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10. Presence of faecal indicator organisms (FIOs) in shellfish waters and mussels *Mytilus galloprovincialis* (Lam.) from the Romanian Black Sea coast

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ABSTRACT. The mussel *Mytilus galloprovincialis* (Lam.) is one of the most popular species with high commercial value and important issue of marine aquaculture in the Black Sea. In Romania, marine waters and shellfish are routinely analyzed for faecal indicator organisms (FIOs) of possible contamination within the national monitoring programme regime required under the EC Shellfish Water Directive (2006/113/EC). The present study focused on evaluating of the present microbiological quality of the Romanian marine mussels and shellfish waters. The microbiological quality (faecal coliforms) was examined in mussel's tissue and seawater by using the current European standard procedures for examination of shellfish and seawaters. The relationship between levels of faecal coliforms (FC) in mussel versus seawater was analysed using a data set of FC results quantified in 110 samples of wild mussel (*M. galloprovincialis*) and water collected from 4 sampling sites within the Romanian Black Sea coastal areas approved for harvesting of shellfish, during the period 2009 - 2013. The levels of faecal indicator organisms were generally higher in mussels than those for Black Sea waters, with FC values ranging from less than 20 MPN/ 100g to 490 MPN/100g mussel tissues. The concentration of FC in mussel's tissue was reduced at low salinity (< 9 psu) and temperature in overlying seawater and much higher at >10 psu and temperatures rising up to 27°C, indicating that salinity and temperature variation in the Black Sea environment can affect the accumulation and survival of enteric bacteria in mussel *Mytilus galloprovincialis*.

Key words: faecal coliforms, environmental factors, *Mytilus galloprovincialis*, coastal water, Black Sea

INTRODUCTION

Microbial contamination in marine shellfish environment is a worldwide phenomenon. Faecal pathogens (i.e. bacteria and viruses) are commonly introduced in the near shore marine shellfish waters from sewage outfalls, nonpoint sources pollution, and river discharges ([***4***]; [***8***]; [***23***]). During filter-feeding, bivalve shellfish (e.g. oysters, clams, mussels) accumulate enteric microbes from water column of the areas where they grow which can pose problems for human and ecosystem health ([***1***]; [***6***]; [***15***]; [***16***]). Thus, an environment free from microbial contamination is required to protect shellfish populations and to assure high quality of shellfish for human consumption ([***10***]; [***13***]; [***19***]).

The faecal indicator organisms (FIOs) are worldwide used as fundamental monitoring tools for assessing the potential presence of pathogenic microorganisms in marine waters and biota. The most commonly referred FIOs are faecal coliforms (FC) and *Escherichia coli* ([***17***]; [***22***]). Based on the EC Shellfish Water Directive (2006/113/EC), enumeration of faecal coliforms in shellfish flesh and waters is the key element in sanitary quality control of live bivalve shellfish and monitoring of shellfish harvesting area ([***31***]).

Although the cultivation of marine bivalve shellfish has no longer tradition in Romania, it becomes more and more popular during the last two decades. Currently, four areas approved as suitable for harvest of shellfish exist along the Romanian Black Sea coast. Following the full implementation of the European Shellfish Water Directive in 2002 ([***28***]), the microbiological quality of these aquatic habitats of shellfish are routinely assessed yearly within the national monitoring programme of the Black Sea marine ecosystem.

The present study focused on evaluating the microbiological quality of the Romanian Black Sea shellfish waters and wild Mediterranean mussels (*Mytilus galloprovincialis*) over a five year period (2009 - 2013). The study also aimed to assess whether differences between shellfish waters and mussels *M. galloprovincialis* occur because of variation of temperature, salinity and FC level in surrounding Black Sea waters, and thus to demonstrate the role of these environmental factors in the controlling the dynamics of FC accumulation by this bivalve shellfish. Numerous studies have investigated comparative bioaccumulation of faecal indicator organisms and pathogens in mussels and have shown the regulative effect of different ecological factors on this physiological process ([***7***]; [***9***]; [***11***]; [***12***]; [***14***]; [***20***]; [***25***]). However, there is general lack of data related to the effect of environmental parameters on the FIOs accumulation by wild *M. galloprovincialis* of the Black Sea, particularly in the Romanian sector. This paper thus not only provides information on the current status of microbial quality of Mediterranean mussel and shellfish water of the Black Sea but also brings effective data to fill this current information gap.

