Monitoring of bivalve mollusk harvesting areas: the relevance of *Salmonella* spp.

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Abstract

The microbiological monitoring of bivalve mollusk harvesting areas in the Marche region is based on the parameters of *Escherichia coli* and *Salmonella* spp. However, Regulation EU/2019/627 stated criteria based on *E. coli* only to determine the health status of these areas. Therefore, the reason for *Salmonella* spp. monitoring, as provided in the Marche region, could be aimed at reducing the risk of placing on the market contaminated bivalve mollusks. This study, using the results of microbiological monitoring carried out in the Marche region from 2015 to 2022 and the methods based on Bayes' theorem and Poisson's distribution, evaluated the effectiveness and efficiency of *Salmonella* spp. monitoring in reducing the risk to the consumer. The results show that i) the use of a single sample unit significantly reduced the possibility of detecting non-compliance with the microbiological safety criterion; ii) the time taken to report positive results (average of approximately 10 days) did not allow the timely implementation of control measures; iii) the prevalence of positive outcomes was quite sporadic: a random trend of positivity is recognizable on a geographical and monthly basis for mussels and a geographical basis for striped clams; iv) considering the predictive value of *E. coli* against *Salmonella* spp., the specificity is very high and the negative predictive value *versus* *Salmonella* spp. would be >80%.

In conclusion, the study shows that the monitoring of *Salmonella* spp. has a limited effect on reducing the risk to the consumer; however, in the cost/benefit assessment, other aspects not covered by this study should be considered.

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Key words: shellfish, *Salmonella* spp., *Escherichia coli,* microbiological monitoring, shellfish harvesting area.

Contributions: all the authors made a substantial intellectual contribution, read and approved the final version of the manuscript, and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare no potential conflict of interest.

Ethics approval and consent to participate: not applicable.

Funding: none.

Availability of data and materials: data and materials are available from the corresponding author upon request.

Conference presentation: this paper was presented at the XXXII National Conference of the Italian Association of Veterinary Food Hygienists (AIVI), Maierato-Vibo Valentia, September 13-14-15, 2023.

Received: 28 November 2023.
Accepted: 4 March 2024
Early access: 15 March 2024

Italian Journal of Food Safety 2024; 13:12142
doi:10.4081/ijfs.2024.12142

Introduction

The Marche region, as well as other Italian regions, has provided for microbiological monitoring of the bivalve mollusk harvesting areas based on both the parameters *Escherichia coli* and *Salmonella* spp. However, Regulation EU/2019/627 (European Commission, 2019) does not indicate the parameters subjected to control and requires only the results of *E. coli* monitoring as an indicator of fecal contamination to determine the health status of harvesting areas. Both the European Community Guidance on Microbiological Monitoring (European Commission, 2021) and the Food and Agriculture Organization and the World Health Organization Technical Guidance (FAO and WHO, 2021) do not take into account *Salmonella* spp.

The Marche region has not explicitly stated the reason for monitoring *Salmonella* spp., but it could be traced back to the aim of reducing the risk of placing on the market live bivalve mollusks contaminated with *Salmonella* spp.

In fact, Regulation CE/2073/2005 (European Commission, 2005) has provided two microbiological safety criteria for live bivalve mollusks: *E. coli* with a 3-class sampling plan (n=5, c=1, m=230 MPN/100 g, M=700 MPN/100 g) and *Salmonella* spp. with a 2-class sampling plan (n=5, c=0, m=absence/25 g).

The regional monitoring plan requires, for both parameters, the test on a single sample unit (s.u.), approximately monthly, with a minimum number of eight samples per year.

The finding of the presence of *Salmonella* spp., as *E. coli* values

>230 MPN/100 g in classified areas, requires the adoption of administrative control measures to protect the consumer, such as restrictions on the use of shellfish from the involved area, mandatory purification of bivalve mollusks, withdrawal/recall of products placed on the market, *etc*.

For many years now, it has been highlighted that the correlation between the results of *E. coli* and *Salmonella* spp. in bivalve mollusks is not significant (Hood *et al.*, 1983). This has been attributed to many factors, *e.g.*, feces of all warm-blooded animals contain *E. coli,* but only in a few cases *Salmonella* spp. is present; the endurance of the two species in the external environment is different (Martinez-Urtaza *et al.*, 2004); there are different ecological niches, *etc*. (Rozen and Belkin, 2001; Winfield and Groisman, 2003; Cioffi *et al.*, 2021).

