NOVEL SURROGATES FOR VIRAL LOAD: BACTERIOPHAGE INFECTING E. FAECALIS. PRESENCE AND SURVIVAL IN DIFFERENT MATRICES

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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 Puerto 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.

The results of this study have revealed several more characteristics of bacteriophages infecting *E. faecalis;* however further studies on enterophages are indispensable.

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. 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, especially those of gastrointestinal (GI) tract [2, 10, 11, 12]. The numerous outbreaks, reported throughout the world, induced epidemiologists and microbiologists to search for best way for prevention from waterborne microbial contamination [2, 3, 9, 12, 13, 14, 15].

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 [16, 17, 18]. 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 [18, 19, 20]. MST methods are useful, however they possess several drawbacks, and there is no ideal MST technique that may be suggested as a standard for source tracking [16, 17, 18].

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 [23, 24, 25].

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, 21, 22]. 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, 22]. The majority of phages infecting *E. faecalis* are double- stranded DNA tailed phages, corresponding to *Siphoviridae* family [5, 21]. 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 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 Puerto Rican for recovery of enterophages.

MATERIALS AND METHODS

Detection of enterophages in untreated and treated wastewaters

The sample of untreated or treated wastewater was filtered to remove contamination. Subsequently, 5 mL of freshly regrown host *E. faecalis* and 1 mL of both $CaCl_2$ (Sigma) and NaN_3 (Sigma) salts were added to the sample. The presence of NaN_3 allowed inhibiting the background microbiota, whereas $CaCl_2$ treatment of bacterial cells produced competent cells which easily uptakes virus DNA. The sample was mixed slowly with 50 mL of liquefied 2 strength (2x) Tryptic Soy Broth [TSB] with agar (1.5 % w/v) (BD), taking care not to create bubbles. The mixture was poured into four sterile Petri dishes and allowed to solidify. Plates were incubated for 48 h at 37 °C or 41 °C, in order to detect different enterophages can replicate at various temperatures. After incubation viral plaques were counted.

Isolation of enterophages

The single viral plaques were transferred into eppendorf-like tubes containing 0.5 mL of PBS and the tubes were centrifuged (eppendorf miniSpin plus) at 14.000 rpm for 10 min at 10 °C. The supernatants were transferred into new sterile eppendorf-like tubes and the double layer method was applied. The supernatant in volume of 300 µL was taken and 1mL of freshly regrown host *E. faecalis* was added. The mixture was combined with 4ml of 1x TSB with agar (0.75% w/v) (BD) and poured into Petri dishes containing TSA (Merck) agar. The plates were incubated for 48 h at 37 °C or 41 °C. After incubation the plates showing total lysis were treated with 5 mL of PBS and agitated slowly for 10 min. The top agar was removed from the plates, transferred to Oak ridge tubes and centrifuged (Sigma) at 14.000 rpm for 10 min at 10 °C. The obtained supernatants were transferred to new sterile tubes and frozen at -80°C.

Determination of enterophages' resistance in different types of water

The samples of distilled, tap and wastewater were sterilized in autoclave for 15 min at 121 °C. To each water sample the enterophage isolate in volume of 225 µL was added and the samples were incubated at 37 °C with agitation; agitating might maintain uniform distribution of enterophages in the sample. In order to determine the enterophages' resistance the double layer

was performed. To 500 µL of water sample 1 mL of freshly regrown host *E. faecalis* was added together with $CaCl_2$ (Sigma) and NaN_3 (Sigma) salts in volume of 50 µL each. The sample was mixed with 4 mL of 1x TSB with agar (0.75% w/v) (BD) agar and the mixture was poured into sterile Petri dishes containing TSA medium. After 48 h incubation at 37 °C viral plaques were counted.

