Research Paper

Depuration Times of *Donax trunculus* and *Tapes decussatus*

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Abstract

The present study was performed to determine the depuration time and ability of *Donax trunculus* (Wedge Clam) and *Tapes decussatus* (Carpet Shell) contaminated with *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Vibrio parahaemolyticus*. Clams were contaminated with each bacterium at the level of 7.0 - 8.0 Log_{10} cfu/g. After contamination, clams were analyzed every 3 h in the first 24 h time period and every 6 h until the 72nd hour. During the depuration process of both clams, the level of bacteria decreased quickly to 40% of initial load in the first 12 h. The results of this study indicate that the depuration time of carpet shells for all bacteria is 66 h. The depuration process of the wedge clam was different from the carpet shell; *S. typhimurium* and *E. coli* can be depurated in 66 and 78 h, respectively, while *V. parahaemolyticus* was present after 72 h at the level of 1.7 Log_{10} cfu/g.

Key words: clam, purification, *Vibrio parahaemolyticus*, *Escherichia coli*, *Salmonella typhimurium*.

Introduction

Clams have high-quality animal protein content which is similar to that of milk and eggs, making them nutritional foods and important components in the human diet worldwide (Fauconneau, 2002; Murchie *et al.*, 2005; Oliveira *et al.*, 2011). Extensively harvested clam species in the world belong to the *Veneridae* family. *Donax trunculus* and *Tapes decussatus* are two important members of this family (FAO, 2012).

Donax trunculus (Wedge Clam) is an Atlantic Mediterranean warm-temperate species. In the Atlantic, it has been recorded from Senegal to the French coast (Tebble, 1966). It inhabits the high-energy environment of exposed sandy beaches, where it forms extensive, dense beds. *Tapes decussatus* (Carpet Shell) is naturally found from the south and west coast of the British Isles to the Mediterranean Sea and along the Atlantic coast from Norway to Senegal (Tebble, 1966; Breber, 1985). It lives in muddy-sand sediments of tidal flats or shallow coastal areas (Parache, 1982). Both of these clams are also common species found along Turkish shores and are harvested intensively from natural beds in the Aegean to Black Seas.

Shellfishes are filter-feeding mollusks which accumulate microorganisms, including human pathogenic bacteria and viruses, when grown in sewage-polluted waters. Because of this, in the Turkish Fisheries Legislation and the Council Directive of European Commission (EC) laying down the health conditions for the production and placing on the market of live bivalve molluscs, production areas are classified on the basis of the *E. coli* level, as category A, B, and C. Bivalves in category A, which contain maximum 230 cfu/100 g *E. coli*, are suitable for consumption. Bivalves collected from category B areas (< 4600 cfu/100 g *E. coli*), can only be destined for human consumption after a depuration process to meet category A standards. Bivalves found in category C areas can be placed on the market only after a delay of a long period (at least two months) whether or not combined with depuration, or after an intensive depuration for a period to be determined (EC, 1991).

Controlled self-purification (depuration) of shellfishes is a common practice which is utilized to reduce

loads of microorganisms. It is done in controlled seawater of which temperature, salinity, oxygenation, and flow rate are monitored permanently. The seawater should also be cleaned and disinfected by various treatments such as UV, ozonation, and filtration to achieve successful purification of shellfishes. Moreover, effectiveness of the depuration process also depends on the diversity and physiology of shellfishes along with characteristics of water and the system (Schneider *et al.*, 2009).

The present work was carried out to determine the depuration ability of *Donax trunculus* and *Tapes decussatus* contaminated with *Escherichia coli*, *Salmonella enterica* subsp. *enterica* serovar Typhimurium and *Vibrio parahaemolyticus*.

Material and Methods

Collection of samples

The *Donax trunculus* and *Tapes decussatus* clams were harvested by diving to offshore natural beds located in the Çanakkale Strait and Marmara Sea (Turkey). After landing, each sample (about 10 kg) was immediately refrigerated (4 °C) and transported to the depuration plant, where they were inspected; dead or damaged specimens were eliminated. The remaining clams were then divided into aliquots of 2.5 kg each and enclosed in special wire mesh bags.