Even the obtained results in the Marche region do not seem to show a correlation between the two parameters, and the prevalence of *Salmonella* spp., despite the high number of performed tests, does not seem particularly relevant.

This study, using statistical methods based on Bayes' theorem and Poisson's distribution, aims to assess the effectiveness and efficiency of monitoring *Salmonella* spp. to prevent the placing on the market of contaminated live bivalve mollusks. In particular, considering the results of microbiological monitoring carried out in the Marche region from 2015 to 2022, as available on the SINVSA informative system database, managed by the Italian Health Ministry, this study aims: i) to determine the efficiency of *Salmonella* spp. monitoring, by comparing the performance of the monitoring plan based on a single s.u. with that based on five s.u.; ii) to determine the effectiveness of monitoring in reducing the marketing of contaminated bivalve mollusks, by measuring the timeliness in adopting measures following positive outcomes; iii) to evaluate the trend and distribution of positive outcomes to recognize any characteristics useful for increasing the predictive value against *Salmonella* spp.; iv) to evaluate, using equations based on Bayes' Theorem, to what extent the monitoring results for *E. coli* may also have a predictive value against *Salmonella* spp.

Materials and Methods

The coastal system of the Marche region, on the west coast of the Adriatic Sea, extends from the mouth of the Tronto River to the Gabicce promontory, for approximately 180 km, characterized mainly by a low, sandy, or gravelly coastline and with a few points of high and rocky coast, Mount Conero and Mount San Bartolo. Almost along the entire coast, there are natural beds of striped clams (*Chamelea gallina*), with 68 distinct classified harvesting areas, and 7 classified natural banks of mussels (*Mytilus galloprovincialis*), present along the rocky coast. The shellfish farming areas are located along the entire coast, at a distance between 2.5 nm and 3 nm from the shoreline and are currently classified as 23 mussel farms (*Mytilus galloprovincialis*), including a relay area, and only one oyster farm (*Crassostrea gigas* and *Ostrea edulis*). Microbiological monitoring of these areas is based on both *E. coli* and *Salmonella* spp. parameters and is carried out by taking single s.u on a monthly basis from each of the 104 geo-referenced sampling stations. Gastropods of the *Nassarius mutabilis* and *Murex brandaris* species, collected in unclassified areas, are also included in the microbiological monitoring of *Salmonella* spp.; in this case, the samples are taken upon landing and with a reduced frequency compared to bivalve mollusks. All analyses are carried out by an official laboratory, the Experimental Zooprophylactic Institute of Umbria and Marche, using the EN ISO 16649-3 method (ISO, 2015) for *E. coli* and the EN ISO 6579-1 method (ISO, 2017) or *Salmonella* spp.

For this study, we used the data from the results of microbiological monitoring carried out from 2015 to 2022, both for bivalve mollusks and for gastropods, as found on the SINVSA database, the Informative System of the Italian Health Ministry.

The Poisson's distribution, a discrete distribution that measures the probability of a given number of events happening in a specified time or space interval, was used to evaluate the efficiency of *Salmonella* spp. monitoring, making a comparison between the results obtained from sampling plans based on a different number of s.u. The Poisson's distribution equation can be expressed as in Equation 1:

$$P_i = \frac{\mu^i}{i} e^{\mu} \qquad \text{[Eq. 1]}$$

with μ=np and where i is the number of positive cases, n is the number of units that constitute the examined quantity expressed in grams, and p is the prevalence of *Salmonella* spp. The probability of obtaining no positive outcomes, *i.e.*, i=0 can be expressed as in Equation 2:

$$P_0=e^{-np} \quad \text{then} \quad p = \frac{lnP_0}{n} \qquad \text{[Eq. 2]}$$

For values from n=25, corresponding to one s.u., up to n=125, for five s.u. and tabulating for P_0 values from 0.01 to 0.99, the related sampling operating characteristic (SOC) curves were constructed (Ciccarelli *et al.*, 2023).

Since the SINVSA database does not contain data relating to control measures issued following positive outcomes, the time intervals between the sample collection and its delivery to the laboratory and between the delivery and the issuing of the test report were calculated. This reporting time interval represents the shortest possible reaction time for adopting control measures, which is useful to evaluate the effectiveness of monitoring in protecting against the placing on the market of contaminated bivalve mollusks. The values were classified for two different outcomes: compliance (≤230 MPN/100 g for *E. coli* and absence/25 g for *Salmonella* spp.) or non-compliance. The main descriptive statistics parameters were calculated, and the relative distributions were represented graphically using box plots.

