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Model development and initial characterization of *Escherichia coli* in the shellfish-producing area of Butrinti Lagoon

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Abstract

The purpose of this research was to estimate the correlation between hydrochemicals and *Escherichia coli* contamination in *Mytilus galloprovincialis* by using multi-linear regression and statistically processing the monthly mean results. This study was conducted in the traditional cultivation of *M. galloprovincialis*, sampled and analyzed (n=136) for *E. coli* microbial analysis with ISO 16649-3. During the years 2015-2017, seawater was measured with a multiparameter apparatus, where four variables [dissolved oxygen (n=115), temperature (n=127), pH (n=115), salinity (n=127), and local area rainfall monitoring (n=23)] were taken into consideration. The results were compared and shown to have a significant correlation, allowing for the quantification of the impact resulting from adjustments made to the monthly mean computation. During the study period, statistical performance for each year was estimated R^2=94.4% (2015), R^2=46.8%, and R^2=97.5% (2017).

Introduction

Various studies have concluded that *Escherichia coli* contamination can be predicted if there are robust historical precipitation and hydrochemical water data at different depths with statistical models. We considered multiple linear regression (MLR) a useful tool to significantly improve the ability to predict hydrochemical parameters and contamination of *E. coli*. Nowadays, early warning approaches are a current topic in the field of fecal contamination in shellfish (Eregno, 2014).

Consumption of raw or insufficiently cooked bivalve mollusks can result in illness due to the presence of pathogenic microorganisms. The evaluation of sources and the potential impact of fecal contamination (both human and animal) near the production and relay areas could provide important findings for determining the extent of the production zone and the sampling plan on which ongoing monitoring is based.

Monitoring using *E. coli* as a microorganism that indicates the presence of fecal contamination could scientifically support the risk associated with contamination with bacterial or viral pathogens (Cefas, 2014). Human pathogens can enter the shellfish beds through a non-functioning water system or in connection with flooding. Additionally, animal pathogens can contaminate the beds *via* runoff from fields filled with animal waste (Westrell *et al*., 2010).

Regulation 2019/627, established by the European Commission (2019), introduced the classification of shellfish harvesting areas. This classification determines the appropriate post-harvest treatment that must be applied to shellfish before they can be considered safe for human consumption. The relevant European Union law, Regulation 2073/2005 (European Commission, 2015a), requires compliance with microbiological criteria for the final product.

In the literature, there are mentioned four main ways that environmental factors can affect the level of fecal contamination that occurs in the bivalve mollusks in the harvesting areas: by altering the number of micro-organisms discharged into the environment (including season, air temperature, and rainfall); by altering (usually reducing) the concentration of micro-organisms in the seawater (which includes the action of sunlight, seawater temperature, and salinity); by altering the way that tides and currents take the contamination to the harvesting area; and by altering the uptake and retention of microorganisms by the bivalve mollusks (CEFAS, 2018). Seasonal effects include the impact of tourism on increasing the loading of sewage to treatment plants and differences in the extent and route of contamination by farm animals. Air temperature could affect the survival of microorganisms on land and the efficiency of biological sewage treatment processes (Kay *et al*., 2010). Increased rainfall tends to reduce the efficiency of biological sewage treatment processes and increase the amount of contamination arising from combined sewer overflows, surface water overflows, and land run-off.

Mathematical models simulating the movement and dispersion of test bacteria of fecal contamination (*E. coli*) have been used in France on occasion as a tool in the positioning of sampling points or even in the choice of the sampling frequency if the results of the simulations are concordant with data observed by the REMI monitoring network.

Starting in 2010, the shellfish production area located in Butrinti Lagoon has been officially designated by competent authorities as a class B production area. As a result, mollusks collected from this area must undergo the depuration process before being introduced to the market.

As far as Butriti Lagoon is concerned, there are no consistent studies to establish the causes that lead to *E. coli fecal* contamination values, or even studies that show that statistical models can help predict fecal contamination when climatic and hydrochemical indicators change. In live bivalve mollusks, the accumulation of *E. coli* and other enteric bacteria is considered a dynamic process. However, the dependent effects of temperature, salinity, and other driven factors have been poorly investigated on the accumulation of enteric bacteria. Rainfall has been previously described as a common environmental factor associated with controlling *E. coli* concentrations in receiving waters (Kelsey *et al*., 2004; Coulliette *et al*., 2009; Campos *et al*., 2011; Schmidt *et al*., 2018). However, the degree of response of *E. coli* concentrations to rainfall can vary considerably between sampling points in a given sampling area (Schmidt *et al*., 2018).