Bacterial strains

E. coli ATCC 25922, *Salmonella enterica* subsp. *enterica* serovar Typhimurium ATCC 14028 and *V. parahaemolyticus* ATCC 17802 were used for the trial. Prior to use in the contamination process, lyophilized strains of *E. coli*, *S. typhimurium,* and *V. parahaemolyticus* were enriched at 37 °C for 24 h in EC Broth (Merck), Brain Heart Infusion Broth (Merck) and Tryptic Soy Broth (Merck) with 7% NaCl, respectively.

Acclimation of the clams

Prior to the experiment, the clams were acclimatized for 24 h in tanks containing 1000 L of artificial seawater to confirm that specimens were alive and actively filtering (Power and Collins, 1989). The conditions in the acclimation tanks were; salinity 2.8-3.3%; temperature 10-12 °C; oxygen values > 90% of air saturation, measured using a Dissolved Oxygen Meter YSI 58.

Contamination of samples

Sample contamination was conducted in 100 liter polyethylene tanks filled with artificial seawater, which was inoculated with specific bacterial suspensions to reach the concentration of 8.0 Log_{10} cfu/mL. The used inoculums were fresh overnight cultures of each organism (*E. coli*, *S. typhimurium*, *V. parahaemolyticus*). The bacteria were grown at 37 °C on plate count agar (PCA, Merck). The cells

were harvested and then suspended in physiological saline solution. Appropriate amounts of the suspension were added to the uptake tank. The clams were removed from the contamination tanks after four hours (Marino *et al.*, 2005).

Depuration of clams

Depuration was carried out under the controlled physicochemical conditions previously described using commercial baths (length of bath, 9.8 m; width, 1.3 m; depth of water, 1.2 m). The system, with an open-circuit seawater-disinfection system that uses both filtration and ozone treatment system, and the ozone treatment tank at a flow rate of approximately 6 L/min needed about 14 h for a complete water cycle. The ozone generator yielded approximately 50 mg/h O_3. The water temperature was regulated by thermostat. In the conditioning tank, water parameters were monitored and controlled to produce a `standard conditioned seawater' of 18 parts per thousand (ppt) salinity, pH 7.1, and 6.0 to 7.0 mg/L of dissolved oxygen (DO). DO levels were compared, though levels were maintained and monitored with a DO meter. DO of 5 mg/L is the lower limit recommended for depuration, and 7 mg/L is the approximate solubility limit of oxygen in seawater having 18 ppt salinity at 14 °C. The clams were placed on a perforated plastic case. Samples were removed at 0, 3, 6, 9, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66, 72, and 78 h for analyses. Uncontaminated clams were used as controls. Each experiment was carried out in triplicate.

Microbiological analyses

Clams were washed and scrubbed free of dirt. The contents (meat and liquor) of samples comprising 50 clams from each site were randomly subdivided into five subsamples of 10 clams each. The subsamples were scrubbed with a sterile knife, and microbial groups were enumerated using the plate count method as described by the Food and Drug Administration (FDA, 1998). The surfaces of the samples were cleaned with alcohol. Meat (10 g) was diluted in 90 mL physiological saline solution (peptone water). Samples were placed in sterile stomacher bags and homogenized in Stomacher 400 Circulator (Seward, UK) for 2 min at low speed. The homogenates of samples were subjected to serial dilution with peptone water (0.1% bacteriological peptone). For *V. parahaemolyticus*, a pre-enrichment procedure was applied at 37 °C for 1 h in peptone water before diluting samples. Portions of 0.1 mL from each dilution were spread onto plates of the appropriate selective media; ENDO agar (Merck) for *E. coli*, thiosulfate citrate bile sucrose agar (TCBS, Merck) for *V. parahaemolyticus*, and xylose lysine deoxycholate agar (XLD, Merck) for *S. typhimurium*, which were incubated at 37 °C for 24-48 h. After incubation, typical colonies were counted and the results were expressed as Log_{10} cfu/g of sample.

Statistical analyses

Descriptive statistics (mean and standard deviation) for quantitative variables, such as length, weight, and levels of bacteria; and T test for determining the depuration ability of clams for bacterial strains were performed with using Microsoft Office Excel 2007 software (Seattle, USA).