Comparison of different Enterococcus faecalis strains

The Porto Rican strain was tested against *E. faecalis* strains isolated from sewage samples collected in Portugal. The wastewater sample of volume between 10-20 mL was filtered for decontamination (0.2 µm filters). The freshly regrown host *E. faecalis* (5 mL) and 500µL of $CaCl_2$ (Sigma) and NaN_3 (Sigma) salts were added to the sample. The sample was then mixed slowly with 30 mL of liquefied 2x TSB with agar (1.5 % w/v) (BD), the mixture was poured into sterile Petri dishes and allowed to solidify. After 48 h incubation at 37 °C viral plaques are counted.

In order to isolate Portuguese *E. faecalis,* the samples were filtered using vacuum pump with membranes (0.22 µm, 47 mm Ø, Millipore®) and the membranes were placed on Slanetz-Bartley (S-B) (Bio-Rad) medium. After 48 h incubation at 37 °C, the single colonies of brownish-pinkish colour were passed to TSA (Merck) medium and incubated for 24 h at 37 °C. Finally, the Bile Aesculin test was performed. The isolated colonies were passed to Bile Aesculin (BEA) (Biokar Diagnosis) medium and incubated for 2 h at 44°C. The strains that presented best results in Bile Aesculin test were passed back to Slanetz-Barley (S-B) medium and kept in refrigerator at (5±3) °C for further analysis.

Nucleic acid analysis

For the purpose of nucleic acid analysis the enterophage nucleic acid was extracted using RTP® Bacteria DNA Mini Kit. The enteophage isolate was centrifuged at 10,000rpm for 3 min and 200 µL of supernatant was transferred carefully into new sterile eppendorf-like tube and the isolation of genetic material was performed as described by manufacturer.

The extracted nucleic acid of particular isolate in volume of 100 µL was treated with 10 µL of DNase, while another 100 µL of the same isolate remained untreated. Both samples were incubated for 30 min at 37 °C and subsequently at 95 °C for 15 min. After incubation, 3 µL of bromophenol blue were added to the samples in order to monitor progress of the samples through the agarose gel (SeaKem® LE Agarose, Cambrex) electrophoresis. The electrophoresis was conducted at 60 V with 1X TAE buffer; when finished, the gel was placed into ethidium bromide for 30-40 min. The visualized bands have being observed under UV lamp and recorded using G. Box (SynGene).

RESULTS AND DISCUSSION

Occurrence and quantification of enterophages in raw wastewater

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. 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. 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 (data not shown). 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 1).

Table 1. 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.

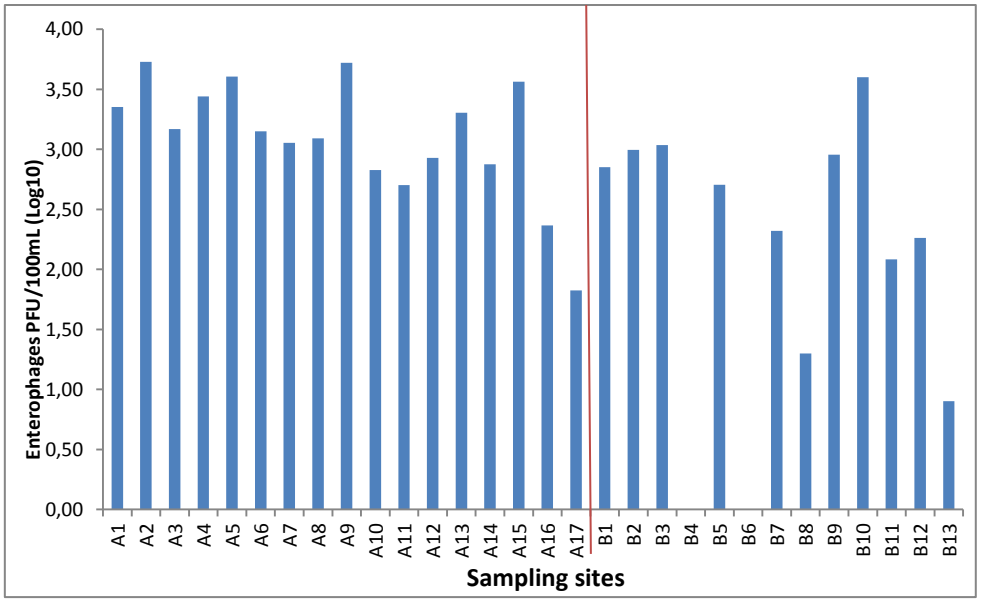

Figure 1. Average concentrations of enterophages at different sampling sites (touristic: A1-A17; non- touristic: B1-B13) over investigation period

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.