MATERIALS AND METHODS

Study sites and sample collection

Four stations located along the Romanian coastline from the northern to the southern Black Sea sector were selected for this study (**Fig. 1**). Each sampling site belongs to only one of the four Romanian Black Sea coastal areas approved as suitable for harvesting of shellfish. Stations Mila 9 (44°99'N, 29°73'E) and Portita (44°66'N, 29°29'E) were located inside of the northern authorized shellfish growing areas: zone 1 covering a total area of 142 Mm^2 in coastal region neighbouring to the Danube fresh river outflow, and respectively zone 2 with a total surface of 215 Mm^2 near the Lake Sinoe and Razelm lagoon complex. The others two sampling stations, namely Mamaia Bay (44°23'N, 28°70'E) and Costinesti (43°93'N, 28°66'E) were located within the zone 3 and zone 4 approved as suitable for growing of shellfish in the southern Romanian Black Sea sector, under the influence of both point and non-point sewage discharges and other sources of anthropogenic pollution such us tourism, industry, and harbours ([***26***]; [***28***]; [***33***]).

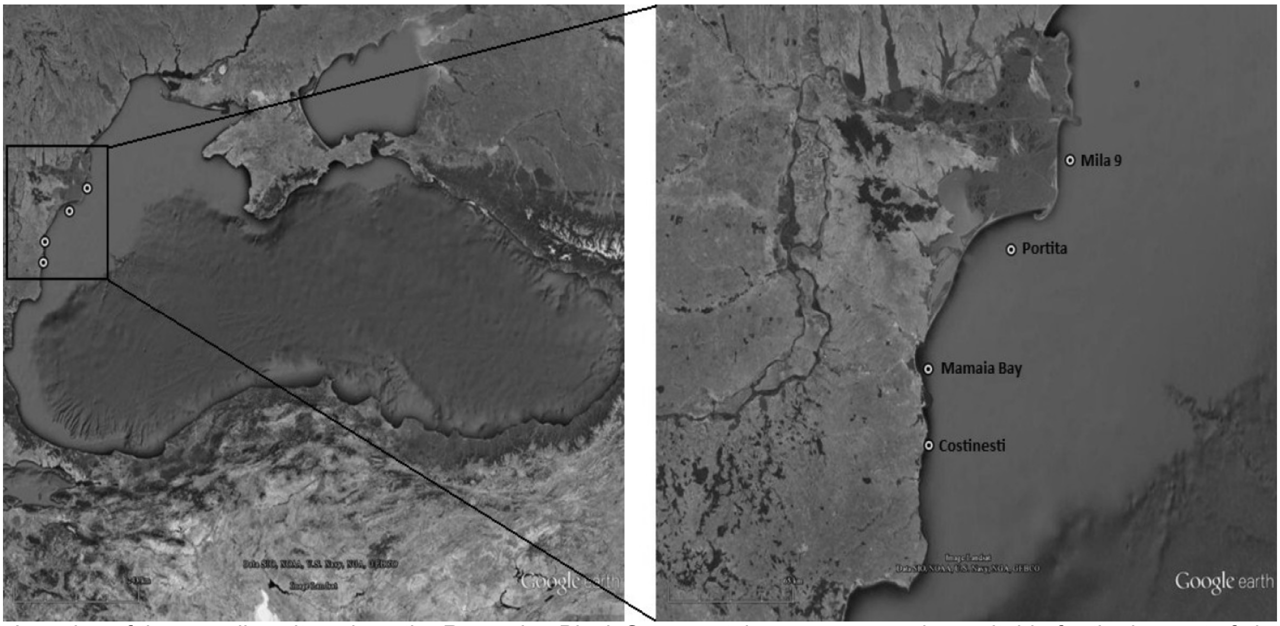

Fig.1. Location of the sampling sites along the Romanian Black Sea coastal zones approved as suitable for the harvest of shellfish.

