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DETERMINATION THE CHANGES OF *E. coli* DEPURATION TIMES IN *Ruditapes decussatus* AND *Venus verrucosa* BY PRODUCTION AREA AND SPECIES DIFFERENCES

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Abstract

In this study, two important economic bivalve mollusc species, grooved carpet shell (*Ruditapes decussatus*) obtained from two different production areas (batch-A and batch-B) and warty venus (*Venus verrucosa*) were contaminated with *Escherichia coli* (*E. coli*), and the time-dependent changes in their ability to depuration process were determined. At the same time, after the natural *E. coli* levels of these two species were determined, the natural depuration times were also investigated by subjecting them directly to depuration process separately.

The natural *E. coli* loads of the carpet shell and warty venus samples used in the research were determined as 1500 MPN/100 g in batch-A samples, 430 MPN/100 g in batch-B samples while the level was determined as 74 MPN/100 g warty venus samples. The natural sample depuration process was completed 18 hours after the start of the process for batch-A samples and 6 hours after the start of the process for batch-B and warty venus samples, and it was determined that the products reached Class A production area standards. Depuration time of samples after the contamination of samples with *E. coli* at the level of 2900 MPN was determined as 12 hours for warty venus, 18 hours for carpet shell. As for the samples contaminated at the level of 4600 MPN, carpet shell (batch-A) and warty venus samples reached the Class A production area standards after 24 hours and warty venus (batch-B) samples reached the Class A production area standard after 48 hours. As a conclusion, species, and sample collection sites may have effect on depuration characteristics of *E. coli* and this may be related to physiological conditions of the specimens during the collection sites.

Keywords: *Ruditapes decussatus*, *Venus verrucosa*, *E. coli*, depuration, bivalve, carpet shell, warty venus

Introduction

Bivalve molluscs are among the seafood with high economic value all over the world due to their nutritional and sensory properties However, since these products are not fed by selective filtration, they directly accumulate biological and chemical risk factors in the marine environment where they grow (Çolakoğlu et al., 2020; Künili et al., 2021). Belong to the biological risk factors, microorganisms including pathogenic bacteria are main issues concerning the food safety and public health. The control of the presence of pathogens in bivalves is planned by monitoring the microbiological quality of seawater where natural production beds or aquaculture systems are located. In this way, instead of direct monitoring of pathogenic bacteria, the presence and levels of indicator microorganisms are monitored, and depuration is applied when necessary to ensure safe bivalve production in a systematic manner. *Escherichia coli* is considered as indicator microorganisms in terms of showing the potential presence of other pathogenic bacteria (Künili & Ateş, 2021) and is included in the legal criteria of the bivalve mollusc production area of the European Union and Türkiye (EC, 1991; Anonymous, 1995). In these criteria, production areas are classified as A, B and C classes with *E. coli* counts <230 MPN/100g, 2600 EMS/100g and 4600 EMS/100g (>6000 EMS/100 g faecal coliforms) respectively. According to these legislations, when the level of *E. coli* exceeds the A class criteria, depuration process must be applied to bivalves until the level reaches equivalent or under this classification limit. For this reason, depuration process is an important step for marketing without getting banned, and public health issues in terms of food safety.

Depuration, self-purification of bivalves under controlled conditions, is a common practice used in the live bivalve processing industry to reduce the number of microorganism risks. Depuration systems utilise controlled seawater provided by continuous monitoring of temperature, salinity, oxygenation, and flow rate. Furthermore, the effectiveness of the system is maintained by cleaning and disinfecting it through various treatments such as addition of chlorine, UV light, ozone, active oxygen, iodophor and filtrations (Künili and Çolakoğlu, 2019).

In this study, two important economic bivalve mollusc species, grooved carpet shell (*Ruditapes decussatus*) and warty venus (*Venus verrucosa*) were contaminated with *Escherichia coli* (*E. coli*) bacteria at levels classified as B and C classes in the European Union standards (EC, 1991) and Turkish Fisheries Legislation (Anonymous, 1995), and the time-dependent changes in their ability to depuration until they reach the acceptable level of A class were investigated. At the same time, after the natural *E. coli* loads of these two species were found, their natural depuration times were also determined by subjecting them to depuration process separately. Furthermore, an attempt was made to determine whether differences in bacterial load and production area influence depuration time. This was achieved by comparing the species grown in production areas with different commercial fishing permits for the carpet shell, which may contain unacceptable levels of *E. coli* during certain production periods, leading to export halts in some of previous year.

