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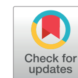
Evaluation of short purification cycles in naturally contaminated Mediterranean mussels (*Mytilus galloprovincialis*) harvested in Sardinia (Italy)



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ABSTRACT

The aim of the present study was to investigate the effect of short purification cycles on the safety of naturally contaminated *Mytilus galloprovincialis* from harvesting areas of the Gulf of Olbia (Sardinia, Italy). Samples from ten batches of mussels were collected before, during and after purification treatment at two purification centres (A-B). All the samples were analysed for *Escherichia coli* and *Salmonella* spp according to Council Regulation (EC) 2285/2015. Detection and enumeration of *Vibrio* spp were performed according to previously published methods. Presumptive identification of *Vibrio* spp isolates were performed by means of conventional biochemical tests and polymerase chain reaction. The presence of Hepatitis A virus was detected by nested reverse transcriptase-polymerase chain reaction. Environmental parameters (water temperature and salinity) were also recorded. The results of *Escherichia coli* counts showed the overall efficacy of the short purification cycles; a purification cycle of 8 h led to a rapid decline in the concentration. The decrease in *Escherichia coli* counts does not correlate with the presence of naturally occurring vibrios, the decline of which occurs at an even slower rate. The average contamination levels for *Vibrio* spp before purification were 8.20 ± 0.47 and 7.99 ± 0.62 Log_{10} CFU/g in samples collected at purification plants A and B, respectively. After purification, the average contamination levels were 8.10 ± 0.60 Log_{10} CFU/g at purification plant A and 7.85 ± 0.57 Log_{10} CFU/g at purification plant B. The contaminated samples revealed the presence of *Vibrio alginolyticus (n=21)*, *Vibrio fluvialis (n=12)*, *Vibrio cholerae (n=4)*, *Vibrio parahaemolyticus* (*n*=*2*) and *Vibrio vulnificus (n=1)*. The *Vibrio parahaemolyticus* isolates carried the *tdh* or the *trh* genes. None of the isolates was *tdh*+/*trh*+. *Salmonella* spp and Hepatitis A virus were not detected. The adoption of short purification cycles for *Mytilus galloprovincialis* in the presence of pathogenic vibrios might not be sufficient to guarantee the safety of consumers.

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1. Introduction

Italy is the third largest European producer of bivalve molluscs after Spain and France (Billé et al., 2013). In Italy, bivalve molluscs are the most important farmed seafood resource, representing more than half of the total national aquaculture production (Meloni et al., 2010); this production is mainly composed of Japanese carpet shells (*Ruditapes philippinarum*) and Mediterranean mussels (*Mytilus galloprovincialis*). Italy is the leading European producer of Japanese carpet shells and the second in the world after China. Moreover, it is the third largest worldwide producer of mussels, after China and Spain (Billé et al., 2013). Japanese carpet shells are mainly farmed in the regions of Veneto and Emilia Romagna, while the production of Mediterranean mussels *M. galloprovincialis* is typical of Emilia Romagna, Veneto, Sardinia and Puglia (Meloni et al., 2010). In Sardinia, the regional shellfish sector is well consolidated: annual production accounts for 83% of the aquaculture species, and it almost exclusively rests on Mediterranean

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mussels *M. galloprovincialis* (Sardegna Agricoltura/Laore, 2009). According to the European Union (EU) shellfish harvesting area classification criteria, most of the Sardinian production areas are classified as class B (Council Regulation (EC) 853/2004). Live bivalve molluscs from these areas must not exceed the health standards reported in Reg. (EC) 2073/2005 and following amendments, including recent Reg. (EC) 2285/2015. Shellfish may be placed on the market for human consumption after purification, relaying in class A areas or cooking by an approved method. Purification is a post-harvest processing strategy intended to reduce the likelihood of transmitting infectious agents to consumers (Polo et al., 2014). Bivalve molluscs are held in tanks with clean seawater under conditions that maximize their natural pumping activity to purge the contaminants in a rapid and efficient manner (Lee et al., 2008). Shellfish contamination occurs because of their nature as suspension feeders, which selectively filter small particles of phytoplankton, zooplankton, viruses, bacteria and inorganic matter from the surrounding water (Dunphy et al., 2006). Furthermore, viruses and naturally occurring bacteria are the most often cited causative agents of disease and death related to shellfish consumption (Croci et al., 2002). Shellfish transmitted illness may occur due to bacteria found naturally in the marine environment and that are consequently a part of the normal biota (Huss, 2000), while other types of contamination can be human-generated before or after shellfish harvesting. Pre-harvest microbial contamination (occurring naturally because of human activities) includes a wide variety of viruses and pathogenic bacteria (Huss, 2000). Purification is effective at removing only faecal bacterial contaminants, such as *Escherichia coli* or *Arcobacter butzleri,* from bivalve molluscs (Gallina et al., 2013; Leoni et al., 2017; Serratore et al., 2014). Generally, the decrease in the number of *E. coli* does not correlate with the presence of seawater autochthonous vibrios (*e.g., Vibrio parahaemolyticus* and *Vibrio vulnificus*), the decline of which occurs at an even slower rate (Kong et al., 2002; Leoni et al., 2016; Normanno et al., 2006; Ripabelli et al., 1999; Serratore et al., 2014). As currently commercially practised, purification is also less effective at removing protozoa and viral contaminants such as norovirus and Hepatitis A virus (HAV) (Chironna et al., 2002; Gallina et al., 2013; Polo et al., 2014; Prato et al., 2013). Purification is not consistently effective nor ineffective at removing other contaminants such as marine biotoxins, *e.g.*, those causing paralytic shellfish poisoning (PSP), diarrhetic shellfish poisoning (DSP) and amnesic shellfsh poisoning (ASP), heavy metals or organic chemicals (Lee et al., 2008). According to Reg. (EC) 2285/2015, the evaluation of shellfish safety is based entirely on the use of *E. coli* as an indicator of faecal contamination and does not consider the occurrence of naturally occurring pathogenic vibrios, such as *V. parahaemolyticus*, for which further studies are necessary to fill the data gap on levels in EU shellfish production areas and to define specific criteria (Suffredini et al., 2014). Faecal indicators provide an inadequate index of microbiological safety for naturally occurring vibrios and underestimate the efficiency of the purification process. Moreover, to develop measures for human health protection based on a robust risk analysis, the acquisition of data on the prevalence of other pathogenic vibrios, such as *Vibrio cholerae*, *V. vulnificus*, and their potential pathogenicity traits, is fundamental (Passalacqua et al., 2016). Recent literature about the occurrence of *E. coli*, *Salmonella* spp, *Vibrio* spp and HAV in the Sardinian bivalve molluscs supply chain (one of the nationally relevant Italian production areas) is limited. Therefore, the aims of this study were *(a)* to verify their occurrence in two Sardinian harvesting areas and *(b)* to investigate the efficacy of short purification cycles carried out by Food Business Operators (FBOs) on the safety of naturally contaminated Mediterranean mussels *(M. galloprovincialis).*

