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Review

Microbial contamination and purification of bivalve shellfish: Crucial aspects in monitoring and future perspectives – A mini-review

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ABSTRACT

Shellfish are a nutritious food source whose consumption and commercial value has risen dramatically worldwide. Although bivalve's consumption can contribute to a healthy diet, some can cause foodborne illnesses. Microbial contamination is chronic and pervasive in harvesting areas and may be passed on to the consumers. Current food safety programs intend to protect consumers. Nevertheless, bivalve's microbial contamination is underestimated and undermanaged, which can pose a potential public health risk. We intend to provide an updated overview of the microbial assessment of bivalves and emerging alternatives or complementary perspectives for the elimination of microbial contamination. Further research is needed for the improvement of public health control and the enhancement of shellfish safety.

Contents

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1. Introduction

It is believed that less processed or natural foods are healthier. Nevertheless, for some products this may be an oversimplification and represents a greater risk to consumers. Bivalve shellfish fit this description (Murchie et al., 2005). For dietetic, traditional or food availability reasons, consumption of bivalves has been rising dramatically worldwide (Fauconneau, 2002; Johnson & Hayasaka, 1988; Murchie et al., 2005). On the other hand, microbial contamination is chronic and pervasive in growing and harvesting areas. By filter-feeding from the surrounding water, bivalves bioaccumulate natural occurring or anthropogenic contaminants, arising this contamination to the consumer (Lees, 2000). Contamination includes pathogenic species capable of producing diseases outbreaks (WHO, 2010). In general, HACCP procedures and product processing applied to food products are sufficient to protect consumers from the risk of diseases. However, shellfish, because of their unique nature have their own distinct aspects of harvesting, processing and handling. Furthermore, bivalves are minimally processed, and traditionally consumed raw or lightly cooked as a whole (visceras included) (Lees, 2000; Murchie et al., 2005; Romalde et al., 1994). Recently, there has been observed an increasing concern regarding food safety, particularly in molluscan shellfish products. Extensive efforts have been pursued to assure a safe supply of bivalves, but disease and death due to viruses and naturally occurring bacteria have been observed. This might be a result of underestimated and undermanaged microbial contamination.

This mini-review focuses on critical aspects related to shellfish safety for human consumption with the aim of serving as a general reference in future investigations. The drawbacks in depuration and relaying processes, encountering potential indicators for human enteric viruses as well as indigenous marine bacteria and the methodology applied to quantify conventional indicators are pointed out. Emerging perspectives regarding the elimination of microbial contamination and the enhancement of shellfish safety are also discussed providing guidelines for future work in monitoring the health of bivalves.

2. Importance of bivalves

Bivalves, as a food component, are characteristically tender, easily digested, additive-free and minimally processed. These characteristics make them a product that almost completely fulfils the demands of consumers (Murchie et al., 2005). These animals also have high-quality animal protein content which is similar to that of milk and eggs making them a nutritive food and an important component in the human diet worldwide (Bernardino, 2000; FAO, 2006; Fauconneau, 2002; Murchie et al., 2005; Sapkota et al., 2008). This is particularly relevant in developing countries where aquatic products are often the only source of animal protein (Fauconneau, 2002).

The importance of bivalve shellfish as a food supply increases if we attend to the natural resource that shellfish growing areas may represent (Johnson & Hayasaka, 1988). Dense beds of bivalve shellfish (epifaunal or infaunal species) occur in inshore estuaries with high primary productivity and have been an important source of food since prehistory (Lees, 2000). However, the aquatic environment is becoming over-exploited and as a consequence of overcatching the depletion of stocks is leading to the reduction of natural shellfish beds and to the need of human intervention in its production (Pillay & Kutty, 2005). The outcome is the development of artificial bivalve shellfish production and exploitation by the food industry (Hernroth, Conden-Hansson, Rehnstam-Holm, Girones, & Allard, 2002). Aquaculture production has been exponentially increasing and becoming one of the fastest-growing food industries, especially in Asia (Defoirdt, Boon, Bossier, & Verstraete, 2004; FAO, 2006; Sapkota et al., 2008). Fig. 1 shows aquaculture production both in quantity and in economic significance for fishes, molluscs, crustaceans and other aquatic animals in 2006 (FAO, 2009). Freshwater finfish represented half of global aquaculture production (54%) and molluscs were the second largest aquaculture product produced worldwide (24%) (FAO, 2009). The oyster culture, particularly *Crassostrea gigas*, dominates the global production of molluscs (Berthe, 2005; FAO, 2006). The Manila clam (*Ruditapes philippinarum*), the Yesso scallop (*Patinopecten yessoensis*), the blue mussel (*Mytilus edulis*) and the blood cockle (*Anadara granosa*) are also widely produced species (Berthe, 2005). Crustaceans come next in relevance, in terms of production, represented mostly by penaeid shrimps and grapsid crabs (FAO, 2006, 2009).

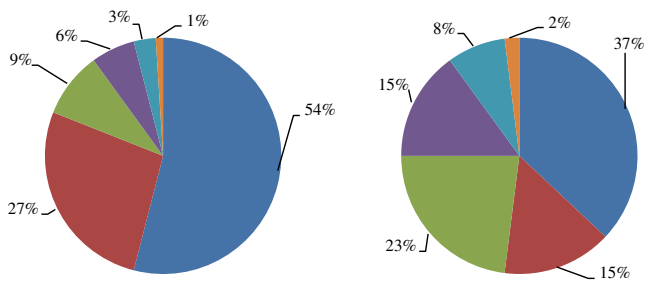

Fig. 1. World aquaculture production in quantity (left) and respective economic significance (right) of major taxonomic families groups in 2006 (FAO, 2009).

3. Bivalves contamination and their risk as vehicles of disease

Contamination of bivalve shellfish occurs mainly because they are suspension feeders that selectively filter small particles of phytoplankton, zooplankton, viruses, bacteria and inorganic matter from the surrounding water (Burkhardt & Calci, 2000; Defossez & Hawkins, 1997; Dunphy, Hall, Jeffs, & Wells, 2006; Lees, 2000).

For the majority of foods, proper refrigeration, storage, handling, cleaning and cooking procedures helps the consumer to control microbial risk and assure product safety. The hazards related to the contamination of bivalves by harmful microorganisms are due to their traditional cooking procedure which may not be enough to ensure consumer's safety. These circumstances make them an important vector of foodborne disease (Lees, 2000). The control of the disease risk associated with bivalves, thus, requires Hazard Analysis by Critical Control Point (HACCP) procedures together with water environment quality management of growing and harvesting areas and post-harvest product processing which might involve depuration and/or heat treatment where appropriate (WHO, 2010).

The true incidence of foodborne disease outbreaks is not known. Even thought there are routine surveillance systems worldwide that compiles the existing information on foodborne diseases, the collected information varies widely between diseases and between countries, not allowing for the numerical comparison of data on foodborne disease. Furthermore, a higher number of reported cases can be the result of a well performing surveillance system and not necessarily that people are more often sick from contaminated food. In addition, the reported number of cases for a country can include cases acquired domestically as well as acquired abroad after travel. No comparison between surveillance systems in term of their efficiency can therefore be made in a realistic way, and subsequently, trying to compare various countries data according

to their surveillance systems is not informative (Rocourt, Moy, Vierk, & Schlundt, 2003).

