Research article

DETERMINING THE PRESENCE OF *ESCHERICHIA COLI* IN MUSSELS IN MONTENEGRO IN THE PERIOD 2022-2023

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This paper explores the biological diversity of the mussel *Mytilus galloprovincialis*, its
ecological functions, and its economic importance for local communities. By analyzing
the ecological roles of mussels in the preservation of ecosystems, as well as their
contribution to the local economy through fishing and aquaculture, the work provides a
comprehensive insight into their importance and the threats they face. The aim of this
study is to analyze the presence of the bacterium *Escherichia coli* (*E. coli*) and determine
statistical significance in mussels collected from three different locations in Montenegro:
Herceg Novi, Tivat, and Kotor. The analyses were carried out during different seasons,
in a period of two years, 2022 and 2023. This approach enabled a detailed analysis of
seasonal variations in the level of mussel contamination. Preliminary results show that
the highest concentrations of *E. coli* were recorded during the summer months, which
can be associated with increased tourist activities and a greater load on the sewage
system. The lowest levels of contamination were registered during the winter months.
This reduction occurs because tourist and industrial activity decreases, resulting in less
waste and wastewater production. Furthermore, precipitation and winds that dilute
pollutants increase, while lower temperatures slow down biological processes. Among
the localities analyzed, samples from Kotor showed the highest average concentration
of *E. coli*, while samples from Herceg Novi had the lowest values. These results indicate
significant differences in the degree of pollution between localities and seasons, which
can have serious consequences for human health, and the ecosystem. The presence of *E.
coli* in mussels directly threatens the safety of food products, because the consumption
of contaminated mussels can lead to various gastrointestinal diseases. Also, long-term
pollution can disrupt the balance of marine ecosystems, affecting biodiversity and the
stability of mussel populations.

Keywords: Mussels *(Mytilus galloprovincialis)*, *Escherichia coli,* Montenegrin coast

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INTRODUCTION

The Montenegrin coast, located in the western part of the Balkan Peninsula, is rich in biodiversity and represents an important ecosystem for various types of mussels. Understanding these aspects is key to preserving these ecosystems and supporting sustainable development [1].

The Montenegrin coast is an area of particular interest for the study of microbiological contamination. Although renowned for its shellfish production, including mussels and oysters, growing pressures from urbanization, agricultural activities, and tourism development may increase pollution risks, particularly during the summer months [1,2]. Seasonal variations in water temperature and air temperature, as well as rainfall, significantly influence the presence of bacteria in seawater [3].

In the Adriatic Sea, mussels are an important source of food and an export branch, so quality and safety control of marine products is of great importance. The microbiological safety of seafood, especially mussels, is becoming an increasingly important topic due to the growing awareness for public health and ecosystem conservation. Also, they can be indicators of the fecal contamination of the environment in which they are located [2,4]. In the European Union, microbiological monitoring of harvesting areas is based on the presence of *Escherichia coli* in molluscs [5]. *Escherichia coli (E. coli)*, is a gram-negative bacterium commonly found in the intestines of humans and warm-blooded animals [6-8]. Mussels, like filters, accumulate microorganisms from the water in which they are found. Faecal contamination, including *E. coli,* can occur due to untreated wastewater or poor hygiene conditions in aquaculture areas [2,9-11]. Its presence in mussels can pose a serious health risk to people who consume these mussels raw or undercooked [8,12].

Mussel production areas are classified according to the levels of *E. coli* detected in mussel meat, which determines the required treatment before they may be marketed for human consumption. According to the current EU regulations (854/2004/EC, 2004) [8,13], these areas have to be classified according to their suitability in terms of microbiological and chemical water quality. Depending on the content of *E. coli* in mussels, all localities should be affiliated as Class A (<230 MPN) *E. coli*/100 g mussel), Class B (<4,600 MPN *E. coli*/100 g mussel), or Class C (4,600–46,000 MPN *E. coli*/100 g mussel) areas.

The aim of this research is to examine the level of presence of *E. coli* in mussels collected from different locations along the Montenegrin coast, to identify potential pollution sources, and to determine the relationship between the contamination levels and environmental factors. In addition, the research relies on standardized microbiological methods for the detection and quantification of *E. coli,* and the results will be analyzed using statistical methods to obtain relevant contamination data. The results of this research can serve as a basis for making informed decisions and

improving practices in the seafood production sector, which would reduce risks to consumer health and preserve biological diversity in coastal ecosystems.