Wild mussels (*Mytilus galloprovincialis*) and surrounding seawaters were collected seasonally from March to September from the selected sites onboard the R/V "Steaua de Mare" during the National Coastal Monitoring Program Expeditions, between 2009 and 2013. Water samples were collected in sterile bottles both from surface (approximately 0.5m depth) and bottom overlying layers, following the standard protocol for aseptic water sampling techniques ([***17***]; [***26***]).Live wilds mussels were collected off of the bottom on natural mussel beds by means of a bivalve dredge. Depth ranges at the four collection sites were 17 - 20 m. All samples were stored at 4°C and on ice and processed within 8 - 12 hours after collection.

Environmental factors

Seawater temperature and salinity from each investigated stations were measured using a multiparameter analyser (Consort C6030T, Belgium) and an YSI CastAway-CTD.

Detection of faecal indicator organisms (FIOs) in mussels and seawaters

The microbiological quality was determined in wild mussel *Mytilus galloprovincialis* and surrounding waters by using the current European standard procedures for examination of shellfish and seawaters (2006/113/EC and 2006/7/EC). Thus, detection and enumeration of faecal coliforms (FC) in mussels and seawater samples and was performed using the 5-tube 3-dilution MPN method employing liquid culture medium ([***17***]; [***26***]).The results were expressed as most probable number per 100 ml (MPN/100 ml) of seawater and most probable number per 100 g (MPN/100 g) shellfish tissue and were scored using the software tool calculating the Most Probable Number (version 3/2013-01-13).

Statistical analysis

A single factor, one-way ANOVA was firstly used to statistically test the differences in faecal coliform (FC) levels in wild mussels, environmental parameters, and faecal coliform (FC) levels in surface seawaters across the fours sampling sites. The results of the faecal coliform (FC) counts were processed by $\log_{10}$ transformation to appropriately normalize the data for ANOVA. Statistical analyses were performed using the statistical software Tanagra (version 1.4.36) with an alpha level of significance of 0.05 ([**21**]).

Since the ANOVA analysis showed no significant differences between sampling sites (**Table 1**), Tanagra version 1.4.36 was further used to create a multiple regression model using its regression tool on the pooled data across the sites. We also calculated standardized regression coefficients and semi-partial (part) correlations to quantify the relative importance of individual parameters on the variation of FC levels in the wild mussels. Variables included in this study that were thought to influence FC concentrations in mussel tissues include salinity in overlaying waters, temperature in overlying waters, FC levels in shellfish surface waters, salinity in shellfish surface waters, and temperature in shellfish surface waters. Only those sampling events with complete information on all considered environmental variables were included in the multiple regression analysis.

Table 1.

Statistical test differences between the four sampling sites (Mila 9, Portita, Mamaia Bay and Costinesti) between years of 2009 – 2013 for each considered variables

Variable	F-value	P-value	F-critical value
Temperature in shellfish surface water	0.4660	0.7078 (P > 0.05)	2.8663
Temperature in bottom overlying water	0.5188	0.6720 (P > 0.05)	2.8663
Salinity in shellfish surface water	1.9469	0.1395 (P > 0.05)	2.8663
Salinity in bottom overlaying water	0.2141	0.8859 (P > 0.05)	2.8663
FC level in shellfish surface water	0.3521	0.7879 (P > 0.05)	2.8663
FC level in wild mussel's tissue	0.4397	0.7260 (P > 0.05)	2.8663

RESULTS AND DISCUSSION

Environmental factors

The results concerning the mean values of investigated abiotic factors are shown in **Table 2.** Overall, the surface seawater temperature registered across all four sites ranged from 3.1°C (March) to 27.1°C (September), whereas the range of temperature in bottom overlaying waters was 2.7°C (March) - 24.8°C (September). Results of ANOVA analysis showed no significant site differences (P > 0.05) in the temperature either in surface and bottom waters from the four sampling sites (Mila 9, Portita, Mamaia Bay and Costinesti; **Table 1**). Similar, a no significant difference was found between temperature of surface and bottom waters at these sites (F = 2.526, P = 0.115).

Table 2.