Material and Method

Ruditapes decussatus (batch-1 and batch-2) and *Venus verrucosa* were used as research material. Batch-1 (A) and Batch-2 (B) of *R. decussatus* are represents different production areas which are characterized different water qualities and geographical areas. *V. verrucosa* were obtained as single batch from different production area. A total of 6 kg for each batch *(R. decussatus*-A, *R. decussatus*-B, and *V. verrucosa*) were obtained for the trials and their natural levels of *E. coli* were determined prior the experiments. The mean lengths and weights were 3.24±0.38 and 17.12±8.42 for *R. decussatus*, and 3.85±0.52 and 20.54±10.11 for *V. verrucosa*, respectively.

Preparation of Bacterial Strain

Lyophilized *Escherichia coli* ATCC 25922 (American Type Culture Collection, USA) strain was used in trials. Before using the strain, overnight culture was prepared in Muller Hinton Broth at 35°C. Prior contamination process, bacterial peptone water (1% w/v) and salt (8.5% w/v) was used to prepare decimal dilutions. A McFarland densitometer (Biosan, Türkiye) was used for adjusting the bacterial load in peptone waters which were used in contamination tanks for the target contamination levels of mussels.

Acclimation and contamination of bivalves

R. decussatus and *V. verrucosa* samples were loosely packed in wire mesh and placed in 100 L polyethylene tanks filled with fresh and filtered seawater with a temperature of 12°C, salinity of 2.7%, pH of 7.1-7.2 and dissolved oxygen of 6.6 mg/L for 24 hours. After confirming that the samples were alive and actively filtering, they were subjected to contamination process in the same tanks replaced with fresh seawater containing adjusted levels of *E. coli* strains. For each species, after their initial loads determined, contamination with *E. coli* at the level of 4600 and 2500 MPN/100g were performed for 2 hours separately, and contaminated bivalves were then subjected to depuration process.

Depuration of bivalves

Depuration process was carried out in four tiled pools approximately 5 metres long, 2 metres wide and 1 metre deep each. The pool filled fresh seawater and used for depuration after twice circulations of the water completed through standard filtration systems containing mechanical and biological filtrations units, and UV lamb and a thermostat. Physicochemical properties of seawater in depuration pool were stabilized with a temperature of 12°C, salinity of 2.3 %, pH of 7.1-7.2 and dissolved oxygen of 6.8 mg/ L. The specimens divided by species and placed in pools within polyethylene cases. When the siphons of the species became visible that indicates active water filtration of the species, the depuration process was then considered as commenced. After the process was started, live specimens collected in six-hour periods and *E. coli* levels determined. The study was terminated when the level of *E. coli* in class A product type quality was reached (<230 MPN).

The depuration process of natural samples was carried out in pool 2 for carpet shell (batch A and B) samples and in pool 1 for warty venus samples. After the natural sample depuration processes were completed for 24 hours, pools 1 and 2 were drained, disinfected, filled with fresh filtered clean water, and prepared for the next treatment processes.

After the depuration of natural samples, pool 1 was used for the depuration of 2900 MPN loaded warty venus and pool 2 was used for the depuration of 2900 loaded carpet shell (batch-A and batch-B), pool 3 was used for the depuration of 4600 loaded carpet shell (batch-A and batch-B), pool 4 was used for the depuration of 4600 loaded warty venus samples.