In general, countries who are members of the Organisation for Economic Co-operation and Development (OECD), meat (8.53%), poultry (4.14%), eggs and dairy products (14.62%) and seafood (6.63%) account for most of the foodborne diseases (Rocourt et al., 2003). When compared with these highly-consumed food products, seafood represents a quite alarming vehicle for foodborne diseases Shellfish are identified as one of the mediums of seafoodborne diseases. In New York, from 1980 to 1994, 339 seafood-associated outbreaks were reported, resulting in 3959 illnesses, 76 hospitalizations, and 4 deaths. Seafood-associated outbreaks accounted for 19% of all reported foodborne outbreaks and 10% of foodborne illnesses. Shellfish, the most frequently implicated seafood item, accounted for 64% of seafood outbreaks. The etiologic agent was confirmed for 654 (36%) of 1802 foodborne outbreaks and 148 (44%) of 339 seafood-associated outbreaks. Of the seafood-associated outbreaks, 14 (9%) were attributed to bacteria, 69 (47%) to viruses, and 65 (44%) to chemical agents. Three of the 4 seafood-associated deaths were caused by *Clostridium botulinum*; the remaining death was caused by *Vibrio vulnificus* (Wallace, Guzewich, Cambridge, Altekruse, & Morse, 1999). From 1993 to 1997, a total of 2751 outbreaks of foodborne disease involving 86,058 people were reported to the Centre for Disease Control (CDC), in Atlanta. The food vehicle was identified in only 1/3 of the outbreaks. Shellfish were often implicated in disease but did not, as opposed to some other foods, result in death. Since meat (66 outbreaks; 3205 cases) and poultry (52 outbreaks; 1871 cases) are food products that are consumed in a much larger amount, when compared to seafood, the number of cases related to shellfish (47 outbreaks; 1868 cases) is rather alarming (Olsen, MacKinon, Goulding, Bean, & Slutsker, 2000). When compared to fish (140 outbreaks; 696 cases), molluscan shellfish caused double the number of cases even though being responsible for a much lower number of outbreaks (Huss, Ababouch, & Gram, 2004; Olsen et al., 2000). In the majority of food outbreaks (67.8%) the disease agent was not identified. In 44.7% of the outbreaks caused by shellfish, the etiological agent was identified and viruses were the most frequent causative agent (23.4%) (Olsen et al., 2000). Between 1995 and 1996, 1919 outbreaks of infectious intestinal disease, affecting more than 40,000 people in England and Wales were reported to the PHLS Communicable Disease Surveillance Centre (CDSC). The food vehicle was identified for 301 outbreaks, 24 of which were reported to be due to shellfish, including 12 outbreaks attributed to eating oysters (Evans et al., 1998).

The risk of disease or death due to contaminated shellfish consumption is inherent to all consumers but the risk increases in those that suffer from underlying health disorders and are exposed to the consumption of raw bivalves. Among the high-risk population are individuals with immunosuppressive disorders (cancer patients, AIDS), achlorhydria and epilepsy, patients with diabetes mellitus, liver and chronic kidney disease and steroid dependent patients (for treatment of asthma). Pregnancy, age and alcohol abuse are also factors that may enhance the development of seafood diseases (Butt, Aldridge, & Sanders, 2004; Ripabelli et al., 1999).

3.1. Microbial contamination and human health

Foodborne disease is a public health problem which comprises a broad group of illnesses. Among them, gastroenteritis is the most frequent clinical syndrome which can be attributed to a wide range of microorganisms (Molnar, Wels, & Adley, 2006). Table 1 summarises some of the biological agents found in shellfish that can cause foodborne diseases. The risk of human intoxications is linked to the ingestion of bivalves contaminated with chemicals and biotoxins. On the other hand, the risk of human infections is related to the ingestion of bivalves contaminated with protozoan parasites, viruses and bacteria.

Chemical hazards (heavy metals, pesticides and drug-residues) are usually associated with aquaculture products or with bivalves caught from polluted waters but, in general, are uncommon in commercially harvested shellfish (Huss, Reilly, & Karim Ben Embarek, 2000; Richards, 1988).

Biotoxins, produced by dinoflagellates and diatoms (domoic acid), on the other hand are a serious health problem. These toxins, usually linked to the unpredictable growth of those microalgae (microalgae blooms), are heat resistant which means that even well cooked bivalves might still present a risk to consumer's safety. Accumulation of toxic marine algae in raw or light cooked shellfish has been associated to Paralytic Shellfish Poisoning (PSP), Diarrhetic Shellfish Poisoning (DSP), Neurotoxic Shellfish Poisoning (NSP), Amnesic Shellfish Poisoning (ASP) and Azaspiracid Poisoning (AZP) occurrences (Botana, 2008; FAO, 2004; Hallegraeff, Anderson, & Cembella, 2003; Huss et al., 2000). The level at which PSP intoxications occur in humans varies considerably according to individual sensitivity and fluctuation in the method of determination. For instance, an oral consumption of 300 μg PSP toxin per person was in some cases reported as fatal, whereas others noted the absence of toxic symptoms after an oral dose of 320 μg PSP toxin per person (Botana, 2008; FAO, 2004). Shellfish containing more than 2 μg Okadaic acid/g hepatopancreas are considered unfit for human consumption and capable of causing DSP (FAO, 2004). No mortality or chronic symptoms associated with NSP were reported and treatment is primarily supportive (FAO, 2004). For ASP the amounts of domoic acid consumed, ranged from 15 to 20 mg/person for an unaffected person to 295 mg/person for a case with severe neurological symptoms (Botana, 2008; FAO, 2004). Mild symptoms were showed after consuming 60–110 mg DA (0.9–2.0 mg domoic acid/kg body weight) and most serious cases were associated with consumption of 135–295 mg of domoic acid (1.9–4.2 mg domoic acid/kg body weight) (Botana, 2008; FAO, 2004). The lowest-observed-effect-level (LOEL) for AZP was 23–86 μg per person with a mean value of 51.7 μg/person (Botana, 2008; FAO, 2004).

The actual public health threat caused by parasites via shellfish consumption is uncertain, largely because there is minimal evidence of the transmission of infection (Robertson, 2007).

Microbial contamination is chronic and pervasive in harvesting areas. Furthermore, viruses and naturally occurring bacteria are the most often cited causative agents of disease and death related to shellfish consumption (Croci, Suffredini, Cozzi, & Toti, 2002; Huss et al., 2000; Lees, 2000; Wittman & Flick, 1995).

Shellfish-derived illnesses can have natural causes due to contaminants that are available in the environment and consequently a part of the normal biota (Shumway & Rodrick, 2009), while others can be human-generated before or after shellfish harvesting. Pre-harvesting microbial contamination (occurring naturally or as a result of human activities) includes a wide variety of viruses and pathogenic bacteria (Huss et al., 2000; Lees, 2000). Regardless of the higher abundance of indigenous marine viruses, only viruses derived from anthropogenic contamination are associated with illness in seafood consumers. Noroviruses, hepatitis A viruses, enteroviruses and adenoviruses are the viruses that are more often linked to shellfish contamination (Le Guyader, Atmar, & Albert, 2007; Lees, 2000; Muniain-Mujika, Calvo, Lucena, & Girones, 2003). Shellfish may also be contaminated post-harvesting. Potential hazard due to sub-lethally injured microbiota that may recover and multiply during later storage must be considered. Contaminant agents may also be introduced through cross-contamination, recontamination or faulty handling and processing (Huss et al., 2000; Shumway & Rodrick, 2009).

Table 1
Some biological agents implicated in seafood-related illness (Adapted from Botana, 2008; Brands et al., 2005; Butt et al., 2004; FAO, 2004; Hallegraeff et al., 2003; Huss et al., 2004; Huss et al., 2000; Muniain-Mujika et al., 2003; Ripabelli et al., 1999; Robertson, 2007).