Environmental factors of Black Sea shellfish waters at the sampling sites (2009 - 2013)

		Mila 9 (shellfish growing zone 1)	Portita (shellfish growing zone 2)	Mamaia Bay (shellfish growing zone 3)	Costinesti (shellfish growing zone 4)
Location (Latitude/Longitude)		44°99'N, 29°73'E	44°66'N, 29°29'E	44°23'N, 28°70'E	43°93'N, 28°66'E
Bottom depth (m)		17 - 20	17 - 20	17 - 20	17 - 20
Temperature (°C) surface waters	2009	23.20 ± 1.10	24.6 ± 2.2	14.46 ± 0.81	15.25 ± 1.25
	2010	14.00 ± 8.00	14.80 ± 8.70	13.65 ± 1.66	13.47 ± 1.34
	2011	17.10 ± 3.20	18.55 ± 1.25	15.45 ± 1.07	15.00 ± 0.99
	2012	11.44 ± 7.99	12.24 ± 9.60	14.67 ± 1.41	15.14 ± 1.93
	2013	22.29 ± 1.71	22.39 ± 1.41	12.39 ± 3.61	15.10 ± 1.99
Temperature (°C) overlying waters	2009	21.10 ± 0.80	23.25 ± 0.95	20.8 ± 3.20	20.52 ± 2.48
	2010	13.15 ± 7.95	13.30 ± 9.20	19.54 ± 2.56	19.63 ± 3.11
	2011	15.15 ± 2.34	13.92 ± 0.77	16.63 ± 3.23	15.74 ± 4.35
	2012	9.74 ± 6.55	10.44 ± 7.75	15.43 ± 7.28	14.16 ± 5.93
	2013	20.36 ± 2.49	17.68 ± 0.23	20.83 ± 3.22	21.51 ± 3.19
Salinity (psu) surface waters	2009	9.35 ± 0.04	11.23 ± 1.42	21.96 ± 3.84	22.50 ± 1.99
	2010	12.27 ± 0.56	16.58 ± 0.21	22.93 ± 2.46	22.88 ± 2.73
	2011	10.48 ± 2.54	12.90 ± 4.21	20.15 ± 3.55	18.35 ± 3.95
	2012	10.30 ± 0.90	12.48 ± 3.08	16.87 ± 7.20	15.82 ± 7.12
	2013	9.90 ± 3.66	9.21 ± 1.76	22.374 ± 3.21	22.4 ± 2.66
Salinity (psu) overlying waters	2009	12.22 ± 2.91	15.20 ± 1.05	16.53 ± 3.37	16.81 ± 2.29
	2010	14.43 ± 0.63	16.81 ± 0.68	11.40 ± 2.66	11.72 ± 2.89
	2011	13.45 ± 2.75	13.99 ± 3.32	14.55 ± 1.25	14.08 ± 1.05
	2012	13.93 ± 3.81	14.06 ± 3.22	14.28 ± 0.87	15.30 ± 1.62
	2013	11.86 ± 5.18	12.79 ± 4.82	10.44 ± 1.79	11.34 ± 2.65

NOTE. Temperature and salinities values are seasonal (March - September) means ± SEM

The range of shellfish seawater salinity across all sites was 6.24 psu (May) - 19.9 psu (September) in surface layers and 6.68 psu (May) – 18.07psu (September) in bottom overlaying layers. The single factor ANOVA showed no significant site differences in the temperature of surface and overlying shellfish waters (**Table 1**). However, a significant difference was found between salinity of surface and mussel beds overlying waters across all these sites (F = 6.193, P = 0.014), reflecting strong haline stratification of the seawaters at the sampling sites.

Coliform levels in shellfish seawater

The faecal coliform (FC) counts in surface Black Sea water at the four sites located within the Romanian authorized shellfish growing zones from July 2009 to August 2013 are shown in **Fig. 2**.

Overall, faecal coliforms were detected in all shellfish water samples with the levels ranged from 1.2 MPN/100 ml to 460 MPN/100 ml, and did not exceed the national and European (2006/7/EC) standards for bathing, coastal waters and transitional waters, at any of the four sample sites, during the investigated period ([***29***]; [***30***]; [***32***]). The level of FC higher than 10 MPN/100 ml was detected in 69% of the seawater samples collected from all four sites and followed a seasonal trend, with higher counts in warmer months (Fig. 2). Results of ANOVA analysis showed no significant site differences in the mean log10 density of FC in shellfish waters from the all investigates sites (P > 0.05; **Table 1**).