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 (data not shown). 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 (data not shown).

Table 2. 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.

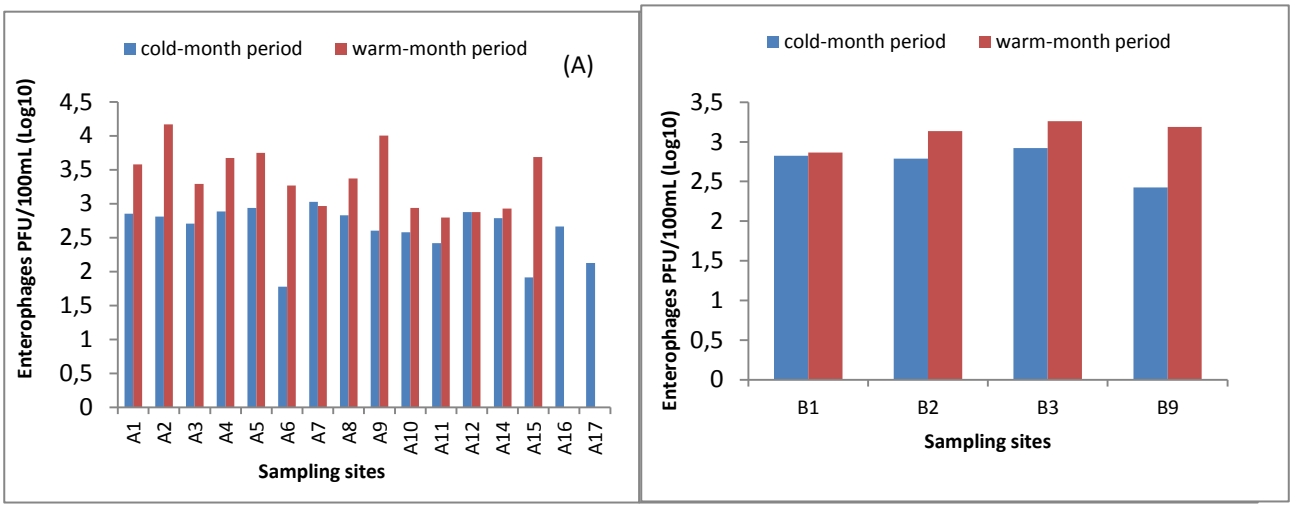

Figure 2. The comparison of enterophages' concentration at touristic (A) and non-touristic (B) sampling sites over warm- and cold-month periods

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 number of enterophages increased also for non-touristic 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.

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.

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. 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 2).

Table 3. 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

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].

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. The concentrations of enterophages in treated wastewaters varied from 3 PFU to 589 PFU/25mL (data not shown).

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.

Enterophage stability in different types of water

The analysis of enterophage stability was performed in distilled, tap and sewage water using double layer method. 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 (Figure 3 (A and B)). The T_{90} in these waters was equalled to 7 days (Table 10).