Risk	Ethiology		Incubation period	Duration of pathology	Illness, symptoms and signs
Infection	Bacteria	*Salmonella* spp.	1–3 days	4–7 days	Gastroenteritis and Enteric (typhoid) fever. Diarrhea, fever, vomiting, abdominal cramps.
		Shigella spp.	24–28 h	4–7 days	Diarrhea, fever, abdominal cramps.
		Enterotoxigenic *E. coli*	1–3 days	3–7 days	Watery diarrhea, abdominal cramps, fever, vomiting.
		Campylobacter jejuni	2–5 days	2–10 days	Diarrhea, cramps, fever, vomiting.
		Staphylococcus aureus	1–6 h	24–48 h	Nausea, vomiting, abdominal cramps, fever, vomiting.
		Listeria monocytogenes	9–48 h 2–6 weeks	Variable	Listeriosis, septicaemia, central nervous system infections (meningitis), gastroenteritis, endocarditis, arthritis, encephalitis, osteomyelitis, pulmonary infections. Fever, muscle aches, nausea, diarrhea, violent or bursting headache and convulsions.
		Vibrio vulnificus	1–7days	2–8days	Wound infections, septicaemia, gastroenteritis. Vomiting, diarrhea, abdominal pain.
		Vibrio parahaemolyticus	2–48 h	2–5days	Wound infections, septicaemia, gastroenteritis. Nausea, abdominal cramps, watery diarrhea, vomiting.
		Vibrio cholera	24–72 h	3–7days	Epidemic and non-epidemic gastroenteritis. Profuse watery diarrhea, vomiting and dehydration causing death with hours.
	Viruses	Noroviruses	24–48 h	24–60 h	Nausea, vomiting, watery large-volume diarrhea.
		Hepatitis A virus	15–50days	2 weeks to 3 months	Diarrhea, dark urine, flu-like symptoms.
		Enteroviruses	10–70 h	2–9days	Nausea, vomiting, abdominal pain, malaise, headache, fever.
		Adenoviruses	10–70 h	2–9days	
	Protozoa parasites	*Cryptosporidium* spp.	2–28days	Days to weeks	Cramping, abdominal pain, watery diarrhea, fever, vomiting.
		Giardia lamblia	1–4weeks	Weeks	Acute or chronic diarrhea, flatulence, bloating.
		Toxoplasma gondii	6–10days	Months	Assymptomatic.
Intoxication	Biotoxins	Several species of the dinoflagellates genus *Alexandrium* spp. and the freshwater cyanophyte *Aphanizomenon flos-aquae*	30 min to 3 h	Hours to 2–3 days	Paralytic Shellfish Poisoning (PSP). Diarrhea, dizziness, nausea leading to parethesias of mouth, lips, weakness, dysphasia, dysphonia, respiratory paralysis.
		Dinoflagellates *Dinophysis* spp. and *Prorocentrum* spp.	30 min to 2 h		Diarrhetic Shellfish Poisoning (DSP). Nausea, vomiting, diarrhea, abdominal pain, chills, headache, fever.
		Dinoflagellate *Gymnodinium breve* (also called *Ptychodiscus breve,* since 2000 called *Karenia brevis*)	30 min to 3 h		Neurotoxic Shellfish Poisoning (NSP). Tingling and numbness of lips, tongue, throat, dizziness, diarrhea, vomiting, nausea, chills, sweats, reversal of temperature, hypotension, arrhythmias, cramps, bronchoconstriction, paralysis, seizures and coma.
		Mainly the Diatom *Pseudo-nitzschia pungens* f. *multiseries* and other *Pseudo-nitzschia* species	24–48 h		Amnesic Shellfish Poisoning (ASP). Vomiting, diarrhea, abdominal pain, neurological problems such as, confusion, memory loss, disorientation, seizure, coma.
		Dinoflagellate *Protoperidinium crassipes*	30 min to 24 h		Azaspiracid poisoning (AZP). Nausea, vomiting, severe diarrhea, and stomach cramps.

Viruses are frequently the cause of seafood-related infections, but hospitalizations and deaths are especially and generally related with bacteria (Butt et al., 2004).

Among the indigenous microbiota of coastal environments, the family Vibrionaceae, particularly *Vibrio parahaemolyticus*, *V. vulnificus* and *Vibrio cholerae*, is targeted as a causative agent of human disease due to the consumption of shellfish (Butt et al., 2004; Hood & Ness, 1982; Normanno et al., 2006; Ripabelli et al., 1999). These natural pathogens remain viable and cultivable in water, even in the absence of organic matter (Croci et al., 2002; Marino et al., 2005; Pruzzo, Gallo, & Canesi, 2005).

Several reports of human disease caused by *Listeria* spp., namely listeriosis, were related to seafood consumption but inconsistent results were observed (probably as a consequence of distinct coast contamination or different efficiencies in the detection and quantification methods). Furthermore, the contamination source (marine environment and processing/handling) and the seasonal fluctuations of the occurrence of these bacteria were not investigated effectively (Butt et al., 2004; Monfort, Minet, Rocourt, Piclet, & Cormier, 1998; Rodas-Suárez, Flores-Pedroche, Betancourt-Rule, Quinones-Ramirez, & Vazquez-Salinas, 2006). It is worth highlighting that there is growing evidence of the emergence of multi-resistant *Listeria monocytogenes* strains, due to the constant use of antimicrobial agents, thus representing a potential threat to human health (Rodas-Suárez et al., 2006).

The presence of *Salmonella* spp. in seafood and water may cause salmonellosis, characterized by enteric (or typhoid) fever along with gastroenteritis, abdominal cramps and diarrhea (Brands et al., 2005). *Salmonella enterica* serovar *Enteritidis* and serovar *Typhimurium* are the most common salmonella that cause infection and death (Butt et al., 2004). Enterotoxigenic *Escherichia coli*, *Campylobacter jejuni* and *Staphylococcus aureus* are also among bivalve bacterial contaminants and agents responsible for human disease (Brands et al., 2005; Butt et al., 2004).

3.2. Microbial contamination sources

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

The increase in population density has increased the vulnerability of shellfish growing areas through shellfish exposure to human and industrial contaminants (Brands et al., 2005; Lees, 2000). Sources of human and animal fecal pollution include pet and wildlife waste, rainfall events, and river flows. Uncontrolled sewage disposal or performed without previous appropriated treatment, small river outlets or diffuse land runoff of contaminants derived from agricultural activities and septic tank leakages may also produce sporadic contamination (Hernroth et al., 2002). Shellfish growing areas are usually close to wastewater discharges or in polluted estuarine systems and bivalve contamination is usually linked to the accumulation of human and animal pathogens of fecal origin. Nevertheless, in the process of filter-feeding, bivalve shellfish are likely to accumulate a diversity of microbiological contaminants (Burkhardt & Calci, 2000; Croci et al., 2002). Considering that fecal associated pathogens available in the marine environment accumulate in bivalves by filter-feeding, thus sewage contaminants may be recycled into the human community (Hernroth et al., 2002). This gains particular importance due to the fact that bivalves may have been exposed to persistent antibiotic residues and to multi-resistant pathogens as a result of an increased use of antibiotics by humans, in aquaculture and livestock. These multi-resistant pathogens may exist in the environment and may re-enter the food chain (Hektoen, Berge, Hormazabal, & Yndestad, 1995; Lees, 2000; Rodas-Suárez et al., 2006; Sapkota et al., 2008). Furthermore, a nonculturable but viable and latent bacteria species of sanitary importance may be present in water besides the existence of various processes that control the levels of microorganisms in coastal marine environments (Troussellier et al., 1998).

