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

C. Pereira [a], L. Santos [a], A.P. Silva [a], Y.J. Silva [a], A. Cunha [a], J.L. Romalde [b], M.L. Nunes [c], A. Almeida [a,*]

[a] Department of Biology and CESAM, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
[b] Departamento de Microbiología e Parasitología e CIBUS e Facultad de Biologia, Universidade de Santiago de Compostela e Campus Universitario Sur., 15782 Santiago de Compostela (A Coruña), Spain
[c] National Institute of Biological Resources (INRB, IP/L-IPIMAR), Av. Brasília, 1449-006 Lisbon, Portugal

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ABSTRACT

The recurrent emergence of infections outbreaks associated with shellfish consumption is an important health problem, which results in substantial economic losses to the seafood industry. Even after depuration, shellfish is still involved in outbreaks caused by pathogenic bacteria, which increases the demand for new efficient strategies to control the shellfish infection transmission. Phage therapy during the shellfish depuration is a promising approach, but its success depends on a detailed understanding of the dynamics of bacterial communities in the harvesting waters. This study intends to evaluate the seasonal dynamics of the overall bacterial communities, disease-causing bacterial populations and bacterial sanitary quality indicators in two authorized harvesting-zones at Ria de Aveiro.

During the hot season, the total bacterial community presented high complexity and new prevalent populations of the main shellfish pathogenic bacteria emerged. These results indicate that the spring/summer season is a critical period during which phage therapy should be applied.

1. Introduction

Bivalve shellfish is a nutritive food source whose consumption and commercial value have risen dramatically worldwide. Bivalves, such as mussels, clams and oysters, as filter feeding organisms, can concentrate contaminants from the surrounding water, including microorganisms that can cause several infectious diseases to humans (Huss et al., 2000; Muniain-Mujika et al., 2003; Butt et al., 2004; FAO, 2004; Huss et al., 2004; Brands et al., 2005; Robertson, 2007). Moreover, shellfish like oysters and sometimes clams or cockles, is often consumed raw or just lightly cooked (Bosch et al., 2009). This cooking habit, together with the fact that the whole animal, including viscera, is consumed, represents a major public health concern since shellfish act like passive carriers of human pathogenic microorganisms (Lees, 2000; Romalde et al., 2002; Murchie et al., 2005; Bosch et al., 2009).

Pathogenic bacteria associated with seafood such as bivalves can be categorized into three general groups: indigenous, non-indigenous and processing contamination (Reilly and Käferstein, 1997; Reilly, 1998; Feldhusen, 1999; Iwamoto et al., 2010). *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio vulnificus*, *Clostridium botulinum* and *Aeromonas hydrophila* are indigenous bacteria of marine or estuarine environments. Non-indigenous enteric bacteria such as *Salmonella* spp., pathogenic *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., and *Yersinia enterocolitica* result from faecal contamination. Some bacteria, such as *Bacillus cereus*, *Listeria monocytogenes*, *Staphylococcus aureus* and *Clostridium perfringens*, are introduced during processing (Feldhusen, 2000).

The microbiological control of shellfish products, as well as the classification of growing areas according to the sanitary quality, assumes particular importance (Anonymous, 2010). Adequate safeguards can be valuable in minimizing the probability of shellfish microbial contamination, from harvesting to consumption, and in the protection of public health. In Europe, the Directives 2006/113/CE (Anonymous, 2006) and 2004/41/CE (Anonymous, 2004) are guidelines to control the levels of microbiological indicators of shellfish. The classifications of bivalves harvesting areas in Europe are based on the *E. coli* concentration measured in 100 g of flesh bivalve and intra-valvular liquid (FIL). The harvesting zones are group in four classes, from A through D. The sequence corresponds to healthy, non-healthy, highly non-healthy and forbidden harvesting when the most probable number (MPN) of *E. coli* per 100 g of FIL is $\leqslant$230, $\leqslant$4600, $\leqslant$46,000 and >46,000, respectively. This classification defines whether shellfish can be sent directly for consumption or needs prior treatment before its commercialization (Lees, 2000). All shellfish sent for direct human consumption without any additional processing must comply with the

* Corresponding author. Tel.: +351 234370350; fax: +351 234372587.
E-mail address: aalmeida@ua.pt (A. Almeida).

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standard of less than 230 *E. coli* in 100 g of FIL in more than 90% of samples. Harvesting from polluted (category B and C) areas is allowed but shellfish must undergo treatment, before being commercialized. In this case, shellfish can be placed on the market for human consumption following controlled self-purification in tanks of clean seawater (commercial depuration), prolonged relaying in clean seawater or commercial heat treatment or processing by any other acceptable method (Jones et al., 1991; Lees, 2000; Murchie et al., 2005). Where *E. coli* concentrations exceed 46,000 per 100 g of FIL (category D) harvesting is forbidden.

