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PAPER

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

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The increasing problem of antibiotic resistance in common pathogenic bacteria and the concern about the spreading of antibiotics in the environment bring the need to find new methods to control fish pathogens. Phage therapy represents a potential alternative to antibiotics, but its use in aquaculture requires a detailed understanding of bacterial communities, namely of fish pathogenic bacteria. Therefore, in this study the seasonal dynamics of the overall bacterial communities, microbiological water quality and disease-causing bacteria were followed in a marine aquaculture system of Ria de Aveiro (Portugal). Analysis of the bacterial diversity of the water samples by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments indicates that the bacterial community structure varied seasonally, showing a higher complexity during the warm season. The diversity of the main fish pathogenic bacteria, assessed by DGGE targeting the *Vibrio* genus, showed lower seasonal variation, with new dominating populations appearing mainly in the spring. Bacterial indicators, faecal coliforms and *enterococci*, enumerated by the filter-membrane method, also varied seasonally. The fluorescent *in situ* hybridization (FISH) results showed that the specific groups of bacteria varied during the study period and that the non-indigenous Enterobactereaceae family was the most abundant group followed by *Vibrio* and *Aeromonas*. The seasonal variation detected in terms of density and structure of total and pathogenic bacterial communities demonstrates the need for a careful monitoring of water through the year in order to select the suitable phages to inactivate fish pathogenic bacteria. The spring season seems to be the critical time period when phage therapy should be applied.

Introduction

The increasing importance of aquaculture to compensate progressive worldwide reductions in the amount and quality of natural fish populations has contributed to aquaculture becoming one of the fastest growing productive sectors, providing nearly one-third of the world's seafood supplies. However, the growth and even the survival of the aquaculture industry are threatened by uncontrolled microbial diseases that cause extensive losses.[1]

Bacterial diseases are a major problem in the expanding aquaculture industry.[2,3] The level of contamination of aquaculture products will depend on the environment and the bacteriological quality of the water where the fish is cultured. There are

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Environmental impact

Bacterial diseases are a major problem in the expanding aquaculture. Although vaccination is the ideal method for preventing infectious diseases, commercially available vaccines are still very limited in the aquaculture field. Chemotherapy is a rapid and effective method to treat or prevent bacterial infections, but frequent use of antibiotics has allowed drug-resistant strains of bacteria to develop. This problem may be serious because few drugs are licensed for fisheries use. To reduce the risk of development and spreading of antibiotic resistant bacteria, other more environmentally friendly methods for controlling fish disease in aquaculture must be developed. In line with this idea the use of bacteriophage therapy in aquaculture seems to be very promising. There are several potential advantages of phage therapy over chemotherapy: (1) specific target, (2) limited resistance development, (3) limited impact, unlike antibiotics, phages are self-replicating as well as self-limiting, (4) regulatory approval, (5) high resistance of phages to environmental conditions, and (6) technologically flexible, fast and cheap.

two broad groups of bacteria of public health significance that contaminate products of aquaculture: those naturally present in the environment—indigenous microflora (*e.g. Aeromonas hydrophila*, *Clostridium botulinum*, *Vibrio parahemolyticus*, *Vibrio cholerae*, *Vibrio vulnificus* and *Listeria monocytogenes*) and those introduced through environmental contamination by domestic animals excreta and/or human wastes—non-indigenous microflora (*e.g.* Enterobactereaceae such as *Salmonella*, *Shigella*, and *Escherichia coli*).[4–6] These non-indigenous bacteria can be originated from point source discharges such as raw sewage, storm water, effluent from wastewater treatment plants and industrial sources. In addition, non-point source discharges such as agriculture, forestry, wildlife and urban run-off can also impair water quality.[7] Vibriosis and photobacteriosis (formerly pasteurellosis), caused by indigenous bacteria, are primarily diseases of marine and estuarine fish, both in natural and commercial production systems throughout the world, occurring only occasionally in freshwater fish. Both diseases can cause significant mortality in fish, reaching values of up to 100% in infected facilities, being currently responsible for most outbreaks in fish farming plants. Vibriosis and photobacteriosis are caused by bacteria from the family Vibrionaceae. Vibriosis is caused by species from the genera *Photobacterium* (namely *P. damselae* subsp. *damselae*, formerly *Vibrio damselae*) and *Vibrio* (namely *V. anguillarum*, *V. vulnificus*, *V. alginolyticus*, *V. parahaemolyticus* and *V. salmonicida*). Photobacteriosis is caused by *P. damselae* subsp. *piscicida* (formerly *Pasteurella piscicida*) that is a highly pathogenic bacterium that does not seem to have host specificity, infecting an ample range of fish species.[8,9] Other indigenous bacteria such as *Aeromonas salmonicida*, *Rickettsia*-like bacteria, *Cytophaga marina*, *Flavobacterium psychrophilum* and *Pseudomonas plecoglossicida* are also important groups of fish pathogens, affecting a variety of fish species from diverse geographical aquatic environments.[10] Although there is a rapid die-off of these bacteria in managed farm fish,[11,12] significant numbers of non-indigenous bacteria remain on the skin and in the guts of fish, causing disease to fish and constituting also a health risk to consumers.[13]