Our study was designed to improve the ability to forecast *E. coli* concentration in live bivalve mollusks (*Mytilus galloprovincialis*) in estuarine waters at the Butrinti Lagoon area using monthly rainfall mean and hydrochemical water measurements in combination with meteorological data. For this purpose, the results of the National Control Program for the *E. coli* indicators were converted into logarithmic values. At this scale, this conversion always explains why the values of precipitation change between microbiological and hydrochemical parameters. This approach has been used in similar studies to speed up this phenomenon (Bougeard *et al*., 2011).

Materials and Methods

Study area

Butrinti Lagoon has an extent of 16 km^2; it reaches a maximum of 5.4 km in length and 1.4 km in width. It is located on the southwest coast of Albania, connected with the Ionian Sea (eastern Mediterranean) through the natural Vivari channel. It is surrounded by the Vurgu Plain to the north, the Mile Mountain to the east, the Vrina Plain to the south, and the mountainous Ksamil peninsula to the west (Moisiu and Durmishi, 2015). The maximum depth is 21.4 m, with an average of 11 m (Hodges, 2011). This lagoon lies within the Mediterranean climatic zone. The average rainfall is between 1200 and 1300 mm, concentrated at 95–100 days per year. Two-thirds of this situation occurs in November-March, reaching a peak in October and November. Winter is mild, with January being the coldest month of the year (average temperature 11.9°C) and July being the hottest one (average temperature 27°C). This plant is considered the second production center for mussels in Albania. In 1989, production had increased to 5000 tons per year. It dropped dramatically in the 1990s due to a foodborne illness with *Vibrio cholerae* and a subsequent ban on the export of mussels by EU countries (Kumar, 2015). In recent years, the annual production of Butrinti mussels has been estimated at around 1500 tons, a quantity that goes mainly to the domestic market.

Data collection and processing

This study was designed to prove the ability to predict *E. coli* levels for B sanitary class in *M. galloprovincialis* in the estuarine waters of the Butrinti Lagoon production zone by using dependent variables of hydro-chemical water parameters (salinity, dissolved oxygen, temperature, and pH). For this purpose, data on *E. coli* were obtained from the National Monitoring Plan of Live Bivalve Mollusks from 2015 to 2017. The conversion to the logarithmic value of the monthly average of *E. coli* MPN/100 g mollusks will consistently explain the fluctuations of precipitation values and hydro-chemical parameters. Our observed dataset was classified into one microbiological parameter, four hydro-chemical parameters, and one meteorological parameter.

The period of monitoring was limited from February to July of each month due to production practices. *E. coli* data was collected at three sampling sites: North, West, and South. All the samples were analyzed for β-D-glucuronidase-positive *E. coli* at a small number, according to Commission Regulation 2073/2005 (European Commission, 2005), and examined using the five-tube most probable number (MPN) following the reference method ISO 16649-3:2015 (ISO, 2015). Results are reported as MPN of *E. coli*/100 g of flesh. Safety evaluation of shellfish is based entirely on the use of food safety criteria laid down in Regulation 2073/2005 and Regulation 2285/2015 (European Commission, 2005, 2015b) such as the absence of *Salmonella* spp. 25 g of flesh and intervalvular liquid and an upper limit of 230 MPN *E. coli*/100 g of flesh and intervalvular liquid in 80% of the samples. 20% of the samples may contain *E. coli* between 230 and 700 MPN/100 g. It is well known that shellfish contamination occurs because LBM are filter-feeding animals that selectively filter and accumulate small particles of phytoplankton, zooplankton, viruses, bacteria, and inorganic matter from the environment (Leoni *et al*., 2017).

Statistical approach: multiple linear regression development

All data exploration and modeling analyses were conducted using MLR in Python 3.8 (Python Software Foundation, Wilmington, DE, USA) and SPSS v.20 (IBM, Armonk, NY, USA). The assembled data set was split into three parts. To elaborate R^2, all the data were preprocessed for significance $p<0.05$. The omnibus test, likelihood ratio, and chi-square test were calculated, indicating statistical significance in the model that contains our predictors and has a significant improvement in fit compared with the no-model. Due to the non-Gaussian nature of the response variables (concentrations of *E. coli* in LBM), they were $\log_{10}$ transformed before use (Kay *et al.*, 2010). We decided to test a hypothetical model where four hydrochemical parameters (salinity, dissolved oxygen, temperature, and pH) were included as explanatory variables against *E. coli*.