MATERIAL AND METHODS

Research period and locations

The research was conducted during two consecutive years (2022 and 2023) at three locations on the Montenegrin coast: Herceg Novi, Tivat, and Kotor (Figure 1). These localities were selected due to their ecological, economic, and touristic importance, as well as for the intensive cultivation of mussels. Sampling was done seasonally (spring, summer, autumn, and winter) to assess seasonal variations in the presence of *E. coli*.

Figure 1. Study area (Herceg Novi, Tivat, Kotor)

Mussel sampling

Mussel (*Mytilus galloprovincialis*) samples were taken directly from the nesting site at a depth of 1 to 3 meters at each location (Figure 2). Samples were collected in sterile containers and transported on ice to the laboratory within 6 hours of sampling. A certain number of samples were collected from each location per season. Each sample included 19 to 23 individuals, which corresponded to a mass of 100 grams of homogenized sample to achieve representativeness. After collection, the samples were transported to the laboratory at a temperature of 4°C.

Figure 2. Threatened mussel species (*Mytilus galloprovincialis*)

Microbiological Analysis

Mussel samples were subjected to the following steps in the laboratory: the mussels were rinsed under cold water and aseptically opened with a sterile knife. Tissue and intervalvular liquids were homogenized and diluted with peptone water to decimal dilutions. The sample used for analysis (10 g of tissue and intervalvular liquid) was obtained by homogenizing 10-15 mussels. Samples were analyzed according to the ISO/TS 16649-3 most probable number (MPN) technique using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide (International Organization for Standardization 2015). The results were recorded as MPN/100 g of mussel tissue and intervalvular fluid.

Statistical processing and data sources

In the statistical analysis of the obtained results of the performed experiment, we used descriptive statistical indicators as the basic statistical method. These indicators enabled us to describe the obtained experimental results and their interpretation. From the descriptive statistical indicators we used: measure of central tendency, standard deviation, standard error of the arithmetic mean, interval of variation and coefficient of variation. Further statistical analysis takes place depending on whether the analyzed data is normally distributed or not. Testing for normality was performed using the Kolmogorov-Smirnov test. In case of normal data distribution for comparing significant differences between samples (the sample is a subset of the basic set, that is, the population) we used parametric analysis of variance (One way analysis of variances). In case the data distribution is not normal, the non-parametric Kruskal-Wallis analysis of variance (Kruskal Wallis Analysis of Variance on Ranks) was used.

In case there are statistically significant differences between groups, pairs of groups will be compared with each other based on the parametric Tukey's test, i.e. the non-parametric Dunn's Multiple Comparison test. The significance of the differences was established at the significance levels of 5 and 1%. All obtained results are shown in tabular and graphical form. Statistical analysis of the results was elaborated using software GraphPad Prism version 6.00 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com and Microsoft Excel.

RESULTS

Analyzing the data obtained from mussel samples collected in 2022, along the Montenegrin coast, using ANOVA post – hoc Tukey test – that compares the average values between three or more groups and examines whether the differences between them are random or statistically significant, it was found that samples collected in spring significantly deviates from the samples collected in other seasons, with significant differences (F=8.378, p<0.001) compared to summer, autumn, and winter. On the other hand, the differences between samples collected in summer, autumn and winter are not statistically significant (Table 1). This indicates a pronounced seasonal effect in spring, while other seasons show more similar characteristics.

Table 1. Descriptive statistical parameters for bacteria E. coli (log MPN/100 g) in mussels in 2022.

	n	$\overline{X}$	SD	SE	CV (%)	X max	X min
spring	22	1.47^{ABC}	0.36	0.0770	24.51	2.23	1.25
summer	23	2.33^A	0.88	0.1843	37.91	4.20	1.25
autumn	23	2.35^B	0.70	0.1476	30.04	3.54	1.25
winter	21	2.14^C	0.61	0.1337	28.56	3.54	1.25

Statistical significance is shown with the same letters: **A,B,C p<0.001**

The letters A, B and C indicate statistical significance between groups. Values with the same letter are statistically significantly different from each other (F=5.902, p<0.001), while values with different letters are not significantly different (p>0.001).

