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Risk valuation for *E. coli* contamination in Campania region shellfish from 2016 to 2021

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A R T I C L E I N F O

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A B S T R A C T

This study set out to assess the microbiological quality of shellfish collected over a six-year period of time in the Campania Region Sea. A total of 1459 samples were examined in order to determine whether *Escherichia coli* was present. To investigate potential correlations between the *E. coli* counts and environmental parameters (salinity, pH, dissolved oxygen, seawater temperature, turbidity, rainfall) and pollution variables (density and distance of heavy and light discharges), data were gathered. With only roughly 19% of the samples not meeting European and Italian criteria (*E. coli* counts more than 230 most likely number MPN per 100 g of pulp and intravalvar liquid), the results showed that the microbiological quality of the shellfish was good.

A correlation between microbial contamination, season, rainfall, and dissolved oxygen was found using statistical analysis. However, the discharge density along the coast per spatial unit (a 200 × 200 MT cell), which was determined using the "quartic" Kernel function, showed found to be the primary factor determining the *E. coli* concentration in the shellfish.

An increase in rain millimeters was found to be associated with a higher risk of heightened *E. coli* contamination, according to a model that was fitted to assess the probability of detecting a higher *E. coli* count in connection to environmental parameters. This outcome could be explained by the discharge density near the coast as well as the increased availability of coliforms, particularly *E. coli*, and nutrients during periods of heavier rainfall.

1. Introduction

Shellfish represent a food of great nutritive value in the Mediterranean diet, especially for the inhabitants of the coastal areas where there is a high consumption [1,2]. As it is well known, shellfish are filter-feeding organisms capable of accumulating and concentrating a large amount of particles from the surrounding water including microorganisms some of which are pathogens (bacteria and viruses), these are considered guards of the health of coastal marine waters (Costa R A., 2013).

In addition, due to these characteristics, shellfish, especially if eaten raw or undercooked, could spread numerous diseases, mainly caused by enterobacteria, vibriobacteria and viruses.

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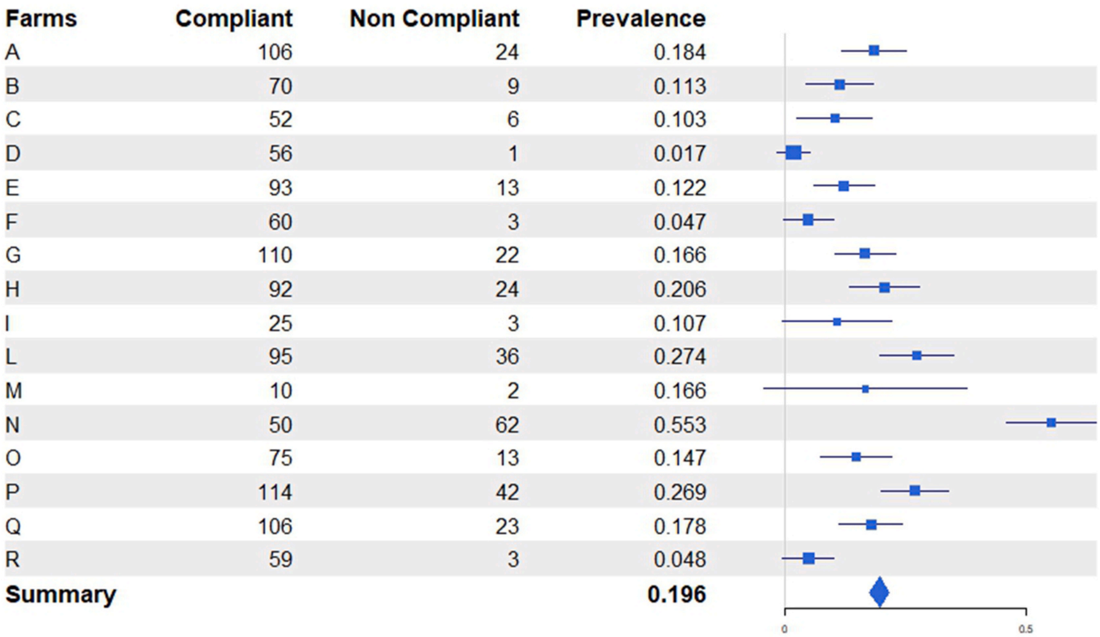

Fig. 1. Forest plot for prevalence per cities.

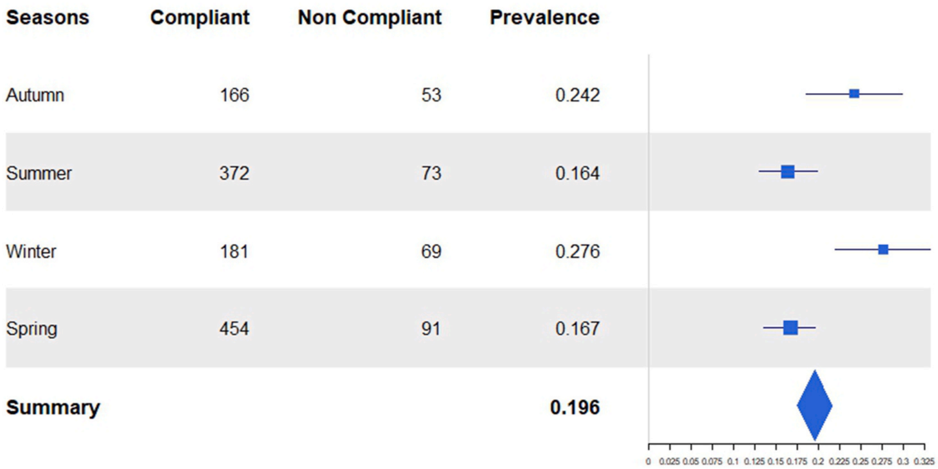

Fig. 2. Forest plot for prevalence per farms.