Multiple linear regression: equation

Linear regression is used to understand the linear relationship between a dependent variable, y, and several independent variables by fitting a linear equation to observed data samples (Coelho-Barros *et al.*, 2008) [Eq. 1].

$$y_i=\beta_0+\beta_1x_1i+\beta_2x_2i+\ldots+\beta_kx_ki+\epsilon i=1,2.. \text{ for i = 1,2,...,N} \qquad [1]$$

In this equation, the components are: y, the dependent variable; x_1,x_2,x_k, the independent or explanatory variables; i, index the n sample observation; ϵ, a random error term.

Results

Overall values of *E. coli* in three consecutive years ranged from a lower bound [limit of detection (LOD); <18 MPN] to 3.61 $\log_{10}$MPN, while the results of salinity had the highest point of 8.81 in the mean value. The water temperature in the year 2015 ranged between 10.2 and 31.8 in mean value. Table 1 highlights descriptive statistics for three consecutive years and the *E. coli* contamination used for modeling. The summary of descriptive statistics is presented by specifying the count, mean, standard deviation, minimum, 1st quartile, median, 3rd quartile, and maximum value for each parameter.

In March 2016, we recorded the highest mean value of dissolved oxygen (8.7 mg/L), and in July 2017, the minimum value of dissolved oxygen (3.18 mg/L). Describing our trends, we could say that the reduction of salinity values due to rainfall and watersheds is significantly linked with increased monthly values of *E. coli* as a result, reaching 230 MPN *E. coli*. According to our data calculated by the Pearson correlation coefficient (Table 2), there is a positive correlation between the *E. coli* values and rainfall. Moreover, the correlation between *E. coli* and water oxygen was calculated as a strong positive relationship, and such a relationship is important during wet months.

Correlation analysis is a statistical method used to discover if there is a relationship between variables. The utilization of Pearson correlation analysis clarifies the complex associations existing between environmental factors and the presence of *E. coli* contamination in shellfish. In 2015, a clear positive correlation existed between levels of dissolved oxygen and *E. coli*, indicating that an increase in dissolved oxygen is associated with a higher degree of contamination of shellfish by *E. coli*. On the contrary, a notable negative relationship existed between water lagoon temperature and *E. coli*, indicating that elevated levels of *E. coli* contamination are linked to lower water temperatures. In addition, salinity (expressed in parts per thousand, ‰) exhibits a significant influence, displaying a strong negative relationship with the presence of *E. coli* contamination. We could hypothesize that a salinity value below 24 ‰ in combination with lower lagoon temperature values could be considered an acceptable threshold for samples exceeding 230 MPN *E. coli* or 2.36 $\log_{10}$ MPN *E. coli*.

Figure 1 shows the graph for observed *E. coli* load *versus* predicted *E. coli* and their residual plots, respectively, for the years 2015-2017 with a 95% confidence interval. Following on from our interpretation, increasing the rainfall period by more than 200 mm of precipitation per month could significantly reach the legal limit of *E. coli* contamination, as a result corresponding to B-class quality sanitary standards. A 3-dimensional plot of multiple regression analysis is shown in Figures 2-4. In these graphs, we have shown relationships between *E. coli* contamination and explanatory variables: the first plot utilizing temperature and oxygen, the second plot with salinity and pH, and the third plot utilizing temperature *versus* salinity. The dependent variables are shown on the y-axis for each graph.

In January 2015, *E. coli* levels were 2.23 log10MPN/100 g. These levels then declined until May, when they dropped significantly. The decline coincides with the rise in lagoon water temperature from 11.97°C in January to 22.45°C in May. The previous analysis's negative correlation matches *E. coli*'s negative relationship with temperature. Salinity levels increased during this timeframe, which may have contributed to the decrease in *E. coli* contamination (Table 3).

In February 2016, log10MPN/100 g=3.03 *E. coli* levels rose. This upward trend continued through March before stabilizing in subsequent months. The peak in February coincided with a drop in seawater temperature and an increase in precipitation, suggesting a link between these variables and *E. coli* contamination. July and August 2016 saw a drop in *E. coli* levels. High temperatures during this period may have caused this decline, which is consistent with the negative correlation between temperature and *E. coli* (Table 3).

In 2017, *E. coli* levels peaked in June and July. The June peak is positively correlated with seawater temperature, while the July peak is associated with increased precipitation and a decrease in salinity. The fluctuations show a negative correlation between *E. coli* and salinity (Table 3).