Table 2. Descriptive statistical parameters for bacteria *E. coli* (log MPN/100 g) bacteria in mussels in 2023.

	n	$\overline{X}$	SD	SE	CV (%)	X max	X min
spring	21	1.90	0.48	0.1058	25.41	2.52	1.25
summer	22	1.65[A]	0.62	0.1341	38.10	3.54	1.25
autumn	19	2.33[AB]	0.57	0.1327	24.82	3.54	1.30
winter	19	1.74[B]	0.48	0.1116	27.90	2.89	1.00

Statistical significance is shown with the same letters: **A,B p<0.05**

The letters A and B indicate statistical significance between groups. Values with the same letter are statistically significantly different from each other (p<0.05), while values with different letters are not significantly different (p>0.05).

Statistical analysis of the tested samples of *E. coli* in mussels along the Montenegrin coast by season in 2023 shows that there are significant differences between some seasonal pairs, specifically between summer and autumn (p<0.05), as well as autumn and winter (p<0.05). However, significant differences between samples from other seasons were not established (Table 2).
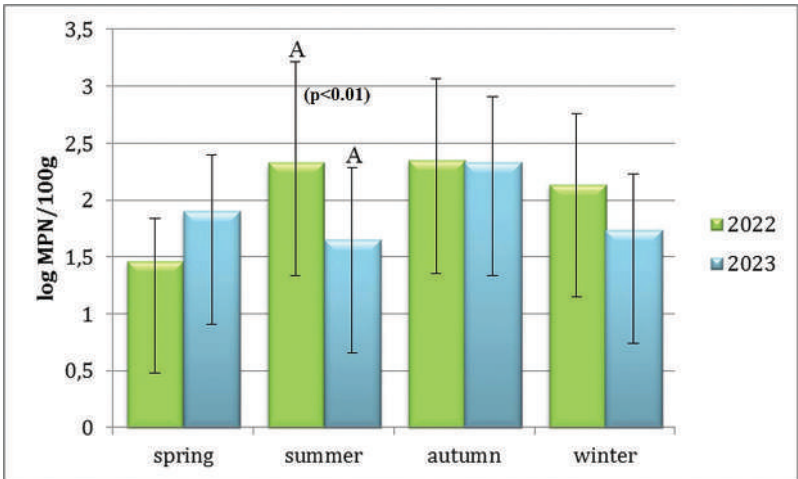

Figure 3. Comparison of seasons on the presence of *E. coli* in mussels

By comparing seasonal variations in the presence of *E. coli* in mussels from the Montenegrin coast for 2022 and 2023, it can be concluded that there were no statistically significant differences in contamination between samples from spring,

autumn and winter. However, during the summer period, a statistically significant difference between the two years (F=6.807, p<0.01) was recorded, which indicates changes in environmental conditions or in the way waste water is managed (Figure 3). A significant difference in the presence of *E. coli* during the summer season between 2022 and 2023 indicates the need for additional by researching the factors that influence contamination.
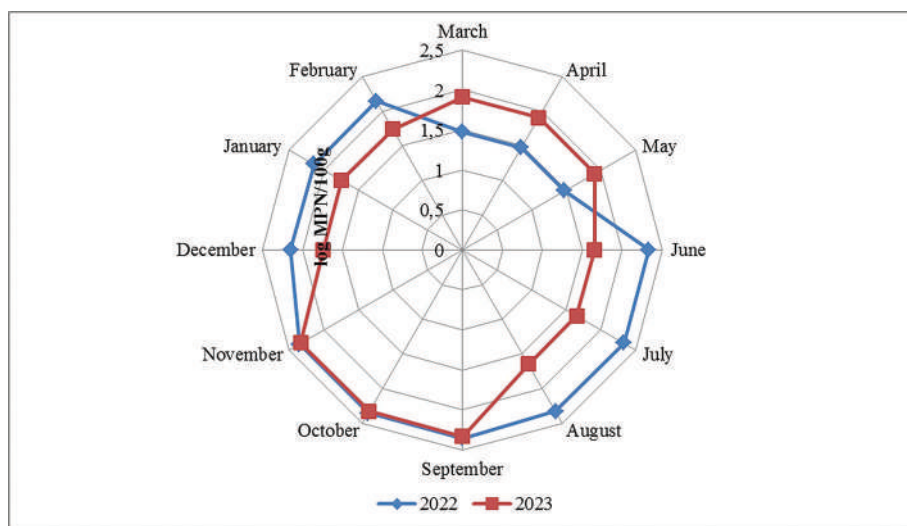