2. Materials and methods

2.1. Sampling

The survey was conducted on samples (*n=300*) of Mediterranean mussels from class B harvesting areas of the Gulf of Olbia (Sardinia). Live bivalve molluscs from these areas must not exceed the limits of a Most Probable Number (MPN) test of 4600 *E. coli*/100 g of flesh and intravalvular liquid in more than 10% of samples. Shellfish may be placed on the market for human consumption after purification, relaying in class A areas or cooking by an approved method. Samples from batches (*n=10*) of mussels were collected before (*T0*), during (*T4, after 4 h*) and at the end (*T8, after 8 h*) of purification treatment from purification centres A and B located near the harvesting areas. A batch is a quantity of live bivalve molluscs collected from the same production area and subsequently intended for delivery to an approved dispatch centre, purification centre, relaying area, or processing plant as appropriate. Five samples per batch were taken from *T0*, *T4* and *T8*. To evaluate the effect of seasonality, sampling was scheduled in two different seasons of the year: batches 1–5 were collected between January and February, while batches 6–10 between June and July. Sampling was performed by the authors and by the local veterinary services of the National Health System, and recordings of environmental conditions (temperature, pH and salinity) of the water used for purification were included.

2.2. Purification treatment

Purification centre A can be described as “recirculating”; clean saltwater (≤15 NTU, Nephelometric Turbidity Units) was supplied directly from an intake point located in an area in compliance with the requirements for a class B production area. Bivalve molluscs (~50 kg/m^2) were placed in one or more high density polyethylene (HDPE) tanks (1100 L each) stacked on top of each other and supplied by a common seawater source in parallel (water flow rate ≥18 L/min). The flow of water disinfected by ozone (≤0.5 mg/l for ≤ 10 min) and/or UV (254 nm, ≥12 Mw/cm^2) was introduced into the tank by means of a spray bar on the surface of the water. A suction bar a few centimetres off the base of the tank (to avoid taking up sedimentary materials) allowed discharge of contaminated water. Prior to disinfection processes, additional treatments (protein skimmers and biofilters) were applied to recirculated seawater to reduce concentrations of metabolic by-products from the bivalve molluscs (such as proteins and ammonia). Recirculating water was then passed through the pump and UV unit (254 nm, ≥12 Mw/cm^2) back to the spray bar. Purification centre B is defined as “flow-though”: natural seawater to be used in the purification process was supplied in the same way as described above for “recirculating” systems. The water was then treated with chlorine dioxide (3 mg/l) and subsequently subjected to filtration with sand and activated carbon units to lower the level of faecal contamination. Salt water was then conveyed to specific tanks, in which the bivalve molluscs could undertake their normal pumping activity to get rid of intravalvular sand and faecal bacteria. To avoid recontamination, at the end of the “flow-through” purification cycles, the seawater that had been used was subjected to UV disinfection (254 nm, ≥12 Mw/cm^2); this process prevented the release of shellfish pathogens or release of toxin-producing phytoplankton from imported bivalve molluscs. During the observed period, the pH values (mean ± s.d.) of water were 6.32 ± 0.01 (*T0*), 6.24 ± 0.02 (*T4*), and 6.29 ± 0.02 (*T8*) at purification centre A and 6.36 ± 0.09 (*T0*), 6.26 ± 0.12 (*T4*), and 6.25 ± 0.01 (*T8*) at purification centre B.

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