Table 4. 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

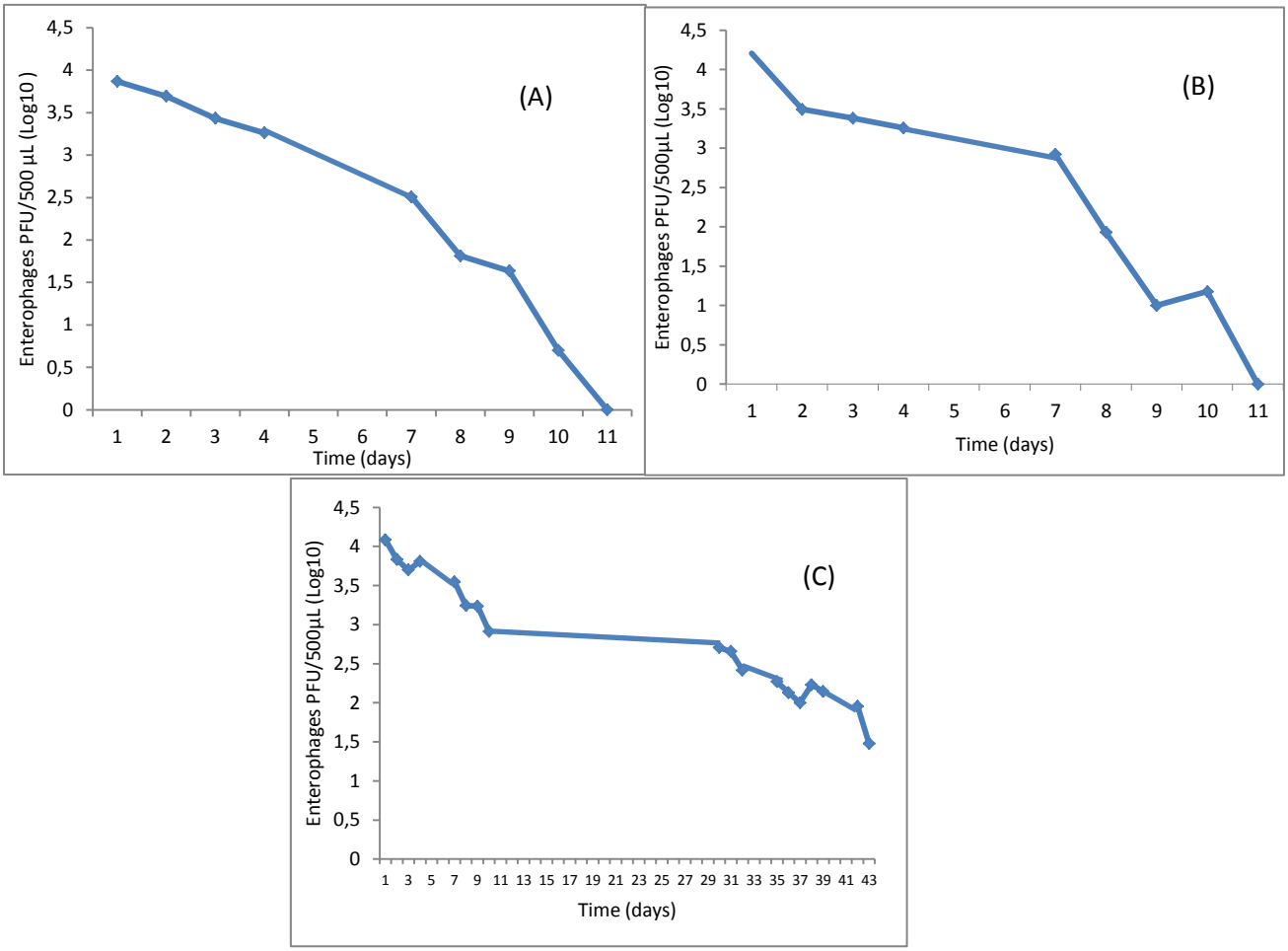

Figure 3. Curves of enterophages' stability in sewage (A), tap (B) and distilled (C) water

On the other hand, the stability of enterophages was significantly higher in wastewater (Figure 3 (C)). In this matrix, enterophages were detected up to 43th day, when their concentration equalled 30 PFU/500 µL (data not shown). The T_{90} for enterophages amounted to 50 days (Table 3). The highest stability of enterophages in wastewater can result from presence of organic matter.