Analytical studies highlight that the main source of contamination in shellfish is represented by human fecal contamination. Phenomena of clogging and overflowing of sewage systems following heavy rainfall have often been linked to outbreaks of diseases associated with the consumption of seafood, especially linked to the presence of viruses [3].

To provide healthy food, Commission Implementing Regulations 627/2019 and EU Reg. 2073/2005 govern the breeding, production and marketing of these foods. Particularly, the maximum limit allowed for the presence of *E. coli* is 230 most probable number (MPN) per 100 g of pulp and intravalvar liquid and Salmonella total absence.

Shellfishes are a crucial part of the Italian culinary tradition specifically in Campania Region there are some species which become main ingredients of many local dishes [4].

The Campania Region's coastline stretches 512 km along the eastern edge of the Tyrrhenian Sea, forming three main bays that include the Gulfs of Gaeta, Naples, and Salerno, which run north to south. The coastline of the region is heavily populated, has a lot of commercial discharges, industrial and harbor activity, as well as pristine or protected areas [5–7]. Consequently, the trophic features of coastal water vary based on both natural and artificial (i.e., discharges) land inputs.

Along the coast of Campania there are 19 shellfish farms and 8 natural beds.

Of the 19 shellfish farms, 18 are located in Naples district, while only one is located in Caserta district. In particular, shellfish

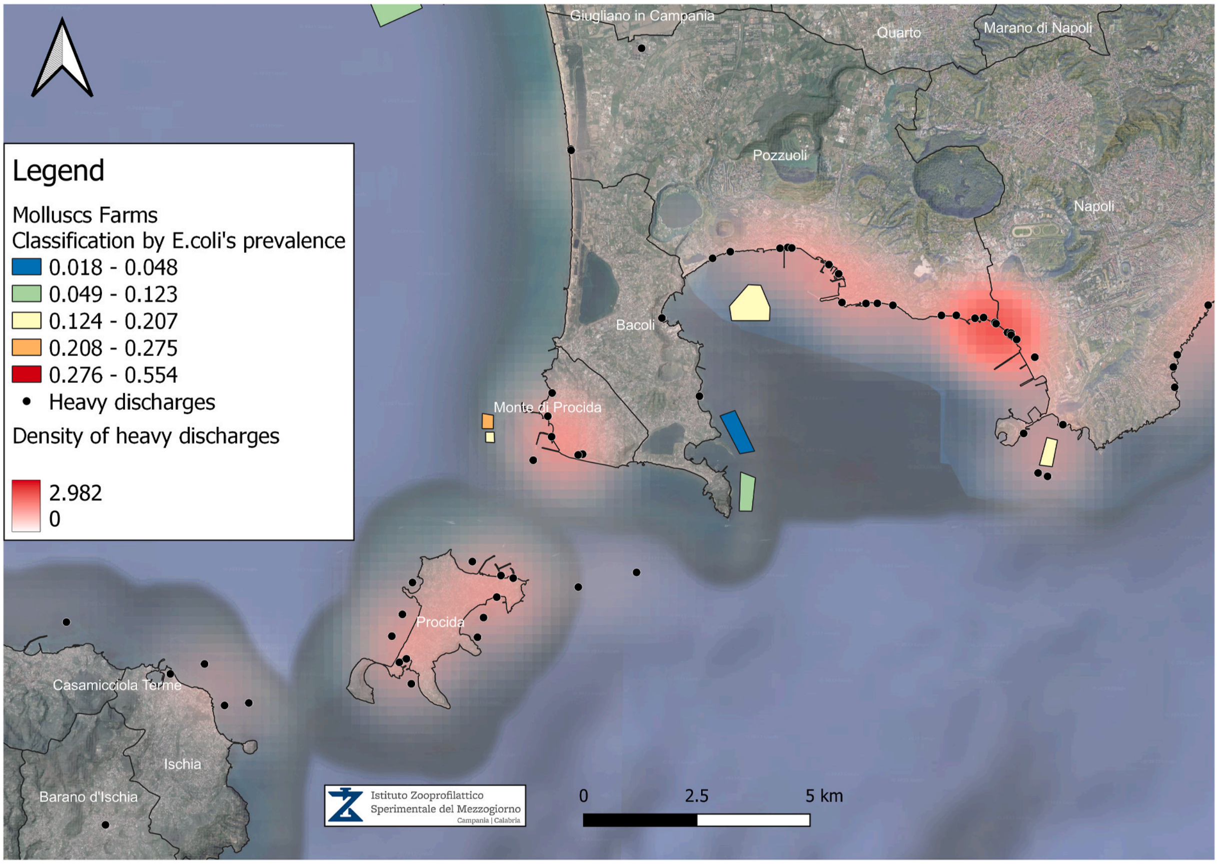

Fig. 3. Kernel density of heavy discharges along the Pozzuoli coast.

farming in Campania is located above all in the Gulf of Pozzuoli (Baia, Bacoli, Capo Miseno, Arco Felice, Monte di Procida), in the Gulf of Naples (Nisida and Castel del'Ovo), in the brackish lagoon area of Lake Fusaro, along the Domitian coast and in the eastern area of Naples (Ercolano, Torre del Greco and Castellammare di Stabia). The main species of shellfish farmed in Campania are mussels (*Mytilus galloprovincialis*), common clam (*Chamelea gallina*), razor clams (*Ensis minor*), clams (*Callista chiane*), cockles (*Donax trunculus*), truffles (*Venus verrucosa*) and clams (Ruditapes spp.).