The trend of positive outcomes for *Salmonella* spp. was evaluated by classifying the results both on a temporal basis as well as on a geographical basis. To evaluate whether the distribution of positive outcomes had a random trend following the Poissonian law, the prevalence of positivity and the average of such events (μ) were calculated; the related distributions were represented in graphs and compared with the corresponding theoretical Poisson's distribution (Equation 1). The *l*og-likelihood ratio test for goodness of fit (G) was used to compare the given and theoretical distributions, along with Williams' correction for samples smaller than 200 units (G_{adj}).

To evaluate the predictive value of the *E. coli* result, considered a test for the presence of *Salmonella* spp., 2×2 contingency tables were drawn up separately for striped clams and mussels, comprising all sample results, for which both tests for *E. coli* and *Salmonella* spp. were carried out. The sensitivity and specificity of the test were also calculated.

The following equations (Equations 2 and 3) based on Bayes' theorem, the conditional probability theorem, were used to calculate the corresponding positive predictive value (PPV) and negative predictive value (NPV) of the test, taking into account a wide range of prevalence of *Salmonella* spp. (from 0.001 to 0.1).

$$PPV = \frac{sens \cdot prev}{sens \cdot prev + (1 - spec) \cdot (1 - prev)} \quad \text{[Eq. 3]}$$

$$NPV = \frac{spec \cdot (1 - prev)}{spec \cdot (1 - prev) + (1 - sens) \cdot prev} \quad \text{[Eq. 4]}$$

Results and Discussion

The theoretical performance of different sampling plans, including that of the safety criterion stated by Regulation CE/2073/2005 (European Commission, 2005) based on five s.u. and that of the monitoring plan based on a single s.u., were graphically summarized in the SOC curves shown in Figure 1.

As expected, the efficiency of the sampling plan with only one s.u. is lower than that of the other plans, and the comparison of the SOC curves allows for a quantitative evaluation of these differences. For example, using only one s.u., the presence of a *Salmonella* spp. in 25g (the lowest value that does not meet the microbiological safety criterion) would not be detected in 40% of samples, while with five s.u., the rate of undetected *Salmonella* spp. would be 0. This clearly demonstrates the reduced efficiency of monitoring harvesting areas based on a single s.u., and to obtain comparable results, it would be necessary to use at least three s.u., effectively tripling the number of tests to be performed.

For both *E. coli* and *Salmonella* spp., the time interval, expressed in days, between the sampling and delivery to the laboratory and between the sampling and the issuing of the test report was determined. The data was classified according to the outcome of the two parameters, compliant or not. The main descriptive statistical characteristics of the relative distributions are summarized in Table 1. The report's time distributions were graphically represented in *Supplementary Figure 1* with the Mean and Whiskers method: 95% of the data are included within the "whiskers".

The average time interval between sampling and delivery to the laboratory is very close to 0, *i.e.*, the sample delivery usually took place on the same day as the collection. However, the time of issuing the test report showed greater variability, with relevant differences depending on the considered parameter and the type of outcome, a condition certainly related to the timing dictated by the analytical methods: for *E. coli,* the EN ISO 16649-3 method (ISO, 2015), and for *Salmonella* spp., the EN ISO 6579-1 method (ISO, 2017).

The interval of time to obtain positive outcomes for *Salmonella* spp. was very long, with an average and median of approximately 10 days. This could lead to a great amount of potentially contaminated bivalve mollusks being placed on the market, as more time is required to implement control measures. Differently from *Salmonella* spp., the reaction time following *E. coli* >230 MPN 100/g outcomes was shorter, with a median of 5 days.

The trend of *Salmonella* spp. monitoring results is reported in *Supplementary Tables 1-3,* respectively, on an annual, monthly, and geographical basis, at the provincial level. Furthermore, a positive outcome was never followed by a second positivity in the subsequent sample.

