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Abstract. This study reports the occurrence of *Escherichia coli* in 243 mussel samples collected along the Boka Kotorska Bay (six harvesting areas), which is located in the Southern Adriatic Sea. Bivalve molluscs can concentrate contaminants from their water environment, so because of that, determination of *E. coli* levels is an important parameter for microbial pollution assessment in the investigated areas. The number of *E. coli* ranged between <18 MPN/100 g to 16 x 10^3 MPN/100 g of mussel soft tissues. In 243 bivalve mollusc samples, analysed during the period 2018-2019, 68.3% of them had low contamination levels, i.e. ≤ 230 MPN *E. coli*/100 g; 31.3% had between 230 and 4600 MPN *E. coli*/100 g, and 0.4% had > 4600 MPN *E. coli*/100 g. Statistical analysis of the number of *E. coli* in mussels established that the mussel farm vl. Duško Vlahović (M5) had the highest mean *E. coli* levels (949.00 ± 2541 MPN *E. coli*/100 g), while the lowest mean level was recorded in Boka mussels (M1) (149.20 ± 258.80 MPN *E. coli*/100 g). Boka Kotorska bay is classified as a Class B mussel production area because it has 32.9% of samples with *E. coli* MPN values between the 20-230 MPN/100g.

1. Introduction

Mussels are filter-feeding animals that process large volumes of water and small particles of phytoplankton, zooplankton, viruses, bacteria, and inorganic matter from the surrounding water they live in to obtain food. At the same time, various pathogens are stored in them, and therefore they can be indicators of pollution in the environment [1]. Consumption of raw or inadequately cooked mussels poses a potential risk for consumers because of many bacterial infections, especially *Escherichia coli* [2]. According to the current EU regulations (854/2004/EC, 2004) [5], mussel sampling sites have to be classified according to their suitability in terms of microbiological and chemical water quality [3]. Depending on the content of *E. coli* in mussels, all localities should be affiliated as Class A (<230 MPN *E. coli*/100 g mussel), B (<4600 MPN *E. coli*/100 g mussel), or C (4600–46,000 MPN *E. coli*/100 g mussel) areas [2].

The contamination of mussels is influenced by different factors, such as pollution sources, rainfall, salinity, temperature, and many others. In recent years, the Montenegrin coast, especially the coast of the Bay of Kotor, has been exposed to constant spills of waste from various industries, shipyards, hotels, and hospitals that are discharged into the sea and lead to pollution the aquatic environment [4]. This study aimed to examine the presence of *E. coli* in *Mytilus galloprovincialis* from farms in Boka Kotorska

Bay, Southern Adriatic Sea, during a one-year period to classify the production area and determine the existence of correlations between six sites from which samples were taken.

2. Materials and Methods

During the period 2018-2019, 243 samples (about 40 samples from each sampling site) of *Mytilus galloprovincialis* were collected from six harvesting areas: Boka mussels (M1), Cogi mar (M2), Goran Biga (M3), Slobo Vujovic (M4), Dusko Vlahovic (M5), Bosko Supica (M6). Each of these farms is located in Bay Kotorska Bay. Samples were taken randomly, packed in sterile bags, and sent to the laboratory. The sample used for analysis (10 g of flesh and intravalvular liquid) was obtained by homogenizing 10-15 mussels.

E. coli was quantified following ISO/TS 16649–3(2005) [7]. The most probable number (MPN) of *E. coli* was obtained using the 5-tubes and 3 dilutions method. From the whole mussel, we used flesh and intervalvular liquid (FIL), of which we measured a 75 g amount, diluted with the Tryptone salt water (1:3), and homogenized in Stomacher (2 min). Seventy mL of Tryptone salt water were added to 30 mL of this mix and were homogenized until 1:10 dilution. A 10 mL of the dilution was further added to 5 tubes of double-concentrated MMGB (Minerals Modified Glutamate Broth). Next, 1 mL of the 1:10 dilution was added in 5 tubes of single concentrated MMGB, while in the remaining 5 tubes a 1 mL of a 1:100 dilution per tube was added. All the tubes were incubated at 37°C for 24 h. Confirmation of *E. coli* was based on culturing 1 µL of positive tubes onto plates TBX agar (Tryptone Bile X-Glucuronide agar, Oxoid, Wesel, Germany) which were incubated at 44°C for 24 h. The presence of *E. coli* was confirmed by the growth of blue colonies. The number of *E. coli* in 100 g of sample was calculated based on the number of positive results in three dilutions, using MPN tables [7]. Statistical analysis of the results was elaborated using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com) and MS Excel. Descriptive statistics of each data set were computed. Statistical significance levels were set at 5 and 1%. All the results are presented in tables, figures, and graphs.

