - May be useful to differentiate host source	- Requires reference library - Requires cultivation of target organism - Libraries geographically and/or temporally specific
RAPD	- Rapid and easy to perform - May be useful to differentiate host source	- Requires reference library - Requires cultivation of target organism - Libraries geographically and/or temporally specific - Has not been used extensively for source tracking
AFLP	- Highly reproducible - May be useful to differentiate host source	- Requires reference library - Requires cultivation of target organism - Requires specialized training of personnel - Labour-intensive - Libraries geographically and/or temporally specific - Variations in methods used in different studies

Table 3 Continued. Review of advantages and disadvantages of MST methods [23, 51]

PFGE	- Highly reproducible - May be useful to differentiate host source	- Requires reference library - Requires cultivation of target organism - Requires specialized training of personnel - Labour-intensive - Libraries geographically and/or temporally specific
Ribotyping	- Highly reproducible - May be useful to differentiate host source - Can be automated	- Requires reference library - Requires cultivation of target organism - Requires specialized training of personnel - Labour-intensive (unless automated system used) - Libraries geographically and/or temporally specific
F+ RNA coliphage	- Distinguishes human from animals - Subtypes are stable characteristics - Easy to perform - Does not require a reference library	- Requires cultivation of coliphages - Subtypes do not exhibit absolute host specificity - Low in numbers in some environments
Gene specific PCR	- Can be adapted to quantify gene copy number - Virulence genes may be targeted, providing direct evidence that potentially harmful organisms are present - Does not require reference library	- Require enrichment of target organism - Sufficient quantity of target genes may not be available requiring enrichment or large quantity of sample - Requires training of personnel - Primers currently not available for all relevant hosts
Community fingerprinting	- Does not require cultivation of target organism - Rapid and easy to perform - Relatively inexpensive - Does not require reference library - Host specific	- Portion of community that can be linked to host specificity may be very small compared to indigenous microbial community - Has not been widely used for MST
Host-specific markers	- Does not require cultivation of target organism - Rapid and easy to perform -Does not require a reference library	- Little is known about survival and distribution in water systems - Primers currently not available for all relevant hosts - Control measures required to avoid cross-contamination
Virus specific markers	- Host specific - Easy to perform - Does not require reference library	- Low in numbers, requires large sample size - not always present even when humans present

The selection of most appropriate method for tracking of faecal pollution source depends on several factors, such as: complexity of watershed, multiple potential sources of contamination, bacterial strains applied for tracking, character of investigation (human/non-human or differentiation between animal species), availability of resources (funds, time constraints, personnel professional skills). Currently, there is no ideal method that may be suggested as a standard method for source tracking. More research needs to be addressed to minimize the issues related to the available techniques. The use of a toolbox of methodologies rather than a single approach is also being studied [21, 22, 23, 52].

4. Enterophages

The first application of bacteriophages as microbiological indicators for the presence of pathogenic enteric bacteria has been reported in the 1930s. In the course of years the correlations between intensity of faecal contamination and the presence of bacteriophages have been found [1].

Bacteriophages are bacterial parasites that play important role in population dynamics, genetics and evolution of their hosts. They are morphologically categorized into tailed phages, including *Podoviridae*, *Siphoviridae*, and *Myoviridae* families, as well as non-tailed phages, characterized by filamentous (i.e. *Inoviridae*), polyhedral (i.e. *Leviviridae*) and pleomorphic (i.e. *Guttaviridae* and *Fuselloviridae*) morphologies. The genomes of phages may consist of single- or double-stranded DNA or RNA, which varies in size from few to several hundred Kb. Additionally, bacteriophages may be classified as lysogenic (temperate) and lytic phages [27].

Enterophages have been found as a novel group of phages that specifically infect *Enterococcus spp*, particularly intestinal *E. faecalis*, but also *E. faecium*, *E. durans* and *E. hirae* [1, 5]. *Enterococcus faecalis* is gram-positive bacterium, inhabiting human and other warm-blooded mammalian gastrointestinal tracts [27]. Enterophages may be present in untreated and treated wastewaters [5]. They may occur as lytic or lysogenic phages and possess relatively narrow range of hosts [27]. Enterophages were found to replicate at 22 °C, 37 °C and 41 °C in the presence of two salts: sodium azide (NaN_3) and calcium chloride ($CaCl_2$). However, there is still unknown how many groups of enterophages exist [5]. The studies on enterophages' morphology and genetics have proved that majority of enterophages are dsDNA tailed phages, corresponding to *Siphoviridae* family, however those belonging to *Myoviridae* and *Podoviridae* have been also found. They are usually icosahedral capsids varying in diameter (from 12 to 93 nm) with tails of different length (60-204 nm).

Nevertheless, other isolates of filamentous, polyhedral and pleomorphic (PFP) morphologies were also detected [5, 27].

The occurrence of enterophages does not restrict to particular geographical areas, thus they can be tested as faecal indicators in different types of water. Due to the fact that enterophages can be isolated at environmental temperatures, they may be detectable in places without the facility of incubator. Preliminary studies indicated superiority of resistance of enterophages to removal treatment over resistance of other bacteriophages, such as coliphages. Moreover, the similarity in die-off rates in fresh and marine waters between enterophages and enteric viruses has been found [5]. It may suggest that they could be more useful as indicators for enteric viruses in treated wastewater. In large watershed, enterophages were found in smaller concentrations than coliphages, thermotolerant coliforms and enterococci. High level of bacterial indicator in large water body implies the faecal contamination of animal origin [5]. The absence of enterophages in cattle faeces may suggest that they are specific to human faecal contamination and could be used for indication of human enteric viruses' presence in recreational waters. Additionally, the die-off rate of enterophages in water sources and beach sand may be correlated with recent faecal contamination [5].

Enterophages seem to be promising viral indicators of human faecal contamination. However, there is still relatively little known about their specific characteristics and further examinations within this field are indispensable.

5. Experimental procedure

5.1. Detection of enterophages in untreated and treated wastewaters

Materials and reagents

- 2 strength (2x) Tryptic Soy Broth [TSB] with agar (1.5 % w/v) (point B.1 in Appendix B)
- $CaCl_2$ (final concentration of 5.2 mg/mL) (point B.3 in Appendix B)
- NaN_3 (final concentration of 0.4 mg/mL) (point B.4 in Appendix B)
- Syringe filters for decontamination with pores of 0.2 µm in diameter (Whatman)
- Water bath at 50 °C
- Incubators at 37 °C and 41 °C

Procedure

1) Raw sewage and treated wastewater were filtered in order to remove contamination; if necessary, in the case of untreated wastewater, a dilution 1:2 was made;
2) 5 mL of freshly regrown host *E. faecalis* (point A.1 in Appendix A), and 1 mL of each salt ($CaCl_2$ and NaN_3) were added to the sample; the presence of NaN_3 allows inhibiting the background microbiota, whereas $CaCl_2$ treatment of bacterial cells produces competent cells which easily uptakes virus DNA;
3) The sample was mixed slowly with 50 mL of liquefied 2x Tryptic Soy Broth with agar (1.5% w/v), taking care not to create bubbles;
4) The mixture was then poured into four sterile Petri dishes and allowed to solidify;
5) Plates were incubated for 48 hours at 37 °C or 41 °C;
6) After 48-hour incubation viral plaques were counted.

5.2. Isolation of enterophages

Materials and reagents

- PBS buffer (point B.5 in Appendix B)
- Trypticase Soy Agar [TSA] medium (point B.6 in Appendix B)
- 1x TSB with agar (0.75% w/v) (point B.2 in Appendix B)
- Sterile Pasteur pipettes
- Sterile eppendorf-like tubes
- Eppendorf-like centrifuge (eppendorf miniSpin plus)

Procedure

1) Single viral plaques were isolated and plucked using sterile Pasteur pipettes. Differences in size and transparency were taking into consideration for the isolation.
2) The plugs were transferred into eppendorf-like tubes containing 0.5 mL of PBS; each plaque was gently dislodged by pipetting up and down in order to avoid a breakdown of tailed phages, thus making them non-infective;
3) The viral plaques were centrifuged at 14.000 rpm for 10 min at 10 °C and the supernatants were transferred into new sterile eppendorf-like tubes;
4) The double layer method was applied; for that, 4 mL of 1x TSB with agar (0.75% w/v) was added to a mixture containing 300 µL of the supernatant and 1mL of freshly regrown *E. faecalis* and poured into Petri dishes containing TSA agar;
5) The plates were incubated for 48 h at 37°C or 41°C;
6) After 48 h of incubation the plates showing total lysis were harvested.

5.3. Harvesting of enterophages

Materials and reagents

- PBS buffer (point B.5 in Appendix B)
- Bent glass rod (hockey stick)
- Sterile Oak ridge tubes
- Syringe filters for decontamination with pores of 0.2µm in diameter (Whatman)
- Orbital incubator at 37°C
- Centrifuge (Sigma)
- Deep-freezer at -80°C

Procedure

1) The plates showing total lysis were treated with 5 mL of PBS and agitated slowly for 10 min;
2) The top agar was removed from the plates using a hockey stick and transferred to Oak ridge tubes;
3) The tubes were centrifuged at 14.000 rpm for 10 min at 10 °C;
4) The supernatants were filtered to remove any bacterial contamination, transferred to new sterile tubes and frozen at -80 °C.