The aim of this study is to identify the possible risk factors that could have conditioned the presence of high *E. coli* values in shellfish taken within production plants located on the coasts of Campania region. This is a retrospective longitudinal observational study. The identification of these factors would allow the planning of containment and mitigation measures capable of preventing the spread of outbreaks of communicable diseases. The approach to the risk, according to the new European regulations, should no longer be based only on the control of contamination by monitoring the finished product, but on all those predictive systems capable of managing the risk upstream of the process with a view to optimizing economic resources. In this context, the aim of this work is to develop a predictive model with the goal of sustainable exploitation of the coastal areas of Campania in favor of shellfish farming.

2. Materials and methods

The study has collected data on monitoring of shellfish in the period from 2016 to 2021 in classified regional areas from 16 farms.

Measurements of *E. Coli* were performed by means of analytical methods validated in compliance with UNI CEI EN ISO/IEC 17025 [2005. General requirements for the competence of testing and calibration laboratories. Milano (Italy): UNI Italian National Authority for Unification].

2.1. Sample collection and E.coli analysis

As required from Regulation (EU) 2019/627, shellfish were collected only by authorised sampling officers (the Veterinary Services of the A.S.L.) at the frequency specified by FSA in monitoring plans, unless sampling can be rescheduled by agreement or where circumstances are outside of the sampling officers' control.

Each sample must consist of at least 10 subjects capable of providing the laboratory with between 75 and 100 g of pulp and intravalvar liquid.

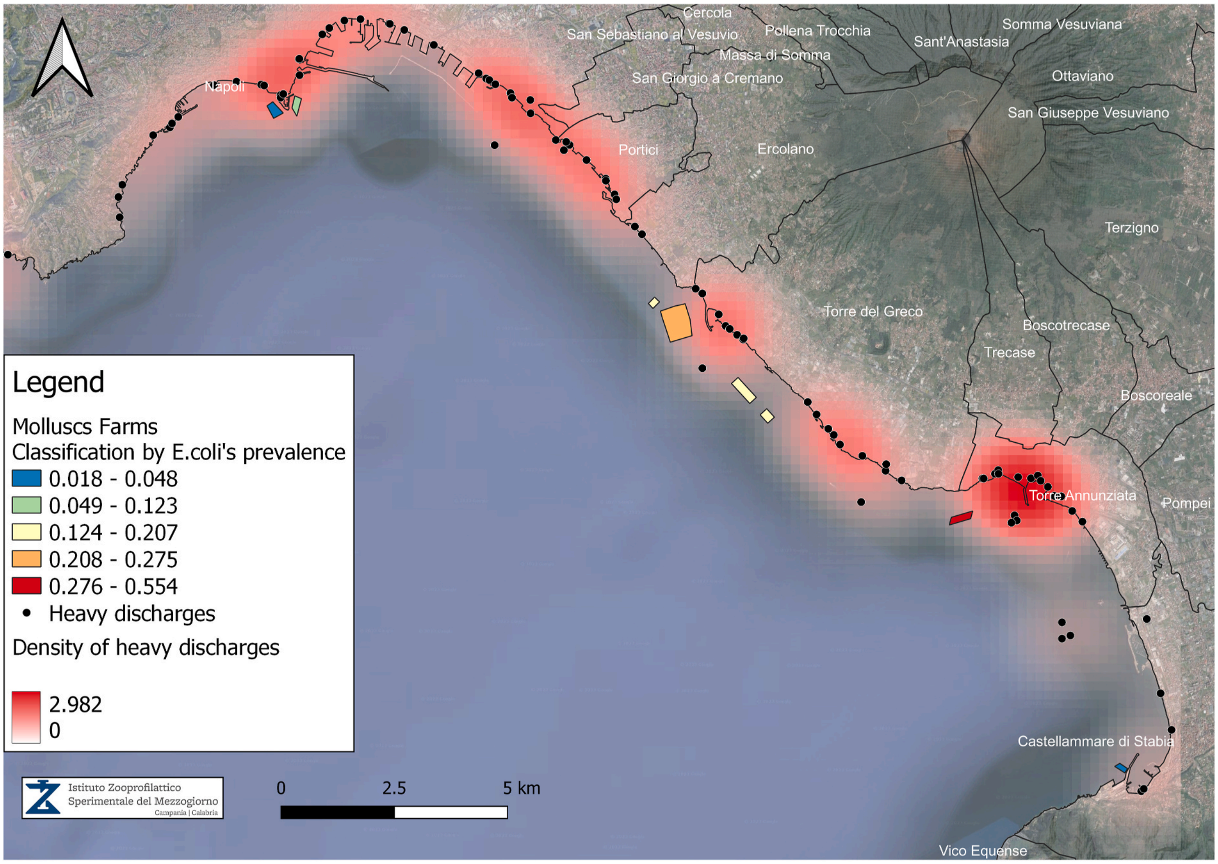

Fig. 4. Kernel density of heavy discharges along the Gulf of Naples.