Microbiological analysis

Shells of samples were washed and scrubbed free of dirt, and surfaces were cleaned with alcohol. Samples were then scrubbed with a sterile knife. Meat and intervalvular liquid of samples (a total of 10 g) was diluted in 90 ml physiological saline solution (peptone water), and homogenized in Stomacher 400 Circulator (Seward, UK) for 2 minutes at low speed. The homogenates of samples were then subjected to a series of serial dilutions with physiological saline solution. *E. coli* levels of each diluted samples were determined using most probable number (MPN) method as described by the Food and Drug Administration (FDA, 1998). Enumeration was performed using EC broth (Merck) with an incubation at 44.5°C ± 0.5°C for 48 hours, and confirmation the presence of *E. coli* in randomly selected tubes was performed

dropping Kovac's reagent (Merck) onto the tubes and subjecting to an incubation for 37.5°C ± 0.5°C for 24 hours after spreading onto Endo agar (Merck) plate.

Results

In this study, natural *E. coli* levels and depuration times of the three different groups separated according to production areas and species presented in Figure 1.

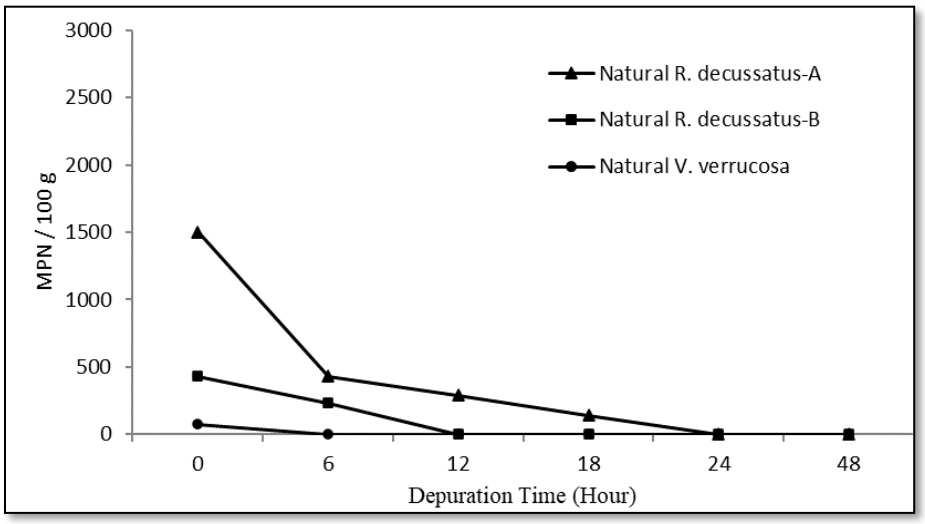

Fig. 1. Natural *E. coli* levels and depuration times of species divided by production areas.

Natural *E. coli* levels of *R. decussatus* and *V. verrucosa* ranged between 74 and 1500 MPN/100 g. The highest *E. coli* levels determined in samples of batch-A which is one of the allowed production areas for *R. decussatus*. Depuration times of *R. decussatus* for excreting *E. coli* was determined 24 hours for batch-A samples (as the maximum 1500 MPN/100 g for initial load), and 12 hours for batch-B samples (as the maximum 430 MPN/100 g for initial load). The lowest level of *E. coli* (74 MPN/100g) was determined in *V. verrucosa* that depuration time determined as 6 hours.

Depuration times of experimentally contaminated *R. decussatus* and *V. verrucosa* were presented in Figure 2 and 3.