5.4. Determination of enterophages resistance in different types of water (raw sewage, distilled and tap water)

Materials and reagents

- 1x TSB with agar (0.75% w/v) (point B.2 in Appendix B)
- TSA (point B.6 in Appendix B)
- $CaCl_2$ (final concentration of 5.2 mg/mL) (point B.3 in Appendix B)
- NaN_3 (final concentration of 0.4 mg/mL) (point B.4 in Appendix B)
- Distilled water
- Syringe filters for decontamination with pores of 0.2 µm in diameter
- Vortex
- Water bath at 50 °C
- Incubator at 37 °C
- Orbital incubator at 37 °C

Procedure

1) Serial dilutions (up to 10^{-8}) of a 37 °C isolated enterophage (as decribed at 5.1-5.3) were made and the double layer method was applied; 500 µL of each dilution was mixed with 1mL of freshly grown *E. faecalis,* and 4 mL of liquefied 1x TSB plus agar. The mixture was then poured into Petri dishes and incubated for 48 h at 37 °C;

2) After 48 h of incubation the viral plaques were counted in order to determine the concentration of enterophages per 1 mL and the volume of enterophage isolate needed to obtain a final concentration of 10^4 PFU/100 mL was calculated;

3) Sewage water sample was filtered to remove suspended solids and all three samples were sterilized in autoclave for 15 min at 121 °C;

4) 225 µL of enterophage isolate was added to each water sample in order to have the final concentration of 10^4 PFU/100 mL. The water samples were then incubated at 37 °C with agitation; agitating would maintain a uniform distribution of enterophages in the sample;

5) Serial dilutions of each sample were performed (up to 10^{-3}) and the double layer was carried out three times a day;

6) The plates were incubated for 48 h at 37°C;

7) After incubation viral plaques were counted;

8) The enterophages' stability analysis in distilled and tap waters was performed up to complete extinction of enterophages; however, in the case of raw sewage, the test was stopped, when there was no more sample for further analysis.

5.5. Isolation of enterophage genetic material from supernatant

Materials and reagents

- RTP® Bacteria DNA Mini Kit
- Eppendorf-like tubes
- Eppendorf-like centrifuge (eppendorf miniSpin plus)
- Vortex
- Water bath at 37 °C and 65 °C
- Block heater at 95 °C

Procedure

1) The enterophage isolate (obtained as described at 5.1-5.3) was placed into sterile eppendorf-like tubes and centrifuged at 10,000 rpm for 3 min;
2) 200 µL of the supernatant was carefully transferred into new sterile eppendorf-like tube and the isolation of genetic material was performed as described by manufacturer (point D.3. in Appendix D);
3) 200 µL of extracted genetic material was divided into two parts: half of the volume was subjected directly to electrophoresis, whereas the remaining half was subjected to treatment with DNase.

5.6. Nucleic acid analysis

Materials and reagents

- SeaKem® Agarose (point D.1 in Appendix D)
- DNase enzyme (Roche)
- 1X TAE Buffer (point D.2 in Appendix D)
- Bromophenol blue
- Ethidium bromide (Sigma)
- Water bath at 37 °C
- Block heater at 95 °C
- Electrophoretic apparatus (GE Healthcare)

- UV lamp
- G. Box (SynGene)

Procedure

200 µL of extracted DNA was divided into two parts.

1) 100 µL of extracted genetic material was treated with 10 µL of DNase and incubated at 37 °C for 30 min;
2) Subsequently, the solution was incubated in block heater at 95 °C for 15 min;
3) After incubation, 3 µL of bromophenol blue were added to the samples in order to monitor the progress of the samples through the agarose gel;
4) The molten agarose was poured onto electrophoretic tray and allowed to solidify;
5) After agarose gel solidification the comb was gently removed and the samples with bromophenol blue marker were loaded into the application slots;
6) The gel was placed in the electrophoretic apparatus and submerged in the buffer;
7) The electrophoresis was carried out at 60V voltage;
8) When the process was finished, the gel was removed from the apparatus and placed in ethidium bromide for 30-40 min;

The bands were visualized under UV and recorded.

5.7. Isolation of *Enterococcus faecalis* strain

Materials and reagents

- Slanetz-Bartley (S-B) medium (point C.1 in Appendix C)
- Bile Aesculin Agar (BEA) (point C.2 in Appendix C)
- TSA medium (point B.6 in Appendix B)
- Membrane filters (0.22 µm, 47 mm Ø, Millipore®)
- Distilled water
- Inoculating loop
- Vacuum pump
- Incubators at 37°C and 44°C
- Refrigerator at (5±3) °C

Procedure

1) Untreated wastewater samples were diluted (up to 10^{-4}). Dilutions of 10^{-3} and 10^{-4} were filtered using a vacuum pump and 0.22 µm pore-size membranes filters. The filters were placed on S-B medium and incubated for 48 h at 37 °C;

2) After 48 h of incubation, single dark colonies were spread in TSA medium with the help of inoculating loop and incubated for 24 h at 37 °C;

3) After 24 hours the Bile Aesculin test was performed; isolated bacteria were passed to Esculin medium and incubated for 2 h at 44 °C; the test is considered positive if more than half the medium is dark brown or black after incubation;

4) The strains that presented best results for Bile Aesculin test were passed to S-B medium and kept in refrigerator at (5±3) °C for further analysis.

5.8. Comparison of different strains of *E. faecalis*

Materials and reagents

- 2x TSB plus agar (1.5% w/v) (point B.1 in Appendix B)
- $CaCl_2$ (final concentration of 5.2 mg/mL) (point B.3 in Appendix B)
- NaN_3 (final concentration of 0.4 mg/mL) (point B.4 in Appendix B)
- Syringe filters for decontamination with 0.2 µm size-pore
- Water bath at 50°C
- Incubator at 37°C

Procedure

1) 100 mL of raw sewage sample was filtered in order to remove contamination;

2) 5 mL of freshly grown *E. faecalis* and 500µL of $CaCl_2$ and NaN_3 were added to the sample;

3) The mixture was combined slowly with 30ml of liquefied 2x TSB + agar (1.5% w/v), taking care not to create bubbles;

4) The mixture was poured into sterile Petri dishes and allowed to solidify;

5) Plates were incubated for 48 h at 37°C;

6) After 48 h of incubation viral plaques were counted.

6. Results and discussion

For the purpose of current study, samples were tested in the period between February and July 2011. The samples monitored belonged to wastewater treatment plants (WWTPs) from touristic (A) and non-touristic (B) sites in Portugal.

6.1. Occurrence and quantification of enterophages in untreated wastewater

Enterophages have been proposed as potential indicators of faecal pollution [5]. In this study, samples were tested from locations receiving high numbers of tourists during warm months and from locations with low input from tourists. The enterophages were detected and isolated from wastewater in February and March (hereafter cold months), as well as in June and July (hereafter warm months).

93 out of the 97 samples tested were positive for enterophages. The percentage of positive samples according to sampling site is presented in the Table 4.

Table 4. Enterophage detection frequency as percentage of total isolates with respect to associated sampling sites

Sampling site	N^{O} of samples investigated	N^{O} of positive samples	Enterophage positive samples [%]
Touristic	72	70	97
Non-touristic	25	23	92
Overall	97	93	96

An overall of 4 samples were negative for enterophages. Analysing and breaking down the sites between touristic and non-touristic sites, 2 samples were negative, in each, for the presence of enterophages (Table 26 in Appendix E).

The overall average concentration of enterophages in wastewaters was 1837 PFU/100mL. Considering the touristic and non-touristic sites separately the results were 2160 PFU/100mL and 908 PFU/100mL, respectively (Table 5).

Table 5. Average concentration of enterophage at urban and rural sampling sites

Sampling site	N^{O} of samples investigated	Average concentration of enterophages [PFU/100mL]
Touristic	72	2160
Non-touristic	25	908
Overall	97	1837

Figure 1 represents the distribution of average enterophage concentrations in analysed samples of sewage water, collected at all sampling sites over the investigation period.

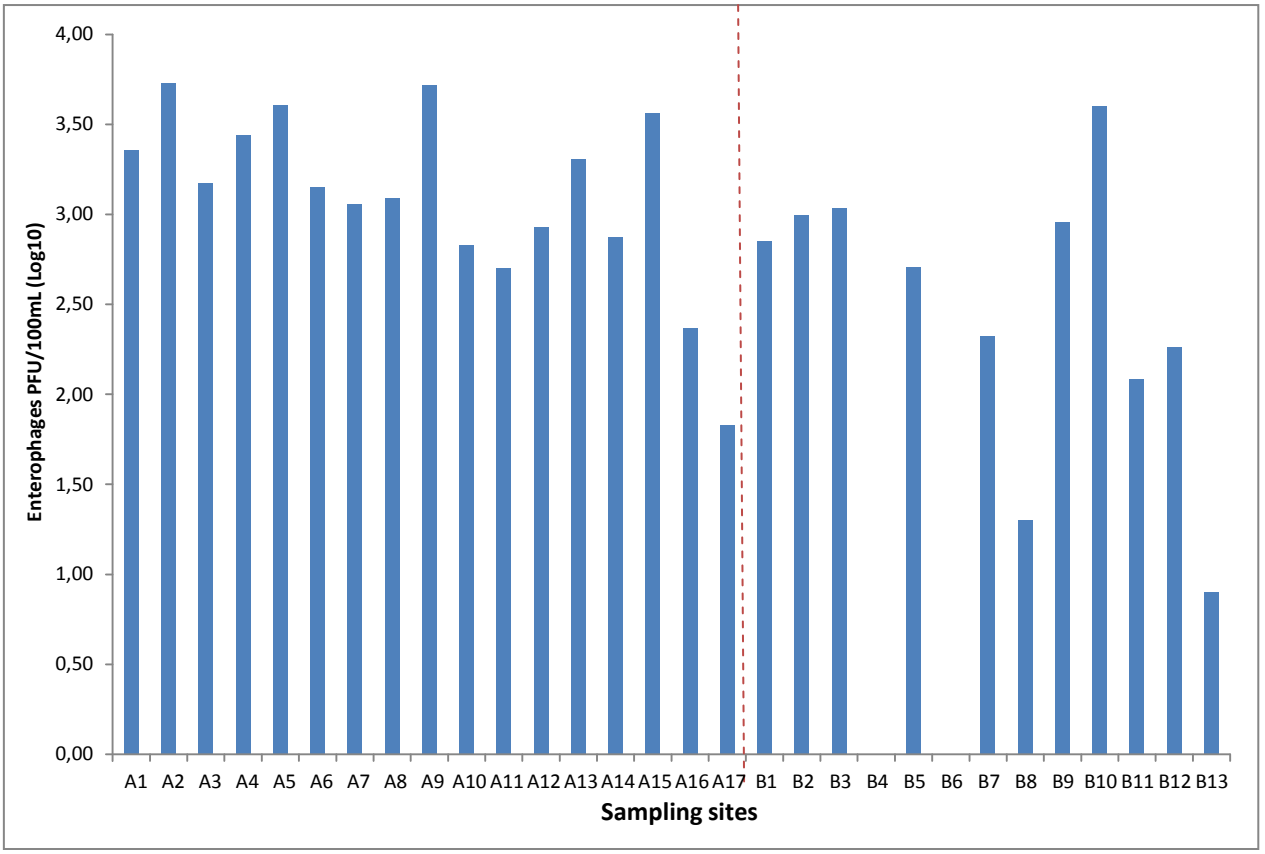


Figure 1. Average concentrations of enterophages at touristic: A1-A17 and non-touristic: B1-B13 sampling sites over investigation period