2.2. Sampling method

Shellfish were sampled by the method normally used for commercial harvesting as this can influence the degree of contamination. The temperature of the surrounding seawater at the time of sampling was recorded.

Where intertidal shellfish were sampled dry, the temperature of the shellfish sample was recorded immediately after collection. To do this the temperature probe was placed in the center of the bagged shellfish sample.

2.3. Sample transport

The samples were delivered to the relevant laboratory (the IZSM lab) for analysis whithin 24 h of collection in cool boxes at a temperature between 2°C and 10 °C.

To avoid contamination, the samples were rinsed with clean sea water.

As regards the preparation of the sample, the recommended reference ISO is 68873:2003 part 3 “Specific rule for the preparation of fish and fishery product”.

2.4. Horizontal method for the enumeration of beta-glucuronidase-positive Escherichia coli

ISO 16649–3:2015/EC1:2017 specifies a horizontal method for the detection and enumeration of β-glucuronidase positive *Escherichia coli*, by means of the liquid-medium culture technique and calculation of the most probable number (MPN) after incubation at (37 ± 1) °C, then at (44 ± 1) °C. This part of ISO 16649 is applicable to the following:

- products intended for human consumption and the feeding of animals;
- environmental samples in the area of food production and food handling.

The method is suitable for the enumeration of cells of *E. coli* that might have been subjected to stress arising from dehydration, freezing, and exposure to a saline (such as marine) environment or damage by disinfectants such as chlorine-containing products.

The number of *E. coli* in shellfish is given as the result of a five-tube per dilution MPN series. The tubes contained 9 mL of mineral-

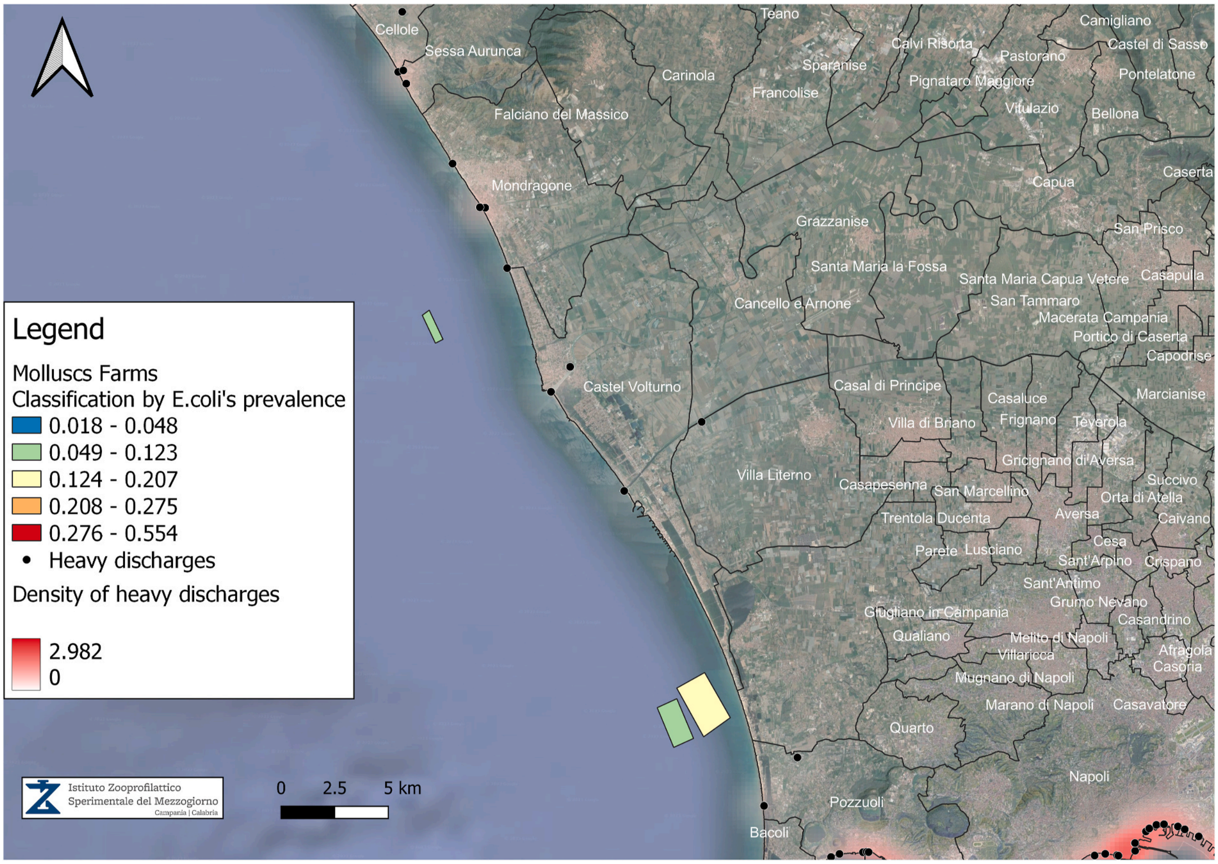

Fig. 5. Kernel density of heavy discharges in Caserta.