Allochthonous microorganism's number may be reduced in the natural environment because of physiological, hydrodynamic and biotic factors. Some of these are: pH, temperature, salinity, oxygen concentration, amount of organic matter, sunlight, water dispersion, re-suspension, sedimentation, competition of autochthonous bacterial community for nutrients and, finally, microbial predation by planktonic organisms (Ho & Tam, 2000; Hood & Ness, 1982; Troussellier et al., 1998). The same factors cannot be applied straightforward to microorganisms naturally present in water that also constitute a health problem (Croci et al., 2002; Pruzzo et al., 2005).

3.3. Factors with influence on microbial contamination of bivalves

Environmental conditions influence the occurrence of microorganisms in seawater and, consequently, their contact with shellfish. Burkhardt and his colleagues showed that temperatures outside the range of 13–22 °C and salinities greater than 25 ppt reduce the survival of *V. vulnificus* in seawater (Burkhardt, III, Watkins, & Rippey, 1992). Annual variation of water temperature and salinity influence shellfish's physiological state and capacity of siphoning and therefore affects the bivalve's ability to selectively accumulate microbial species. Kaspar and Tamplin described that the greatest accumulation of microorganisms in hard-shelled clams occurred during certain periods in the spring, at temperatures ranging from 11.5 to 21.5 °C (Kaspar & Tamplin, 1993).

Furthermore, bivalve's inter- and intra-specie variations determine the amount of water filtered, which is between twenty and one hundred liters of water a day, independently of the environmental conditions (Richards, 1988; Robertson, 2007). This means that, bivalve molluscs feeding physiology determines the accumulation of pathogenic microorganisms filtered from the overlying water (Burkhardt & Calci, 2000; Ho & Tam, 2000). These phenomena may partially explain seasonal and geographical differences in microbial content of bivalves (Hernroth et al., 2002). The availability of edible shellfish depends on the fluctuation of microorganism (type and quantity) in the marine environment as contamination results from ingestion of accessible contaminants. The ability of accumulated microorganisms to persist and multiply in bivalve tissues, despite the natural protection of the shellfish by the bactericidal activity of the haemolymph, also influences the existence of unhealthy shellfish (Johnson & Hayasaka, 1988; Power & Collins, 1990; Pruzzo et al., 2005).

4. Ensuring safe human consumption

4.1. Controlling harvesting areas

A few years ago, investment in sewage treatment processes still had many barriers to overcome. The geographical location of the shellfish industry was used as an argumentative factor to justify the difficult and expensive task in achieving and maintain high standards of water quality. Investing in adequate sewage treatment systems was considered disproportionate in terms of the value of the shellfish industry (Lees, 2000). Environmental concerns have contributed in recent years, to the increased investment in sewage infrastructure. However, important improvements are still needed, namely appropriate discharge locations for treated water, adequate arrangements for storm water storage and treatment, tertiary treatment of effluents and adequate evaluation methodologies of the effluent microbial quality (Lees, 2000). The location of pollution inputs must be previously well identified in order to assure that quality-monitoring programs take them into consideration. This may result in the expansion of sewage infrastructures even to sparsely populated areas or other areas which represent a low sewage input (Lees, 2000). Risk management strategies for shellfish harvesting areas must be improved in other to prevent shellfish contamination (Shumway & Rodrick, 2009).

4.2. Legislation for safeguarding consumers

Adequate safeguards can be useful in minimizing the probability of shellfish microbial contamination, from harvesting to consumption, and in the protection of public health. The European Directive 2006/113/CE (Anonymous, 2006) and the European Directive 2004/41/CE (Anonymous, 2004d), the US interstate agreement set out by the Food and Drug Administration (Anonymous, 1993) or the UK Advisory Committee on Microbiological Safety of Food (Anonymous, 1998) are guidelines, based on the levels of microbiological indicators for both shellfish and overlying waters. The legislation employs a classification to the seafood harvesting areas according to bacterial indicators of sanitary quality (*E. coli*), quantified through a 5-tube 3-dilution most probable number (MPN) test. This classification determines whether shellfish can be sent for direct consumption or must be treated previously to commercialization (Lees, 2000). Table 2 summarises the European standards for bivalve shellfish beads. All shellfish sent for direct human consumption without any further processing must comply with a standard of less than 230 *E. coli* in 100 g of shellfish meat in more than 90% of samples. Shellfish harvesting from polluted (category B and C) areas is allowed when shellfish undergo previous treatment, before being commercialized. Bivalve molluscs harvested from growing areas exceeding Category A standards 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, Howell, & O'Neill, 1991; Lees, 2000; Murchie et al., 2005). Shellfish from category C areas may, if necessary, be depurated before commercialization. However, some processes may not be effective at high levels of

Table 2
European classification of bivalves growing areas according of *Escherichia coli* (Lees, 2000).

Category	MPN of *Escherichia coli* per 100 g of seafood	Treatment required
A	≤230	Direct human consumption.
B	[230; 4600]	Depuration or relaying, to meet category A.
C	[4600; 46,000]	Protracted relaying to meet category A. Relaying to meet category B and depuration.
D	>46,000	Harvesting prohibited.

contamination, so another category is defined as D. Shellfish from those harvesting areas cannot be treated by any of the procedures previously mentioned.

The final product is sealed, labelled for traceability and commercialized giving distributors and consumers the confidence of a safe certified product (Jones et al., 1991; Lees, 2000; Shumway & Rodrick, 2009).

The U.S. Food and Drug Administration control procedures similarly rely on microorganism indicators for monitoring harvest waters in order to determine approved and restricted harvest areas and the treatment requirements prior to being released for human consumption (Lees, 2000). Category A defines the cleanest growing areas from which shellfish can be harvested and these areas are classified as "approved". Bivalve growing areas that do not comply with satisfying criteria, or without classification due to the lack of sanitary surveys, cannot be harvested for human consumption and are defined as "restricted". Harvest restriction can also be employed for short periods of time as a result of predictable or sporadic pollution. Such areas are classified as "conditionally approved" or "conditionally restricted" (Lees, 2000). The frequency of sample collection is dependent on the degree of contamination of the harvesting areas (Richards, 1988).

In many countries, these standard guidelines become very important for the regulation of shellfish harvesting and routine monitoring of overlying waters (Jones et al., 1991). However, when authorized shell fishing harvesting areas decrease, non-ethical activities such as illegal harvesting from polluted and restricted areas, wet storage of harvested shellfish in polluted waters, and other violations of legislation become problematic (Jones et al., 1991).