From the observation of Figure 1, it is noticeable that the wastewaters from touristic places presented higher levels than the ones found in non-touristic places. The difference in enterophage detection is significant, since the average concentration found in samples from touristic areas is more than twice higher than that from non-touristic. The prevalence of enterophages at touristic sites over non-touristic is the result of tourists influx to holiday resorts during warm months.

6.2. Comparison of enterophages' concentration between touristic and non-touristic sites over cold- and warm -month periods

The isolation of enterophages during cold- and warm-month periods revealed variations in their concentration, especially in untreated wastewaters from touristic locations. 43 samples were analysed during the cold-month period and 54 samples during the warm-month period (Table 26 in Appendix E). The number of enterophages detected in samples from touristic sites over cold-month period was 607 PFU/100mL, whereas during warmer months it was 3402 PFU/mL, more than five times higher. Considering the non-touristic areas an increase, even though not so marked, was also observed for warm months. The number of enterophages at non-touristic sampling site in cold-month period was 514 PFU/100mL against 1217 PFU/100mL in warm months (Table 6).

Table 6. Average enterophage concentrations at touristic and non-touristic sampling sites over warm- and cold-month periods

Sampling site	Average concentration of enterophages in cold-month period [PFU/100mL]	Average concentration of enterophages in warm-month period [PFU/100mL]
Touristic	607	3402
Non-touristic	514	1217

The Figure 2 represents the comparison of enterophages' concentration in samples collected from touristic areas over warm- and cold-month periods.

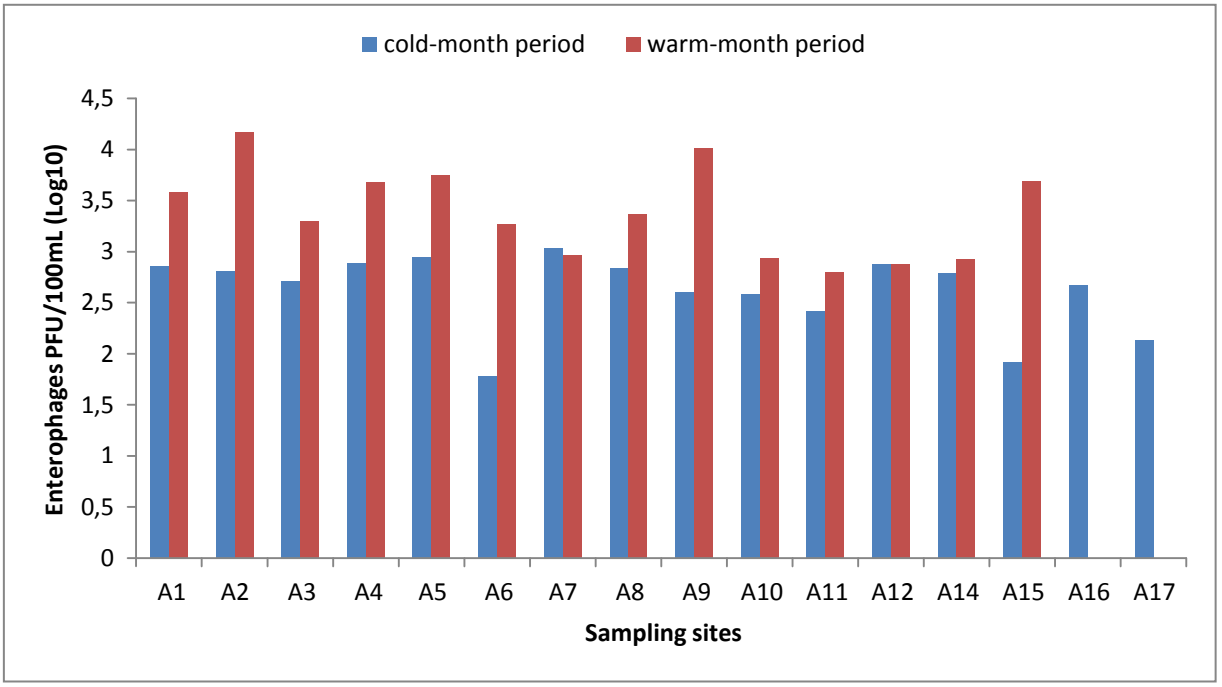


Figure 2. Comparison of enterophages' concentration at touristic sampling sites over cold and warm-month periods

From the observation of Figure 2, there is perceptible a steep increase on enterophages' number during warm months. The most significant differences in concentrations occur at A2, A6, A9 and A15 sites. The substantial increment on the number of enterophages during warm-month period occurs due to the flow of tourists in June and July.

The Figure 3 shows the levels of enterophages in non-touristic sites in warm- and cold-month periods.

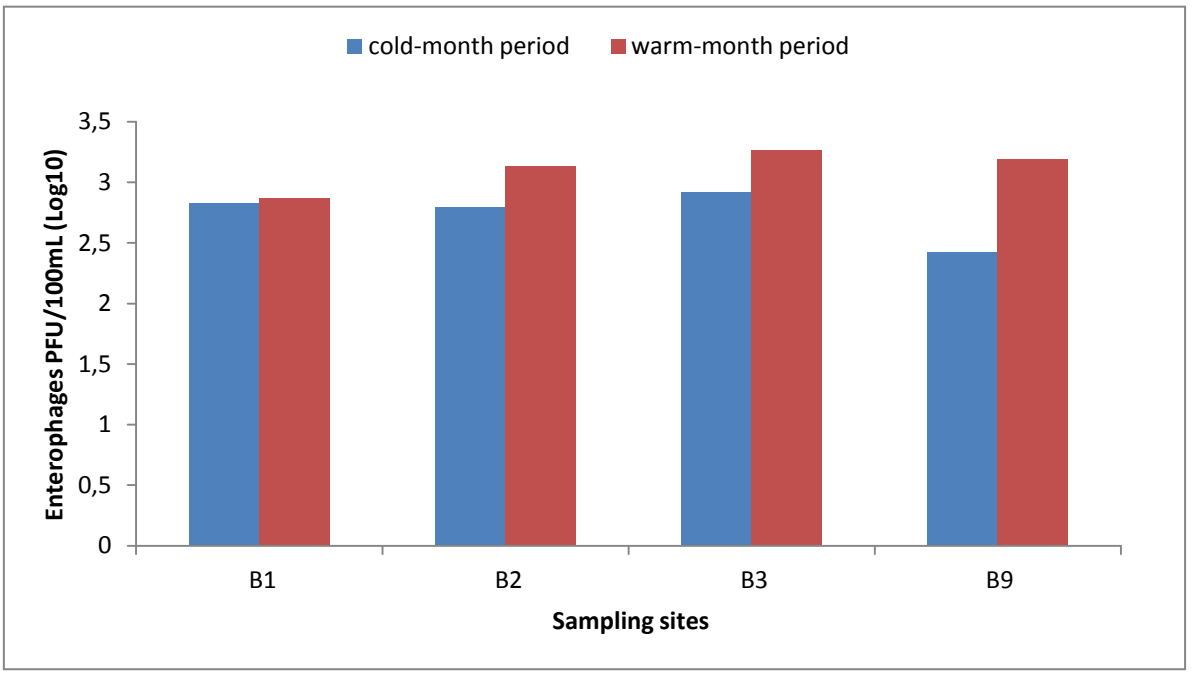


Figure 3. Comparison of enterophages' concentration at non-touristic sampling sites over cold- and warm-month periods

The number of enterophages increased also for these locations, albeit a less pronounced one. The slight increment in enterophages' concentration in non-touristic sites might also occur due to increase in human population in these areas during warm months.