Figure 4. Comparison by months of the presence of *E. coli* in mussels

The results of monitoring *E. coli* presence in mussels over two consecutive years on the Montenegrin coast, shown that there are no significant differences (p>0.05) were found between the same months, indicate stable conditions within the ecosystem (Figure 4).
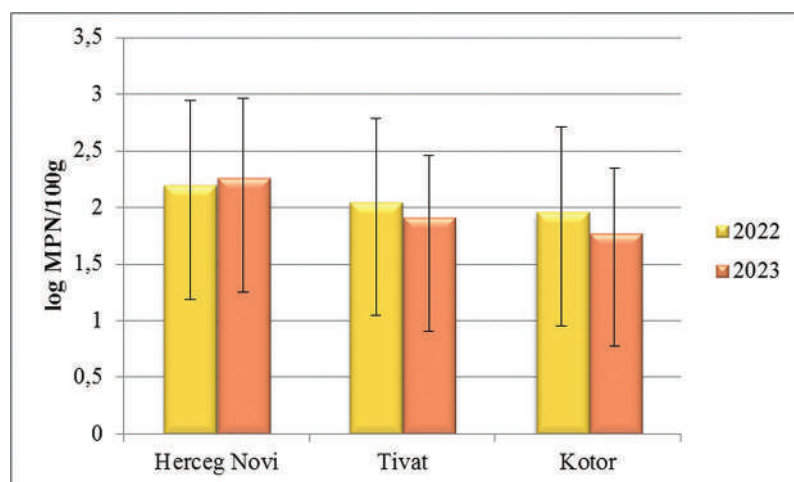

Figure 5. Comparison of municipalities on the presence of *E. coli* in mussels

Statistical analysis shows that there were no statistically significant differences between the same municipalities Herceg Novi, Tivat and Kotor, (F=1.670, p>0.05) on the Montenegrin coast in the presence of *E. coli* in mussels between 2022 and 2023 (Figure 5). The results obtained, despite the fact that they did not show a statistically significant difference, indicate the relative stability of seawater quality in the observed area, which may be a consequence of constant anthropogenic and natural influences during the studied period.

Although the analyzed data did not show statistically significant differences, it can be reasonably assumed that expanding the research to a larger number of mussel farms in several different municipalities, as well as conducting sampling in several time periods over the years, would contribute to the identification of potential spatial and seasonal trends that were not evident in the current analysis of the obtained data. Such an approach would achieve a deeper and more comprehensive understanding of the functioning of the ecosystem and the factors that influence its stability.

DISCUSSION

Escherichia coli is a common component of the intestinal microbiota, but certain virulent strains can cause colibacillosis [14]. The research results show clear seasonal and spatial differences in the presence of *E. coli* bacteria in mussels from three locations along the Montenegrin coast during 2022 and 2023. The analysis revealed pronounced seasonal variations, as well as significant differences in the patterns between two years. The year 2022 was characterized by a greater number of statistically significant differences between seasons, which indicates more pronounced fluctuations in the presence of *E. coli*. On the other hand, the results from 2023 suggest a more stable ecological balance and a reduction in seasonal differences, with significance recorded only between summer and autumn, and autumn and winter. This difference between the two years can be attributed to a combination of climatic and environmental factors, as well as improvements in coastal water and mussel quality management. In 2022, likely more abundant rainfall, more intense tourist activity and less developed wastewater treatment infrastructure could have contributed to increased contamination, especially in seasonal transitions [15,16]. On the other hand, the year 2023 could have been marked by more favorable weather conditions, such as less inflow of nutrient-rich inland waters and pollution, leading to a more stable state and reduced differences between seasons. The ecological dynamics of the coastal sea, including the biological processes within mussels, as well as the natural regeneration of the ecosystem, probably played a key role in these changes [16]. In addition, improving mussels and water quality management practices such as better wastewater treatment, establishing sanitary zones, and adapting farming areas could have contributed to reducing contamination. This particularly applies to localities such as Kotor and Tivat, where anthropogenic pressure is higher, while Herceg Novi, thanks to its more open location and better water circulation, shows a more favorable contamination pattern [1].

In both years of analysis, the presence of *E. coli* exhibited pronounced seasonal fluctuations: spring is the key seasonal period with the most pronounced changes compared to other seasons. These changes may be related to the increase in activity in nature (vegetation, faunal cycles) or to the specifics of the climatic conditions in spring on the Montenegrin coast. Spring was identified as the season with the biggest differences compared to the other seasons. The other three seasons (summer, autumn, and winter) show less seasonal variability, which may indicate a more gradual transition between warm and cold periods [15,16]. These results can be useful for further analysis of climate trends, environmental changes, or even economic activities that depend on seasonal factors (tourism, agriculture). This indicates specific seasonal variations, which may be related to the analyzed parameters (weather conditions, biological factors, activities during that period).