In general, it is easy to recognize that the positive outcomes were sporadic, with a prevalence that has settled at values close to 1%; *Salmonella* spp. was never detected in oysters and only once in gastropods. These results comply with the literature indicating

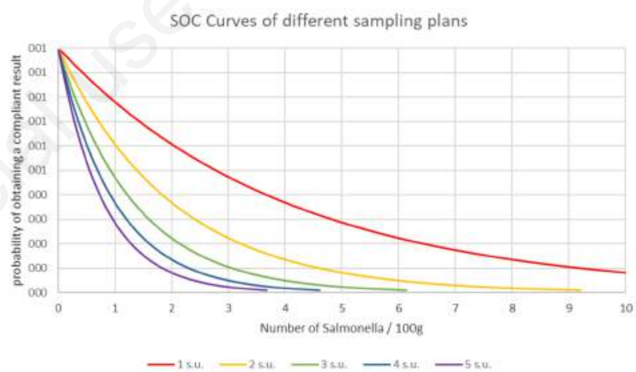

Figure 1. Performance of different sampling plans: the sampling operating characteristic curves show the probability of obtaining compliant (negative) outcomes related to a different amount of *Salmonella* spp.; the ordinates represent the probability, and the abscissas indicate the number of *Salmonella* spp. in the matrix. SOC, sampling operating characteristic; s.u, sample unit.

Table 1. Main descriptive statistical characteristics of the distributions relating to the time from sampling to delivery of samples and from delivery to the issuing of the test report for *Escherichia coli* and *Salmonella* spp. expressed in days.

	Delivery of sample				Issuing of the test report			
	Salmonella (-)	*Salmonella* (+)	*Escherichia coli* ≤230	*Escherichia coli* >230	*Salmonella* (-)	*Salmonella* (+)	*Escherichia coli* ≤230	*Escherichia coli* >230
Nr of outcomes	9104	100	9596	990	9104	100	9596	990
Mean μ	0.04	0.06	0.04	0.01	6.66	10.39	7.89	8.62
Stand. Dev. σ	0.42	0.34	0.46	0.12	15.00	6.76	18.43	19.71
Median	0	0	0	0	5	10	5	5
25° percentile	0	0	0	0	3	7	3	3
75° percentile	0	0	0	0	7	13	8	9

Nr, number; Stand. Dev., standard deviation; (+), positive outcomes intended as *Salmonella* spp. detected; (-), negative outcomes intended as *Salmonella* spp. not found.

low levels of *Salmonella* spp. presence in samples from marine areas in temperate regions (Simental and Martinez-Urtaza, 2008; Rubini *et al.*, 2018; Bazzoni *et al.*, 2019; Rincé *et al.*, 2019). A modest decreasing trend can be recognized on an annual basis; on a monthly basis, the positive outcomes were concentrated in the spring period, from March to June, exceeding 50% of the total; and finally, the differences on a provincial geographical basis did not appear relevant.

In the hypothesis of their random distribution, the trend of positive outcomes, independently for striped clams and mussels and on a temporal and geographical basis, was compared with the expected frequencies, according to a random trend following Poisson's distribution. Table 2 summarizes the relative numerical values and the results of the G_{adj} test used to evaluate the significance of the differences between the observed and expected distributions in the H_0 hypothesis with α=0.05, that the distribution of positive outcomes has a random trend. *Supplementary Figures 2-5* display the trend of the classes of observed and expected events and their relative frequencies.

These graphs allow us to recognize similarities for all the tested conditions, on a temporal and geographical basis, and for both considered species. The G_{adj} test allows us to reject the H_0 hypothesis only for the striped clam geographically based option. Instead, in the other three tested cases, the differences between observed and expected distributions are not significant, and it is possible to recognize their random trend.

This random pattern, coupled with the discovery that positive results were never followed by a positive for the next sample, may be consistent with the hypothesis that detected *Salmonella* spp. were not associated with specific sources of contamination on the coast (Winfield and Groisman, 2003). It might depend on the widespread contamination level of that marine environment, predominantly governed by the presence of persistent rains (Martinez-Urtaza *et al.*, 2004; Simental and Martinez-Urtaza, 2008; Setti *et al.*, 2009), influenced by environmental factors such as variations in temperature, ultraviolet radiation, competition and predation, turbidity, and salinity, and maybe also connected to the different ability of *Salmonella* spp. serovars to survive in seawater (Martinez-Urtaza *et al.*, 2004; Korajkic *et al.*, 2019). This behavior was more evident in mussels that, in general, are further from the sources of contamination or areas of great anthropogenic pressure. For striped clams instead, the monthly basis trend of positive outcomes, not recognized as random, may have been related to the seasonal trend of the rainfall, as suggested by *Supplementary Table 2*, which showed a trend to concentrate in the spring period of rains.