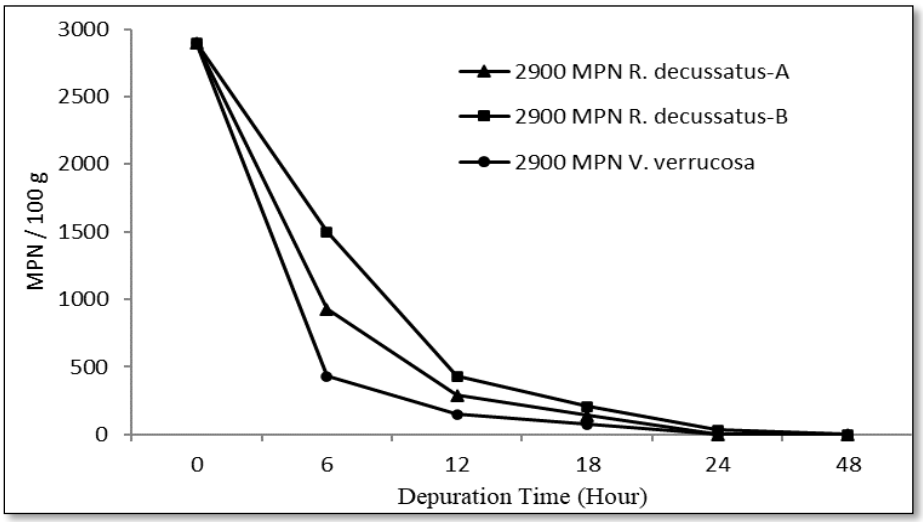

Figure 2. Depuration times of *R. decussatus* (batch-A and B) and *V. verrucosa* contaminated with *E. coli* at 2900 MPN/100g representing class B production areas.

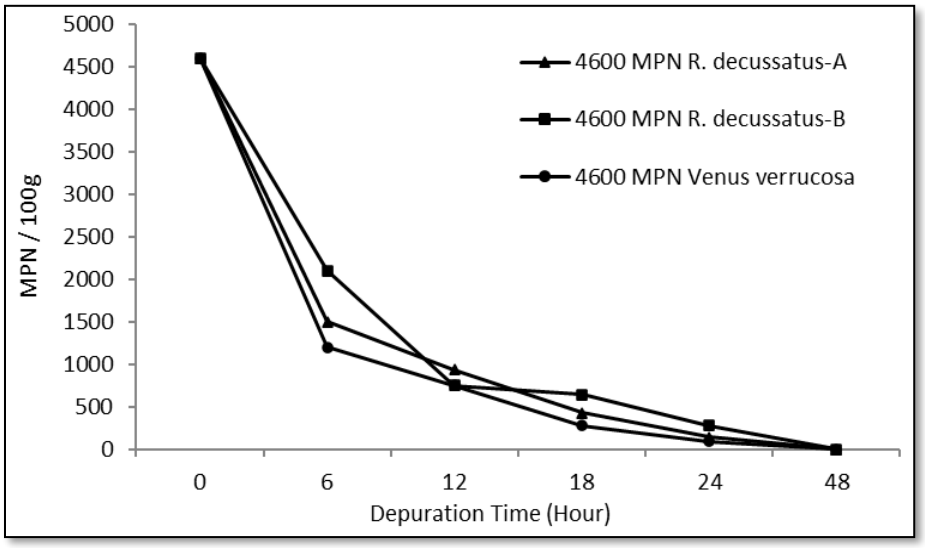

Figure 3. Depuration times of *R. decussatus* (batch-A and B) and *V. verrucosa* contaminated with *E. coli* at 4600 MPN/100g representing class B-C production areas.

In order to represent Class B and Class C production areas, clam species were attempted to be contaminated with E. coli higher than 2600 EMS/100 g and 4600 EMS/100 g, respectively. According to the Class B representative level, the initial loadings of contaminated samples were set at 2900 EMS/100 for each species group. A rapid decrease was observed in the first 6 hours of depuration. The logarithmic decrease slowed down after 12 hours of depuration and Class A production criteria were reached after 12 hours of depuration. To represent Class C, an attempt was made to contaminate species with approximately 4600 EMS/100 g *E. coli*. A similar depuration characteristic observed in Class B experiments was also determined for Class C. A rapid decrease in initial load occurred in the first 6 hours of depuration and the logarithmic decrease slowed down after 18 hours. For this group, Class A criteria were met after 24 hours of depuration. For both Class B and Class C representative groups, *E. coli* levels were not detectable after 48 hours of depuration.

Discussion

Bivalve molluscs can accumulate contaminants in seawater, and this becomes even more important when environmental conditions expose them to stress that makes them more susceptible to accumulation. Among these contaminants, pathogenic microorganisms, the presence of which is controlled by the examination of indicator microorganisms such as *E. coli*, pose a public health concern in terms of food safety. For this reason, as stated in the legislation, *E. coli* levels are periodically checked in bivalve mollusc production areas and these production areas are divided into three classes according to *E. coli* presence. Class A products containing less than 230 MPN 100/g E. coli can be marketed directly, while Class B (containing 230 - 4600 EMS/100 g E. coli) and Class C products (containing more than 4600 EMS/100 g E. coli) are subjected to a series of practices such as soaking in clean coastal sea waters, depuration process after pasteurisation and freezing to ensure marketable product conditions. For this reason, production area and contamination levels, are important issues when considering production of a bivalve mollusc species. In this study, we aimed to determine the effects of the production areas and the species differences of two economic bivalve molluscs on the depuration abilities of *E. coli* that its contamination levels adjusted according to class A, B and C production areas described in the Legislations (EC, 1991; Anonymous, 1995).