modified glutamate medium. After 24 ± 2 h at 37 °C, an aliquot was taken from each tube that showed the presence of acid, indicated by yellow coloration, and was subcultured with a loop onto a plate of tryptone bile glucoronide agar; the plates were then incubated for 24 h at 44 °C. The *E. coli* was enumerated for the presence of colonies that showed any shade of dark blue, light blue, or blue-green, indicating the presence of b-glucoronidase–positive *E. coli.*

2.5. Statistical analysis

The dataset was a combination of data from different institutional sources:

1. Laboratory Management System (SIGLA) of the Istituto Zooprofilattico Sperimentale del Mezzogiorno (IZSM) for microbiological analysis;
2. Regional agency for the environmental protection (ARPA Campania) for environmental parameters of marine waters and coastal discharge georeferenced database;
3. Multi-risk functional center of the Campania Region Civil Protection for rainfall data;

The georeferenced coastal discharge database provided the coordinates of all discharges of various kinds present along the Campania coast.

The discharge density along the coast per spatial unit was calculated based on the "quartic" Kernel function described by Silverman (1986), a mathematical model that considers the distance among all discharges. The spatial resolution chosen for the Kernel density model was the average distance among the discharges (a square of 200 × 200 m). Two types of discharges were identified based on the amount of pollutants released into the water: the light discharges category which included rainfall, rivers, lakes and streams, swimming pools and other light waste; the heavy discharges category including wastewater treatment plant discharges into underwater pipelines, wastewater treatment plant discharges along the coast, discharges without purification into underwater pipelines, discharges without purification along the coast, overflow pipes of sewage collectors, overflow pipes of lifting systems.

Rainfall is the sum of the daily rainfall four days before the sampling day [8–10].

The *E. coli* variable was categorized as non-compliant if the values are greater than 230 MPN/100 g and compliant for values less than or equal to 230 MPN/100 g. The exploratory analysis was conducted on environmental parameters.

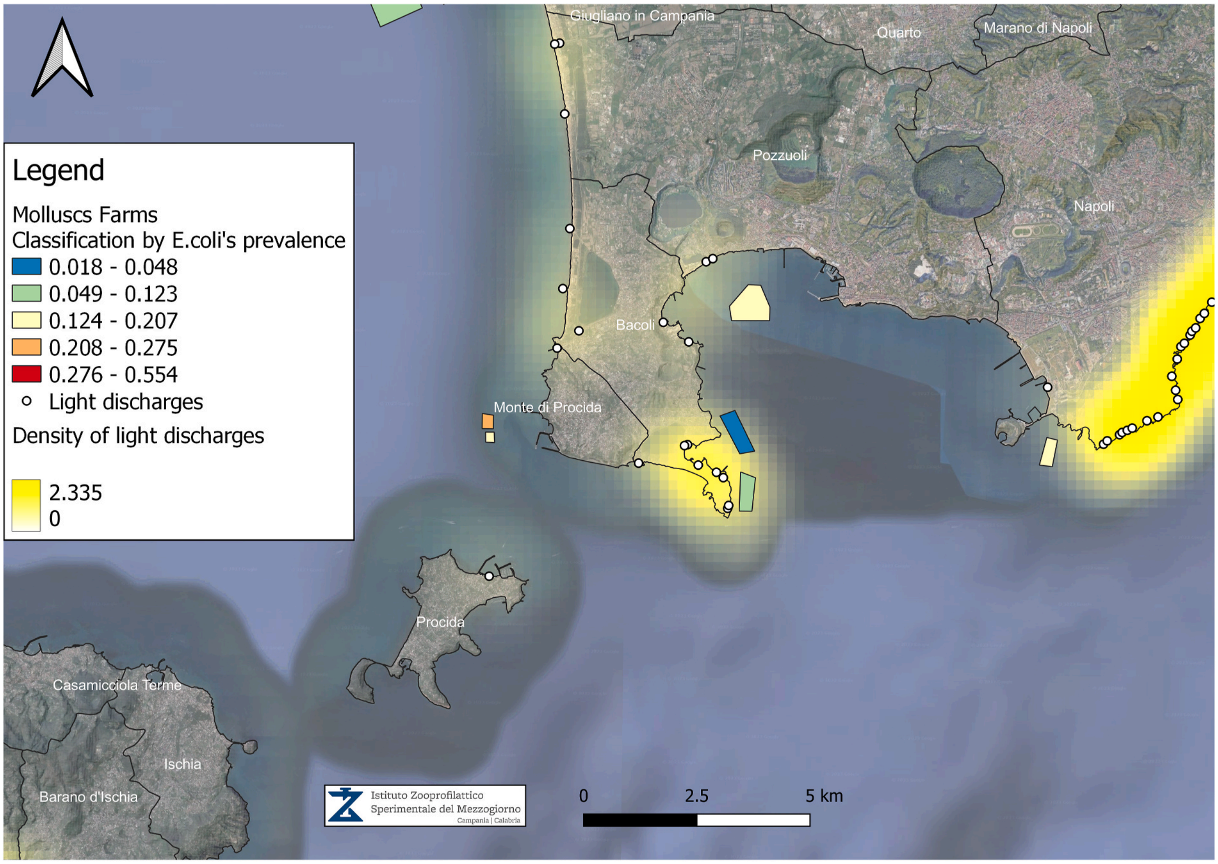

Fig. 6. Kernel density of light discharges along the Pozzuoli coast.