Other important aspects, other than the classification of growing areas, must be considered in order to reduce shellfish contamination. To achieve consumer protection and to minimize the inherent risks of shellfish consumption, legislation also sets requirements for sample collection, wet storage, bivalve self-purification by depuration and/or relaying (tank construction and operation, packaging, labelling), shellfish processing, laboratory analytical methodologies and product distribution. Regulations on food hygiene (Regulation N° 852/2004/EC) and on living bivalve molluscs (Regulation N° 853/2004/EC-Annex III Section VII) are well understood. Other regulations impose microbiological criteria for foodstuffs that set acceptable microbiological limits for all foods including live bivalve shellfish (Regulation N° 2073/2005) (Anonymous, 2004a, 2004b, 2005). Regulation N° 854/204/EC establishes specific attributes on the organisation of official controls on products of animal origin intended for human consumption (Anonymous, 2004c). At all stages, starting from the moment that the shellfish is collected until its consumption, good handling practices by applying Good Manufacturing Practice (GMP), Good Hygiene Practice (GHP) and a well designed HACCP programme are needed to prevent contamination and ensure a safe product (Huss et al., 2000; Lees, 2000; Marino et al., 2005; Shumway & Rodrick, 2009). Despite all regulations and guidelines. Sagoo and his colleagues showed that, in the UK during 2003, molluscan shellfish from retail and production premises found that 4% of 682 batches were unsatisfactory due to the presence of high levels of *Escherichia coli* (3.3%; 102 to 106 cfu g^{-1}), *V. parahaemolyticus* (0.4%; 102 to 106 cfu g^{-1}), and *Staphylococcus aureus* (0.3%; >103 cfu g^{-1}) (Sagoo, Little, & Greenwood, 2007).

5. Purification methods

Sanitary regulations rely on bacterial indicators of sewage contamination to classify shellfish harvesting waters and to estimate the efficiency of purification methods (Murchie et al., 2005). These purification procedures, used to reduce anthropogenic or natural microbial contamination of bivalve molluscs, have been used since the 1920s and are now extensively used worldwide (Lees, 2000). Unhealthy harvested bivalves purge contaminants when transferred into clean natural shellfish beds (relaying) or into tanks (depuration) (Richards, 1988; Shumway & Rodrick, 2009). Depuration consists of a flow-through or recirculation system of chemically (chlorine, ozone, iodophores, and activated oxygen) or physically (UV irradiation) disinfected water to allow purification under controlled conditions (Lees, 2000; Richards, 1988; Son & Fleet, 1980). This process usually occurs in 2 days (Lees, 2000). Relaying consists of transferring contaminated harvested bivalves to cleaner areas allowing self-purification in the natural environment for longer periods, at least two months for category C shellfish, according to EU standards (Lees, 2000; Richards, 1988). Purification processes are based on the assumption that if by filtering polluted water shellfish can become contaminated, they may also purge the contaminants by filtering clean water. Thus, microbial depuration decreases the risk for potential infections due to shellfish consumption. In fact, most consumers prefer to buy depurated products, not only because they are safer in terms of contamination, but also because they are less gritty and more palatable (Richards, 1988).

5.1. Depuration – practical considerations

Depuration efficiency is primarily related to bivalve's size, siphoning activity, and physiological conditions (Jones et al., 1991; Richards, 1988).

The type and quantity of initial contamination also accounts for depuration efficiency as more contaminated bivalves require longer depuration times and different microorganisms respond differently to the purification process. Likely, seeded (laboratory-induced) and natural-contaminated bivalves present dissimilar kinetics of contaminant elimination (Son & Fleet, 1980). Artificially contaminated molluscs depurate more rapidly than environmentally contaminated ones (Croci et al., 2002; Jones et al., 1991; Richards, 1988). Different rates of elimination also occur when bivalves are contaminated with individual or several bacteria (Son & Fleet, 1980).

Temperature and salinity are two important parameters to consider in the purification process according to the type of shellfish. Variations in environmental requirements among bivalves may reflect shellfish adaptation to *in situ* conditions. Animal stress induced by differences in water temperature, from that of the *in situ* shellfish growing areas to the process water, also influence purification time and efficiency. Lowering the temperature may help in keeping bivalves alive longer and maintain lower bacterial concentrations, however, this would also extend the period of time required for effective depuration. Shellfish conditioning, that allows shellfish to acclimate to the temperature and salinity of the water, seems necessary to ensure maximum depuration (Johnson & Hayasaka, 1988; Richards, 1988). Specific studies are required to

determine optimal conditions for shellfish microbial depuration accordingly to geographical characteristics (Johnson & Hayasaka, 1988). Differences in experimental design, such as commercial or laboratory-scale depuration systems must also be considered, as the time needed for bivalve purification differ (Jones et al., 1991). Susceptibility to temperature fluctuations is less likely in thermostatically controlled systems. Also, water volume and shellfish loading rates will affect the pH and the dissolved oxygen levels in the system. The number of bivalve layers in depuration recipients can promote increases in the microbial load as result of recontamination, obstruction of water flow and restrictions of shell opening (Richards, 1988). Depuration has a great potential as a means of purging shellfish, at least partially, of microbiological contaminants. Nevertheless, more detailed studies are needed to determine the effect of physiologically parameters, such as food availability, temperature, salinity, dissolved oxygen and shellfish state. This would allow the development of an improved depuration method (Jones et al., 1991).

5.2. Relaying – drawbacks

In contrast to depuration, where bivalves can only be held for a short period of time (maximum of 48 h), in the relaying method, molluscs can be kept for longer periods (at least two months) (Lees, 2000; Richards, 1988). In fact, in controlled purification, extended periods will reduce palatability and quality of bivalves and might even, cause bivalve mortality due to the unavailability of food. This will obviously result in a negative economic impact, due to delayed marketing and commercialization (Jones et al., 1991).

Drawbacks of relaying include: lack or availability of acceptable sanitary shellfish growing waters, early harvesting from fishermen and economical considerations namely regarding ownership rights (Lees, 2000; Richards, 1988). In addition, bivalves are more susceptible to environmental disturbances that cannot be controlled such as temperature fluctuations, water movements (tides and storms) and weather (Lees, 2000; Richards, 1988; Son & Fleet, 1980). Smothering and clogging by sediments, physiological stress, shell damage and predation are very likely to occur during the relaying process (Richards, 1988). Furthermore, water quality of relaying areas is difficult to assure. The possibility of recontamination by seasonal variations of naturally occurring bacteria populations or transient pollution (due to heavy rains and associated land runoff), may contaminate acceptable relay areas, leading to an ineffective microbial reduction (Croci et al., 2002; Ho & Tam, 2000; Lees, 2000; Richards, 1988). Assessing the efficiency of the relaying process is also difficult because the indicator microorganism's levels may fluctuate erratically during the exposure period (Ho & Tam, 2000; Richards, 1988).

In summary, eating raw or lightly steamed shellfish harvested from contaminated areas, but purified in acceptable marine waters or in artificial tanks, can still cause infection and disease in a significant percentage of the exposed population (Lees, 2000; Richards, 1988).

5.3. Microorganisms indicators – important considerations

Conventional depuration can be a viable alternative for molluscs that have been exposed to polluted waters improving their quality as a food resource, especially for those that are sold alive for raw consumption – it reduces the bacteria levels present in mollusc meat without heat processing (Johnson & Hayasaka, 1988; Jones et al., 1991; Lees, 2000).

However, the efficiency of these purification practices is questionable since it is based on bacterial indicator standards to ensure shellfish safety. The use of such indicators was made necessary by the difficulty in detecting many human pathogenic bacteria and viruses. Additionally, they avoid the need to screen for individual fecal pathogens (Scott, Rose, Jenkins, Farrah, & Lukasik, 2002). Nevertheless, there is a well known lack of correlation between the presence of bacterial indicators and viral pathogens (which are tightly attached to the internal tissues) in both shellfish and harvesting waters. Dissimilar elimination rates of indicator bacteria compared to viruses and indigenous marine bacteria are also well documented (Marino et al., 2005; Murchie et al., 2005; Romalde et al., 2002; Son & Fleet, 1980). Hence, more representative and accurate indicators are sought in order to improve the microbial control of shellfish (Formiga-Cruz et al., 2003).