The enumeration of fish pathogens has been mainly carried out by culture-dependent methods and, consequently, data about the presence and abundance of the different bacterial groups of pathogens in aquaculture systems, related to the total bacterial community, are scarce. In addition, cultivation dependent analyses of pathogenic bacteria often require more than 3 days before completion of the assay. Recently, molecular tools have emerged as a powerful approach for fast detection and quantification of genes coding virulence factors and bacterial fish pathogens.

Due to the fast worldwide emergence of antibiotic-resistant bacterial pathogens in humans, medicine, agriculture and aquaculture regulators are severely limiting antibiotic usage. Phage therapy represents a potentially viable alternative to antibiotics and other antimicrobials. When compared to other methods based on the direct addition of antibiotic/disinfectant into the aquaculture systems, phage therapy presents a lower risk for fish,[6,14] inactivating pathogenic bacteria and avoiding fish contamination. The safety of the phage therapy approach is additionally increased by the fact that phages do not induce the selection of resistant bacterial strains.[14] The possibility to replace at least some of the currently used antimicrobials (*e.g.*, antibiotics bronopol, hydrogen peroxide, and formalin) that are potentially dangerous for fish and consumers and invariably cause some degree of environmental pollution adds further value to the phage therapy approach by minimizing the risk of undesired side effects and allowing a less risky repetition of the treatment. However, the success of phage therapy in aquaculture systems depends on a detailed understanding of the temporal dynamics of the most important pathogenic bacteria. The main goal of this work was to study the seasonal variation of overall bacterial community composition of microbiological water quality and quantify the prevalence of disease-causing bacteria in a marine aquaculture system of the Ria de Aveiro (Portugal). The data collected were used to identify critical time periods when phage therapy should be applied.

Experimental

Study area and sampling

This study was conducted in the semi-intensive aquaculture system Corte das Freiras located in the estuarine system Ria de Aveiro (latitude: 40°37′51.44″N, longitude 8°40′31.75″W) on the north-western coast of Portugal. Since the aquaculture is located near the city of Aveiro it is subjected to some contamination introduced by human wastes and, therefore subjected to chemotherapy treatment. The aquaculture is divided in ten earth ponds of approximately 2500 m^2 each, which are supplied with water from Ria de Aveiro. The gilthead seabream is stocked at 12 000 fish ha^{-1}. Fish semi-intensive culture for human consumption is an important economic activity in Ria de Aveiro.

Water samples were collected at early morning two hours before low tide, in mild weather conditions, from a culture tank of *Sparus aurata* (gilthead bream). Temperature salinity, dissolved oxygen and pH were measured in the field.

Samples from surface water were taken directly into sterile glass bottles and kept cold and in the shade during transport to the laboratory where they were processed within the next 1–2 hours.

In a first phase it was quantified the main pathogenic bacteria of fish in water samples collected on four dates: April 2007, October 2007, December 2007 and February 2008. In a second phase, the seasonal dynamics of the bacterial community structure was evaluated on November 2008, January 2009, March 2009, May 2009 and July 2009. The bacterial indicators (faecal coliforms and faecal *enterococci*) were analyzed during the entire study period, on eight different dates: October 2007, December 2007, February 2008, June 2008, November 2008, March 2009, May 2009 and July 2009. The quantification of these bacteria was done in order to evaluate the seasonal variation of contamination by non-indigenous bacteria in the aquaculture system.