Since the monitoring of *E. coli* represents a legal requirement, we checked the possibility of using these results as a predictive test for the presence of *Salmonella* spp. In fact, the two tests were generally performed on the same sample, and the time to obtain the *E. coli* test report was significantly shorter than that for the non-compliant outcomes of *Salmonella* spp. The results are summarized in Table 3, and Figure 2 shows the results of the calculated PPV and NPV of the

Table 2. Comparison between observed and expected rates of positive outcomes, independently for striped clams (*Chamelea gallina*) and mussels (*Mytilus galloprovincialis*). The log-likelihood ratio test for goodness of fit with the Williams correction assessed the goodness of fit of the two competing distributions: the observed positive outcomes and those expected.

Event classes			0	1	2	3	4	5	6	7	8
Positive striped clam cases on a monthly basis		Observed events	61	13	13	5	0	1	1	0	1
Prevalence	1.123	Observed rate	0.642	0.137	0.137	0.053	0.000	0.011	0.011	0.000	0.011
Mean (μ)	0.768	Expected events	44	34	13	3	1	0	0	0	0.000
Log-likelihood ratio test $G_{adj(8gdl)}$	39.084	Expected rate	0.464	0.356	0.137	0.035	0.007	0.001	0.000	0.000	0.000
*Positive mussel cases on a monthly basis		Observed events	75	15	5	1	---	---	---	---	---
Prevalence	1.031	Observed rate	0.781	0.156	0.052	0.010	---	---	---	---	---
Mean (μ)	0.292	Expected events	72	21	3	0	---	---	---	---	---
Log-likelihood ratio test $G_{adj(8gdL)}$	6.663	Expected rate	0.747	0.218	0.032	0.003	---	---	---	---	---
*Positive striped clam cases by harvesting areas		Observed events	30	20	10	9	1	---	---	---	---
Prevalence	1.095	Observed rate	0.429	0.286	0.143	0.129	0.014	---	---	---	---
Mean (μ)	0.986	Expected events	26	26	13	4	1	---	---	---	---
Log-likelihood ratio test $G_{adj(8gdL)}$	8.200	Expected rate	0.373	0.368	0.181	0.060	0.015	---	---	---	---
*Positive mussel cases by harvesting areas		Observed events	20	10	7	1	---	---	---	---	---
Prevalence	1.069	Observed rate	0.526	0.263	0.184	0.026	---	---	---	---	---
Mean (μ)	0.711	Expected events	19	13	5	1	---	---	---	---	---
Log-likelihood ratio test $G_{adj(8gdL)}$	2.688	Expected rate	0.491	0.349	0.124	0.029	---	---	---	---	---

$G_{adj,}$ log-likelihood ratio test for goodness of fit with the Williams correction; *conditions in which it is possible to accept the hypothesis H_0 with α=0.05, recognizing that the differences are linked to chance.

Table 3. The 2×2 contingency tables, and related sensitivity and specificity, of *Escherichia coli* and *Salmonella* spp. results for tests performed on the same sample, distinct for striped clams and mussels.

	Striped clams		Mussels	
	Escherichia coli >230	*Escherichia coli* ≤230	*Escherichia coli* >230	*Escherichia coli* ≤230
Salmonella spp (+)	35	19	3	17
Salmonella spp (-)	562	4484	71	2060
Sensibility	0.059		0.041	
Specificity	0.996		0.992	

(+), positive outcomes intended as *Salmonella* spp. detected; (-), negative outcomes intended as *Salmonella* spp. not found.

test. As expected due to the limited sensitivity, the PPV never assumed relevant values, while when the prevalence of *Salmonella* spp. is ≤0.01, a situation that occurred in the period taken into account, the NPV remained at values >80%. These values could support the monitoring option only for *E. coli* since when the result of *E. coli* is ≤230 MPN 100/g it becomes very likely to obtain a favorable result for *Salmonella* spp. These results are compliant with the finding that the relative risk of isolating *Salmonella* spp. is lower when *E. coli* concentration does not exceed the median (Rincé *et al.*, 2018).

