Some risk factors that affect contamination of mussels (*Mytilus galloprovincialis*) from the Bay of Kotor, Montenegro

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N Grković[1], B Velebit[2], V Teodorović[1], N Karabasil[1], D Vasilev[1], V Đorđević[2] and M Dimitrijević[1]

[1] Faculty of Veterinary Medicine, University of Belgrade, Bulevar Oslobodjenja 18, 11000 Belgrade, Serbia
[2] Institute of Meat Hygiene and Technology, Kacanskog 13, 11040 Belgrade, Serbia

E-mail: nevena.ilic@vet.bg.ac.rs

Abstract. Pollution and contamination of the Bay of Kotor ecosystem arise from both anthropogenic sources and natural weathering. In recent decades, a need has arisen for regular control of marine organisms, which are used in human nutrition, because the entire bay is constantly and increasingly exposed to negative anthropogenic impact. Molluscs, including mussels (*Mytilus galloprovincialis*), can be involved in foodborne disease. They are filter feeding organisms, able to retain and concentrate in their bodies the bacteria, parasites, viruses and biotoxins of marine algae present in their external environment. A structured field study was undertaken in the Bay of Kotor, Montenegro, in order to investigate plausible influence of environmental factors, like rainfall and temperature, on the variability of *Escherichia coli* and norovirus (NoV). This study focuses on human-derived pathogens that are abundant in sewage-related sources. We proved the negative correlation between outside temperature and the number of *E.coli* and the presents of Norovirus in Bay of Kotor mussel. We used this data from the sampling site to discuss options to better manage the risk of contamination of shellfish. From the aspect of food safety, an upgrade of monitoring plans in the future could lead to obtaining safer products.

1. Introduction

With the increase in the consumption of seafood in recent years, marine mussels (*Mytilus galloprovincialis*) have become commercially more important seafood species worldwide. Also they are available throughout the year, and are reasonably tolerant to environmental change and pollution [1]. In recent decades, growing urbanization and industrialization led to extensive settlement of the Montenegrin coast, and caused pollution of the environment of the Bay of Kotor, especially the sea water and organisms that live in it. Wastes from various industries, shipyards, hotels and hospitals near the bay discharge into the sea and are a constant source of pollution of the aquatic environment.

Although seawater temperature is a good predictor of the temporal degree of contamination, especially of noroviruses, within a site it clearly cannot, on its own, predict the overall degree of contamination occurring. For this, the role of other variables was investigated.

Pollutant material, which can be rich in protozoa, viruses or bacteria, will originate from a variety of point sources such as sewage discharges and rainfall. Near the Bay of Kotor lies Crkvice, the area with the highest average precipitation in Europe. This water brings a large amount of suspended particles into the bay that affect the ecological conditions in seawater. The amount of pollutants that come into the sea due to large amounts of precipitation is not inappreciable [2].

Current legislation specifies that an evaluation of the sources and types of faecal contamination impacting shellfish production areas combined with monitoring of faecal indicator organisms (*Escherichia coli*) in shellfish flesh is undertaken to provide an indication of the risk of contamination with bacterial and viral pathogens [3]. Food safety agencies are trying to mitigate the risk of pathogens in aquatic environments, but still there is no comprehensive indicator system for their monitoring [4].

This study was undertaken to investigate the significance of some of environmental factors on the microbiological contamination of shellfish, as determined by *E. coli* concentration in the shellfish and the presence of norovirus. The potential for rapid diagnosis, high throughput, as well as sensitivity and quantitative nature of the real-time PCR test all make it highly suitable to replace virus isolation for both surveillance and diagnostic and research purposes [5]. The object was to identify whether such factors need to be taken into account when assessing the potential effects of proposed sewage discharges on commercial shellfish.

2. Materials and Methods

We used an existing database of positive samples of NoV (genogroups I and II) and *E.coli* (reported as most probable number/100 g) quantified in mussels (*Mytilus galloprovincialis*) from 6 sampling points within 6 production areas around the coast of the Bay of Kotor was used in this study. The research was done in a one-year period, from July 2015 to July 2016, on a monthly basis. The NoV and *E. coli* dataset analysed has been previously reported by Ilić et al. [6].

Levels of *E. coli* and NoV were quantified using appropriate ISO methods for most probable number [7] and quantitative Real-Time reverse transcription (RT)-PCR [8], respectively.

Information on potential risk factors includes rainfall, outside temperature and average daily quantity of waste water assumed to influence NoV and *E.coli* contamination. The outside temperature was measured at the moment of mussel sampling, and precipitation was measured seven days earlier. Sewage discharges impacting shellfish beds was from the database of consented water company and private sewage discharges to controlled waters. The dataset (rainfall and temperature) was produced by Environment Agency.

We investigated the relationships between microbiological parameters (NoV and *E. coli*) in mussels and the risk factors using Spearman's correlation. This coefficient is a statistical measure of the strength of the relationship between pairs of data and is denoted by r.

3. Results and Discussion

The results of environmental monitoring showed that the Bay of Kotor experienced variable, seasonal rainfall in harvesting areas that were investigated. The rainfall fluctuated between 64.1 mm in August and 122 mm in September (average=93.05 mm) (Fig. 1). The outside temperature, during all monitored seasons, varied between -0.4±1.0°C in January and 20.0±1.0°C in August (Fig. 2). All production areas were commercially harvested and classified under Regulation (EC) No 854/2004.

Analysis of correlation between rainfall in Bay of Kotor and the prevalence of norovirus in marine mussels, harvested in the same location, showed that there was a poor positive correlation (r=0.47) which was not statistically significant (p>0.05). Also, analysis of correlation between rainfall and the number

of *E. coli* in mussels showed there was a mean positive correlation (r=0.57), which was not statistically significant (p>0.05) (Fig. 3).