3. Results and Discussion

During the one-year study period, a total of 243 mussel samples were examined for *E. coli.* Results showed that the number of *E. coli* in 86 samples (35.4%) was < 18 MPN/100 g, in 80 samples (32.9%) was ≤ 230 MPN/100 g; (Class A areas), in 76 samples (31.3%) was between 230 and 4600 MPN/100 g (Class B areas) and in 1 sample (0.4%) was > 4600 MPN/100 g (Class C areas) (Fig 1.). The highest detected number of *E. coli* was 1.6×10^3 MPN/100g, found in one sample from Dusko Vlahovic (M5). If only one sample is out of range 20 - 230 MPN/100g, this harvesting area can not be classified as a Class A area (Reg. (EC) No 854/2004) [5]. Similar results were obtained in studies conducted in South Albania [8]. We recorded a slightly higher number of samples in this area where the *E. coli* MPN was > 4300/100g. In contrast, a study by Henigman et al. [9] reported much better results. On the Slovenian coast, during the tested period, 88.2% of samples did not exceed 230 MPN *E. coli*/100 g.

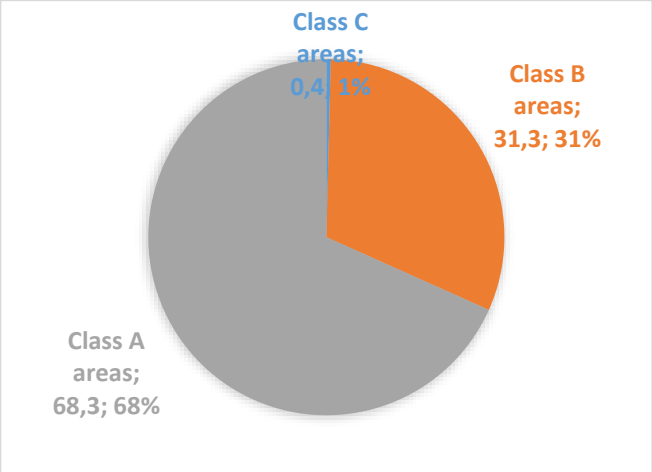

Figure 1. Classification of bivalve mollusks according to production areas

Numbers of *E. coli* in mussels from harvesting areas in Boka Kotorska Bay are shown in Table 1. Statistical analysis of the number of *E. coli* in mussels from six farms on the Montenegrin coast established that the mussel farm Dusko Vlahovic (M5) had the highest *E. coli* MPN values (949.00 ± 2541), while the lowest *E. coli* MPN value was recorded in Boka mussels (M1) (149.20 ± 258.80).

Table 1. Number of *E. coli* (MPN/100g) in Boka Kotorska Bay cultivated mussels

	n	$\bar{x}$	SD	SE	CV (%)	X max	X min
M1	41	149.20[a]	258.80	40.4200	173.42	1100	18
M2	41	149.40[b]	386.10	60.3000	258.43	2400	18
M3	41	238.00[c]	582.00	90.8900	244.48	3500	18
M4	40	658.50	1191	188.3000	180.85	5400	18
M5	40	949.00[abc]	2541	401.8000	267.80	16000	18
M6	40	294.90	447.70	70.7900	151.85	2400	18

The same letters indicate significant differences between groups: a, b, c, p<0,

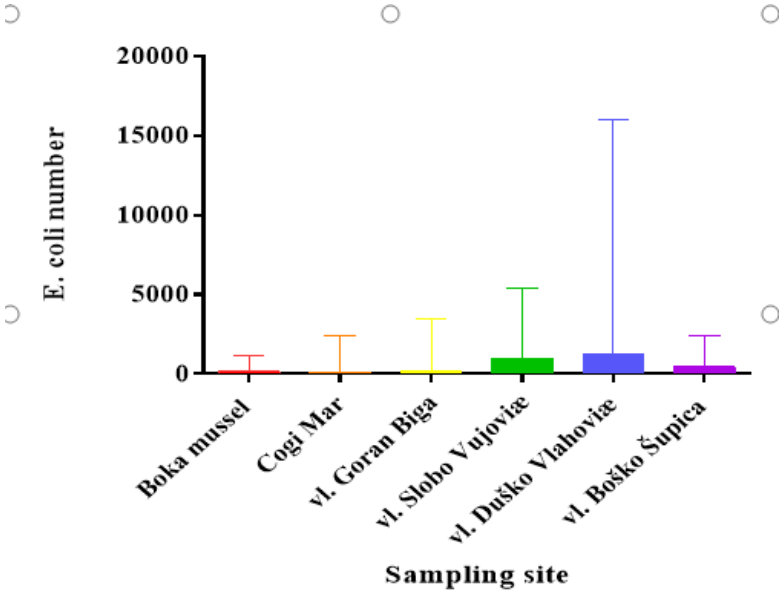

Figure 2. *E. coli* MPN in mussels by farms

Statistically significant differences were found between M1 and M5; M2 and M5; M3 and M5 ($p < 0.05$).

4. Conclusion

Montenegrin shellfish breeders have just recently begun to implement EU classification on shellfish harvesting areas. This research study demonstrated that the South Adriatic area of Boka Kotorska bay had been classified as a Class B mussel production area due to a high percentage of samples with *E. coli* MPN values less than 4600 MPN/100 g. Although the results obtained in six tested harvesting areas did not exceed upper limits laid down by the EU Regulation No 854/2004, all mussels caught in the area must undergo a depuration process before being placed on the market.

Since *E.coli* is a primary indicator of the microbiological quality of shellfish, this study will contribute to risk assessment in human consumption of this type of seafood originating from Montenegro.

Acknowledgment

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