E. coli depuration times in different clam species were previously reported by Çolakoğlu et al. (2014). In that study, *Donax trunculus* and *Tapes decussatus* were used as material and the initial *E. coli* load (10^7 cfu/g) were reported as rapidly decreasing by 40% of initial load, and completely depurated after in 66 hours. In another study in which *Mytilus galloprovincialis* used as material, the initial load of *E. coli* with 10^4 - 10^5 cfu/g was decreased by 2 folds in 6 hours (Künili & Çolakoğlu, 2019). Similarly, it was reported that even the initial load of *E. coli* exceeds the category B, *Paphia undulata* meets the standards of marketable live bivalve mollusc criteria after 72 hours of depuration (El-gamal, 2011). In a previous study, a shorter depuration time for *Chamelea gallina* was reported as 48 hours even the initial load as higher as 10^4 cfu/g of *E. coli* (Barile et al., 2009). Moreover, in the same study, it was reported that depuration process of *M. galloprovincialis* under the same conditions was less than 36 hours. Shorter depuration times for *M. galloprovincialis* than clams as the species in our study are also supported in another study which reports that *E. coli* levels could be dropped to detectable levels in *M. galloprovincialis* contaminated with 10^5 cfu/g after 44 hours of depuration (Croci et al., 2002). As seen for *M. galloprovincialis*, the depuration time of Crassostrea virginica (26 hours) contaminated with *E. coli* can be shorter than that of hard-shell clam *Mercinaria mercinaria* (68 hours) (Love et al., 2010).

In this study, it was determined that *E. coli* depuration characteristics showed similar characteristics with those hard-shell bivalves, and showed differences with those oysters and mussels, reported previously. According to this, 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 other microorganisms like protozoans, environmental conditions (temperature, salinity, pH, etc.), and environmental pollutants are the other important parameters affecting physiological conditions of the bivalve molluscs (Künili et al., 2021; Künili, et al., 2021a; Çolakoğlu et al., 2023) that may also lead to changes in the depuration process (Çolakoğlu et al., 2014; Künili & Çolakoğlu, 2019). In specific to our study scope, differences in species, and sample collection sites may still have effect on depuration characteristics of *E. coli* since 4600 MPN/100g contaminated batch A and B samples of *R. decussatus* showed slight differences. This may be also related to physiological conditions of the specimens during the sample collection and differences in the environmental factors of production areas.

Conclusion

Depuration is an important process in the live bivalve mollusc trade because it is a measure both to prevent economic loss and to protect the consumer from food poisoning. *E. coli* is an indicator microorganism for reliable and rapid assessment of food safety risks in the live bivalve mollusc sector. Depuration process, which can be affected various factors including species differences and production areas, is the essential processing procedure for live bivalve species. Therefore, periodic monitoring in production areas as well as determination of depuration times and adequacy for *E. coli* or other specific microorganisms in different bivalve species and according to different seawater environmental parameters may be essential for sustainable production and safe consumption.

Ethical approval

"Not applicable"

Informed consent

Not available.

Data availability statement
The authors declare that data can be provided by corresponding author upon reasonable request.

Conflicts of interest
There is no conflict of interests for publishing this study.

Funding organizations
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Contribution of authors

İbrahim Ender Künili: Funding acquisition, Supervision, Investigation, Methodology, Conceptualization, Data curation, Formal analysis, Writing original draft, Review, Editing.
Selin Özge Dinç: Formal analysis, Writing original draft, Validation, Visualization, Review, Editing.

All authors have read and agreed to the published version of the manuscript.

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