During the sampling periods, antibiotics were not used in the aquaculture system.

Water properties

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

Evaluation of the seasonal dynamics of bacterial community structure and of *Vibrio* genus

Triplicate water samples of 300 mL were filtered through 0.22 μm pore-size filters (Poretics, USA). Collected cells were resuspended in 2 mL of TE buffer (pH 8.0) and centrifuged. After resuspension in 200 μL TE, 1 mg mL^{-1} lysozyme solution was added to induce cell lysis and incubated at 37 °C for 1 hour.[15] DNA extraction was performed using the genomic DNA purification kit (MBI Fermentas, Lithuania). DNA was resuspended in TE buffer and stored at −20 °C until analysis. The yield and quality of DNA were checked after electrophoresis in a 0.8% (w/v) agarose gel.

PCR amplification of an approximately 400 bp 16S rDNA fragment (V6–V8) was performed using the primer set F968GC and R1401.[16] The reaction was carried in a Multigene Gradient Thermal Cycler from MIDSCI. The 25 μL reaction mixture contained approximately 50 to 100 ng of extracted DNA, 1× PCR buffer, 3.75 mM $MgCl_2$, 0.2 mM of deoxynucleoside triphosphates, 0.1 μM of each primer, 1 U of Taq Polymerase (MBI Fermentas, Lithuania). Acetamide (50%, 0.5 μM) was also added to the reaction mixture. The amplification protocol included a 4 min initial denaturation at 94 °C, 34 cycles of 95 °C for 1 min, 53 °C for 1 min and 72 °C for 1 min 30 s, and a final extension for 7 min at 72 °C.

The diversity of the *Vibrio* genus was analyzed after amplification of bacterial DNA using the primers Vib-F (727) 5′-AGG CGG CCC CCT GGA CAG A-3′ and Vib-R (1423) 5′-ARA CTA CCY RCT TCT TTT GCA GC-3′.[17] Each PCR reaction mixture contained: 1× PCR buffer, 0.2 mM d-NTP's, 2.5 mM $MgCl_2$, 0.5% DMSO; 0.2 μM of each primer, 2.5 U of Taq Polymerase, 50 ng of the DNA template and 13.25 μL of distilled deionized water to a total reaction volume of 25 μL. Thermal cycling was as follows: 1 cycle of 7 min at 94 °C; 30 cycles of 1 min at 94 °C, 1 min at 56 °C and 1 min at 72 °C and a final extension of 72 °C for 10 min. Positive (DNA from a known bacterial species) and negative (water only) controls were included in every PCR reaction. PCR products were checked using standard agarose gel electrophoresis and ethidium bromide staining.

DGGE was performed with a CBS System (CBS, USA). PCR products were loaded onto 6–9% polyacrylamide gel in 1× TAE buffer. The 6–9% polyacrylamide gel (bisacrylamide : acrylamide = 37.5 : 1) was made with a denaturing gradient ranging from 32 to 60% (100% denaturant contains 7 M urea and 40% formamide). A marker standard composed by 11 bands halting at different denaturant concentrations was included in the extremities of each gel. Electrophoresis was performed at 60 °C for 16 h at 150 V. Following electrophoresis, the gels were incubated for silver staining. The solutions used were 0.1% (v/v) ethanol plus 0.005% acetic acid for fixation, 0.3 g silver nitrate for staining, freshly prepared developing solution containing 0.003% (v/v) formaldehyde, 0.33% NaOH (9%), and finally, 0.75% sodium carbonate solution to stop the development. The gels were digitalized and analyzed with the software package Gelcompar 4.0 program (Applied Maths) as previously described by Smalla *et al.*[18] After automatic band search, the bands detected were carefully checked and artefacts were removed. The sets used for band detection were 5% minimal profiling (area along the densitometric curve) and 0.5% minimal area. The positioning and quantification of bands were carried out by setting tolerance and optimization at 5 points, *i.e.* 1.0%. The band positions and their corresponding intensities from each treatment were exported to Excel files and the band surface was converted to relative intensity by dividing its surface by the sum of all band surfaces in a lane. Bray–Curtis similarities were calculated based on the band position and intensity.

