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ZOO-SANITARY SURVEY FOR POTENTIAL MUSSEL AQUACULTURE ZONE DESIGNATION AT THE ROMANIAN COAST

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Abstract

An essential aspect for developing mussel (Mytilus galloprovincialis Lamarck, 1819) aquaculture at the Romanian Black Sea coast is the classification of culture areas from the microbiological point of view, in compliance with European Commission Regulation no. 854/2004. According to this document, the indicator species is the bacteria Escherichia coli and thresholds have been set to classify the bivalve culture areas for human consumption: for class A, no post-harvest treatment is required to reduce microbiological contamination, while for classes B and C purification, relaying or cooking by an approved method are mandatory. In this context, monthly samples were collected from three sampling points along the Romanian coast and the contamination of mussel tissue and intravalvular liquid with E. coli was analyzed. The reference method for analysis was the Most Probable Number (MPN), as mentioned by European standard EN/ISO 16649-3. The aim of this research was to assess the zoo-sanitary and food safety suitability of the most indicated areas for shellfish culture, previously selected taking into account the environmental constraints of the Romanian coast (no sheltered areas, currents, strong winter winds).

Key words*: contamination, E. coli, food safety, microbiological standards, mussels.*

INTRODUCTION

Romanian aquaculture has been historically focusing mainly on freshwater fish species, yet, recently mariculture has started to generate interest. In this context, valuable finfish species, such as turbot (*Psetta maxima*) (Niță and Nenciu, 2017), sturgeons (Russian sturgeon *Acipenser gueldenstaedti* and Siberian sturgeon *Acipenser baerii*) (Niță et al., 2018b) or golden-gray mullet (*Liza aurata*) (Niță et al., 2018a), were found to be suitable for rearing in culture conditions and provide an opportunity for market development. Moreover, in Romania, there has been a slight increase in the consumption of mussels and oysters in public nutrition (Niță et al., 2019), thus offering a great development opportunity of shellfish aquaculture.

However, significant focus should be put on zoo-sanitary conditions and public health, as shellfish can generally be considered to be a safe, healthy and nutritious food, but the consumption of bivalve mollusks harvested from contaminated waters may lead to disease due to the presence of micro-organisms (Nicolae et al., 2019). The assessment of sources and types of human and animal fecal contamination in the proximity of shellfish harvesting areas, combined with microbiological monitoring based on the use of indicator organisms (the bacteria *Escherichia coli* in the European Union) provides an assessment of the risk of contamination with bacterial and viral pathogens and are the basis for public health checks (Nenciu e al., 2020). Equally important, the increase in the demand for bivalves has encouraged the harvesting of mussels from natural populations, growing mussels on floating installations (long-line systems) and acclimatization of high-value bivalves - the Japanese oyster, for instance (Zaharia et al., 2017). The annual quantity of mussels harvested in the Romanian Black Sea coast area amounts to approximately 15 t, and the only existing mariculture farm (with interrupted activity), S.C. MARICULTURA S.R.L., can produce annually approximately 5 t of cultured mussels (Niță et al., 2019).

In the current context of Romanian legislation, the main drawback for developing shellfish aquaculture is the lack of coordination between

institutions (Sanitary Veterinary Directorate, Public Health Directorate, Romanian Waters Administration), but some steps in settling this issue have been made, as a collaboration protocol between several institutions is under elaboration. Moreover, the establishment of the Shellfish Aquaculture Demonstrative Center (S-ADC) in the frame of the National Institute for Marine Research and Development "Grigore Antipa" (NIMRD) - General Fisheries Commission for the Mediterranean (GFCM) collaboration aims at the promotion of scientific, technical and technological bases for bivalve shellfish aquaculture in Romania (Niță et al., 2018c).