Depuration is a useful method to eliminate microorganisms from bivalves when conducted under conditions that maximize the natural filtering activity, which results in expulsion of intestinal contents. However, some pathogenic microorganisms are resistant to this process (FAO, 2008; Martínez et al., 2009; Rong et al., 2014). To reduce the risk of the development and transmission of infections caused by microbial pathogens, including multidrug-resistant bacteria, other technologies associated with depuration, such as phage therapy (application of lytic phages to prevent and/or to treat bacterial infections) must be developed. The association of the phage therapy with the depuration process would contribute to the improvement of the decontamination, reducing the time required, and consequently the production costs, increasing the bivalve safety and quality. As specific pathogen-killers, bacteriophages are effective agents for controlling bacterial infections, without affecting the normal flora (Park and Nakai, 2003; Hawkins et al., 2010). Moreover, this is a relatively inexpensive method (Almeida et al., 2009). The potential of the use of bacteriophages to control bacterial diseases has been reported across diverse fields by numerous researchers (Nakai and Park, 2002; Park and Nakai, 2003; Vinod et al., 2006; Karunasagar et al., 2007; Almeida et al., 2009; Pereira et al., 2011a; Stelios et al., 2011; Steven et al., 2011; Bueno et al., 2012; Lim et al., 2012; Mateus et al., 2014a,b; Rong et al., 2014; Silva et al., 2014). However, the inherent phage specificity, which is one of the major advantages of phage therapy, requires an extensive knowledge of phage bacterial hosts, including their spatial and temporal dynamics. Therefore, in order to apply a successful phage therapy during the depuration of bivalves it is essential to conduct a detailed study of the dynamics of the whole natural microbial community in the harvesting waters, focusing on the most important pathogenic bacteria (Pereira et al., 2011b).

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

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

2. Material and methods

2.1. Study area and sampling

Ria de Aveiro is an estuarine system located in the northwestern coast of Portugal (8″44′W, 40′39′N) with an area of 47 km^2 (Almeida and Alcântara, 1992) connected to the Atlantic by a narrow opening (Fig. 1), where the culture of bivalves is an activity of great socio-economic importance. The most exploited species are: grooved carpet shell clam (*Venerupis decussatus*), pullet carpet shell clam (*Venerupis pullastra*), cockle (*Cerastoderma edule*), blue mussel (*Mytilus edulis*) and grooved razor shell clam (*Solen marginatus*), reaching an annual production of about 5000 tons (Sobral et al., 2000).

Samples were collected in two authorized harvesting zones. One located in Mira channel (40°36′30″N, 8°44′52″W), classified as a statute B (230–4600 MPN *E. coli* per 100 g of FIL) and the other in Ílhavo channel (40°37′50″N, 8°41′9″W) classified as a statute C (4600–46,000 MPN *E. coli* per 100 g of FIL) (Anonymous, 2013). Ílhavo channel (CI) is close to port and industrial facilities and receives the effluent of the sewage treatment plant. Mira channel (CM) is a recreational area which has almost no industrial activity and it is subjected to some anthropogenic contamination. Sampling was performed on six dates in February, April, June, August, October and December 2012. In each sampling site, four water samples were collected at 20 cm below surface with sterile glass bottles. Samples were kept cold in refrigerated boxes and immediately transported to the laboratory, where they were processed within a period of 1–2 h.

2.2. Meteorological conditions and water properties

Precipitation and solar irradiance prior to sampling events were recorded at the meteorological station of the University of Aveiro, located on the vicinity of sampling sites. Temperature and salinity were measured in the field, using a WTW LF 196 Conductivity Meter. Dissolved oxygen was also determined in the field with a

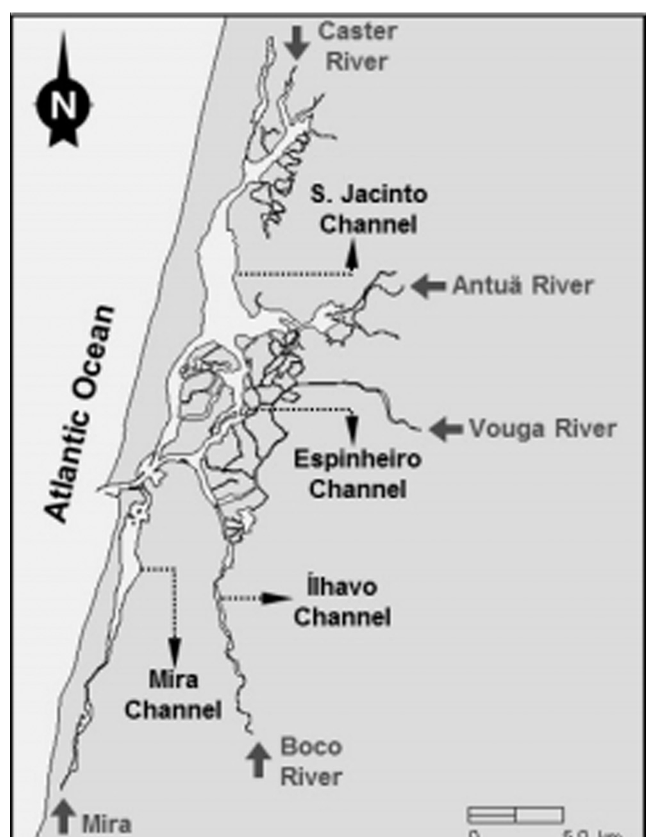

Fig. 1. Ria de Aveiro lagoon (Portugal) with the main channels of the estuarine system, channels indicated with arrows.