Comparison of applicability of Enterococcus 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. faecali*s isolates from Portuguese sewage water, using single layer method.

The Table 4 shows the comparison of effectiveness of Puerto Rican strain and other Portuguese *E. faecalis* host strains for recovery of enterophages.

Table 5. 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 4). Additionally, the PR strain proved substantial sensitivity for recovering of enterophages. There were eight samples, which proved 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 4). The test for applicability of different *E. faecalis* has proved that overall Puerto Rican host strain was the most useful for detection of enterophages in Portuguese wastewaters.

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 4 (A, B and C) represent the results of nucleic acid analysis on overall 27 different enterophage isolates.

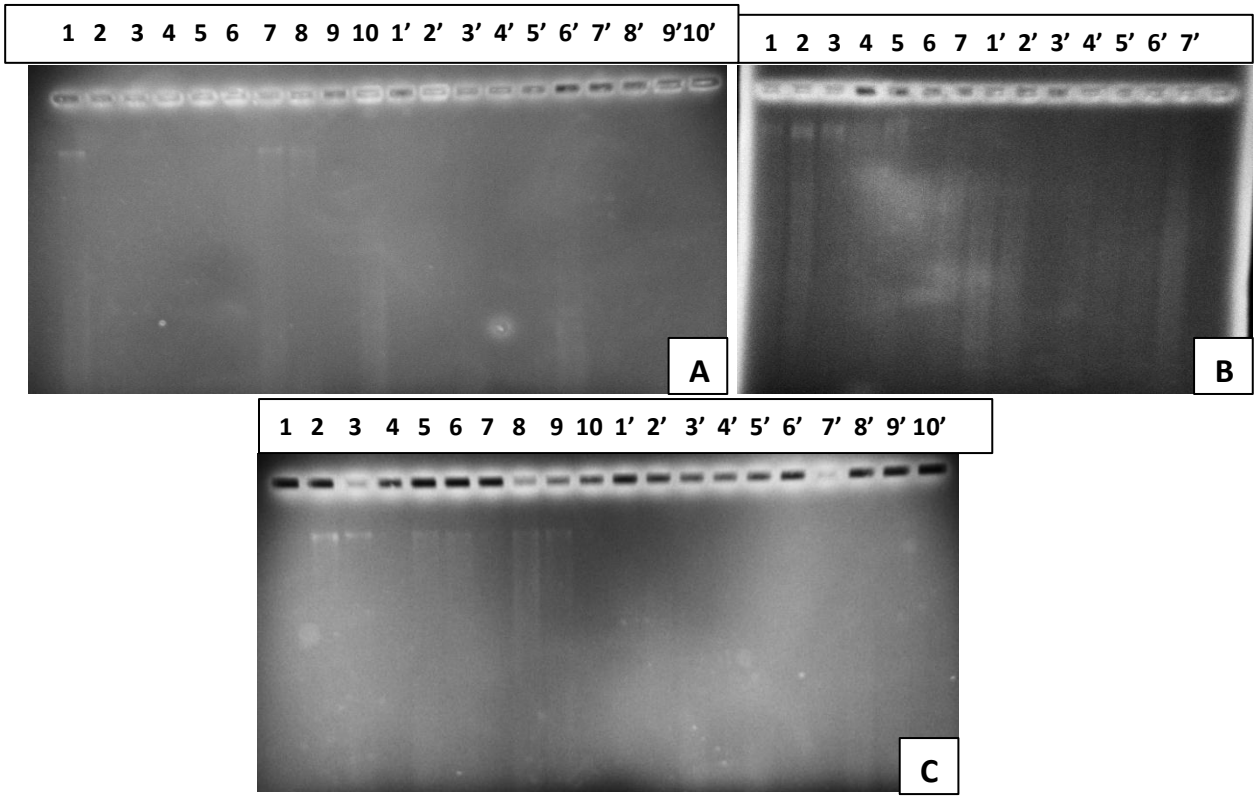

Figure 4. The genetic material of thirteen enterophage isolates in 1% agarose gel (A, B, C)

From the observation of Figure 4 (A) 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. The similar results are observable in the Figure 4 (B), in which the lanes 1', 7' and 8' revealed degradation of nucleic material by DNase, thus indicating dsDNA composition. The Figure 4 (C) 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.