Food safety monitoring of shellfish production areas in the European Community is currently regulated by the "Hygiene Package", which entered into force on 1 January 2006. This legislative package includes Regulations (EC) No. 852/2004 and No. 853/2004, which target industry professionals, Regulation No. 854/2004, aimed at competent authorities, responsible of carrying out official sanitary controls, and Regulations (EC) No. 854/2004, 2073/2005 and No. 882/2004, which refer to end-product standards required for bivalve mollusks (Nenciu et al., 2020).

The detailed implementation of classification and monitoring programs following Regulation (EC) No. 854/2004 is the responsibility of competent authorities and may vary between Member States (European Community, 2004). In Romania, the competent authority is the Sanitary-Veterinary and Food Safety National Authority, yet, up to date, no official classification has been undertaken.

In this context, the aim of this research was to assess the microbiological contamination of mussel samples collected from selected areas, in order to indicate the most suitable potential areas for mussel culture at the Romanian coast, from the zoo-sanitary and food safety perspective.

MATERIALS AND METHODS

The risk of contamination of shellfish is evaluated by reference to the sources and types of fecal contamination (human and animal) in the vicinity of the shellfish production areas (shoreline survey), on the one hand, and the results obtained based on the indicator bacteria *Escherichia coli*, from samples taken in these areas, on the other hand (Table 1). Areas are classified following a full assessment of this risk and the classification given to an area determines whether shellfish harvested in that area require post-processing treatment and, where appropriate, the level of such treatment (Anon, 2017).

Table 1. Criteria for the classification of bivalve mollusk harvesting/culture areas

Class	Criteria for the classification of bivalve mollusk harvesting areas	Post-harvest treatment required to reduce microbiological contamination
A	Samples of live bivalve mollusks from these areas must not exceed, in 80% of samples collected during the review period, **230 *E. coli* per 100 g** of flesh and intravalvular liquid. The remaining 20% of samples must not exceed 700 *E. coli* per 100 g of flesh and intravalvular liquid	None
B	Live bivalve mollusks from these areas must not exceed, in 90% of samples, **4,600 MPN *E. coli* per 100 g** of flesh and intra-valvular liquid. In the remaining 10% of samples, live bivalve mollusks must not exceed 46,000 MPN *E. coli* per 100 g of flesh and intra-valvular liquid	Purification, relaying or cooking by an approved method
C	Live bivalve mollusks from these areas must not exceed the limits of a five-tube, three dilution MPN test of **46,000 *E. coli* per 100 g** of flesh and intravalvular liquid	Relaying or cooking by an approved method

For the current research, in order to assess the risk of contamination of mussels with pathogens, mussel samples were taken from three selected sampling stations (two replicates/station - Figure 1, Table 2), on a monthly basis, during July - December 2019, covering a 6-month time frame.

The three stations (Mangalia - MG1 and MG2, Constanța - CT 1 and CT2, and Năvodari - NV1 and NV2) were selected as most suitable due to their geographical location in sheltered areas (protected by hydrotechnical constructions), which would allow for actual shellfish farms to be established. Moreover, a preliminary analysis indicated no potential land-based contamination sources.

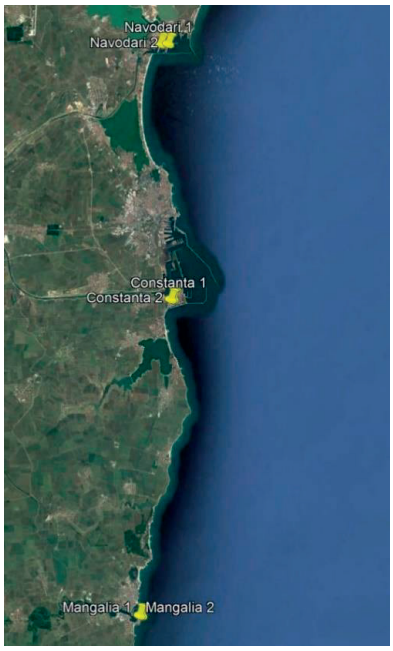

Figure 1. Map of sampling stations

The mussel samples were collected using a custom-made scraper, with stainless-steel handle, frame and blade and a collector sack made of mesh (Figure 2, left side). After scraping from the natural substrate, mussels were placed in appropriately labeled zip-lock bags and cold stored for transportation to the laboratory (Figure 2, right side). All samples were processed within 24 hours after collection, being kept in a refrigerator.