Results and Discussion

Bivalve mollusks accumulate contaminants within the water column they inhabit. The important ingredients of these contaminants, microorganisms, can cause acute gastroenteritis in humans who eat bivalve mollusks raw or undercooked. Therefore, bivalves should be subjected to depuration to eliminate microbiological risks after harvest. According to national and international standards, live mussels should be controlled in terms of *E. coli*, *Salmonella* spp., *Vibrio* cholerae and *V. parahaemolyticus* content. Therefore, in this study bivalve mollusks were contaminated with the most referenced strains of these bacteria in previous studies and they were subjected to the depuration process until the mussel tissue met the standards of < 230 CFU/100 g *E. coli* and absence of *Salmonella* spp., and *V. parahaemolyticus* were reached (Anonymous, 1995; EC, 1991). The observation of excreted bacteria by the clam species was made in a fully controlled closed cycle depuration system. Initial loads of bacteria in clams and average biometry characteristics of clams used in experiments are presented in Table 1.

Contamination of shellfishes via marine water by *E. coli* is mainly due to the disposal of wastewater and it indicates the possible presence of pathogenic microorganisms. Therefore, *E. coli* concentrations are recognized as a shellfish quality indicator by national and international regulations. The present study has shown that clams contaminated with *E. coli* above the threshold level of category C area can be depurated and meet the standards of category A after 72 h of depuration.

Wedge clams excreted about 50% of the initial load of *E. coli* after 36 h. The decrease in *E. coli* loads was rapid until the sixth hour of depuration; however, it remained at the level of Log_{10} 1.7 in wedge clams after 78 h. In Carpet shell,

E. coli was undetectable at the 72th hour of the depuration process (Figure 1). There is no statistical difference between excretion rates of both clams for *E. coli* (p > 0.05).

Many authors have reported depuration times for *E. coli* in various bivalve species. El-Gamal (2011) stated that *E. coli* can be completely excreted by *Paphia undulata* after 72 h of depuration, even if the initial amount is above the threshold level of category B. Barile *et al.* (2009) reported that *Chamelea gallina*, which was contaminated with 4.0 Log_{10} cfu/g *E. coli*, can reach the marketable level after 48 h depuration. Nonetheless, the depuration time of *Mytilus galloprovincialis* under the same conditions was determined to be less than 36 h. In another study, it was observed that *E. coli* was detectable after 44 h of depuration in *M. galloprovincialis* contaminated with 5.0 Log_{10} cfu/g (Croci *et al.*, 2002). The excretion time for *E. coli* in *Crassostrea virginica* (26 h) and *Mercinaria mercinaria* were reported to be shorter than for hard shell clams (68 h) (Love *et al.*, 2010). According to these reported findings, the depuration process for *E. coli* is mostly affected by diversity of bivalve species. Along with this, the size and physical condition of bivalves, initial loads of bacterial strains and environmental conditions (temperature, salinity, pH, etc.), are the other important parameters affecting the depuration process of this bacteria (Joven *et al.*, 2011; Schneider *et al.*, 2009).

The presence of *Salmonella* in the marine environment constitutes a potential threat to human health, since these microorganisms are involved in seafood-borne outbreaks (EFSA, 2010; Norhana *et al.*, 2010). The legal limit of *Salmonella* spp. enforced by Turkish Fisheries Legislation and EC Directives is absence of 25 g in meat and intravalvular liquor of bivalves. In the present study, *S. typhimurium* was the first strain excreted from both clam species at the 66th hour of the process (Figure 2). It was determined that there is no statistical difference between excretion rates of both clam species for *S. typhimurium* (p > 0.05).

In several studies it has been stated that a three day depuration process could be enough for excretion of *Salmonella* (Barile *et al.*, 2009; El-Gamal, 2011). El-Shenawy (2004) has reported that clams containing *Salmonella* in the limits of category B, can be depurated by 90% of initial

Table 1 - Biometry characteristics of two species of clams used in this study and initial bacterial loads per clam.