To verify the normality of the quantitative variables the Shapiro-Wilk statistical test was used. The association with the presence of *Escherichia coli* was evaluated with the chi-square test for the categorical variables while for the continuous variables the student's t-test or the Mann Whitney were used according to the distribution of the variable. The significance level value of the statistical tests was set to alpha 0.05. To evaluate the factors influencing *E. coli* contamination, a multivariate logistic regression model was developed using *E. coli* as the response variable and environmental, microbiological and pollution parameters as covariates. For the construction of the final additive model, we proceeded by progressively introducing the variables that have shown a statistically significant association with the outcome and the model with the lowest Akaike's information criterion (AIC) was preferred.

To evaluate the factors influencing *E. coli* contamination, a multivariate logistic regression model was developed. In the model used, *E. coli* was a dependent variable (categorical dichotomous). Temperature, oxygen, rainfall, kernel density (light discharge), kernel density (heavy discharge), heavy discharge distance (continuous), season and municipality (categorial) were included as independent variables. For the construction of the final additive model, we proceeded by progressively introducing the variables that have shown a statistically significant association with the outcome. The model with the lowest Akaike's information criterion (AIC) was preferred. The variables included in the final model were oxygen, rainfall, kernel density (heavy discharge).

3. Results

From 2016 to 2021, 1459 samples of bivalve shellfish taken from 16 farms located along the Campania coast were examined. Of the total number of samples taken, 1173 were compliant and 286 non-compliant. *Mytilus galloprovincialis*, the most widely bred, was sampled for a total of 1158 samples.

The prevalence of *E. coli*, the ratio between non-compliant samples and total samples taken, was calculated for each farm and it is represented in Fig. 1.

The highest prevalence is recorded in farms located in the municipalities of the Vesuvius area, while the lowest value is recorded in plants of the Phlegrean municipalities. Considering seasonality, the prevalence is higher in the autumn/winter period than in the spring/summer period (Fig. 2) (see Fig. 4) (see Fig. 5) (see Fig. 6) (see Fig. 7) (see Fig. 8) (see Fig. 3).

The shellfish farms on the coast are represented in cartography (Figs. 3–8) as polygons characterized by different colors chosen on the prevalence of *E. coli*. The prevalence classes were identified with the criterion of natural intervals, i.e. minimizing the variance within the group and maximizing the variance among the groups. The cartography showed the Kernel density of light and heavy

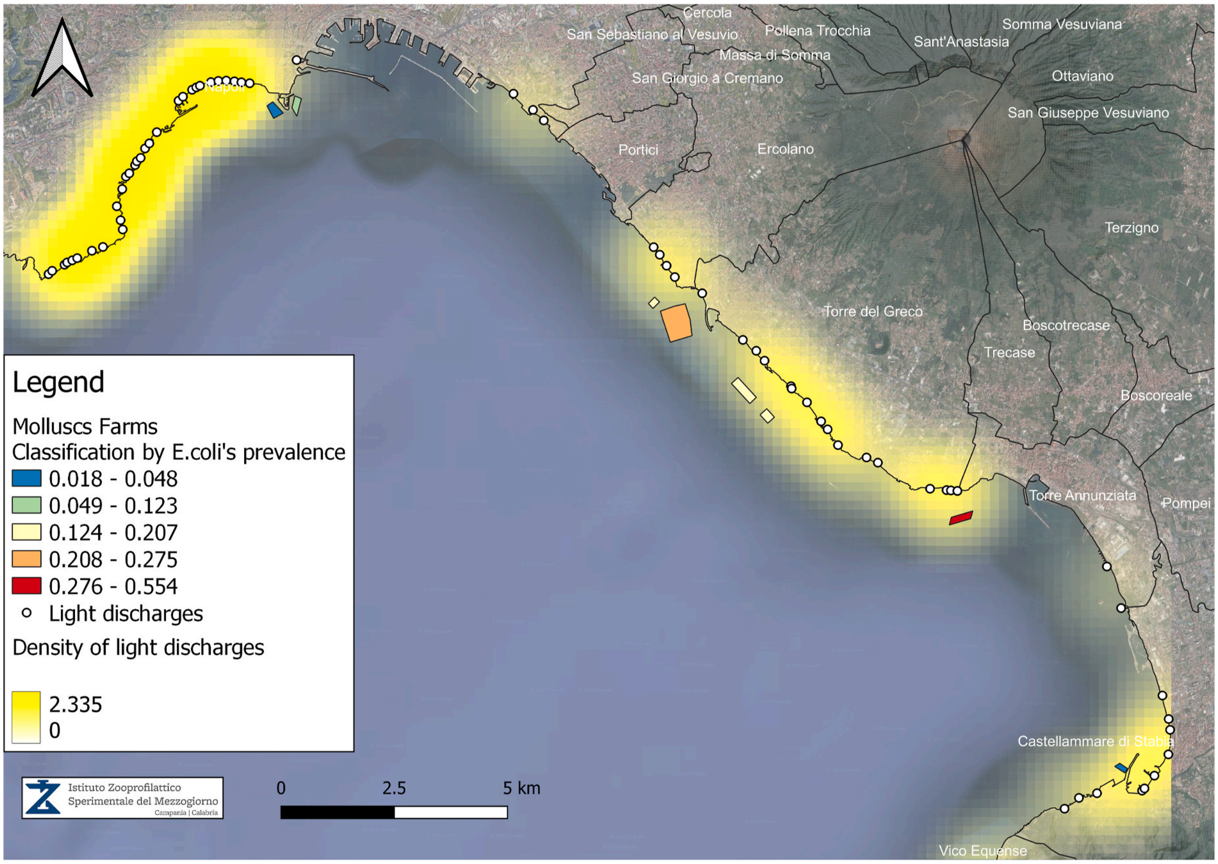

Fig. 7. Kernel density of light discharges along the Gulf of Naples.

discharges too.