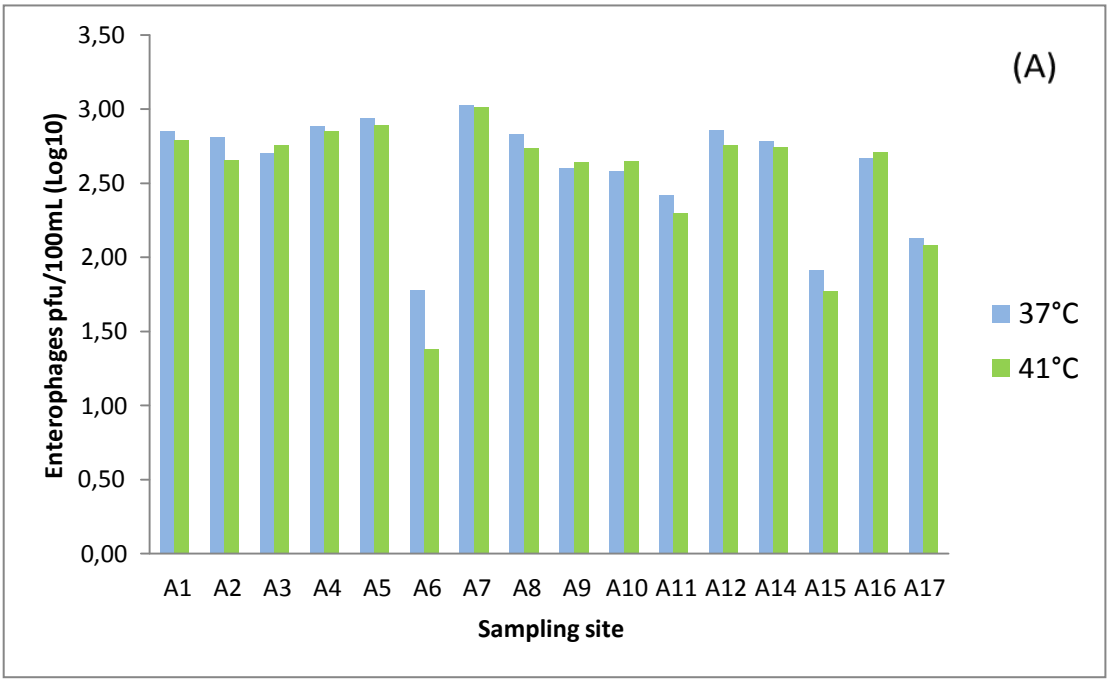
6.3. Discrimination between enterophages replicating at different temperatures

The ability of enterophages to replicate at different temperatures was tested, at 37 °C and 41 °C, in wastewater. The difference in the replication temperature may also be associated to differences in the isolates. In order to perform the replication of enterophages, firstly the single layer method and subsequently the double layer method were applied (as described in points 5.1-5.3).

Table 7. Average concentrations of enterophages isolated at 37°C and 41°C at touristic and non-touristic sites

Sampling site	N^{O} of samples investigated	Enterophages isolated at 37°C [PFU/100mL]	Enterophages isolated at 41°C [PFU/100mL]
Touristic	32	523	478
Non-touristic	11	386	350
Overall	43	583	525

43 samples, from which 32 corresponded to touristic places and 11 to non-touristic places, were analysed. The results revealed a predominance of enterophages detected at 37 °C over those detected at 41 °C, both at touristic and non- touristic sites; however the difference is not significant (Figure 4 (A and B)). In the case of touristic areas, the levels of enterophages were between 523 PFU/100mL and 478 PFU/100mL at 37 °C and 41 °C, respectively. For non-touristic locations, the concentration of enterophages was 386 PFU/100mL and 350 PFU/100mL for 37 °C and 41 °C respectively (Table 7). Nonetheless, 4 samples received from touristic places and 2 from non-touristic places presented higher levels of enterophages at 41 ºC when compared to 37 ºC, as shown in Figure 4.



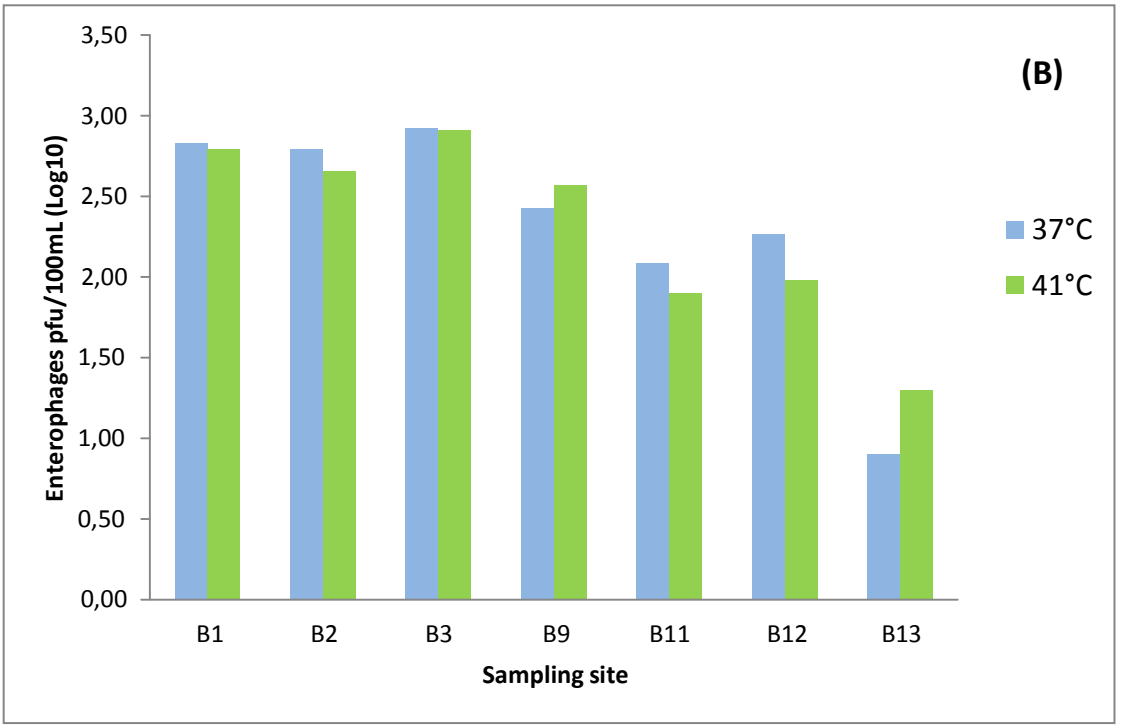


Figure 4. Average concentrations of enterophages isolated at 37°C and 41°C at particular touristic (A) and non-touristic (B) sites

The results obtained demonstrate the ability of enterophages, isolated at 37 °C and 41 °C, to replicate at these two different temperatures. Nevertheless, there is still unknown how many groups of enterophages exist [5].

6.4. Occurrence and quantification of enterophages in treated wastewater

For the purpose of detection and quantification of enterophages in treated wastewater, the samples were analysed using the single layer method.

From among 43 investigated samples, 4 revealed positive results for enterophages' presence, which constituted 9,3 % of overall samples analysed (Table 8). The concentrations of enterophages in treated wastewaters varied from 3 PFU to 589 PFU/25mL (Table 30 in Appendix G).

Table 8. Enterophages' detection frequency in treated wastewater as percentage of total isolates

	Total	N^{O} of positive samples
N^{o} samples	43	4
Percentage [%]		9,3

The results of current assay revealed relatively low frequency of enterophage detection in treated wastewaters. This may imply the considerable sensitivity of enterophages to water treatment, since the treatment processes may significantly decreases the concentration of microorganisms.

6.5. Enterophages' stability in different types of water (raw sewage, distilled and tap water)

The analysis of enterophage stability was performed in distilled, tap and sewage water using double layer method (as described in point 5.4). The quantification of enterophages was carried out three times a day. In order to compare the stability of enterophages in different types of water, the decay constants k_d from enterophages in distilled, tap and wastewater were determined using slopes of linear regressions on $\log_{10}$ plots (PFU versus time [days]). The time of 90% decrease in PFU concentrations (T_{90}) was also determined by diving ln (0.1)/k_d [5].

The stability of enterophages proved to be similar in distilled and tap waters. Enterophages survived up to 11 days in these matrices, until their complete extinction. The T_{90} in these waters was equalled to 7 days (Table 9).

Table 9. Decay constants (k_d) and time of 90% decrease in PFU concentrations (T_{90}) of enterophages in distilled, tap and sewage water

Parameter	Distilled water	Tap water	Sewage water
k_d ($days^{-1}$)	0,337	0,338	0,046
T_{90} (days)	7	7	50

The Figures 5 and 6 represents the stability curves for enterophages in distilled and tap water, respectively.