Quantification of the seasonal variation of bacterial indicators of faecal contamination (non-indigenous bacteria)

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

Relative abundance of the main fish pathogenic bacterial groups

Samples were filtered through 0.22 μm polycarbonate filters (GE Osmonics), fixed with 4% paraformaldehyde for 30 min and rinsed with PBS 1× and MilliQ water. The filters were stored at room temperature until hybridization. The relative abundance of specific groups of bacteria was determined by *Fluorescent in situ hybridization* (FISH) using 16S rRNA target probes[19] labeled with CY3. The probe Eub338-II-III[20] was used to quantify bacteria belonging to the Domain Bacteria. The bacteria belonging to the non-indigenous Enterobactereaceae family and to the indigenous *Vibrio*, *Aeromonas* and *Pseudomonas* genera were detected with the specific probes ENT183,[21] VIB572a,[22] AERO1244[23] and Pae997,[24] respectively.

For each probe, three filter sections were placed on a parafilm-covered glass slide and overlaid with 30 μL hybridization solution with 2.5 ng μL^{-1} of probe. The hybridization solution contained 0.9 M NaCl, 20 mM Tris–HCl (pH 7.4), 0.01% SDS, and the optimum concentration of formamide for each probe.[25] Filters were incubated in sealed chambers at 46 °C for 90 min. After hybridization, filters were washed for 20 min at 48 °C in wash solution (20 mM Tris–HCl pH 7.4, 5 mM ethylenediaminetetraacetic acid, 0.01% SDS, and the appropriate concentration of NaCl).[25] Rinsed and dried filter pieces were counterstained with 2 μg mL^{-1} 4′,6-diamidino-2-phenylindole (DAPI) and mounted on glass slides with Vectashield and Citifluor (1 : 4). The DAPI staining provides a measure of the abundance of total microorganisms. Samples were examined with a Leitz Laborlux K microscope equipped with the appropriate filter sets for DAPI and CY3 fluorescence. At least 15 fields were counted for each of the three replicates.

Statistics

Total bacterial numbers and the relative abundance of specific bacterial groups were tested for normality (Kolmogorov–Smirnov test) before the comparison of means. Parametric analysis of

variance (ANOVA) was performed, providing that data were normally distributed and that the variance of group means was homogeneous (Levene test).

Results

Water properties

The ranges of values of the parameters that describe water properties are summarized in Table 1. In the aquaculture system, salinity varied between 35.7 in October 2007 and 16.7 in December 2007. Temperature oscillated from 10.8 °C in November 2007 to 20.8 °C in June 2008 and the dissolved oxygen ranged between 1.6 mg L^{-1} in July 2009 and 6.0 mg L^{-1} in April 2006. pH varied between 7.4 in April 2006 and 8.2 in May 2009.

Seasonal dynamics of bacterial community structure and of *Vibrio* genus

Bacterial community structure was examined by comparing DGGE profiles of 16S rDNA fragments during the different sampling moments (Fig. 1). Reproducibility of PCR amplification and DGGE was confirmed by similar results obtained for the three sub-samples analyzed at each date (Fig. 1A). DGGE profiles revealed seasonal differences in the structure of bacterial communities, with a higher diversity of ribotypes during the warm season, when the temperature reached the higher values. Bray–Curtis similarity index for bacterial community ranged between ~25% and ~97%, varying widely between sampling moments. Cluster analysis of the band patterns obtained from DGGE analysis (Fig. 1B) revealed the occurrence of weak similarities (as low as 25%) between the bacterial community structures in water samples collected in the different months. The bacterial community in the water samples collected in March showed the lowest value of similarity (~25%) with those detected in the remaining sampling periods. For the other months, the bacterial communities present in the water samples collected in November and January showed the highest similarity index (>85%).