The mean MPN counts of faecal coliforms in the surface shellfish waters from sampling sites Mila 9, Portita, Mamaia Bay and Costinesti were 0.57×10^2, 0.92×10^2, 0.72×10^2 and 0.91×10^2 MPN/100 ml, respectively. At the northern sites Mila 9 and Portita, the highest level of FC was observed in September 2010 (2.18×10^2 MPN/ 100 ml) and August 2013 (3.7×10^2 MPN/ 100 ml), while the highest counts for southern stations Mamaia Bay and Costinesti were observed in August and September 2010 with 2.48×10^2 and 4.6×10^2 MPN/ 100 ml, respectively. Our data are in agreement with the results of previous studies which usually reported high level of faecal coliforms in surface seawater at similar temperatures during the warmer months ([***3***]; [***26***]; [***27***]).

Coliform levels in mussel

Faecal coliforms (FC) were detected in wild mussel *Mytilus galloprovincialis* all over the study period from July 2009 to August 2013 (Fig. 2). The MPN of FC in wild mussels ranged from 1.2 MPN/100 g to 490 MPN/100 g. The level of FC in mussels exceeding the European standards of ≤ 300 MPN/100 g (2006/113/EC) was detected in only 16.7% of the mussels samples collected from all four Romanian shellfish growing coastal zones, during the investigated period. According with ANOVA results, the variations in FC in the mussels were no significant (P > 0.05) between the four investigated sites (**Table 1**).