From the analysis of the cartographies, it can be observed that the plants with higher prevalence are those in correspondence with high Kernel density values for both light and heavy discharges.

Kernel density of light discharges doesn't influence the *E. coli* value because Kernel density median was the same for both compliant and non-compliant group (Table 1).

The influence of the season and municipality variables on the compliant and non-compliant groups was tested using a Chi-squared test.

E. coli results are significantly associated (p-value <0.05) with season and municipality (Table 2).

The density of heavy discharges influenced the outcome of the samples. In fact, the group of non-compliant is associated with a higher average density value.

Considering the distance among the sampling points and the light discharges, we can see that in the samples with compliant results, the mean and median value of the distance is greater than that of the group of non-compliant. The same consideration cannot be made for the distance calculated with respect to heavy discharges (Table 2).

After evaluating all the explanatory variables, we proceeded to identify those useful for constructing an additive logistic regression model. The variables selected were: rainfall, percentage of oxygen present in the water and the density of heavy discharges (Table 3).

The rainfall (odds ratios, 1.03; 95% confidence interval [1.01 to 1,04]) and the density of heavy discharges (odds ratios, 1.38; 95% confidence interval [1.04 to 1,82]) on the coasts appear to be risk factors associated with positivity for *E. coli* in shellfish. For the rainfall variable, it can be stated that with an increase of 1 mm of rainwater, the risk of amplifying *E. coli* is likely of 3%.

As the Kernel density of heavy discharges increases by one unit, there is a 38% increase in the risk of registering a non-compliant for *E. coli* in shellfish.

Conversely, the oxygen covariate (odds ratios, 0.98; 95% confidence interval [0.97 to 0.99]), as a percentage of saturation in sea water, recorded an odd ratio value < 1, allowing the variable to be classified as a protection factor. We can therefore say that as water saturation increases by one percentage point, the risk of developing positivity decreases by about 2%.

In the end, the regression logistic model used, confirmed that the variables (rainfall and density of heavy discharges) play a crucial rule in shellfish contamination event.

Fig. 8. Kernel density of light discharges in Caserta.

4. Discussion

The quality of bivalve shellfish farmed in marine areas is the subject of great attention from the national and European community. The ability of these foods to spread certain pathologies in the population has stimulated numerous studies over time aimed to early identification of risk determinants. Acting on prevention represents the only real resource available to the National Health Service but it also appears essential to promote the commercial growth of the shellfish farming sector by identifying correct containment but not repressive measures.

This study was included in this context, which experimentally restricted the risk areas by contextualizing the indications of the bibliography to the regional reality.

The study confirmed that the prevalence of *E. coli* is higher in areas with higher population density [11] such as the Vesuvius area.

The non-compliant samples tend to concentrate in the autumn/winter period although sampling activity is reduced; in fact, the outcome of the chi-squared test confirms that the association is statistically significant.

It has also been highlighted that rainfall, in terms of rainwater fallen, takes on a critical value in this period and also in the logistic model rainfall acts as a risk factor for the presence and increase of *E. coli* values [12,13].

The geolocation of heavy discharges has shown that farms are not always at least 500 m away from polluting sources, however the model does not identify distance as a risk factor; on the other hand, the model confirmed the density as capable of increasing the possibility of contamination of the shellfish by about 40%.

Light discharges do not appear to affect shellfish contamination either in terms of density or distance of sampling.

Among the environmental parameters considered in the model, dissolved oxygen has been shown to be a protective factor in the contamination of shellfish. In fact, dissolved oxygen is a chemical parameter used to characterize the fitness for life, for living beings that use oxygen, such as fish, and the level of pollution of a marine system. Therefore, high saturation levels are associated with a good quality of the marine system and therefore less polluted waters and consequently a lower presence of pathogens.

The retrospective longitudinal observational study has identified the risk factors that have conditioned high *E. coli* values in shellfish in Campania region. The identification of these factors would allow the planning of containment and mitigation measures capable of preventing the spread of outbreaks of communicable diseases.

A limitation of our study is the need for a long sampling period to obtain statistically significant data a multidisciplinary team for data gathering.

Table 1
Mann Whitney test (pollution variables).