Cluster analysis of the band patterns obtained from DGGE analysis (Fig. 2A) revealed that the diversity of *Vibrio* in water samples collected in November, when temperature reached the lowest values, showed the lowest similarity value (<80%) with the remaining sampling moments (Fig. 2B). The bacterial communities displayed the highest similarity values (~92%) in the

Table 1 Water properties in the aquaculture system during the study period

Sampling dates	Salinity	Temperature/°C	Dissolved oxygen/mg L^{-1}	pH
April 2007	32.1	17.7	6.0	7.4
October 2007	35.8	16.9	4.6	7.9
December 2007	16.7	13.4	2.9	8.0
February 2008	31.0	16.0	3.4	8.1
June 2008	31.4	20.8	5.7	7.7
November 2008	29.5	10.8	2.9	8.1
January 2009	19.7	12.8	2.7	8.0
March 2009	31.0	13.8	2.4	8.1
May 2009	31.4	17.5	2.7	8.2
July 2009	32.8	20.1	1.6	8.0

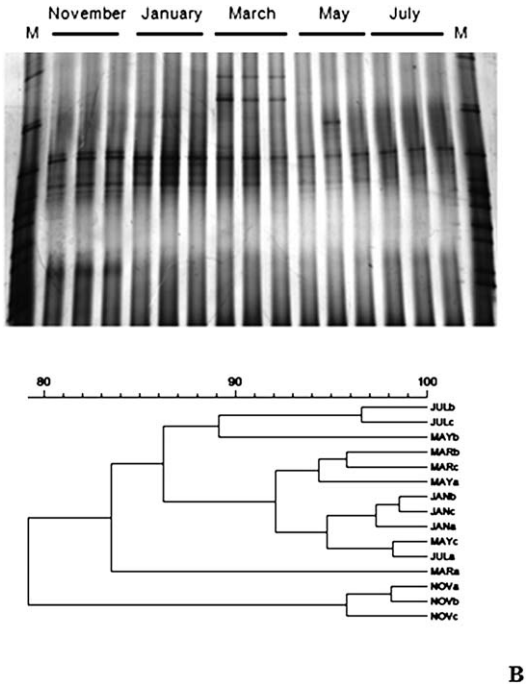

Fig. 1 Seasonal DGGE profile of 16S rDNA of bacterial communities of the aquaculture system (A) and dendrogram generated from the pattern of bands obtained by DGGE (B). M—molecular weights marker.

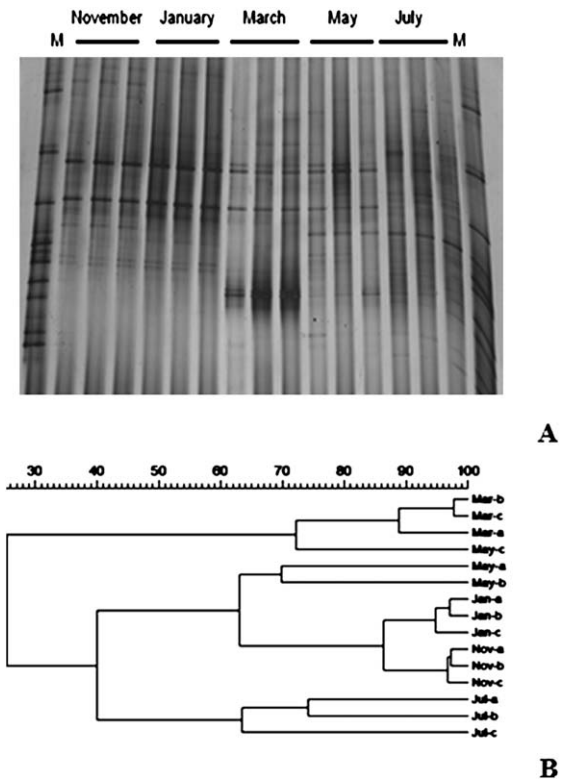

Fig. 2 Seasonal DGGE profile of *Vibrio* 16S rDNA in the aquaculture system (A) and dendrogram generated from the pattern of bands obtained by DGGE (B). M—molecular weights marker.

months of January and March. The highest diversity of ribotypes was observed during the spring season.