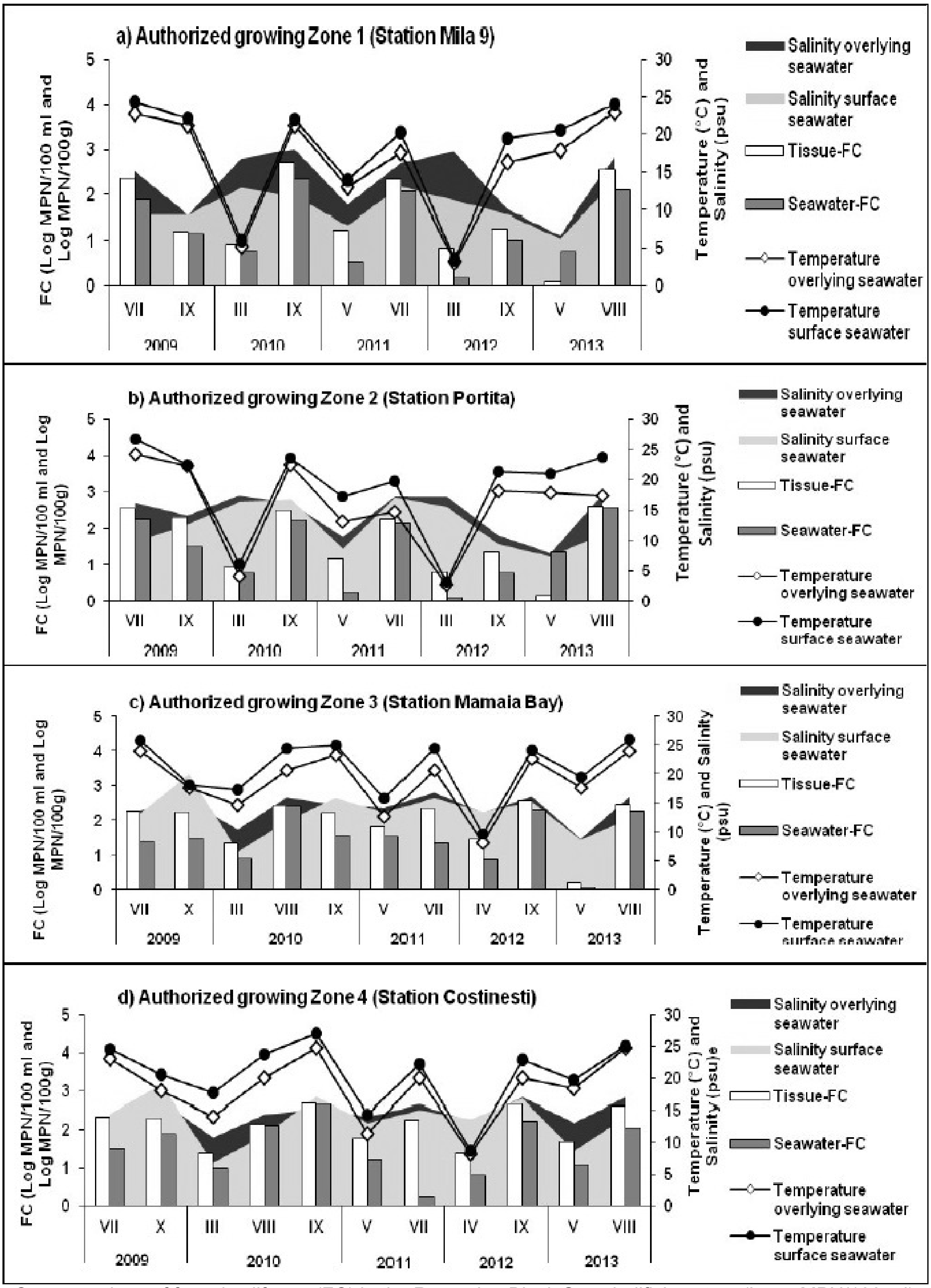

Fig. 2. Concentrations of faecal coliforms (FC) in the Romanian Black Sea shellfish waters (Log_{10} MPN/100 ml) and *M. galloprovincialis* tissue (Log_{10} MPN/100g) assessed over the entire 5-year sampling period with seasonal trends in salinity and temperature.

Note. (a) station 1 - Mila 9; (b) station 2 - Portita; (c) station 3 - Mamaia Bay; (d) station 4 - Costinesti.

The mean MPN counts of faecal coliforms in mussels collected from sampling sites Mila 9, Portita, Mamaia Bay and Costinesti were 1.32×10^2, 1.47×10^2, 1.61×10^2 and 2.02×10^2 MPN/100 g, respectively. The highest log_{10} MPN of FC in the mussel samples were registered at all sites in summer and the lowest log_{10} FC counts in spring (**Fig. 2**). The concentration of FC in mussels was reduced (< 2 MPN/100 g) at low salinity (< 9 psu) and temperature (<10°C) in seawater and much higher (up to 490 MPN/100 g) at salinity >10 psu and temperatures rising up to 27°C.

Our findings are in agreement with that of several previous researchers who usually reported higher levels of FC in mussels during the warmer months and undetectable or lower levels of this bacterium during the colder months ([*2*]; [*5*]; [*7*]; [*10*]). However, no significant seasonal variation (P > 0.05) in FC in mussel samples could be detected in either site, suggesting the Romanian littoral zone is a highly variable environment that significant affects the filter-feeding and enteric bacteria accumulation activity of mussels.

Table 3.
Results of multiple regression analysis of log_{10} MPN faecal coliforms levels in tissues of wild mussel versus a range of environmental factors tested in the four Romanian authorized shellfish growing areas

Independent variables	Regression coefficient	Standardized regression coefficient	P-value	Squared semipartial correlation coefficient
Temperature in surface seawater	0.0362	0.2137	0.041	5.9%
Salinity in surface seawater	0.0201	0.2931	0.036	6.5 %
Temperature in overlying seawater	0.0253	0.2072	0.019	4.3%
Salinity in overlying seawater	0.1308	0.5226	< 0.0001	31.4%
Log FC counts in surface seawater	0.1794	0.3775	0.025	9.7%

Effects of environmental factors on coliform levels

Accumulation and survival of enteric bacteria in seawaters and shellfish is largely influenced by a combination of different environmental factors (citare). In this study, faecal coliforms levels in wild mussel samples were compared with temperature and salinity, as major environmental determinants, and fecal coliforms contents in shellfish surface seawaters. The results of multiple regression analysis of log_{10} FC counts in mussels versus the five factors (abiotic and biotic) tested in the sampling sites corresponding to each Romanian authorized shellfish growing coastal zone are shown in **Table 3**.