Table 2. Coordinates of sampling stations

Sampling Location						
Coord.	**MG1**	**MG2**	**CT1**	**CT2**	**NV1**	**NV2**
Lat. N	43.78951	43.78957	44.08433	44.08473	44.32421	44.32387
Long. E	28.58694	28.58424	28.64386	28.64638	28.64558	28.65562

The quality biological parameters investigated were the following: total number of germs (TNG), total coliforms (TC), fecal coliforms (FC) and *E. coli*.

The methods used for determining the microbial contamination of mussel samples were detection by culture technique on usual and selective media, except for *E. coli*, for which the European reference method was used: detection and Most Probable Number (MPN) technique specified in EN/ISO 16649-3/2015.

Figure 2. Mussel sample collection using a scraper (left side) and zip-lock bagging for cold storage and transportation to the laboratory (right side) (Original photos)

The flesh and intravalvular liquid of the mussels were excised and sampling units (10 g) were homogenized. Analysis for the detection and enumeration of *E. coli* on the homogenate took place by using a five-tube, three dilution (1 g, 0.1 g and 0.01 g) Most Probable Number (MPN) test method according to EN/ISO 16649-3. This is a two-stage, five tube by three dilution MPN method. The first stage of the method is a resuscitation requiring inoculation of minerals modified glutamate broth (MMGB) with a series of diluted bivalve mollusk homogenates (flesh and intravalvular liquid) and incubation at 37±1°C for 24±2 hours (Figure 3).

Figure 3. Preparation of samples for homogeneization of tissue and intravalvular liquid (Original photo)

E. coli was subsequently confirmed by subculturing tubes showing acid production onto tryptone bile glucuronide agar (TBGA) and detecting β-glucuronidase activity by the presence of blue or blue-green colonies.

The detection of the most probable number is made from the combination of positive and negative tubes (Figure 4).

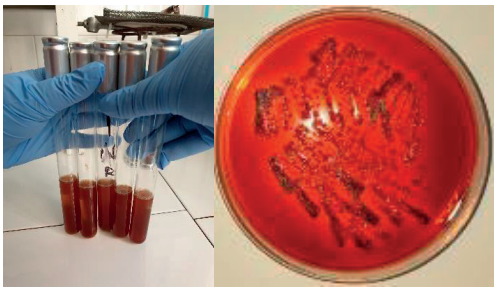

Figure 4. *E. coli* determination using the MPN technique (Original photos)

RESULTS AND DISCUSSIONS

At a preliminary examination, the organoleptic quality of all sampled mussels, related to smell and taste, was appropriate for human consumption. None of the mussels analysed had a smell or taste altered compared to the specificity of these seafood items.

The results obtained after the microbiological analysis of the sampled mussel tissue and intravalvular liquid homogenates are summarised in Tables 3 - 5 below.

In Mangalia, the southernmost station, the overall bacterial contamination (TNG) was the highest in November and December, while total coliforms (TC) recorded the peak value in August. Fecal coliforms (FC), however, reached higher values in July, explainable by the presence of tourists in the area during the summer season. Yet, regulated values for public health (<300 germs/100 g) were not exceeded. (Table 3, Figure 5).

Table 3. Values of microbial contamination of *M. galloprovincialis* samples in Mangalia: total number of germs (TNG), total coliforms (TC), fecal coliforms (FC) and *E. coli*.