Conclusions

In this study, the results of the microbiological monitoring of the bivalve mollusk harvesting areas carried out in the Marche region between 2015 and 2022 were taken into consideration, as reported in the SINVSA informative system database. With statistical methods based on Bayes' theorem and Poisson's distribution, an attempt was made to measure the efficiency and effectiveness of *Salmonella* spp. monitoring in protecting against the placing on the market of contaminated live bivalve mollusks. The study revealed that the following: i) the efficiency of monitoring in protecting against the placing on the market of contaminated bivalve mollusks, compared to the safety criteria for *Salmonella* spp. stated in Regulation CE/2073/2005, appeared very limited. In fact, with the monitoring plan based on a single s.u., a non-negligible number of non-compliant samples could not be recognized; ii) likewise, the effectiveness of monitoring also appeared to be very limited due to the excessively long reporting times for positive outcomes. In that way, the timely implementation of control measures was not allowed, resulting in a decrease in the amount of contaminated bivalve mollusks placed on the market; iii) the positive outcomes found in the considered period were sporadic, with a prevalence close to 1%; they assumed a statistically significant random trend both on a temporal and geographical basis for mussels, but only on a geographical basis for striped clams. Furthermore, consecutive episodes of positivity have never occurred in samples coming from the same sampling station. This trend seemed consistent with the hypothesis that *Salmonella* spp., detected by the monitoring, usually cannot be traced back to a specific and localized source of contamination along the coast, but it may depend on a state of widespread contamination of the marine environment, also related to the ability of different *Salmonella* spp. serovars to survive in seawater. This condition would further reduce the relevance of this form of monitoring; iv) the hypothesis that results for E. *coli* may have a predictive value against *Salmonella* spp. could not be supported since the PPV was generally very low. However, when the prevalence assumes values like those highlighted in the studied period, it was shown that the NPV would not be negligible, with a value >80%. This could be a further element to support the choice to resort to monitoring only *E. coli*.

In conclusion, the study showed that the monitoring of *Salmonella* spp., as well as the implementation from 2015 to 2022 in the Marche region, had limited effectiveness and efficiency and, therefore, a limited impact on the ability to reduce the risk for the consumer. The obtained results underline the need to reconsider the opportunity to maintain this form of monitoring and to proceed with a careful cost/benefit assessment within the scope of risk management activities. However, other aspects that were not taken into account in this study, such as, *e.g.*, the opportunity to have updated epidemiological information on the spread of *Salmonella* spp. serovars in the environment should be considered.

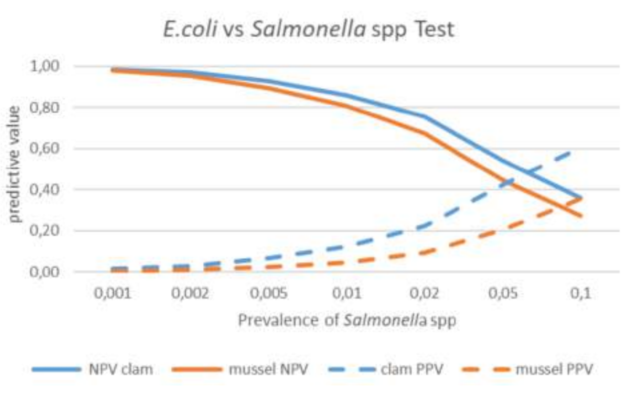

Figure 2. Positive predictive value (PPV) and negative predictive value (NPV) of the *Escherichia coli* outcomes *vs Salmonella* spp. outcomes.

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Online supplementary materials

Supplementary Table 1. Monitoring results and prevalence of Salmonella spp. classified on an annual basis.

Supplementary Table 2. Monitoring results and prevalence of Salmonella spp. classified on a monthly basis.

Supplementary Table 3. Monitoring results and prevalence of Salmonella spp. classified on a district basis, for each of the species subjected to monitoring.

Supplementary Figure 1. Graphic representation, with the Mean and Whiskers method, of the distributions relating to the issuing report time for E. coli and Salmonella spp. (95% of the data are included within the "whiskers"). The data on the abscissa are expressed in days.

Supplementary Figure 2. Trend of positive striped clam outcomes, classified on a monthly basis, compared to those expected in the hypothesis of random distribution.

Supplementary Figure 3. Trend of positive mussel outcomes, classified on a monthly basis, compared to those expected in the hypothesis of random distribution.

Supplementary Figure 4. Trend of positive striped clam outcomes, classified by harvesting area, compared with those expected in the hypothesis of random distribution.

Supplementary Figure 5. Trend of positive mussel outcomes, classified by harvesting area, compared with those expected in the hypothesis of random distribution.