Overall, the multiple regression analysis of log_{10} FC counts in mussels versus environmental parameters indicated a significant positive association between FC counts in mussel samples and the other five parameters considered as predictors including temperature and salinity of both surface and bottom water layers, and respectively the log_{10} FC counts in surface seawater ($P < 0.05$; **Table 3**).

According with the semi-partial correlations, seawater salinity explained most of the variation in log10 FC densities that was unexplained by, or non-associated with the other four parameters. However, most of the variation in log_{10} FC densities in mussels was explained by salinity registered in the bottom overlying waters (31.4%) compared with the shellfish surface waters (6.5%). Squared semi-partial correlation also revealed that surface water temperature explained 5.9% of the variation in log_{10} FC densities in mussels and only 4.3% by temperature in bottom layers (**Table 3**). Our results might be in contrast to several previous studies that usually reported a higher proportion of variance in log_{10} FC counts in mussels attributable to water temperature than salinity ([***10***]; [***11***]; [***14***]). However, most bivalves are very sensitive to dilutions of seawater since the salinity affects the filter-feeding process ([***2***]; [***5***]; [***9***]; [***24***]). Despite of the great tolerance to environmental variability, *M. galloprovincialis* has a fairly low tolerance to environments of decreased salinity such us the brackish coastal waters of the Romanian Black Sea, strongly influenced by the freshwater input from Danube River. Thus, the range of seawater salinities encountered in the Romanian shellfish growing areas over the investigated period might explain the accumulation of FC in wild mussels *Mytilus galloprovincialis* greatly impacted by salinity.

Based on the multiple regression analysis and semi-partial correlation, log FC contents in shellfish surface seawaters was found to be positively associated with log_{10} FC counts in mussels (P < 0.; **Table 3**) and explained 9.7 % of the variation. Thus, in addition to salinity, the density of FC in shellfish 05surface water was the second important factor that also affected the FC concentration in wild mussel. We found that levels of faecal coliforms were generally higher in mussels than those for Black Sea shellfish waters and paralleled that of ambient surface seawater (**Fig. 2**). During the investigated period, the wild mussel *M. galloprovincialis* accumulated faecal coliforms to levels that averaged two times (SD = 1.45) greater than levels of FC present in their surface waters. Our findings are in agreement with that of different former studies who reported strong association between the levels of FC in mussel *M. galloprovin*cialis and overlaying waters based on the high efficiency of these filter-feeders in removing and concentrating of FC from the marine water column ([***10***]; [***12***]; [***18***]).

Even though the sum of all squared semi-partials did not reach 100%, the multiple regressions and semi-partial correlation analysis clearly indicated that data on all five variables are necessary to encompass variation in the total tissue FC level. Furthermore, the relatively moderate proportions of variance attributable to the key abiotic and biotic parameters observed in this study are typical of data obtained under environmental conditions, suggested that beside salinity and temperatures, FC level in wild mussel from the Romanian shellfish areas are influenced by various factors ([***2***]; [***3***]; [***11***]; [***23***]).

CONCLUSIONS

The overall evaluation of faecal contamination of the mussel (*M. galloprovincialis*) and the shellfish environment reveals the occurrence of low levels of FC during the 5 years of microbiological monitoring (2009 - 2013). FC counts from the shellfish seawaters were well below the European limits and the tissue levels of FC were below 300 MPN/100 g in over 75% of samples suggesting no serious contamination of the wild mussel beds in the Romanian Black Sea areas approved as suitable for the harvest of shellfish.

The levels of FC in wild mussels and surface shellfish seawaters were found to be dependent on the environmental conditions present. Our results highlight the importance of salinity as major factor affecting the abundance of FC in wild mussels (*M. galloprovincialis*) from the estuarine and brackish coastal Black Sea waters of the Romanian sector. However, this research only explains about 60% of the variability of FC concentrations within the wild mussels leaving many questions yet unanswered. Further detailed research is needed to fill this gap in knowledge, taking into account the multiple environmental factors acting together on FIOs accumulation and clearance from wild *Mytilus galloprovincialis* of north-western Black Sea shellfish areas.

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