Quantification of bacterial indicators of faecal contamination

The variation of the concentration of faecal coliforms and enterococci in the water of aquaculture system is represented in Fig. 3. The concentrations of faecal coliforms were, in general, slightly higher than those of *enterococci*. The differences observed for faecal coliforms and faecal *enterococci* densities during the sampling period were significant (ANOVA, $p < 0.05$).

In general, the values of the faecal indicators decreased during the sampling period. The highest values of *enterococci* were obtained in October 2007 (45.3 UFC 100 mL^{-1}) and the lowest in May 2009 (5.3 UFC 100 mL^{-1}). Over the cold months (October, November and December) a clear decrease in the abundance of faecal coliforms was noticed, reaching its lower values on May and June. The highest values of faecal coliforms were obtained in July 2009 (36.3 UFC 100 mL^{-1}) and the lowest in June 2008 (4.0 UFC 100 mL^{-1}).

Quantification of main pathogenic bacteria of fish

The variation in the relative abundance of bacteria belonging to *Bacteria* domain as well as Enterobactereaceae family and *Vibrio*, *Aeromonas* and *Pseudomonas* genera detected by FISH is represented in Fig. 4. The relative abundance of the *Bacteria* domain varied between 76.63 ± 1.05% in December 2007 and 95.40 ± 0. 97% in February 2008, relatively to the DAPI counts. Total bacterial numbers were not significantly different during the sampling period (ANOVA, $p > 0.05$).

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

The relative abundance of Enterobactereaceae family was higher in October 2007 (5.77 ± 0.53%), decreasing over the winter and spring seasons and reaching its lower values on April 2007 (2.29 ± 0.7%), when temperature and dissolved oxygen reached the highest values. *Aeromonas* showed the highest relative abundance values in December 2007 (5.55 ± 0.23%) and the lowest on February 2008 (1.05 ± 0.22%). An increase in the relative abundance of *Vibrio* was observed between October 2007 (1.94 ± 0.17%) and February 2008 (3.86 ± 0.04%). The highest values of *Pseudomonas* were obtained in April 2007 (2.29 ± 0.2%) and the lowest in February 2008 (0.96 ± 0.04%).

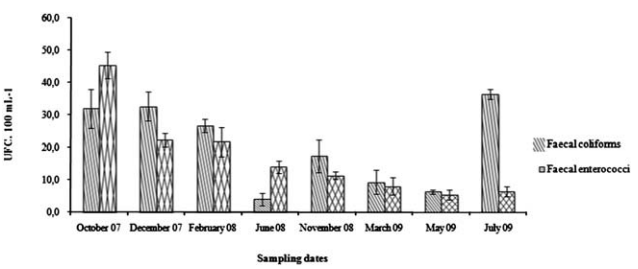

Fig. 3 Seasonal variation of faecal coliforms and faecal *enterococci* in the aquaculture system.

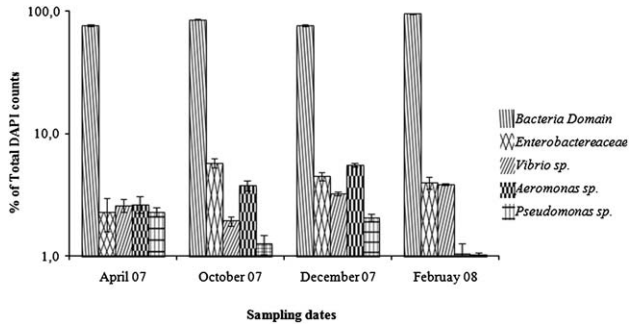

Fig. 4 Relative abundance of *Bacteria* domain, Enterobactereaceae family, *Vibrio*, *Aeromonas* and *Pseudomonas* genera detected by FISH in the aquaculture system.