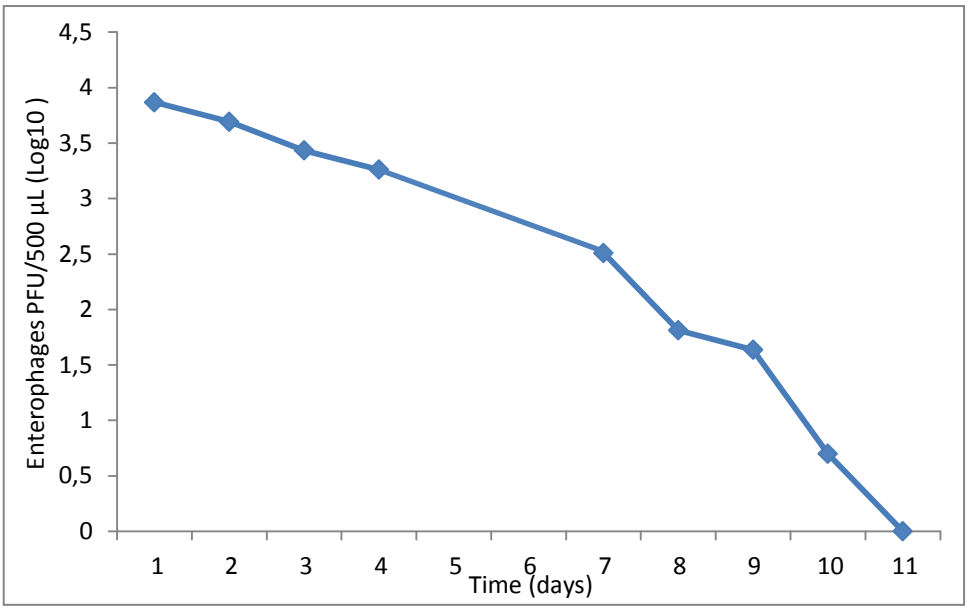


Figure 5. Stability curve for enterophages in distilled water

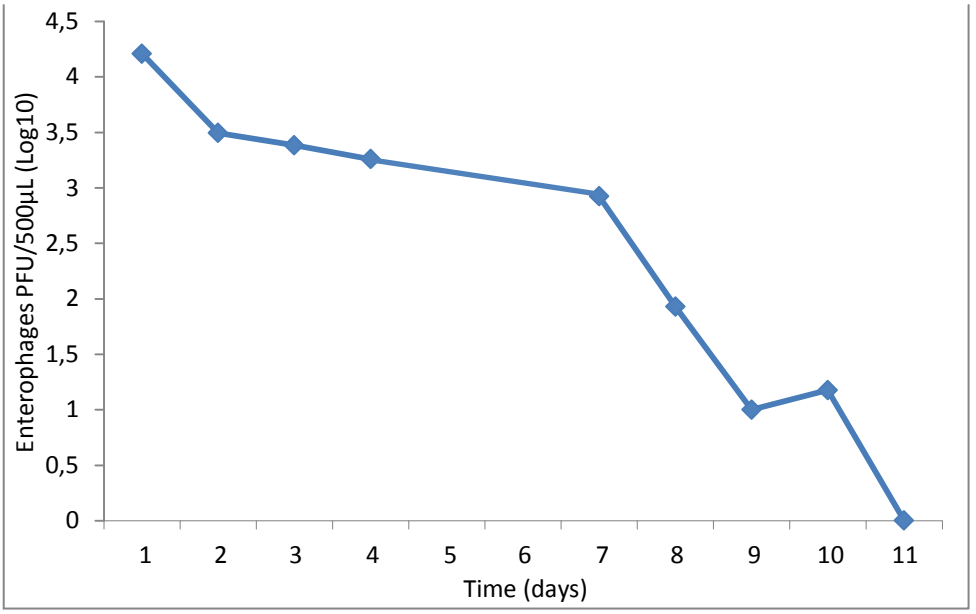


Figure 6. Stability curve for enterophages in tap water

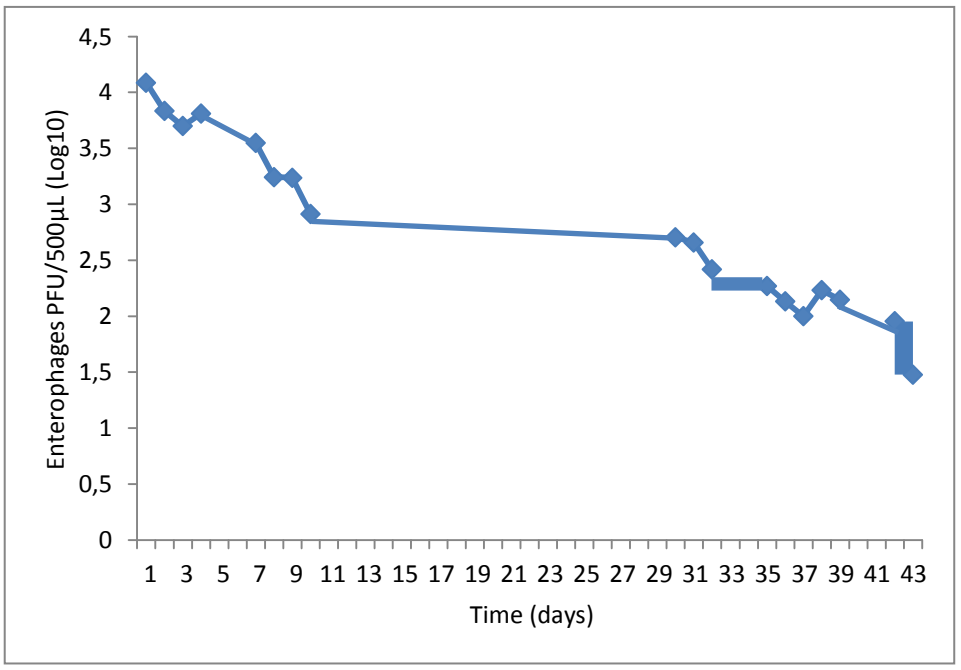


Figure 7. Stability curve for enterophages in wastewater

On the other hand, the stability of enterophages was significantly higher in wastewater (Figure 7). In this matrix, enterophages were detected up to 43th day, when their concentration equalled 30 PFU/500 µL (Table 31 in Appendix H). The T_{90} for enterophages amounted to 50 days (Table 9). The highest stability of enterophages in wastewater can result from presence of organic matter.

6.6. Enterophage nucleic acid analysis

The enterophage nucleic acid analysis was performed using the nucleic acid extracted from enterophage isolates that replicated at both 37 °C and 41 °C. The nucleic acids were treated with DNase enzyme and subjected to gel electrophoresis. The visible bands have being observed after staining with ethidium bromide.

The Figure 8, 9 and 10 represent the results of nucleic acid analysis on overall 27 different enterophage isolates.

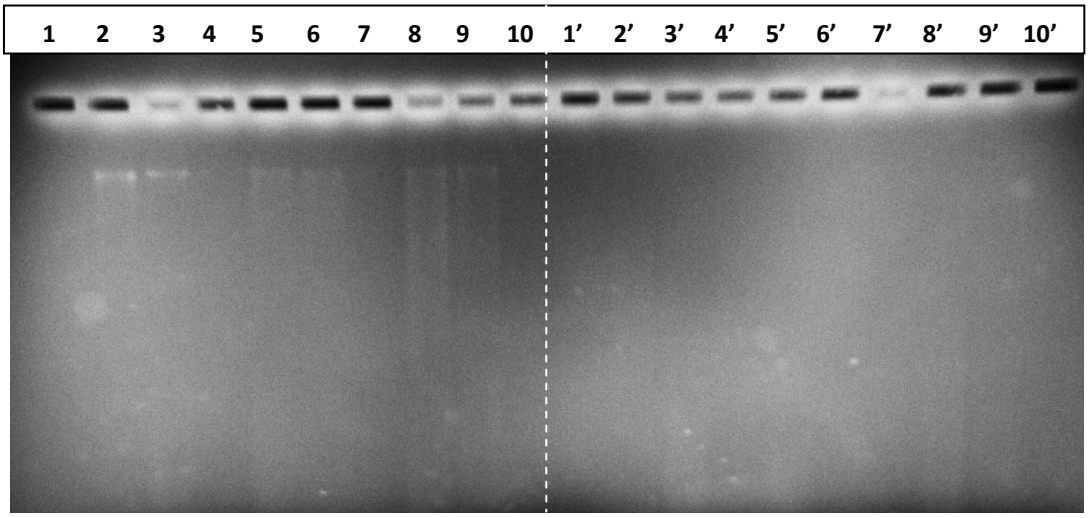


Figure 8. The genetic material of six enterophage isolates in 1% agarose gel

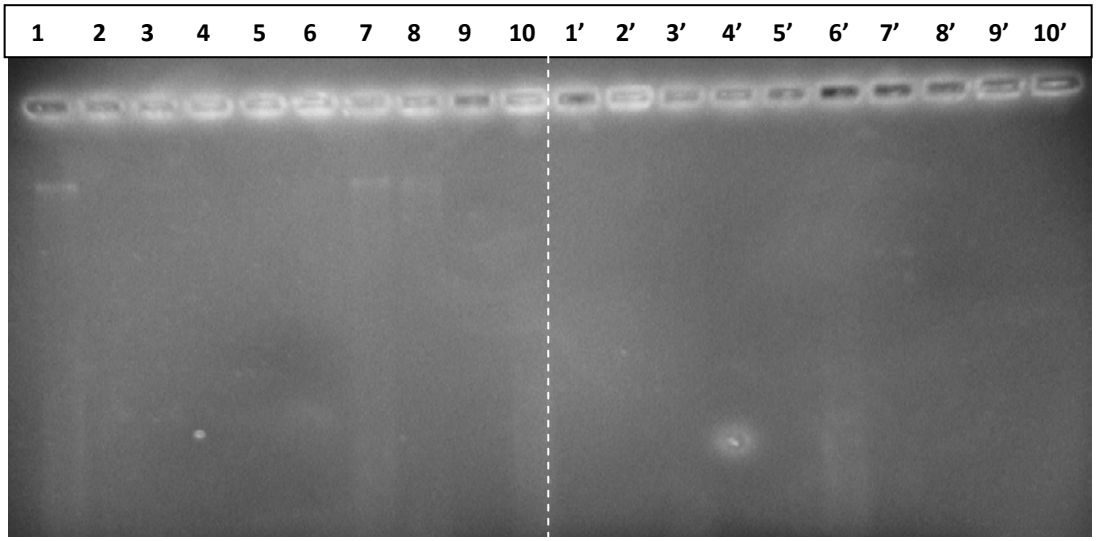


Figure 9. The genetic material of three enterophage isolates in 1% agarose gel

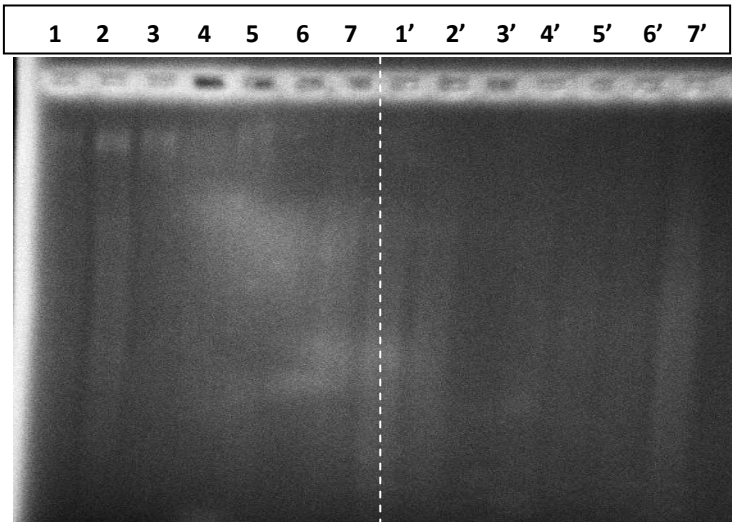


Figure 10. The genetic material of four enterophage isolates in 1% agarose gel

From the observation of Figure 8 it is noticeable that fragments in lanes 2', 3', 5', 6', 8' and 9' were degraded by DNase, showing that genetic material of those six enterophage isolates was composed of double-stranded DNA (Figure 8). The similar results are observable in the Figure 9, in which the lanes 1', 7' and 8' revealed degradation of nucleic material by DNase, thus indicating dsDNA composition. The Figure 10 presents the results of nucleic acid analysis on other enterophages, from which 1, 2, 3 and 5 enterophages proved to contain dsDNA genetic material.