The results of our research do not agree with the results of Campos et.al. [9], who proved that the predictive environmental factor for *E. coli* contamination in mussels in England and Wales was rainfall. In the current study in the Bay of Kotor, rainfall was not found to be associated with NoV contamination in the oysters. At some sites, *E. coli* levels have been found to reduce during high levels of rainfall possibly due to either a suspension of shellfish filtration activity at low salinities or to the dilution of impacting sewage plumes [10], and it is possible that a similar process also influenced the levels of NoV in shellfish.

There was a poor negative correlation between seasonal occurrence of NoV and outside temperature (r=-0.35), which was not statistically significant (p>0.05). Statistically significant (p<0.01) and medium negative correlation (r=-0.77) was obtained between outside temperature and the number of *E. coli* in the mussels (Fig. 4).

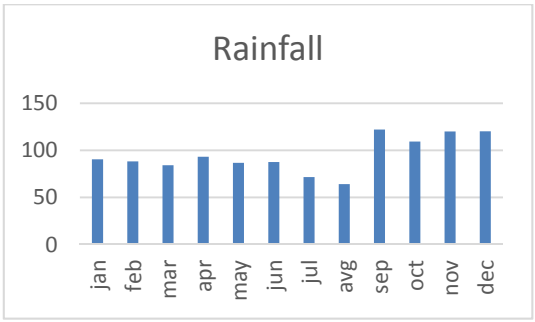

Figure 1. Seasonal variation of rainfall

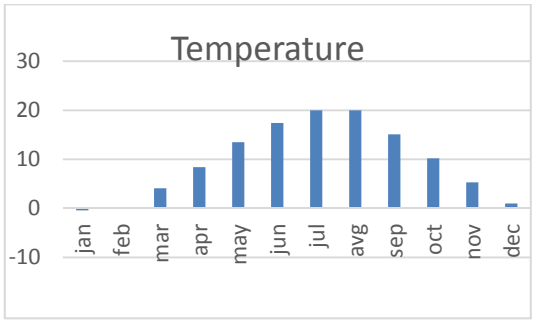

Figure 2. Seasonal variation of temperature

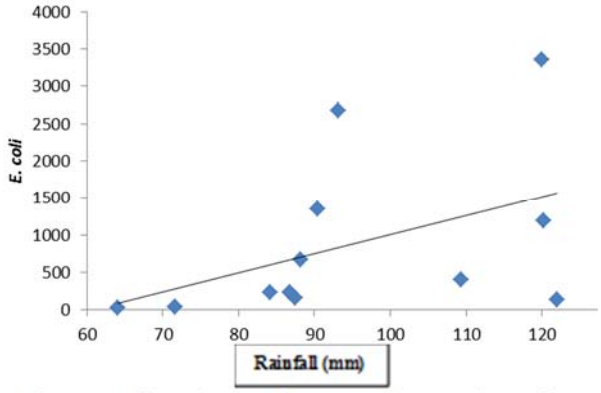

Figure 3. Correlation between the number of *E.coli* (MPN/ 100g shellfish) in Bay of Kotor mussels and rainfall during four seasons.
(n=12, Spearman |r|=0.57, p>0.05)

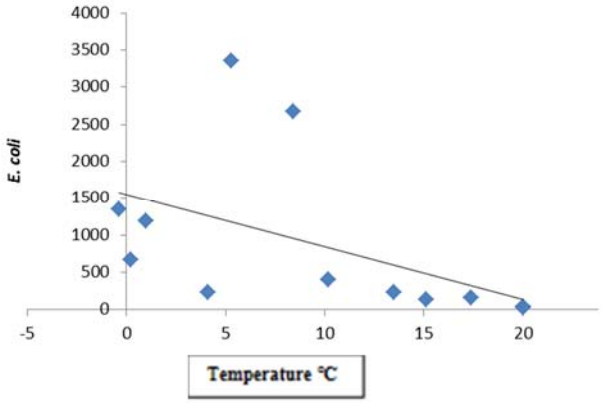

Figure 4. Correlation between the number of *E.coli* (MPN/100g shellfish) in Bay of Kotor mussels and outside temperature during four seasons.
(n=12, Spearman |r|=-0.77, p<0.01)

Overall, the predictive environmental factor for *E.coli* contamination in the mussels was outside temperature, while the predictive factor for total NoV (GI+GII) in the mussels was water temperature [11].

During summer months in the Bay of Kotor, the average daily quantity of sewage discharged was 2600 m^3/day (wastewater from households and from industry), which is much more than average daily quantity of sewage discharged in winter, when it was 2000 m^3/day.

4. Conclusions

From the results shown above, we concluded that, in the harvesting areas, the sources and abundance of human faecal pollution from sewage discharges were the main factor influencing the risk of contamination in mussels. However, it is also apparent that additional site-specific factors can further influence the extent of impact of this contamination on the shellfish.

We also observed that, in the catchments studied, the environmental factors driving the abundance of *E. coli* in shellfish do not operate in a similar way to those driving the abundance of NoV. The strength and significance of the risk factors varies between NoV and the statutory indicator of faecal pollution (*E. coli*).

Therefore, a distinct set of measures is required to manage the risk of NoV contamination of mussels. For all pathogens that can potentially be found in seawater, sewer overflows that catch sewerage water, produce the biggest risk of contamination, as they discharge crude, untreated sewage into the environment**.**

The results obtained in this study should contribute to the introduction of shellfish monitoring programmes, which would be improved with acquisition of all environmental parameters data, including demographic, hydrometric, climatic and pollution source characteristics and Real-Time qRT-PCR tests on cultured areas. Control measures for pathogens in mussels should focus on reducing volumes of sewage contamination in production areas.

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