In 2022, *E. coli* was significantly more abundant in spring compared to summer ($p < 0.01$), autumn ($p < 0.01$), and winter ($p < 0.01$). This seasonal fluctuation may be related to increased runoff of nutrient-rich surface water during spring due to rainfall and snowmelt in coastal regions. In 2023, spring exhibited less pronounced differences compared to other seasons, where only the difference between summer and autumn is statistically significant ($p < 0.01$). Fall and summer are also interesting: in 2022, the differences between summer and autumn are not statistically significant, which suggests stability in microbiological parameters in these periods, when the sea temperature is the highest [10,15]. In the case of results where no statistically significant differences were found, it can be assumed that expanding the research to a larger number of locations and mussel farms could reveal potential trends and significant differences not observed in this sample, thus allowing for a more comprehensive understanding of the state and dynamics of the ecosystem. In 2023, autumn showed a significant difference compared to summer and winter, which may indicate additional contamination factors in this period, such as increased tourist activity or weather conditions [16]. The relative stability of *E. coli* levels in other seasons shows some progress, but summer is still a period that requires special attention and targeted preventive measures to ensure the safety and quality of seafood along the Montenegrin coast. These findings can be explained by relatively constant climatic conditions, without major external factors that would lead to drastic changes in water quality. The stability in bacterial presence may also be due to the continuous impact of moderate pollution sources, such as sewage discharges or wastewater, which did not fluctuate in intensity over the observed years. The marine ecosystem, including natural water flows and the biological resilience of mussels to microbiological challenges, likely contributed to these results. If water quality protection measures were consistently implemented, this could have further maintained similar pollution levels. Seasonal patterns of tourism may also represent a stable factor, not leading to increased or decreased contamination in certain months. All these circumstances collectively provide an explanation for the absence of significant variations in *E. coli* presence. A similar study conducted in Dubrovnik on mussels in 2016 and 2017 also found that *E. coli* contamination was most pronounced in spring

and summer [1,10]. These findings were attributed to increased tourist activity, higher wastewater discharge, and environmental conditions during these seasons, aligning with our observations along the Montenegrin coast.

Although the localities of Herceg Novi, Tivat, and Kotor are subject to similar environmental and climatic influences, local differences in the presence of *E. coli* may be related to the following: the intensity of human activities: a greater number of mussels farms, increased tourist traffic and waste water disposal can contribute to greater pollution in certain localities, such as Tivat and Kotor: coastal hydrodynamics: Herceg Novi, with better water circulation due to its more open location, can show lower contamination values compared to the bays of Tivat and Kotor, where water stagnation and deposition of organic matter are more pronounced. These results indicate the stability of environmental and sanitary conditions affecting contamination levels. However, the absence of significant differences may also indicate that the changes in the presence of *E. coli* were too subtle or that the conditions in both years were almost identical.

The year 2022 exhibited more pronounced seasonal differences, especially in spring, which may be a consequence of the specific climatic conditions of that year (e.g., higher precipitation levels or inland water runoff). The year 2023 shows a more stable pattern in the presence of *E. coli*, with significant differences mainly between autumn and winter. This points to the possible impact of long-term climate and environmental changes, as well as improvements in wastewater treatment or varying conditions at mussels farms. In the future, in order to increase the precision of the analyses, it may be useful to increase the number of samples and the frequency of monitoring or to apply more advanced statistical methods that can better detect smaller but significant changes in contamination. Climatic data collected during the research show that an increase in sea temperature in summer and autumn may contribute to a decrease in the presence of *E. coli* due to greater filtration activity in mussels and a reduced influx of pollution [2]. Precipitation and runoff during the spring increase the level of contamination, while the winter period shows relatively stable values, probably due to reduced activity and greater sea circulation.

Practical implications and recommendations for mussel farmers are that spring is a critical period when it is necessary to intensify monitoring and implement measures to reduce contamination (e.g., adequate wastewater disposal, control of surface runoff). The results indicate the need for specific seasonal water quality management measures in mussels farming areas, especially in the localities of Tivat and Kotor. The consumption of mussels from certain locations and seasons may pose an increased risk to human health; therefore, continuous monitoring of the presence of *E. coli* is necessary in accordance with international standards (ISO 16649-3).