The remaining lanes revealing no bands would contain too little nucleic acid material, thus ethidium bromide was not able to stain it properly. On the other hand, the slightly visible bands, starting in a half of certain lanes, may suggest that the DNase did not degrade the entire DNA molecules.

6.7. Comparison of applicability of *E. faecalis* different strains for enterophage detection

The comparison of different strains of *Enterococcus faecalis* was performed in order to test the applicability of Puerto Rican (hereafter PR) host strain throughout the world. The PR strain was tested against six *E. faecalis* isolates from Portuguese sewage water, namely LAIST_ENT_001, LAIST_ENT_002, LAIST_ENT_003, LAIST_ENT_004, LAIST_ENT_005 and LAIST_ENT_006, using single layer method.

The Table 10 shows the comparison of effectiveness of Puerto Rican strain and other Portuguese *E. faecalis* host strains for recovery of enterophages.

Table 10. Comparison of effectiveness of PR host strain and Portuguese *E. faecalis* host strains.

	Total	**Higher count for PR strain**	**Higher count for Portuguese strains**	**(+) for PR strain and (-) for Portuguese strains**	**(-) for PR strain and (+) for Portuguese strains**
N° samples	55	42	9	8	1
Percentage (%)		76	16	14	2

The results showed that PR strain revealed much higher ability to recover enterophages in Portuguese wastewaters. It was predominant in 42 samples that come to 76% of all of the analysed samples, whereas Portuguese strains indicated higher counts in 16% of the samples (Table 10). Additionally, the PR strain proved substantial sensitivity for recovering of enterophages.

There were eight samples, which proved positive detection of enterophages using PR strain (concentration varying between 20 and 1830 PFU/100mL (Table 32 in Appendix I)) and negative for Portuguese strains, whereas one sample with negative detection when using PR strain and positive with Portuguese ones (concentration from 16 and 72 PFU/100mL (Table 32 in Appendix I)) (Table 10). The test for applicability of different enterococci has proved that overall Puerto Rican host strain was the most useful for detection of enterophages in Portuguese wastewaters.

7. Conclusions

In this study enterophages have been proposed as potential viral indicators of human faecal pollution [5]. The aim of research was to further characterize enterophages, by means of their prevalence in untreated and treated wastewater, ability to replicate at different temperatures, stability in different types of water as well as composition of enterophage genetic material. Moreover, the Porto Rican *E. faecalis* host strain was compared with *E. faecalis* strains isolated from Portuguese wastewaters, in order to assess its possible universality for recovery of enterophages.

The samples monitored belonged to wastewater treatment plants (WWTPs) from touristic (A) and non-touristic (B) sites in Portugal and were analysed in the period between February and July 2011. For the purpose of enterophage detection and isolation, the single layer and double layer methods were performed [5, 28].

The determination of enterophage occurrence in untreated wastewater was performed by detection and enumeration of enterophages in samples from locations receiving high numbers of tourists during warm months and from locations with low influx of tourists. From among overall 97 investigated, 4 samples were negative for enterophages, amounting to 96 % of positive enterophage detection (Table 4). The high frequency of enterophage recovering indicates their prevalence in untreated wastewater and relatively simple detection. Moreover, the single layer method ensures fast detection, since the viral plaques were visible enough for counting after 24 hours [5, 28]. The average concentration of enterophages in wastewaters equalled 1837 PFU/100mL. Considering the touristic and non-touristic sites separately, the results were 2160 PFU/100mL and 908 PFU/100mL, respectively (Table 5). The wastewater samples from touristic places presented higher levels than the ones found in non-touristic places (Figure 1). The difference in enterophage detection was significant, since the average concentration found in samples from touristic areas doubly exceeded that from non-touristic. The prevalence of enterophages at touristic sites over non-touristic is a result of tourists' influx to holiday resorts during warm-month period.

The comparison between enterophages' number during cold- and warm-month periods revealed the substantial increment on enterophage concentration during warm-month period in untreated wastewaters from touristic locations. The number of enterophages detected in samples from touristic sites over cold-month period was 607 PFU/100mL, whereas during warmer months it was 3402 PFU/mL, more than five times higher (Table 6). Considering the non-touristic areas the increase, even though not so marked, was also observed for warm-months. The number of enterophages at non-touristic sampling site in cold-month period was 514 PFU/100mL against

1217 PFU/100mL in warm-months (Table 6). These results corroborate the previous assumption that the increase in enterophages' population is a result of tourists' influx to holiday resorts.

The discrimination between enterophage isolates, which can replicate at 37 °C and 41 °C revealed a predominance of enterophages detected at 37 °C over those detected at 41 °C, both at touristic and non-touristic sites; however, the difference was not significant (Figure 4 (A and B)). In case of touristic site, the levels of enterophages were found between 523 PFU/100mL and 478 PFU/100mL at 37°C and 41°C, respectively (Table 7). For non-touristic locations, the concentrations of enterophages equalled 386 PFU/100mL and 350 PFU/100mL at 37 °C and 41 °C, respectively (Table 7). Nonetheless, 4 samples received from touristic places and 2 from non-touristic places presented subpopulations of enterophages that preferably infected *E. faecalis* at 41 °C rather than at 37 °C (Figure 4). The results of this test prove that enterophages isolated at 37°C and 41°C are able to replicate at these two different temperatures [5, 48]. However, it is still uknown how many groups of enterophages exist.

The detection and quantification of enterophages in treated wastewater exhibited 4 positive results for enterophages presence, which constituted 9,3 % of overall samples analysed (Table 8). The concentrations of enterophages in treated wastewaters varied from 3 PFU to 589 PFU/25mL (Table 30 in Appendix G). The results of enterophage detection in treated wastewater imply considerable sensitivity of enterophages to water treatment, since treatment practises may significantly reduce the concentrations of microorganisms in water [31, 32, 33].

The stability of enterophages was investigated in distilled, tap and sewage water. In order to compare the results, the decay constants k_d from enterophages in distilled, tap and wastewater were determined and time of 90% reduction in PFU concentrations (T_{90}) was calculated [5]. The stability of enterophages proved to be similar in distilled and tap waters. Enterophages survived up to 11 days in these matrices. The T_{90} in these waters was equalled to 7 days (Table 9). On the other hand, the stability of enterophages was significantly higher in wastewater (Figure 7). In this matrix enterophages were detected up to 43th day, when their concentration equalled 30 PFU/500 µL (Table 31 in Appendix H). The T_{90} for enterophages amounted to 50 days (Table 9). The highest resistance of enterophages in wastewater is still not clear; however it can result from the presence of organic matter.

The analysis of enterophage genetic material was conducted using the nucleic acid extracted from enterophage isolates that replicated at both 37°C and 41°C. The nucleic acid molecules were treated with DNase enzyme and subjected to gel electrophoresis. From among 27 analysed isolates, 13 proved the composition of double-stranded DNA genetic material (Figures 8, 9 and 10). The enterophage nucleic acid analysis conducted in this study has confirmed the results of previous

studies on enterophage genetics [5, 27]. Therefore, it can be assumed that enterophages infecting specifically E. faecalis strain contain dsDNA genetic material.

For the purpose of testing the applicability of Puerto Rican host strain throughout the world, the comparison of different strains of *Enterococcus faecalis* was performed. The PR strain was tested against six *E. faecali*s isolates obtained from Portuguese wastewaters. The results proved that overall Puerto Rican strain was the most useful from among tested bacterial strains, for recovering of enterophages in Portuguese wastewaters. The samples investigated while using PR host counted higher average enterophage concentrations in 76 % of all the analysed samples, whereas Portuguese strains indicated higher counts in 16% of the samples (Table 10). Moreover, PR strain proved substantial sensitivity for recovering of enterophages. There were eight samples, which gave positive detection of enterophages using PR strain and negative for Portuguese strains, whereas one sample with negative detection when using PR strain and positive with Portuguese ones (Table 32 in Appendix I). The test for applicability of different *E. faecalis* has revealed the potential universality of Porto Rican host strain for recovery of enterophages. However, these results have to be confirmed in some other geographic areas.

Current research revealed several more characteristics of bacteriophages infecting *E. faecalis.* Nonetheless, further studies on enterophages are necessary, since it is still little known about their specific nature and there is no information on their correlation with human enteric viruses.

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9. Appendices

Appendix A- Reagents for preparation of fresh host strain *Enterococcus faecalis*

A.1. Overnight *Enterococcus faecalis* host strain

Table 11. Reagents and quantities for preparation of freshly grown *E. faecalis*

Reagent	Quantity
Host strain *Enterococcus faecalis*	Single colony
Dextrose Azide Broth medium (Biokar Diagnosis)	2x50 mL

1) The host strain of *E. faecalis* was re-streaked weekly onto fresh Slanetz-Bartley (S-B) medium and Petri dish was kept at 4-7°C;

2) The single colony of host was inoculated in 50 mL of Dextrose Azide Broth and incubated with agitation overnight at 37°C;

3) After overnight incubation 1 mL of *E. faecalis* was regrown by inoculation in 50 mL of Dextrose Azide Broth and incubated with agitation for 3 hours at 37°C and the freshly grown host was ready to use.