Station	MG 1				MG 2			
Values (number of germs/100 g of mussel flesh and intravalvular liquid)								
2019	TNG	TC	FC	*E. coli*	TNG	TC	FC	*E. coli*
July	5000	490	230	**50**	400	5400	220	0
Aug.	1000	3500	20	0	500	230	0	0
Sept.	6000	490	170	0	6000	490	110	**50**
Oct.	2000	280	0	0	1000	79	0	0
Nov.	10000	220	20	0	10000	220	0	0
Dec.	10000	330	20	0	10000	50	0	0
*Limits**			*300*	*230*			*300*	*230*

*as per Regulation (EC) No. 854/2004

Concerning *E. coli* contamination, of all samples collected from this station, only in two the presence of this indicator bacteria was confirmed, in July and September, again most likely correlated with the presence of tourists (Table 3, Figure 5). Similarly to fecal coliforms, the recorded values did not exceed the regulated limit of 230 germs/100 g of mussel flesh and intravalvular liquid, which rates Mangalia as a Class A area for shellfish culture/harvesting.

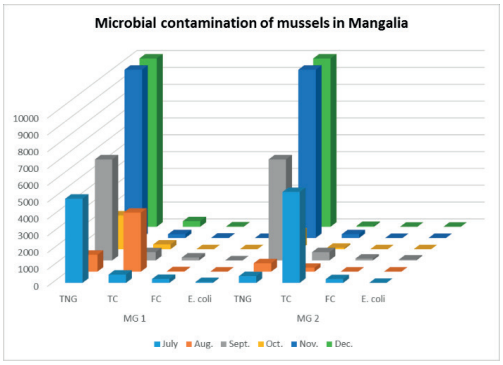

Figure 5. Microbial contamination of *M. galloprovincialis* samples in Mangalia

In the central station, Constanta, overall bacterial contamination (TNG) was higher compared to Mangalia in September, yet total coliforms (TC) and especially fecal coliforms (FC) recorded lower values (Table 4, Figure 6).

Table 4. Values of microbial contamination of *M. galloprovincialis* samples in Constanța: total number of germs (TNG), total coliforms (TC), fecal coliforms (FC) and *E. coli*

Station	CT 1				CT 2			
Values (number of germs/100 g of mussel flesh and intravalvular liquid)								
2019	TNG	TC	FC	*E. coli*	TNG	TC	FC	*E. coli*
July	13000	4300	130	**20**	10000	2300	0	0
Aug.	40000	1700	50	0	60000	1100	50	0
Sept.	100000	3300	20	0	400000	490	170	0
Oct.	400	130	50	0	1000	240	20	0
Nov.	40000	80	0	0	25000	220	0	0
Dec.	100000	230	0	0	150000	490	50	0
*Limits**			*300*	*230*			*300*	*230*

*as per Regulation (EC) No. 854/2004

With reference to *E. coli* contamination, this station recorded the lowest degree of contamination, with only one sample with 20 germs/100 g of mussel flesh and intravalvular liquid, significantly below the allowed limit for Class A (Table 4, Figure 6).

These results, corroborated with the shoreline assessment and general topography of the site, indicated that the Constanta area is the most suitable for establishing and operating a

shellfish farm, as well as for collecting wild mussels from the environment. The southern dike of the Constanta port offers an appropriate habitat for the fixation and growth of mussels, on the hand, and, on the other hand, it provides shelter against currents and storms for any potential farming installation set-up in this location.

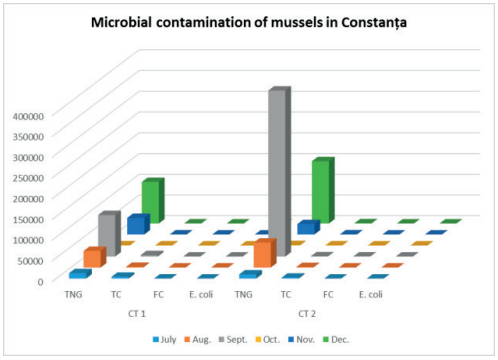

Figure 6. Microbial contamination of *M. galloprovincialis* samples in Constanța

In the Năvodari station, the highest overall microbial contamination (TNG, TC and FC) values of all stations were recorded in July, August and September, most likely due to the proximity to the Mamaia resort, a very busy holiday destination during summer (Table 5, Figure 7).