Discussion

In total, we assessed 134 hydrochemical observations, 136 microbiological testing analyses for *E. coli,* and 23 rainfall data and normalized the mean log scale during modeling. Our data model demonstrates that we found a positive relationship between *E. coli* and rainfalls in 2015 and 2017. However, this work presents some limitations. Results may have been negatively influenced by the uncertainty of the method and the way the LOD values were calculated because we could use the lower or upper bound of LOD instead of the middle bound of LOD as it was used in this study. Thus, it could have reflected the overall results of the monthly mean. This approach was commonly used in this particular field. Moreover, we found that 2016 was less correlated in comparison with the other years. Modeling of *E. coli* is considered a useful tool to improve the ability to predict the concentrations of *E. coli* and other climatic, hydrochemical, and microbiological factors when the shellfish is ready to enter the domestic market or during export. Various studies have concluded that *E. coli* contamination can be predicted if a significant link exists between consistent historical environmental data in association with hydrochemical data from water (Schmidt *et al*., 2018).

Conclusions

Quantification of *E. coli* and its relationship with driven parameters of waters and rainfalls are key indicators to our understanding of microbiological risk assessment because shellfish could expose consumers due to possible bioaccumulation of pathogens such as *Salmonella, Vibrio spp.,* norovirus, hepatitis A, and many other contaminants.

This study has demonstrated that combining environmental data such as precipitation with hydrochemical data can be used to model *E. coli* concentration in molluscan shellfish at Butrinti Lagoon and possibly to forecast classification for temporary closure or reopening of the shellfish production area. Our study demonstrated that by increasing our predictors, like rainfall over 200 mm per month, in combination with other predictors such as salinity, less than 24.0‰, and temperature below 21°C, it is possible to estimate that shellfish with contaminated E. coli are more likely to meet the legal criteria. Our modeling approach contributes to shellfish management and consumer protection by indicating critical values found in risky months or periods as defined by European Union regulations.

These findings demonstrate that environmental modeling could be useful for building accurate statistical models for *E. coli* contamination as a rapid warning system to predict fecal contamination before harvesting and possibly for the development of long-term predictions of *E. coli* in the shellfish industry.

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Table 1. Descriptive statistics of explanatory variables and normalized data used for modeling.

Escherichia coli	O_2	T	pH	Salinity	Rainfalls
N 136*	115**	127*	115**	127*	23***
Mean 2.38	7.16	19.57	8.26	21.88	155.44
STD 0.70	1.30	6.79	0.18	4.44	96.75
MIN 1	3.18	6.53	7.92	16.3	6.8
25% 1.89	7.24	14.54	8.18	18.335	85.295
50% 2.54	7.51	17.69	8.24	20.9	131.62
75% 2.8	7.84	25.285	8.3	23.35	220.63
MAX 3.61	8.7	31.77	8.81	32.67	328.2

N, total number of observations; mean, mean of observation value; STD, standard deviaton; MIN, minimum value; 25%, first quartile; 50%, the median; 75%, third quartile; MAX, maximum; T, temperature; *bi-weekly frequency; **bi-weekly frequency; ***monthly frequency. In February 2015, missing data of dissolved oxygen (n=3) and pH (n=3); in April 2015 missing data of dissolved oxygen (n=3) and pH (n=3); in May 2017 missing data of dissolved oxygen (n=6) and pH (n=6).

Table 2. Matrix of Pearson correlation coefficient for each predicted variables, year 2015.

	E.coli	Dis. Oxy	Temp.	pH	Salin.‰	Rainf.
E.coli	1.00	0.79	-0.92	-0.61	-0.93	0.71
Dis. Oxy		1.00	-0.69	-0.69	-0.92	0.45
Temp.			1.00	0.42	0.88	-0.85
pH				1.00	0.61	-0.42
Salinity‰					1.00	-0.60
Rainfalls						1.00

E. coli, *Escherichia coli*; Dis. Oxy, dissolved oxygen; Temp, temperature; Salinity‰, salinity in ‰ (permil); Rainf., rainfalls in mm/month.

Table 3. Summary dataset of *Escherichia coli* contamination, hydrometric parameters of water and rainfalls in monthly mean values.