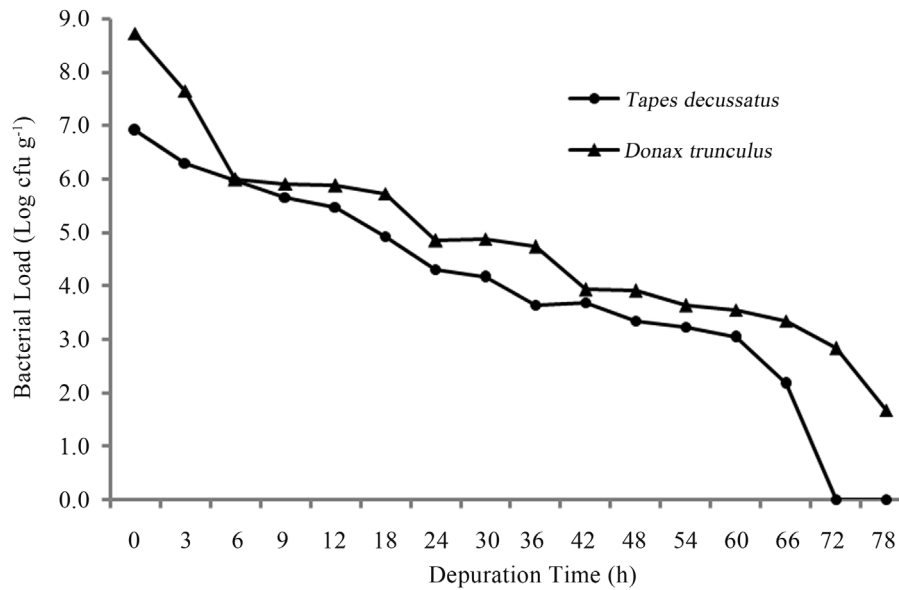| Clam species	Bacterial strains	N	Mean length (mm)	Mean weight (g)	Mean initial bacterial loads (Log_{10} cfu/g)
Donax trunculus	*E. coli*	120	30.6 ± 2.0	3.3 ± 1.1	8.7 ± 0.4
	S. typhimurium	125	29.0 ± 4.5	3.6 ± 1.4	8.8 ± 0.4
	V. parahaemolyticus	117	28.6 ± 3.4	3.6 ± 1.1	8.8 ± 0.2
Tapes decussatus	*E. coli*	80	30.4 ± 4.7	15.2 ± 3.1	6.9 ± 0.5
	S. typhimurium	72	30.7 ± 6.2	16.5 ± 3.6	6.4 ± 0.6
	V. parahaemolyticus	75	31.1 ± 2.2	16.3 ± 3.4	8.9 ± 0.9

Figure 1 - Changes in *E. coli* levels in *Tapes decussatus* and *Donax turunculus* during the depuration process.
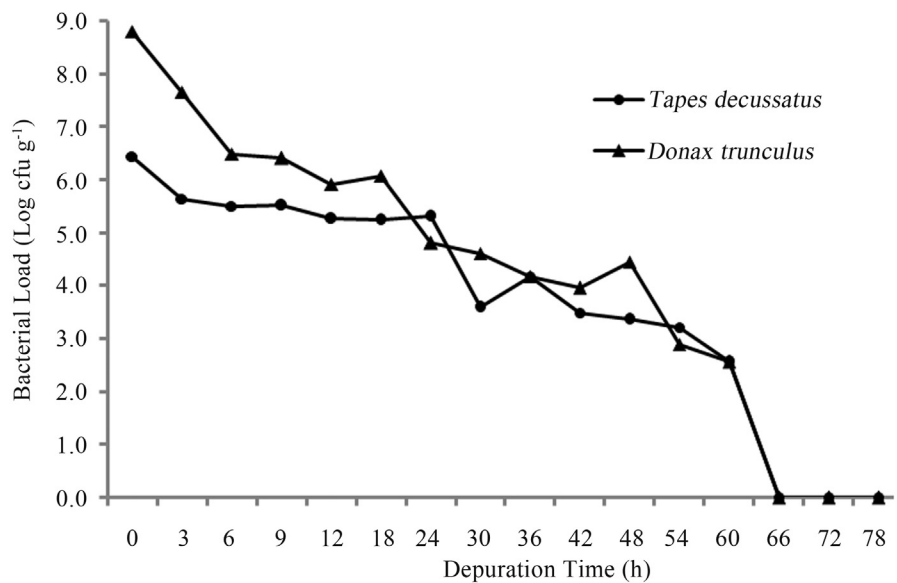

Figure 2 - Changes in *S. typhimurium* levels in *Tapes decussatus* and *Donax turunculus* during the depuration process.

loads after 4 days. However, clams containing *Salmonella* in the limits of category C, reduce initial load less than 50% in the same depuration period. El-Gamal (2011) has noted that *Paphia undulata* can depurate 97% of 3.5 Log_{10} cfu/g *Salmonella* after three days depuration process. In another study, the depuration times for *C. gallina* and *M. galloprovincialis*, which were contaminated with *S. typhimurium* at the level of 4.0 Log_{10} CFU/g, were determined as 72 and 36 h respectively (Barile *et al.*, 2009).