Table 5. Values of microbial contamination of *M. galloprovincialis* samples in Năvodari: total number of germs (TNG), total coliforms (TC), fecal coliforms (FC) and *E. coli*

Station	NV 1				NV 2			
Values (number of germs/100 g of mussel flesh and intravalvular liquid)								
2019	TNG	TC	FC	*E. coli*	TNG	TC	FC	*E. coli*
July	100000	400	70	0	200000	4800	220	**50**
Aug.	150000	5400	230	**20**	100000	540	17	0
Sept.	50000	330	170	0	100000	3300	210	**20**
Oct.	40000	50	0	0	50000	240	0	0
Nov.	30000	230	0	0	25000	220	0	0
Dec.	50000	230	0	0	50000	240	230	**50**
*Limits**			*300*	*230*			*300*	*230*

*as per Regulation (EC) No. 854/2004

Concerning *E. coli* contamination in Năvodari, this station recorded the highest degree of contamination, with four contaminated samples, in July, August, September and December (Table 5, Figure 7). Similarily to the other two stations, the values registered were low, below the limit of 230 germs/100 g of mussel flesh and intravalvular liquid, which rates Năvodari as well as a Class A area for shellfish culture/harvesting.

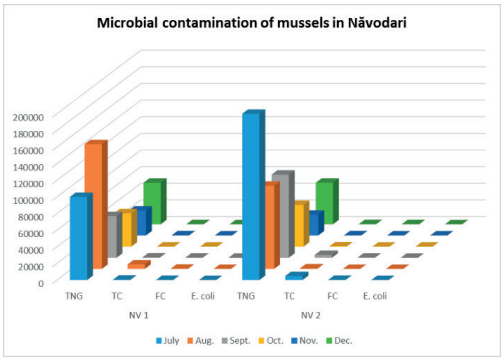

Figure 7. Microbial contamination of *M. galloprovincialis* samples in Năvodari

The results of this zoo-sanitary survey showed that all three selected sampling locations along the Romanian Black Sea coast (Mangalia, Constanța and Năvodari) are suitable for shellfish culture and/or harvesting, as the values recorded by bacterial contaminants do not pose a threat for public health and the shellfish could be safely consumed without any further purification being required.

CONCLUSIONS

The aim of this research was to assess the microbiological contamination of mussel samples collected from three selected areas, taking into account the environmental constraints of the Romanian coast (no sheltered areas, currents, strong winter winds), in order to evaluate the zoo-sanitary and food safety suitability of the most indicated areas for shellfish culture.

The overall bacterial contamination (TNG, TC and FC) in all three sampling stations was low, with values not exceeding the allowed threshold (for TC).

With reference to *E. coli* contamination, the results of this research indicate very low values in all three sampling stations. Consequently, according the criteria for the classification of bivalve mollusk harvesting/culture areas, the mussels collected from these locations can be included in Class A, thus not requiring post-harvest treatment to reduce microbiological contamination (Table 1).