A.2. Dextrose Azide Broth medium

Table 12. Reagents and quantities for preparation of Dextrose Azide Broth medium

Reagent	Quantity
Dextrose Azide Broth medium (Biokar Diagnosis)	35.6 g
Distilled water	1000 mL

1) The Dextrose Azide Broth in weight of 35.6 g was dissolved in 1000 mL of distilled water;

2) The solution was autoclaved at 121°C for 15 minutes;

3) After cooling the medium was kept at 4-7°C.

Appendix B- Solutions for detection and isolation of enterophages

B.1. 2 strength (2x) Tryptic Soy Broth [TSB] with agar (1.5 % w/v)

Table 13. Reagents and quantities for preparation of Tryptic Soy Broth (1.5 % w/v)

Reagent	Quantity
Tryptic Soy Broth (BD)	30.0 g
Agar (Oxoid)	15 g
Distilled water	1000 mL

1) The Tryptic Soy Broth in weight of 30.0 g was suspended in 1000mL of distilled water;

2) 15 g of microbiological agar was added to the solution, to obtain the final concentration of medium of 1.5%;

3) The reagents were mixed for dissolution and the solution was autoclaved in 121°C for 15 minutes;

3) The sterilized medium was cooled and poured into Petri dishes;

4) After solidification the plates were kept at temperature of 5±3°C.

B.2. 1 strength (1x) Tryptic Soy Broth [TSB] with agar (0.75 % w/v)

Table 14. Reagents and quantities for preparation of Tryptic Soy Broth (0.75 % w/v)

Reagent	Quantity
Tryptic Soy Broth (BD)	30.0 g
Agar (Oxoid)	7.5 g
Distilled water	1000 mL

1) The Tryptic Soy Broth in weight of 30.0 g was suspended in 1000mL of distilled water;

2) 7.5 g of microbiological agar was added to the solution, to obtain the final concentration of medium of 0.75%;

3) The reagents were mixed for dissolution and the solution was autoclaved in 121°C for 15 minutes;

3) The sterilized medium was cooled and poured into Petri dishes;

4) After solidification the plates were kept at temperature of 5±3°C.

B.3. $CaCl_2$ (5.2 mg/mL)

Table 15. Reagents and quantities for preparation of $CaCl_2$ (5.2 mg/mL)

Reagent	Quantity
$CaCl_2$(Sigma)	520.0 mg
Distilled water	100 mL

1) $CaCl_2$ in weight of 520.0 mg was dissolved in 100 mL of distilled water;

2) The solution was filtered using syringe filters (Whatman) with pores of 0.2 µm in diameter for decontamination;

3) $CaCl_2$ with a final concentration of 5.2 mg/mL was kept at room temperature.

B.4. NaN_3 (0.4 mg/mL)

Table 16. Reagents and quantities for preparation of NaN_3 (0.4 mg/mL)

Reagent	Quantity
NaN_3 (Sigma)	40.0 mg
Distilled water	100 mL

1) NaN_3 in weight of 40.0 mg was dissolved in 100 mL of distilled water;

2) The solution was filtered using syringe filters (Whatman) with pores of 0.2 µm in diameter for decontamination;

3) NaN_3 with a final concentration of 0.4 mg/mL was kept at room temperature.

B.5. 1X PBS

Table 17. Reagents and quantities for preparation of 1X PBS

Reagent	Quantity
NaCl (Riedel- de Haën)	8 g
KCl (Riedel- de Haën)	0.2 mL
KH_2PO_4 (Sigma)	0.2 mL
$Na_2HPO_4 \cdot 7H_2O$ (Riedel- de Haën)	1.15 g
Distilled water	1000 mL

1) All of the reagents in given weights (Table 20) were dissolved in 1000 mL of distilled water;

2) The solution was autoclaved at 121°C for 20 minutes;

3) After sterilization PBS 1X was kept at room temperature.

B.6. Trypticase Soy Agar (TSA)

Table 18. Reagents and quantities for preparation of Trypticase Soy Agar (TSA)

Reagent	Quantity
Trypticase Soy Agar (Merck)	40.0 g
Distilled water	1000 mL

1) The Trypticase Soy Agar medium in weight of 40.0 g was dissolved in 1000mL of distilled water;

2) The solution was autoclaved in 121°C for 15 minutes;

3) The cooled medium was dispensed into Petri dishes and allowed to solidify;

4) The plates were kept at temperature of 5±3°C.

Appendix C- Solutions for isolation of *Enterococcus faecalis*

C.1. Slanetz-Bartley medium

Table 21. Reagents and quantities for preparation of Slanetz-Bartley medium

Reagent	Quantity
Slanetz-Bartley (Bio-Rad)	41.4 g
Distilled water	1000 mL

1) The Slanetz-Bartley medium in weight of 41.4 g was dissolved in 1000mL of distilled water;

2) The solution was boiled for one minute until complete dissolution, taking care to not overheat;

3) The cooled medium was dispensed into Petri dishes and allowed to solidify;

4) The plates were kept at temperature of 5±3°C.

C.2. Bile Aesculin Agar (BEA)

Table 20. Reagents and quantities for preparation of Bile Aesculin Agar (BEA)

Reagent	Quantity
Bile Esculin Agar (Biokar Diagnosis)	54.6 g
Distilled water	1000 mL

1) The Bile Aesculin Agar in weight of 54.6 g was dissolved in 1000mL of distilled water;

2) The solution was autoclaved in 121°C for 15 minutes;

3) The sterilized medium was cooled and poured into Petri dishes;

4) After solidification the plates were kept at temperature of 5±3°C.

Appendix D- Solutions for nucleic acid analysis

D.1. SeaKem® LE Agarose (1% w/v)

Table 21. Reagents and quantities for preparation of Seakem LE Agarose medium

Reagent	Quantity
SeaKem® LE Agarose (Cambrex)	5 g
Distilled water	500 mL

1) The SeaKem® LE Agarose in weight of 5 g was dissolved in 500 mL of distilled water to a final gel concentration of 1%;

2) The solution was heated until complete dissolution;

3) The agarose prepared was kept at room temperature.

D.2. 1X Tris-Acetate-EDTA (TAE) buffer

Table 22. Reagents and quantities for preparation of TAE buffer (50X concentration)

Reagent	Quantity
Trisma (Sigma)	60.5 g
0.5M NaEDTA (pH 8.0) (Sigma)	25 mL
Glaciar acetic acid (Fluka)	14.28 mL
Distilled water	210.72 mL

1) At first the buffer in final concentration of 50X was prepared; the Trisma in weight of 60.5 g was dissolved in 125 mL of distilled water;

2) Subsequently, the 25 mL of 0.5M NaEDTA and 14.28 mL of glaciar acetic acid were added;

3) The volume of buffer was adjusted to 250 mL with distilled water;

4) The 50x TAE was diluted to final concentration of 1X TAE.

D.3. Nucleic acid extraction (RTP® Bacteria DNA Mini Kit)

Table 23. Reagents and quantities for nucleic acid extraction

Reagent	Quantity
Resuspension Buffer R	400 µL
Binding Buffer B6	400 µL
Wash Buffer I	500 µL
Wash Buffer II	600 µL
Elution Buffer D	200 µL

1) 400 µL of Resuspension Buffer R was added to supernatant and resuspended by pipetting up and down; the resuspended sample was transferred into the Extraction Tube L and vortexed shortly;
2) The sample was placed into water bath at 37°C for 10 min and subsequently at 65°C for 10 min;
3) After incubation at 37°C and 65°C the Extraction Tube L was transferred into block heater and incubated at 95°C for 10 min;
4) 400 µL of Binding Buffer B6 was added to the sample and the mixture was vortexed shortly;
5) The sample was loaded onto RTA Spin Filter Set and incubated for 1 min at room temperature. Afterwards, it was centrifuged at 12.000 rpm for 1 min. The filtrate was discarded and RTA Spin Filter was placed back into the new RTA Receiver Tube;
6) 500 µL of Wash Buffer I was added and sample was centrifuged at 10.000 rpm for 1 min. The filtrate was discarded and RTA Spin Filter was placed back into the new RTA Receiver Tube;
7) 600 µL of Wash Buffer II was added and the sample was centrifuged at 10.000 rpm for 1 min. The filtrate was discarded, RTA Spin Filter was placed back into the new RTA Receiver Tube and RTA Spin Filter Set was centrifuged at max. speed for 3 min in order to remove the ethanol completely;
8) The RTA Spin Filter was placed into new 1,5 mL Receiver Tube, 200 µL of Elution Buffer D was added and the mixture was incubated for 1 min at room temperature;
9) The sample was finally centrifuged at 8.000 rpm for 1 min and the DNA was eluted in Receiver Tube.