CONCLUSION

The findings of this study contribute to a deeper understanding of the ecological processes and management challenges in coastal ecosystems, particularly along the Montenegrin coast. The observed seasonal patterns of contamination emphasize the interconnectedness between environmental dynamics, anthropogenic pressures, and aquaculture sustainability. Rather than viewing contamination events as isolated occurrences, they should be interpreted as indicators of broader ecosystem responses to climatic variability, land-based inputs, and coastal development. These results highlight the necessity of integrating continuous environmental monitoring into coastal management frameworks, ensuring that water quality, mussel health, and public safety remain central priorities. By aligning local policies with adaptive management strategies-such as improved wastewater treatment, spatial planning, and stricter seasonal monitoring-authorities can better mitigate ecological risks while supporting sustainable aquaculture production. Although the 2023 data suggest a reduction in seasonal variability compared to 2022, the persistence of certain contamination patterns underscores the importance of long-term observation and cross-sector collaboration. In the study by Grković et al. (2023), conducted in Boka Kotorska Bay, clear seasonal variations in the nutrient composition of *Mytilus galloprovincialis* were observed, emphasizing the influence of environmental conditions on mussel quality. In contrast, the present research builds upon these findings by focusing on contamination patterns and their ecological and management implications, thus providing a broader perspective on ecosystem stability and coastal water sustainability. In a wider context, these findings reinforce the need to consider global drivers such as climate change, tourism, and urbanization when designing regional sustainability policies. Ultimately, this research demonstrates that effective ecosystem management in dynamic coastal zones requires a balance between environmental protection, economic development, and food security.

Authors' contributions

IZB and NG carried out sample collection and performed microbiological analyses of the samples. SĐ, NG, DB, BV, JJ, and RT conceived the study, participated in its design and coordination, and revised the final version of the manuscript. SĐ, BV, and JJ conducted data analysis and statistical interpretation of data. SĐ, NG, VD, and RT, came up with the draft of the manuscript. All authors read and approved the final manuscript.

Declaration of competing interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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UTVRĐIVANJE PRISUSTVA *ESCHERICHIA COLI* U DAGNJAMA U CRNOJ GORI U PERIODU 2022-2023

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Ovaj rad istražuje biološku raznolikost dagnji (*Mytilus galloprovincialis*), njihove ekološke funkcije i ekonomski značaj za lokalne zajednice. Analizom ekoloških uloga dagnji u očuvanju ekosistema, kao i njihovog doprinosa lokalnoj ekonomiji kroz ribolov i akvakulturu, rad pruža sveobuhvatan uvid u njihov značaj i pretnje sa kojima se suočavaju. Cilj ove studije bio je analiza prisustva bakterije *Escherichia coli (E. coli)* i utvrđivanje statistički značajne razlike između dagnji sakupljenih sa tri različite lokacije u Crnoj Gori: Herceg Novi, Tivat i Kotor. Analize su sprovedene tokom različitih sezona, u periodu od dve godine, 2022. i 2023. Ovaj pristup je omogućio detaljnu analizu sezonskih varijacija u nivou kontaminacije dagnji. Preliminarni rezultati pokazuju da su najveće koncentracije *E. coli* zabeležene tokom letnjih meseci, što se može dovesti u vezu sa povećanim turističkim aktivnostima i većim opterećenjem kanalizacionog sistema. Najniži nivoi kontaminacije zabeleženi su tokom zimskih meseci. Do ovog smanjenja dolazi zbog toga što se turistička i industrijska aktivnost smanjuju, što rezultira manjom proizvodnjom otpada i otpadnih voda. Dalje, padavine i vetrovi koji razblažuju zagađivače pojačavaju se, dok niže temperature usporavaju biološke procese. Među analiziranim lokalitetima, uzorci iz Kotora pokazali su najveću prosečnu koncentraciju *E. coli*, dok su uzorci iz Herceg Novog imali najniže vrednosti. Ovi rezultati ukazuju

na značajne razlike u stepenu zagađenja između lokaliteta i godišnjih doba, što može imati ozbiljne posledice po ljudsko zdravlje i ekosistem. Prisustvo *E. coli* u dagnjama direktno ugrožava bezbednost prehrambenih proizvoda, jer konzumiranje kontaminiranih dagnji može dovesti do raznih gastrointestinalnih bolesti. Takođe, dugoročno zagađenje može narušiti ravnotežu morskih ekosistema, utičući na biodiverzitet i stabilnost populacija dagnji.