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 Puerto 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 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 1). The high frequency of enterophage recovering indicated their prevalence in untreated wastewater and relatively simple detection. Moreover, the single layer method ensured 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 1). 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 might be 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 2). 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 2). These results corroborated the previous assumption that the increase in enterophages' population occurred due to 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 (Table 3). In the 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 3). For non-touristic locations, the concentrations of enterophages equalled 386 PFU/100mL and 350 PFU/100mL at 37 °C and 41 °C, respectively (Table 3). 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 (data not shown). The concentrations of enterophages in treated wastewaters varied from 3 PFU to 589 PFU/25mL (data not shown). The results of enterophage detection in treated wastewater may imply considerable sensitivity of enterophages to water treatment, since treatment practises may significantly reduce the concentrations of microorganisms in water [24, 25, 26].

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 (Figure 3 (A and B). The T_{90} in these waters was equalled to 7 days (Table 4). On the other hand, the stability of enterophages was significantly higher in wastewater (Figure 3 (C)). In this matrix enterophages were detected up to 43^{th} day, when their concentration equalled 30 PFU/500 µL (data not shown). The T_{90} for enterophages amounted to 50 days (Table 4). 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 4 (A, B and C)). 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 majority of 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 5). 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 5). The test for applicability of different *E. faecalis* has revealed the potential universality of Puerto 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.

REFERENCES

1. R.A.E. Barrell, P.R. Hunter, G. Nichols, Microbiological standards for water and their relationship to health risk, Commun Dis Public Health, 2000, vol. 3: pp. 8-13;
2. World Health Organization, Guidelines for safe recreational water environments, Volume 1: Coastal and fresh waters, Geneva, 2003;
3. M. J. Figueras, J. J. Borrego, New Perspectives in Monitoring Drinking Water Microbial Quality, Int J Environ Res Publ Health, 2010, vol. 7, pp. 4179-4202;
4. A. Touron, T. Berthe, G. Gargala, M. Fournier, M. Ratajczak, P. Servais, F. Petit, Assessment of faecal contamination and the relationship between pathogens and faecal bacterial indicators in an estuarine environment (Seine, France), Mar Pollut Bull, 2007, vol. 54, pp. 1441–1450;
5. T. M. Santiago-Rodríguez, C. Dávila, J. González, N. Bonilla, P. Marcos, M. Urdaneta et al., Characterization of Enterococcus faecalis-infecting phages (enterophages) as markers of human fecal pollution in recreational waters, Water Res, 2010, vol. 44, pp. 4716-4725;
6. A. Costán-Longares, M. Montemayor, A. Payán, J.Méndez, J. Jofre, R. Mujeriego et al., Microbial indicators and pathogens: Removal, relationships and predictive capabilities in water reclamation facilities, Water Res, 2008, vol. 42, pp. 4439-4448;
7. D. E. Nnane, J. E. Ebdon, H. D. Taylor, Integrated analysis of water quality parameters for cost-effective faecal pollution management in river catchments, Water Res, 2011, vol. 45, pp. 2235-2246;
8. V. K. Tyagi, A. K. Chopra, A. A. Kazmi, Arvind Kumar, Alternative microbial indicators of faecal pollution, Iran J Environ Health Sci Eng, 2006, Vol. 3, pp. 205-216;
9. A. Smyłła, Zagrożenia bakteryjne wód powierzchniowych, proceeding of: VII OGÓLNOPOLSKIA SESJA POPULARNONAUKOWA „ŚRODOWISKO A ZDROWIE – 2005", Częstochowa, 2-3 June, 2005, Available at: http://www.ietu.katowice.pl/wpr/Aktualnosci/Czestochowa/Referaty/Smylla.pdf (Accessed: 24 September 2011);