Appendix E. Average enterophage concentrations detected in wastewater collected at particular sampling sites

Table 24. Enterophage concentrations in particular samples* at corresponding sampling sites**

Sample	PFU/100mL	Sampling site
Feb1	197	A1
Feb2	82	A15
Feb3	753	A3
Feb4	266	A10
Feb5	262	A11
Feb6	405	B3
Feb7	838	A4
Feb8	728	A5
Feb9	596	A12
Feb10	134	A17
Feb11	804	A7
Feb12	706	A8
Feb13	650	A9
Feb14	740	A2
Feb15	602	B2
Feb16	121	B11
Feb17	183	B12
Mar1	698	A4
Mar2	800	A7
Mar3	563	A8
Mar4	393	A2
Mar5	632	B2
Mar6	800	B1
Mar7	1006	A5
Mar8	714	A14
Mar9	60	A6
Mar10	1600	A10
Mar11	594	A8
Mar12	1228	A1
Mar13	148	A9
Mar14	266	B9
Mar15	540	B1
Mar16	496	B3
Mar17	1600	A7
Mar18	848	A8
Mar19	478	A2
Mar20	260	A3
Mar21	506	A14
Mar22	968	A2
Mar23	1600	B3
Mar24	8	B13
Mar25	464	A16
Mar26	854	A12
Jun1	3050	A5

Jun2	4630	A15
Jun3	8600	A4
Jun4	1130	A13
Jun5	2030	A3
Jun6	350	A10
Jun7	0	A16
Jun8	750	A12
Jun9	0	A17
Jun11	730	A7
Jun12	400	A8
Jun13	610	A14
Jun14	260	A6
Jun15	60	A11
Jun16	1830	B3
Jun17	15830	A5
Jun18	5400	A1
Jun19	18800	A9
Jun20	9500	A15
Jun21	28800	A2
Jun22	4200	A3
Jul1	1736	A7
Jul2	4286	A8
Jul3	2510	B10
Jul4	2490	B2
Jul5	2500	A5
Jul6	1731	A14
Jul7	844	A2
Jul8	1540	B9
Jul9	1188	A11
Jul10	2195	A1
Jul11	1440	A9
Jul12	460	A15
Jul13	5485	B10
Jul14	4630	A6
Jul15	1200	A3
Jul16	339	B1
Jul17	0	B4
Jul18	508	B5
Jul19	2222	A10
Jul20	20	B8
Jul21	1850	B1
Jul22	0	B6
Jul23	20	A10
Jul24	880	A4
Jul25	2910	A13
Jul26	1080	A5
Jul27	190	A14
Jul28	20	B1
Jul29	300	A7
Jul30	210	B7
Jul31	690	A6
Jul32	440	A3

Jul33	240	B2

(*) where: Feb1-Feb17 were collected in February, Mar1-Mar27 in collected March, Jun1-Jun22 collected in June, Jul1-Jul33 in July 2011

(**) where: A1-A17 correspond to touristic sampling sites, B1-B13 correspond to non-touristic sampling sites

Table 25. Average enterophage concentrations over the whole monitoring period at particular sites

Touristic site	PFU/100mL	Non-touristic sites	PFU/100mL
A1	2255	**B1**	710
A2	5371	**B2**	991
A3	1481	**B3**	1083
A4	2754	**B4**	0
A5	4032	**B5**	508
A6	1410	**B6**	0
A7	1134	**B7**	210
A8	1233	**B8**	20
A9	5260	**B9**	903
A10	671	**B10**	3998
A11	503	**B11**	121
A12	847	**B12**	183
A13	2020	**B13**	8
A14	750		
A15	3668		
A16	232		
A17	67		

Table 26. Average enterophage concentrations [PFU/100mL] in cold-month period at touristic (A1-A17) and non-touristic (B1-B13) sampling sites

Touristic site	PFU/100mL	Non-touristic site	PFU/100mL
A1	713	**B1**	670
A2	645	**B2**	617
A3	507	**B3**	834
A4	768	**B4**	n.a.
A5	867	**B5**	n.a.
A6	60	**B6**	n.a.
A7	1068	**B7**	n.a.
A8	678	**B8**	n.a.
A9	399	**B9**	266
A10	381	**B10**	n.a.
A11	262	**B11**	121
A12	750	**B12**	183
A13	n.a.	**B13**	8
A14	610		
A15	82		
A16	464		
A17	134		

Where: (n.a.) - no sample analysed in given month

Table 27. Average enterophage concentrations [PFU/100mL] in warm-month period at touristic (A1-A17) and non- touristic (B1-B13) sampling sites

Touristic site	PFU/100mL	Non-touristic site	PFU/100mL
A1	3798	**B1**	736
A2	14822	**B2**	1365
A3	1968	**B3**	1830
A4	4740	**B4**	0
A5	5615	**B5**	508
A6	1860	**B6**	0
A7	922	**B7**	210
A8	2343	**B8**	20
A9	10120	**B9**	1540
A10	864	**B10**	3998
A11	624	**B11**	736
A12	750	**B12**	1365
A13	2020	**B13**	1830
A14	844		
A15	4863		
A16	0		
A17	0		

Appendix F. Isolation of enterophages at 37°C and 41°C

Table 28. Average concentrations of enterophages recovered at 37°C and 41°C at corresponding sampling sites

Sampling site	Detection at 37°C [PFU/100mL]	Detection at 41°C [PFU/100mL]
A1	713	621
A2	645	452
A3	507	575
A4	768	712
A5	867	783
A6	60	24
A7	1068	1037
A8	678	547
A9	399	441
A10	381	446
A11	262	198
A12	725	567
A13	n.a.	n.a.
A14	610	553
A15	82	59
A16	464	514
A17	134	121
B1	670	623

B2	617	452
B3	834	809
B4	n.a.	n.a.
B5	n.a.	n.a.
B6	n.a.	n.a.
B7	n.a.	n.a.
B8	n.a.	n.a.
B9	266	372
B10	n.a.	n.a.
B11	121	80
B12	183	96
B13	8	20

Where: (A1-A17)- touristic sites, (B1-B13)- non-touristic sites
(n.a.)- no sample analysed for given location

Table 29. Frequency of positive replication of enterophages at 37°C and 41°C

Sample	Replication at 37°C	Replication at 41°C
Feb1	P	P
Feb2	P	P
Feb3	P	P
Feb4	P	P
Feb5	P	P
Feb6	P	N
Feb7	P	P
Feb8	P	N
Feb9	N	N
Feb10	P	P
Feb11	N	P
Feb12	N	P
Feb13	P	P
Feb14	P	P
Feb15	P	P
Feb16	P	P
Feb17	P	P
Mar1	P	P
Mar2	P	P
Mar3	P	N
Mar4	P	P
Mar5	P	P
Mar6	P	P
Mar7	P	P
Mar8	P	P
Mar9	P	P
Mar10	P	P
Mar11	P	P
Mar12	P	P
Mar13	P	P
Mar14	P	P

Mar15	P	P
Mar16	P	P
Mar17	P	P
Mar18	P	P
Mar19	P	P
Mar20	P	P
Mar21	P	P
Mar22	P	P
Mar23	P	P
Mar24	N	N
Mar25	N	N
Mar26	P	P

Where: (P)- positive replication; (N)- negative replication

Appendix G. Average enterophage concentrations detected in effluents collected at particular sampling sites

Table 30. Average enterophage concentrations in effluents (E1-E43) at corresponding sampling sites

Sample	PFU/25mL	Sampling site
E1	0	B9
E2	0	A5
E3	0	A9
E4	0	A13
E5	0	A3
E6	0	A12
E7	0	A15
E8	0	A2
E9	0	A3
E10	0	A7
E11	19	A8
E12	589	B10
E13	0	B2
E14	0	B1
E15	0	A11
E16	0	A1
E17	0	A9
E18	0	A15
E19	517	B10
E20	0	A6
E21	0	A3
E22	0	B1
E23	0	A13
E24	0	B11
E25	0	B14
E26	0	B15
E27	0	B12
E28	0	B16

E29	0	A14
E30	0	B7
E31	0	A3
E32	0	A11
E33	3	B17
E34	0	B18
E35	0	B19
E36	0	B2
E37	0	B1
E38	0	A10
E39	0	A7
E40	0	A14
E41	0	A1
E42	0	A9
E43	0	B9

Appendix H. Results of enterophages' resistance in different types of water

Table 31. Average daily concentration of enterophages in distilled, tap and wastewater

Time [days]	Type of water		
	Sewage [PFU/500µL]	Tap [PFU/500µL]	Distilled [PFU/500µL]
1	12183	16100	7400
2	6800	3117	4933
3	5000	2413	2717
4	6450	1807	1827
7	3529	836	323
8	1748	85	65
9	1725	10	43
10	820	8	5
11	-	1	1
30	507		
31	455		
32	261		
35	186		
36	135		
37	100		
38	170		
39	140		
42	90		
43	30		

Appendix I. Average concentrations of enterophages recovered using different *Enterococcus faecalis* host strains

Table 32. Results of comparison between different strains of *Enterococcus faecalis* strains expressed in concentration of enterophages recovered using particular strain [PFU/100mL]

Sample	PR	LAIST_ENT_001	LAIST_ENT_002	LAIST_ENT_003	LAIST_ENT_004	LAIST_ENT_005	LAIST_ENT_006
Jun1	3050	2875					
Jun2	4630	1525					
Jun3	8600	2125					
Jun4	1130	0					
Jun5	2030	1675					
Jun6	350	0					
Jun7	0	0					
Jun8	750	0					
Jun9	0	0					
Jun10	830	125					
Jun11	730	0					
Jun12	400	0					
Jun13	610	50					
Jun14	260	3040					
Jun15	60	10					
Jun16	1830	0					
Jun17	15830	3150					
Jun18	5400	2425					
Jun19	18800	14950					
Jun20	9500	25					
Jun21	28800	5025					
Jun22	4200	1450					
Jul1	1740	503	947	0	503		
Ju2	4290	C	1136				
Jul3	2510	55	600	0	0		
Jul4	2490	2135	425	8	893		

Jul5	2500	470	150	0	0		
Jul6	1730	825	500	0	0		
Jul7	840	2194	2672	0	0		
Jul8	1540	680	0	0	0		
Jul9	1190	38	128	3	0		
Jul10	2200	940	0	10	0		
Jul11	1440	5005	35	55	40		
Jul12	460	15	0	0	0		
Jul13	5490	50	0	0	0		
Jul14	4630	140	290	0	125		
Jul15	1200	2245	0	0	0		
Jul16	340	125	146	0	0		
Jul17	0	38	53	16	72		
Jul18	510	C	58	C	C		
Jul19	2220	286	831	0	111		
Jul20	20					0	
Jul21	1850					905	
Jul22	0					0	
Jul23	20					0	
Jul24	880					770	626
Jul25	2910					160	529
Jul26	1080					715	804
Jul27	190					10	131
Jul28	20					20	46
Jul29	300					50	225
Jul30	210					240	3260
Jul31	690					360	645
Jul32	440					450	245
Jul33	240					260	1045

Where: C- contaminated sample