The occurrence of few pathogenic bacteria in shellfish does not generally represent a high risk to public health because threshold levels necessary to cause illness far exceed those present. In contrast, viruses are infectious even in very low numbers, which makes total virus depuration essential to ensure public safety (Lees, 2000; Richards, 1988). Disease outbreaks associated with the consumption of shellfish compliant with the *E. coli* standard (less than 230 *E. coli* per 100 g), particularly in relation to viral infections, continues to be reported (Doré, Henshilwood, & Lees, 2000; Lees, 2000). It seems that viruses survive longer both in the marine environment and in the digestive tracts of bivalves compared to *E. coli* (Hernroth et al., 2002). Furthermore, there are studies reporting the detection of viruses in shellfish harvested from areas considered unpolluted, and meeting the current bacteriological standards (Muniain-Mujika et al., 2003; Romalde et al., 2002). Viral pathogens include culturable and nonculturable viruses whose detection methods are complex, laborious, time-consuming and expensive. Consequently, their use in routine monitoring is limited, hindering their establishment as regulatory standards methods (Hernroth et al., 2002; Lees, 2000; Murchie et al., 2005).

5.3.1. Indicator microorganisms – alternatives

The analysis of fecal coliforms and *E. coli* has limited predictive value for viral pathogens such as, noroviruses (NV), hepatitis A viruses (HAV), enteroviruses (EV) and adenoviruses (ADV), and alternative indicators microorganisms have been proposed (Muniain-Mujika et al., 2003). Traditional depuration does not significantly reduce the levels of Male-specific RNA (F-RNA) bacteriophages, somatic coliphages, bacteriophages infecting *Bacteroides fragilis*, or the occurrence of human pathogenic viruses, although its efficiency in reducing *E. coli* levels was confirmed (Formiga-Cruz et al., 2003). Based on these findings, the phages above mentioned have been suggested as putative indicators of viral contamination (Hernroth et al., 2002). F-RNA phages, frequently found in sewage and fecal contaminated waters, are a group of single-stranded RNA viruses that belong to the family Leviviridae and their physical and genomic properties are similar to the NV and HAV (Doré et al., 2000; Doré, Mackie, & Lees, 2003). F-RNA bacteriophages are probably more representative of the pathogenic viral kinetics in shellfish than *E. coli,* either because they are more resistant to environmental stress (U.V. irradiation), or because they have longer retention time in shellfish (due to the differences in the way they are accumulated and eliminated) or even a combination of the two (Doré et al., 2003). Virus depuration is slower than indicator bacteria clearance, requiring more than 48 h and still does not always meet acceptable criteria (Lees, 2000; Richards, 1988). In fact, recent studies suggested a 5-day depuration treatment to ensure elimination of viruses in mussels (Formiga-Cruz et al., 2003). Hence, the slower elimination kinetics of F-RNA bacteriophages in relation to *E. coli,* during depuration, appears to be representative of the kinetics of elimination of human enteric viruses (Hernroth et al., 2002). These properties associated to the simplicity of enumeration, make F-RNA phage an attractive

indicator organism for viral contamination in the marine environment (Doré et al., 2003; Hernroth et al., 2002). However, some authors have presented some reservations in terms of the fact that monitoring through this indicator will increase shellfish safety (Hernroth et al., 2002; Torrado, Henshilwood, Lees, & Romalde, 2002; Vilariño, Ribao, Henshilwood, & Romalde, 2006). Indeed, F-RNA phages have demonstrated a significant relationship to the presence of human viruses in shellfish, although showing very weak predictive capability for EV, HAV and ADV and a stronger predictive capability for NV (Formiga-Cruz et al., 2003). On the other hand, the absence of F-RNA bacteriophages appears to be a reliable indicator that enteric viruses, such as NV, are likely absent (Doré et al., 2000). Similarly to *E. coli*, F-RNA bacteriophages are not human specific, and a contamination with this phage may be associated to animal feces originated by land runoff and may not imply health risk due to NV (Doré et al., 2000). Oligonucleotide probe hybridization methods for geneotyping F-RNA bacteriophages would provide the possibility to differentiate animal-associated from human-associated bacteriophage groups (Doré et al., 2000). Somatic coliphages, viruses that infect *E. coli* bacteria, are constantly present in treated or non-treated sewage, they are non-pathogenic to humans, and are more similar to enteric viruses with respect to physical characteristics, environmental resistance to inactivation in the marine environment and resistance to treatment processes than are indicator bacteria (Cole, Long, & Sobsey, 2003). However, coliphages are able to increase their initial effluent discharge number in marine environment and in shellfish. Furthermore, they are not a specific index for pollution with human enteric viruses, as they are found in both human and other animals (Legnani et al., 1998). Male-specific (F+) coliphage (group II and III) has been pointed out as providing an additional advantage in distinguishing animal and human fecal pollution (Cole et al., 2003; Scott et al., 2002). The *Bacteroides* spp. is present in high numbers in both the human and animal gut and is a major component of human feces (Scott et al., 2002). Several studies have reported that the probability of detecting viruses increases when phages of *B. fragilis* are found, particularly, *B. fragilis* RYC2056 (Muniain-Mujika et al., 2003). The detection of *B. fragilis* phage has the advantage of being highly specific. Additionally, these phages do not replicate in the environment (Scott et al., 2002). This could be a suitable group of bacteriophages to be used as an indicator of the presence of viruses in shellfish (Muniain-Mujika et al., 2003).

Some authors propose human ADV as a molecular index of viral contamination in shellfish (Hernroth et al., 2002; Muniain-Mujika et al., 2003; Pina, Puig, Lucena, Jofre, & Girones, 1998). In fact, this virus was usually detected when EV and HAV were also found (Hernroth et al., 2002). Technical simplicity related to simpler detection methodologies of DNA viruses compared to those of RNA viruses and more sensitive and specific molecular techniques, are the advantages of using human ADV as a molecular indicator of human-specific viral fecal pollution (Hernroth et al., 2002; Lees, 2000; Muniain-Mujika et al., 2003). However, epidemiological studies for EV and ADV are difficult to perform because those infected by the viruses can act as carriers without showing any symptoms. As a result, the disease may only become apparent after the infection of another individual, probably far away from the original source (Hernroth et al., 2002; Muniain-Mujika et al., 2003). However, detection of human ADV by PCR has been proposed as a molecular parameter for monitoring the presence of human viruses in the environment, more studies are required to define the relationship between the level of viral contamination in shellfish and their potential pathogenic effect after consumption (Muniain-Mujika et al., 2003). Furthermore, ADV are present in much higher numbers than HAV or NV and therefore their value as indicators are limited (Torrado et al., 2002).

It is important to notice that environmental conditions play an important role in the accessibility, accumulation and elimination of both viral contaminants and potential indicator organisms from bivalves (Hernroth et al., 2002).