	Compliant (N = 1173)	Non compliant (N = 286)	*P*-value
TEMPERATURE			
Mean (SD)	20.3 (4.02)	19.7 (4.04)	<0.05
Median [Min, Max]	20.1 [13.3, 27.4]	19.3 [13.3, 26.7]	
SALINITY			
Mean (SD)	37.8 (0.253)	37.8 (0.181)	0.37
Median [Min, Max]	37.8 [35.9, 38.3]	37.8 [37.4, 38.4]	
OXIGEN PERCENTAGE SATURATION			
Mean (SD)	107 (9.75)	105 (10.4)	<0.05
Median [Min, Max]	106 [86.9, 188]	104 [86.9, 133]	
SUM RAINFALL			
Mean (SD)	3.34 (8.15)	7.63 (14.9)	<0.05
Median [Min, Max]	0 [0, 69.0]	0 [0, 66.4]	
TURBIDITY			
Mean (SD)	0.708 (1.06)	0.817 (1.07)	0.153
Median [Min, Max]	0.450 [0, 21.0]	0.453 [0.0350, 9.80]	
pH			
Mean (SD)	8.17 (0.0590)	8.17 (0.0605)	0.58
Median [Min, Max]	8.17 [8.03, 8.37]	8.17 [8.03, 8.37]	
KERNEL DENSITY (HEAVY DISCHARGE)			
Mean (SD)	0.572 (0.487)	0.651 (0.426)	<0.05
Median [Min, Max]	0.541 [0, 1.81]	0.695 [0, 1.81]	
KERNEL DENSITY (LIGHT DISCHARGE)			
Mean (SD)	0.410 (0.384)	0.439 (0.392)	0.308
Median [Min, Max]	0.288 [0, 1.11]	0.291 [0, 1.11]	
DISTANCE LIGHT DISCHARGE			
Mean (SD)	1640 (1750)	1450 (1370)	<0.05
Median [Min, Max]	1090 [263, 12100]	1020 [263, 12100]	
DISTANCE HEAVY DISCHARGE			
Mean (SD)	1540 (1620)	1320 (1290)	0.985
Median [Min, Max]	938 [196, 6480]	922 [196, 6480]	

Table 2
Chi square test qualitative variable vs outcome.

	Compliant (N = 1173)	Non compliant (N = 286)	Total	*P*-value
SEASON				
Autumn	166 (14.2%)	53 (18.5%)	219 (15.0%)	<0.05
Summer	372 (31.7%)	73 (25.5%)	445 (30.5%)	
Winter	181 (15.4%)	69 (24.1%)	250 (17.1%)	
Spring	454 (38.7%)	91 (31.8%)	545 (37.4%)	
SPECIES				
other-bivalve	15 (1.3%)	44 (15.4%)	59 (4.0%)	<0.05
mussel	1158 (98.7%)	242 (84.6%)	1400 (96.0%)	
CITY				
Giugliano-Castelvolturno	108 (9.2%)	7 (2.4%)	222 (15.2%)	<0.05
Monte di Procida	59 (5.0%)	3 (1.0%)	285 (19.5%)	
Napoli	25 (2.1%)	3 (1.0%)	272 (18.6%)	
Pozzuoli Baia	10 (0.9%)	2 (0.7%)	247 (16.9%)	
Torre-Ercolano	168 (14.3%)	26 (9.1%)	259 (17.8%)	
Torre Annunziata - Castellammare	220 (18.8%)	65 (22.7%)	174 (11.9%)	

Table 3
Logistic regression model.

	OR	C.I. 95%	p-value
Oxygen	0.98	[0.97; 0.99]	0.03
Rainfall	1.03	[1.01; 1,04]	0.000005
Kernel density (heavy discharge)	1.38	[1.04; 1.82]	0.02

The strength of this study was the quality of sampling data from the procedure established in national monitoring shellfish plan same as the environmental parameters and the data reproducibility.

On the other hand, the weakness of this study is that the shellfish samples are not always taken at the same time as seawater samples, introducing a confounding factor into the model.

4.1. Conclusions

The logistic regression model was developed in support of a Lagrangian WaComM and artificial intelligence model [14] that is already in use by local Health Department (ASL). The Lagrangian WaComM model is capable of predicting the dynamics of pollutants above harvesting areas and the physical processes that favor the phenomena of bio-accumulation.

On the other hand, the logistic regression model identifies the risk factors to be considered potential harmful in shellfish consumption.

The output of our model in association with the output of the WaComM model are both given to the Competent Health Authority so that can take a restrictive measures on the harvesting shellfish with significant benefits on the food safety, resources employed by the competent authority and by the food operators in case of non-compliance according to Article 59 of EU Regulation 2019/627.

In order to implement actions to protect Public Health it is to be hoped the integration of our data with WaComM model that will allow real-time valuation of areas of potential microbiological contamination.

For the first time it has been conducted a study using the data from the monitoring in Campania Region relating different sources of contamination such as microbiological and environmental.

5. Data availability statement

Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

No.

Has data associated with your study been deposited into a publicly available repository?

No.

Sharing research data helps other researchers evaluate your findings, build on your work and to increase trust in your article. We encourage all our authors to make as much of their data publicly available as reasonably possible. Please note that your response to the following questions regarding the public data availability and the reasons for potentially not making data available will be available alongside your article upon publication.

Has data associated with your study been deposited into a publicly available repository?

The data that has been used is confidential.

CRediT authorship contribution statement

Roberta Pellicanò: Writing – original draft, Validation, Supervision, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Roberta Brunetti:** Formal analysis, Data curation. **Tecla Toscano:** Writing – review & editing. **Sonia Smeraldo:** Visualization, Software, Data curation. **Loredana Baldi:** Writing – original draft, Formal analysis, Data curation, Conceptualization. **Stefania Cavallo:** Writing – original draft, Visualization, Methodology, Data curation. **Stefano Capone:** Writing – original draft, Visualization, Supervision, Data curation. **Germana Colarusso:** Writing – original draft, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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