Discussion

The success of phage therapy depends on the knowledge of the density of the main pathogenic bacteria as well as of their seasonal variation. In general, total bacterial numbers were fairly constant over the year, but the relative abundance of specific bacterial groups varied significantly during the sampling period. Among potentially pathogenic bacteria, Enterobactereaceae were the most abundant, indicating that non-indigenous pathogenic bacteria are an importance source of contamination in this aquaculture system. In fact, the indicators of faecal contamination, faecal coliforms and faecal *enterococci*, were present during the entire sampling period in the aquaculture system, confirming the importance of these bacterial groups in aquaculture. Although there is a rapid die-off of these enteric bacteria in managed farm fish,[11,12] significant numbers of those bacteria remain on the skin and in the guts of fish and can cause a health risk to consumers.[13] The occurrence of higher numbers of Enterobactereaceae and of faecal indicators during the cold season when rain is more abundant suggests that these non-indigenous enteric bacteria are transported by runoff from land and by resuspension of bottom sediments. On the other hand, the rise in salinity during the warm season constitutes a difficulty against non-halophilic bacteria growth in aquaculture systems.

Vibrio and *Aeromonas* genera that include *Aeromonas salmonicida*, causative agent of furunculosis,[26] that is capable of infecting a wide range of host species, and members of the family Vibrionaceae that are currently responsible for most outbreaks in fish farming plants,[8,9] were also present at high concentrations in the aquaculture system (corresponding to 10% of DAPI counts). The abundance of *Vibrio* and *Aeromonas* was higher than that observed in a culture tank water of eel, reaching values similar to those obtained for eel slime samples.[27] Consequently, phages of Enterobactereaceae, *Vibrio* and *Aeromonas* groups must be considered as relevant targets of phage therapy. The culture-independent *in situ* hybridization approach using specific probes provides an overview of the real proportion of different cultivable and non-cultivable pathogenic bacterial groups and their

evolution in aquaculture systems. This information, together with the monitoring of faecal contamination, is of great value to decide which phages should be selected for phage therapy.

The DGGE results showed that the bacterial community structure in general and, more specifically, that the diversity of the bacterial group most implicated in fish disease outbreaks (*Vibrio* genus) in fish farming plants varied seasonally, indicating that it is necessary to take in consideration this variation when specific phages are selected to inactivate fish pathogenic bacteria. Although the seasonal variation of the *Vibrio* genus was reduced in comparison to that of the total bacterial community, the primers used to analyze the diversity of fish pathogens belonging to this genus (*e.g. V. anguillarum*, *V. vulnificus*, *V. alginolyticus* and *V. parahaemolyticus*) also detect species from the *Photobacterium* genus (*P. damselae* subsp. *damselae*, formerly *Vibrio damselae*; and *P. damselae* subsp. *piscicida*, formerly *Pasteurella piscicida*) that cause vibriosis and photobacteriosis (formerly pasteurellosis) that are primarily diseases of marine and estuarine fish, both in natural and commercial production systems throughout the world.[8,9] Posterior sequencing of the main bands from the DGGE profiles of the total community and *Vibrio* genus will provide a better understanding of the species present in the samples and their possible pathogenic potential.

Previous work in this aquaculture system[28] showed that some other factors of variation, namely the chemical disinfection of the water, affect the seasonal pattern of variation of bacterial abundance and diversity, emphasizing the importance of monitorization of the bacterial community in order to implement phage therapy. As the aquaculture is located near the city of Aveiro, the water is subjected to some contamination introduced by human wastes and, therefore, chemotherapy treatment is frequently applied. The chemical treatment applied is selective in relation to the bacterial community, affecting differently the bacterial groups.

The overall bacterial community and the disease-causing bacteria as well as the indicators of microbiological water quality of the aquaculture system show clear but distinct patterns of seasonal variation. The higher concentration of the main fish pathogenic bacteria and of bacterial indicators of faecal contamination during the rainy season and the higher diversity of the *Vibrio* genus in spring indicate that the risk of disease outbreak in the aquaculture system may vary throughout the year. However, the springtime, when *Vibrio* genus, the pathogenic bacteria currently responsible for fish infections in the studied fish-farming plant, displayed the highest diversity, seems to be a crucial time for disease outbreaks.

Conclusions

The seasonal variation of the overall bacterial community and of the disease-causing bacteria as well as of the indicators of microbiological water quality demonstrates the need for a careful monitoring of water through the year in order to select the suitable phages to inactivate fish pathogenic bacteria. The higher complexity of the total bacterial community during the warm season and the appearing of new dominating populations of the main fish pathogenic bacteria mainly in the spring suggest that the spring season is the critical time period when phage therapy should be applied.

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