Temperature and UV irradiation are some of the factors affecting the viability and stability of viral particles in seawater and virus removal during depuration (Formiga-Cruz et al., 2003; Lees, 2000). Somatic coliphages have been indicated as ensuring a better marine water quality monitoring than F-RNA phages and fecal coliforms because the formers are less susceptible to longer solar wavelengths, which are predominant in the marine environment (Sinton, Finlay, & Lynch, 1999). It was found that the probability of a positive detection of any of the pathogenic virus decreases as the temperature of shellfish growing waters increases (Muniain-Mujika et al., 2003). The levels of the potential indicators also change with temperature. The distribution of F-RNA bacteriophages has been shown to be seasonal, with higher levels during the winter; this trend was also observed in the identification of typified NV, but not for the detection of ADV, EV, or HAV (Doré et al., 2003; Formiga-Cruz et al., 2003; Hernroth et al., 2002). In fact, NV gastroenteritis has been considered a "winter vomiting disease" (Doré et al., 2000; Hernroth et al., 2002). Phages infecting *B. fragilis*, in contrast to ADV, decrease in number with temperature (Hernroth et al., 2002). The selection of an indicator microorganism is further complicated when focusing on the potential pathogenicity of some indigenous marine bacteria (Murchie et al., 2005). Autochthonous bacteria are not implicitly associated with the presence of fecal contamination. Thus classical indicators of fecal contamination do not predict their presence in shellfish or water (Hood & Ness, 1982). Furthermore, one of the basic criteria for a good indicator organism is that the indicator must survive as long as the pathogen, but *E. coli* does not survive in estuarine water as well as *V. cholera* (Hood & Ness, 1982). Several authors have confirmed the lack of correlation between traditional indicators and the presence of *Vibrio* spp. (Hood & Ness, 1982; Marino et al., 2005; Normanno et al., 2006; Ripabelli et al., 1999). Seasonal variations in the indigenous bacteria populations make it extremely difficult to select safe waters for mollusc harvesting (Croci et al., 2002). Fecal indicators provide an inadequate index of microbiological safety for naturally occurring vibrios and underestimate the efficiency of the depuration process. Like enteric viruses, *Vibrio* spp. has a different response to the depuration process from that of *E. coli*. It is possible to obtain edible shellfish from anthropogenically-contaminated shellfish, but the same measure cannot be used with shellfish contaminated by naturally occurring bacteria. Similarly, it is expected that the elimination of microorganisms derived from fecal contamination and those included in shellfish natural microflora would be different (Croci et al., 2002; Jones et al., 1991). In fact, indigenous marine bacteria do not depurate well and may even multiply in depurating shellfish tanks and pumping systems (Richards, 1988). Therefore, a more appropriate indicator must be developed to reduce seafood illness risk derived from *Vibrio* spp.. Enterococci have been proposed as a more appropriate indicator of the risk from vibrios than *E. coli* (Marino et al., 2005). It is also important to be aware of the fact that none of the current regulations include specific tests for indigenous marine bacteria (Murchie et al., 2005). Thus, the need to improve shellfish-borne disease control strategies must also focus its attention on *Vibrio* spp. (Ripabelli et al., 1999).

5.4. Methodologies for monitoring bivalve's safety – critical points

Present legislation verifies seafood safety according to bacterial indicators of sanitary quality measured through a 5-tube 3-dilution most probable number (MPN) test (Lees, 2000). Besides the wide acceptance, it is recognized that this test presents interpretive,

technical and microbial problems leading to the underestimation of both bacteria indicators and contamination-level and is therefore of limited reliability. The MNP is a statistical estimate of the mean number of bacteria in the sample, thus the result is a semi-quantitative enumeration of bacteria indicators. The precision of the bacteria estimation is low and is dependent on the number of tubes used in the laboratory analysis (Rompré, Servais, Baudart, de-Roubin, & Laurent, 2002). For this reason, this indirect enumeration procedure is intrinsically less accurate than the direct methodologies, unless the population densities are low. MPN method is time-consuming due to the duration of the incubation; it is also tedious and laborious (Hackney, Ray, & Speck, 1979; Rompré et al., 2002). The accuracy of this method is further significantly reduced by the interference of antagonistic bacteria, a certain degree of heterogeneity of the coliform group, the inhibitory nature of the media and weak detection level of slow-growing, stressed or viable or active but nonculturable microorganisms (Rompré et al., 2002). Nonlethal injury may be caused either by temperature, pH, water activity, irradiation, sanitizers, starvation or by a combination of these factors (Hackney et al., 1979). Specially developed media with the appropriated composition may help to recover these stressed or injured cells. Some advantages of this method are: its simplicity, low cost and no need of sophisticated laboratory and equipment. Improvements to the MPN test have been developed over the years. Biochemical tests, based on metabolic reactions, can be used for culturable bacteria identification and enumeration. However, they are not totally specific, and supplementary confirmation tests are necessary. Microbial enzyme profiles can be used to detect indicator bacteria as a complement or alternative to the classical method (Rompré et al., 2002). Nevertheless, innovative methods of bacterial detection and quantification are needed. Molecular methods have appealing characteristics such as sensitivity, specificity, the short time needed to produce results and the fact that they do not require complex culture or additional confirmation procedures, thus allowing for the detection of both culturable and nonculturable bacteria (Hernroth et al., 2002; Pina et al., 1998; Rompré et al., 2002). Additionally, they allow for the detection of more than one microorganism or molecular marker with a single assay (multiplex-PCR) (Scott et al., 2002). Polymerase chain reaction (PCR) or reverse transcriptase-PCR (to detect RNA viral genomes, such as those from viruses) is the most frequently applied nucleic-acid-based method (Le Guyader et al., 2007; Pina et al., 1998; Rompré et al., 2002; Shumway & Rodrick, 2009). Despite the success of PCR and reverse transcriptase (RT)-PCR in detecting minimal starting quantities of nucleic acid (as little as one cell equivalent), the drawbacks of PCR-based assays included low amplification due to the presence of inhibitor substances, and the absence of information about the physiological activity of the bacteria or viruses being studied, because nucleic acids are extracted from viable, dead, culturable or nonculturable microorganisms (Rompré et al., 2002). Some attention must also be paid to results given by methods based on PCR amplification of viruses because they might overestimate the risk for transmission of viable viruses. In addition, molecular approaches can only be performed with highly skilled staff in specialized laboratories providing high-technology services (Hernroth et al., 2002; Le Guyader et al., 2007; Rompré et al., 2002). Real-time PCR overcome the lack of quantification in molecular methods by measuring PCR product accumulation through a dual-labeled fluorogenic probe (i.e., TaqMan Probe). As this method does not require post-PCR sample handling, it also avoids potential contaminations of the PCR product. Real-time quantitative PCR is extremely accurate, reproducible and less labour-intensive than other quantitative PCR methods that also had been designed, and can be applied to both virus and bacteria (Heid, Stevens, Livak, & Williams, 1996).

6. Emerging perspectives

The emergence of *Vibrio* spp. as a human pathogen is of particular concern for shellfish producers. In addition, bivalves contaminated with these bacteria are difficult to recognize since they are not affected in appearance, palatability or smell. Several elimination methods have been proposed: UV depuration, gamma radiation, heat, cold temperatures, tabasco sauce and other horseradish-based sauces. Regardless of their success and limitations, these processes do not represent an alternative for raw seafood (Shehane & Sizemore, 2002).

Bacteriocins (plasmid-derived proteins used as microbial defense systems) have been studied as a method for the removal of *Vibrio* spp. from seafood (Riley & Wertz, 2002; Shehane & Sizemore, 2002). Three bacteriocin-producing strains (IW1, BC1 e BC2), belonging to the group IV bacteriocins of lactic acid bacteria, have been found to exhibit a varied inhibitory spectrum and stability. Bacteriocin IW1 neutralized few strains of *V. vulnificus*, BC1 eliminated several strains of *V. vulnificus*, *V. cholerae* and *V. parahaemolyticus* and, finally, BC2 neutralized *Vibrio* spp. *Plesiomonas shigelloides* and *E. coli*. Taking into account both the broadest inhibitory spectrum for *Vibrio* spp. and bacteriocin stability, BC2 was proposed as a new method of control of *Vibrio* spp. (Shehane & Sizemore, 2002).