Year	Month	*Escherichia coli*	Oxy	T	pH	Salinity	Rainfalls
		$\log_{10}$MPN/100g	mg/L	°C	units	ppt	mm/month
2015	January	2.23	7.71	11.97	8.24	20.67	312.82
	February	2.54	n.a	10.2	n.a	17.23	301.25
	March	2.56	8.64	14.07	7.92	16.3	211.51
	April	1.88	7.72	17.42	8.17	19.72	211.51
	May	1.94	7.9	22.45	8.31	20.47	75.52
	June	1.8	7.51	25.32	8.05	23.88	196.52
	July	1.23	4.9	31.77	8.38	32.67	77.29
2016	February	3.03	7.99	14.33	8.28	17.63	287.42
	March	1.67	8.7	15.47	8.37	17.03	229.75
	April	1	7.9	18.9	8.18	18.15	131.47
	May	2.65	7.46	21.38	8.21	18.52	151.58
	June	1.9	7.41	25.25	8.2	20	131.62
	July	2.11	6.29	28	8.3	23.18	56.84
	August	1.44	5.53	27.93	8.59	27.78	97.01
	November	2.74	7.75	17.47	8.09	17.87	328.2
	December	2.69	7.84	13.03	8.25	22.73	6.8
2017	January	2.57	6.23	6.53	8.17	23.3	151.11
	March	2.86	7.58	14.75	8.22	23.4	93.3
	April	2.48	7.51	17.69	8.25	21.71	98.4
	May	2.86	n.a	21.97	n.a	21.83	257.1
	June	3.61	7.47	25.57	8.21	20.9	101
	July	3.45	3.18	31.05	8.81	32.15	42.3
	October	3.45	7.24	17.6	8.24	26.2	24.9

$\log_{10}$MPN, base-10 logarithm of the most probable number; Oxy, dissolved oxygen; T, temperature; n.a, missing data.

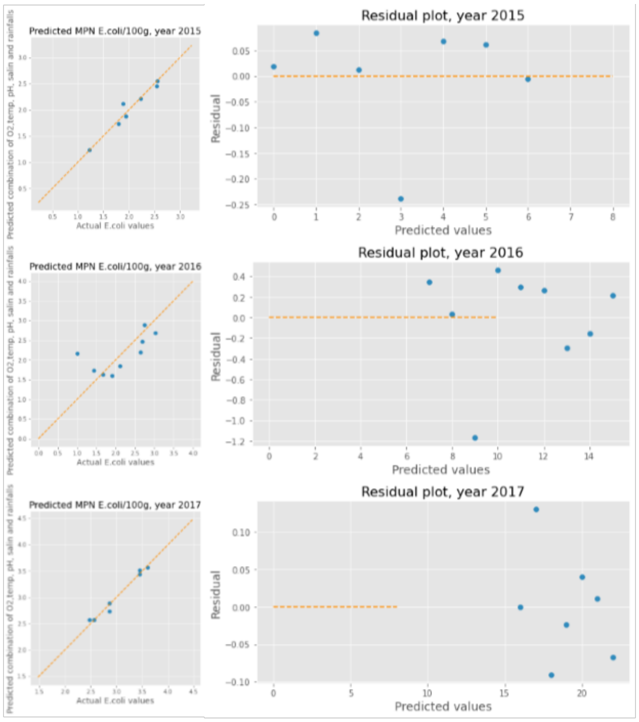

Figure 1. Observed values of *Escherichia coli versus* predicted *E.coli* in the years 2015, 2016, and 2017.

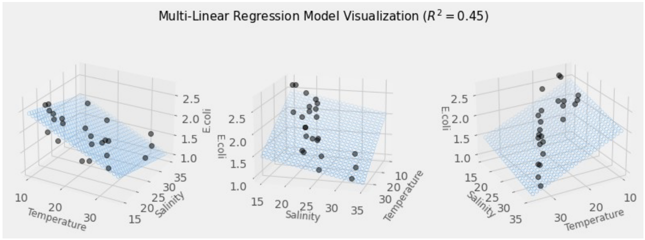

Figure 2. *Escherichia coli* is predicted by temperature and salinity.

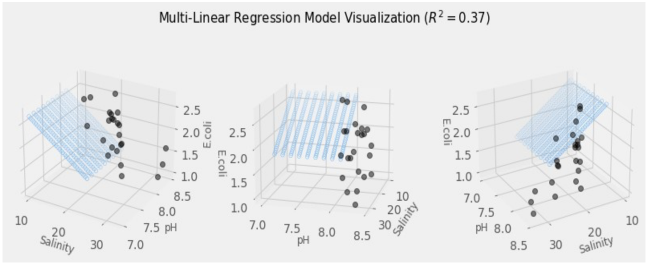

Figure 3. *Escherichia coli* is predicted by salinity and pH.

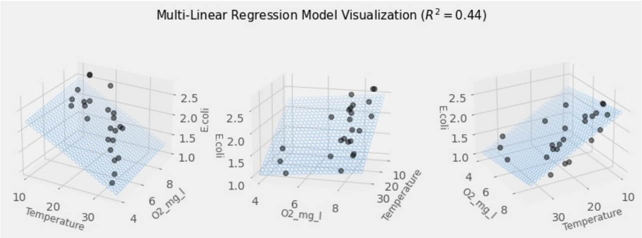

Figure 4. *Escherichia coli* is predicted by temperature and dissolved oxygen.