10. A. Dufour, M. Snozzi, W. Koster, J. Bartram, E. Ronchi, L. Fewtrell, Assessing Microbial Safety of Drinking Water: Improving Approaches and Methods, London, IWA Publishing on behalf of OECD, WHO, 2003;
11. J. A. Soller, M. E. Schoen, T. Bartrand, J. E. Ravenscroft, N. J. Ashbolt, Estimated human health risks from exposure to recreational waters impacted by human and non-human sources of faecal contamination, Water Res, 2010, vol. 44, pp. 4674-4691;
12. K. Pond, Water recreation and disease. Plausibility of Associated Infections: Acute Effects, Sequelae and Mortality, London, IWA publishing on behalf of WHO, 2005;
13. D. Kay, J. Fawell, An FWR Guide to Standards for Recreational Water Quality, Foundation for Water Research, 2007; available at: http://www.fwr.org/guides.htm (Accessed: 24 September 2011);
14. Council Directive 91/271/EEC of 21 May 1991 concerning urban waste-water treatment, European Council, 1991, OJ L 135, 30.05.1991, pp. 40-52;
15. P. Roccaro, G. Mancini, F. G.A. Vagliasindi, Water intended for human consumption-Part I: Compliance with European water quality standards, Desalination, 2005, vol. 176, pp. 1-11;
16. K. G. Field, M. Samadpour, Fecal source tracking, the indicator paradigm, and managing water quality, Water Res, 2007, vol. 41, pp. 3517 – 3538;
17. J. W. Santo Domingo, T. A. Edge, Identification of primary sources of faecal pollution, in: Safe Management of Shellfish and Harvest Waters, London, IWA publishing on behalf of WHO, 2010;
18. National Risk Management Research Laboratory, Microbial Source Tracking Guide Document, U.S. Environmental Protection Agency, 2005, Available at: http://www.epa.gov/nrmrl/pubs/600r05064/600r05064.pdf (Accessed: 24 September 2011);
19. C. L. Meaysa, K. Broersmab, R. Nordina, A. Mazumder, Source tracking fecal bacteria in water: a critical review of current methods, J Environ Manage, 2004, vol. 73, pp. 71-79;
20. C. Hagedorn, L. Xinqiang, Current and future trends in fecal source tracking and deployment in the LakeTaihu Region of China, Phys Chem Earth, 2011, vol. 36, pp. 352–359;
21. R. Mazaheri Nezhad Fard, M. D. Barton, M. W. Heuzenroeder, Novel Bacteriophages in Enterococcus spp., Curr Microbiol, 2010, vol. 60, pp. 400-406;
22. N. Bonilla, T. Santiago, P. Marcos, M. Urdaneta, J. S. Domingo, G.A. Toranzos, Enterophages, a group of phages infecting Enterococcus faecalis, and their potential as alternate indicators of human faecal contamination, Water Sci Technol, 2010, vol. 61, pp. 293-300;
23. Q. S. Meng, C. P. Gerba, Comparative inactivation of enteric adenoviruses, polioviruses and coliphages by ultraviolet irradiation, Water Res, 1996, vol. 30, pp. 2665-2668;

24. R. Sommer, W. Pribil, S. Appelt, P. Gehringer, H. Eschweiler, H. Leth et al., Inactivation of bacteriophages in water by means of non-ionizing (UV-253.7 nm) and ionizing (gamma) radiation: a comparative approach, Water Res, 2001, vol. 35, pp. 3109–3116;
25. C. Tartera, A. Bosch, J. Jofre, The inactivation of bacteriophages infecting *Bacteroides fragilis* by chlorine treatment and UV- irradiation, FEMS Microbiol Lett, 1988, vol. 56, pp. 313-316;