Bacteriocins have been also investigated as an alternative solution to contamination by *L. monocytogenes*. A large number of IIa class bacteriocins were proposed as highly active against these bacteria (Riley & Wertz, 2002).

Bacteriocins have numerous applications as controlling agents in food but the US FDA only recognizes some bacteriocins as safe for the production of fermented foods such as Nisin, a bacteriocin produced by lactic acid bacteria (Riley & Wertz, 2002; Shehane & Sizemore, 2002). Despite their relatively narrow spectrum of activity against specific bacterial pathogens, bacteriocin's use for the preservation of food creates the dilemma of selecting resistant strains or cross-resistant strains (Riley & Wertz, 2002).

Naturally occurring bacteriophages have been used as biocontrol agents in aquatic environments for fish diseases and other infections (Nakai & Park, 2002). It has been suggested that phage treatment could be useful in controlling *Vibrio splendidus* infection (Sugumar, Nakai, Hirata, Matsubara, & Muroga, 1998) in cultured larvae of the Pacific oyster (*C. gigas*) (Park & Nakai, 2003). Berthe (2005) suggested bacteriophages for the treatment of bacterial infections in molluscan aquaculture production (Berthe, 2005). Although these reports focus on bivalve's pathogens, a similar application could be given to human pathogens. However, reports on microbial control with phages are not available for any bivalve specie or bacterial infection.

Due to the drawbacks associated with obtaining edible shellfish, additional post-harvest processing methods are also being investigated as an alternative for ensuring shellfish safety for human consumption. Since 1992, high pressure processing (HPP) has been proposed as a physical method for food preservation and has already found several commercial food applications, including oyster processing (Murchie et al., 2005). HPP technology makes the inactivation of numerous microorganisms possible by exposing molluscan shellfish to relatively high hydrostatic pressure, for a short period of time at ambient temperatures, while retaining the raw taste, appearance, texture and nutritional properties of the raw shellfish. The same process can be used for shucking oysters without any mechanical force (Kingsley, Holliman, Calci, Chen, & Flick, 2007). These characteristics favour both the shellfish processing industry and consumers. Even though HPP treatment offers advantages over conventional processing techniques in enhancing food safety, the protection is dependent on the composition of food and on the target

microbiota. Microorganisms can differ widely in their intrinsic sensitivities to HPP (Murchie et al., 2005). There is experimental evidence that *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* are reduced by HPP (Calci, Meade, Tezloff, & Kingsley, 2005; Murchie et al., 2005). However, other bacteria reveal a wide range of resistance to HPP depending on the strain (Gram-negative bacteria are, generally, more susceptible than Gram-positive species), growth phase, growth temperature and the composition of surrounding matrices (Murchie et al., 2005). Reports on the use of HHP treatment on raw shellfish showed a reduction of infectious HAV (Calci et al., 2005). However, similarly to bacteria, viruses also differ widely in their vulnerability to HPP (Murchie et al., 2005). Algal toxins will probably be less affected by HPP, but further studies are needed.

The efficiency of HPP-inactivation of microorganisms in shellfish needs further investigation that must include different internal locations of bacteria and viruses in the bivalve, the seasonal and geographical variations in shellfish physiology and composition and lastly the isotonic strength of the harvest waters. Also, additional investigation is needed to determine the mechanisms of inactivation, the reason for the different resistance of viruses and the potential hazard of sub-lethally injured microbiota that may recover and multiply during subsequent storage and may lead to an over-estimation of microbial inactivation. The effects of HPP on both microorganisms and seafood are highly dependent on processing parameters that also need further investigation (Murchie et al., 2005).

In contrast to the previously mentioned, porphyrins present a distinct way of improving shellfish quality since it is focused on the reduction of water contamination rather than in the bivalve. Porphyrins are compounds of natural origin which, when irradiated, generate some hyper-reactive and highly cytotoxic oxygen species (mainly, singlet oxygen) attacking different cellular components. Recently, porphyrins were synthesized to attack several types of microbial cells. The irradiation of the porphyrin causes mortality of a variety of pathogenic agents including Gram-positive and Gram-negative bacteria and parasites in either the cystic or the vegetative stage. These compounds were pointed as a novel photochemical technique for the treatment of microbiologically polluted aquaculture waters (Magaraggia, Faccenda, Gandolfi, & Jori, 2006).

7. Conclusions

The nutritional and economical value of shellfish is acknowledged worldwide. Similarly, filter-feeding bivalves are well known as efficient transmitters of seafood-born disease. Over a long period of time, the high-risk nature of this product and the underestimation factors, have been well documented in many investigative reports and international agencies.

Preventive measures to enhance the quality of living bivalve shellfish when commercialized have included the monitoring and improvement of the water quality found at the harvesting areas. Nevertheless, bacterial indicators used for shellfish health evaluation were announced, in different reports, as inadequate predictors of the presence of autochthonous bacteria and human enteric viruses. Considering the results of these findings, in order to ensure public health, more accurate indexes of water quality and bivalve microbiological safety are required since they are still not available. Also the predictive value of putative indicators needs further evaluation, as specific disadvantages and contradictory results in their use have been pointed out by past studies. Indeed, the overwhelming findings of these studies suggest that the potential indicators may complement the use of *E. coli* for a better guarantee of sanitary safety. However, the development of a local diagnostic scheme for direct detection and identification of the existing pathogens for monitoring bivalve health is probably a future tendency. Future investigations should address the relationships between indicator microorganisms survival with regard to that of the pathogens they are designed to predict. Further work is required to establish a scientific agreement among those considered potential indicators, or others to be discovered, and also to understand the implications of their introduction into legislation. Different threshold levels necessary to cause illness between pathogenic bacteria and viruses must also be considered.

Conventional methodology, applied to predict the level of contamination by quantifying bacterial indicators, needs to be improved in specificity and reduced in time. Detection by new molecular methods may be more sensitive and specific, which will allow for a faster response to health safety problems. The adjustment of the threshold levels of contamination for bacteria and viruses in relation to the risk of occurrence of disease must also be considered. Methods of detecting several pathogens should be implemented so that the assessment of microbial contamination can be more closely associated with the results produced by epidemiological studies.

Depuration and relaying helps to improve shellfish quality but if prevention of human or animal-induced pre-harvest contamination can be achieved, natural causes will always be present. A better knowledge of the parameters affecting the kinetics of the processes of depuration is still needed. More sensitive, reliable, and universally accepted depuration procedures must be developed, so that standardized methodologies can enable the comparison between the experimental results. Technological advances should also be employed.

Reoccurrence of seafood-borne diseases lead to the investigation of alternative methods to eliminate microbial contamination. Bacteriocins, bacteriophages, HPP and porphyrins may be future approaches to control shellfish microbiological contamination. The increased use of antibiotics for the treatment of disease has lead to the emergence of multi-resistant bacteria, which can be released to the environment re-entering the food chain, and consequently, represent a higher risk to consumers. Particular attention should be given to multi-resistant pathogenic bacteria in order to ensure that present or new indicators will be correlated with pathogen occurrence and that methodologies assure the elimination of these bacteria.

Consumer protection involves both the knowledge of the risk associated to the ingestion of raw shellfish and the preventive actions that take into account shellfish specificity, shellfish contamination and adequate regulations. The combination of new depuration approaches and a more accurate quality assessment will help to relieve public concern regarding foodborne diseases associated with shellfish products.

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