Of all investigated stations during this 6 month zoo-sanitary survey, the Constanta area seems to be the most suitable for establishing and operating a shellfish farm, as well as for collecting wild mussels from the environment.
However, the trial classification itself of a certain production/culture area is not sufficient to guarantee safe human consumption. As such, the first step in an official control programme is establishing a sampling programme of bivalve mollusks in the production area (6 months). Subsequently, classified relaying and production areas must be periodically monitored to check the microbiological quality of live bivalve mollusks in relation to the production and relaying areas (weekly samples).
As far as the sampling requirements are concerned, sampling plans must be drawn-up providing for such checks to take place at regular intervals or on a case-by-case basis if harvesting periods are irregular. The geographical distribution of the representative sampling points and the sampling frequency must ensure that the results of the analysis are as representative as possible for the area considered.
The trial analyses performed on mussel samples collected from the Mangalia, Constanța and Năvodari areas were performed in NIMRD's laboratory using the reference technique, yet, in order to comply with EU regulations, the Competent Authority (namely the Romanian Sanitary-Veterinary and Food Safety National Authority) shall designate accredited laboratories for the EN/ISO 16649-3 standard that may carry out the analyses of samples taken during official controls.
It must be underlined that classification is not permanent and, once regular monitoring indicates non-compliance with the set-parameters, classification shall be suspended and the entire process must be re-run, in order to allow safe marketing on the local and European market.

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REFERENCES

Anon (2017). Microbiological monitoring of bivalve mollusc harvesting areas - Guide to good practice. Technical Application, Retrieved December 27, 2019, from www.eurlcefas.org.

European Community (2004). Regulation (EC) No. 854/2004 of the European Parliament and of the Council of 29 April 2004 Laying Down Specific Rules for the Organisation of Official Controls on Products of Animal Origin Intended for Human Consumption. *Off J Eur Union L*. 226, 83-127. Retrieved December 27, 2019, from https://eur-lex.europa.eu/.

EN ISO 16649-3 (2015). Microbiology of the Food Chain - Horizontal Method for the Enumeration of Beta-Glucuronidase-Positive *Escherichia coli* - Part 3: Detection and Most Probable Number Technique Using 5-Bromo-4-chloro-3-indolyl-ß-D-glucuronide. International Organization for Standardization, Geneva. Retrieved December 27, 2019, from https://www.iso.org/standard/56824.html.

Nenciu, M.I., Niţă, V., Ţoţoiu, A., Hamza H. (2020). Framework for setting-up a classification and monitoring program for shellfish at the Romanian Black Sea coast. *Journal of Environmental Protection and Ecology*, 21(1) (in press).

Nicolae, C.G., Popescu, A., Nenciu, M.I., Costache, M. (2018). EU regulations for organic aquaculture - a key for producing organic food. *Scientific Papers. Series D. Animal Science*, LXI (1), 333-336.

Niță, V., Nenciu, M.I. (2017). Using the recirculating technology in a pilot-system for mariculture at the Romanian Black Sea coast. *Journal of Environmental Protection and Ecology,* 18(1), 255-263.

Niță, V., Nenciu, M.I., Nicolae, C.G. (2018a). Experimental rearing of the Golden gray mullet *Liza aurata* (Risso, 1810) in a recirculating system at the Black Sea. *Agriculture for Life, Life for Agriculture Conference Proceedings*, 1(1), 149-154. DOI: 10.2478/alife-2018-0022.

Niță, V., Nenciu, M.I., Raykov, V., Nicolae, C.G. (2018b). First attempt of rearing the Siberian sturgeon (*Acipenser baerii* Brandt, 1869) in Black Sea water. *AgroLife Scientific Journal*, 7(1), 97-102.

Niță, V., Theodorou, J., Nicolaev, S., Maximov, V., Nenciu, M.I. (2018c). Capacity building and expert training in the frame of the Constanta Shellfish Aquaculture Demonstrative Center. *Cercetări Marine/Recherches Marines*, 48, 92-99.

Niță, V., Theodorou, J., Nicolaev, S., Nenciu, M.-I. (2019). Advancing shellfish aquaculture as a sustainable food procurement option in emerging Black Sea riparian countries: Romania Country Report. *Scientific Papers. Series D. Animal Science*, LXII (2), 364-370.

Zaharia, T., Niță, V., Nenciu, M.I. (2017). *Background of Romanian marine aquaculture*. Bucharest, RO: CD Press Publishing House (in Romanian).