Vibrio species are inhabitants in marine coastal waters and remain viable in this environment for a very long time, even in the absence of organic matter (Su and Liu,

2007). Among these species, *V. parahaemolyticus* is one of the major seafood-borne gastroenteritis agents and is frequently associated with the consumption of improperly cooked shellfishes (Chae *et al.*, 2009). According to Turkish legislation on fisheries, *V. parahaemolyticus* should be negative in 25 g of meat and intravalvular liquid of marketed live shellfishes (Anonymous, 1995).

In the present study, *V. parahaemolyticus* was depurated at about similar times to the other bacteria in the carpet shell; however, it was detectable even at the 78th hour of the depuration process in the wedge clam (Figure 3).
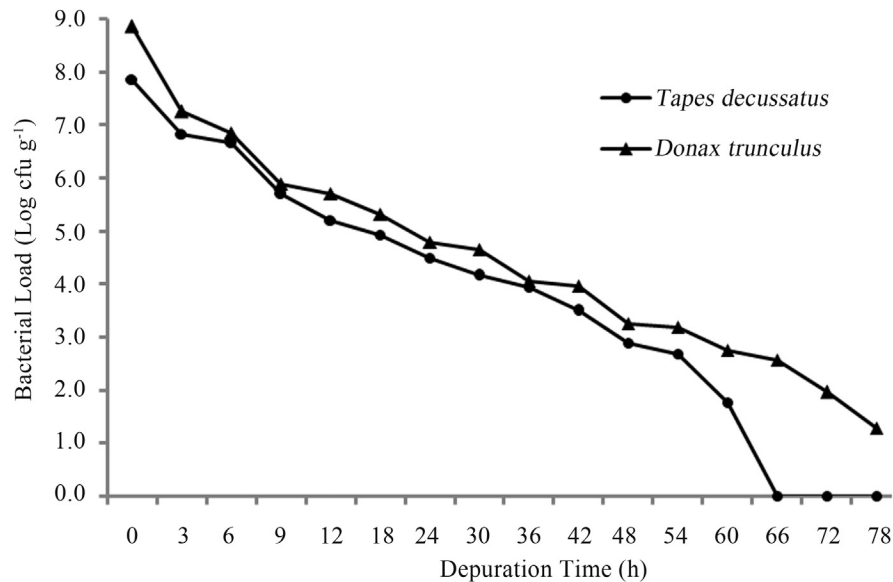
Figure 3 - Changes in *V. parahaemolyticus* levels in *Tapes decussatus* and *Donax turunculus* during the depuration process.

The results presented here have similarities with the findings of previous studies. Croci *et al.* (2002) determined that *V. parahaemolyticus* at the concentration of 4.9 Log_{10} cfu/g remained in *M. galloprovincialis* after 44 h depuration, even though the majority of the reduction occurred in the first 5 h of treatment. This observation was made in another study which highlighted that 13.6% of the initial concentration of *V. parahaemolyticus* was retained after 48 h depuration in *Crassostrea gigas* (Wang *et al.*, 2010). Barile *et al.* (2009) also notified that *V. parahaemolyticus* decreased 75% of initial load in first 24 h and remained even after 60 h in *C. gallina.* In these studies, researchers also concluded that *V. parahaemolyticus* has different depuration behavior compared to the other bacteria. The reason for this behavior can be explained by the fact that Vibrios might affect the filtration capacity of clams (Barile *et al.*, 2009).

In conclusion, wedge clam and carpet shell, which were contaminated above the threshold level of category C, reached the standards of category A after the depuration process; however, *V. parahaemolyticus* was detectable after the process in the carpet shell. Therefore, it has emerged from the results of this study that *V. parahaemolyticus* should be considered while categorizing the bivalve production beds and determining bivalves for direct human consumption harvested from category A.

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