Environmental Influence on *Rapana venosa* Shell Morphotypes and Phenotypes from the Romanian Black Sea Coast

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Rapana venosa (Valenciennes, 1846) is a muricid gastropod originating from the Sea of Japan and occurring in abundance into the Black Sea. The aim of the present paper is to report differences displayed by the shell of rapana in terms of aspect in tight connection with seawater pollution and marine benthic substrates. For this purpose, whole individuals were collected from three representative sites of the Romanian Black Sea Coast. Morphological and phenotypical characteristics were registered for each shell. Furthermore, a chemical analysis of shell composition was performed with an inductively coupled plasma spectrometer in order to identify composition differences among sampling areas.

Keywords: Rapana venosa, Rapana thomasiana, shell morphotypes, shell phenotypes

The veined rapana shell (*Rapana venosa*, Valenciennes, 1846) or *Rapana thomasiana* (Crosse, 1861) is a predatory gastropod native to the East-Asian Pacific seas [1-4] and firstly noticed in the Black Sea in the early 1940s by Drapkin, at Novorossiysky Bay. The most common vector of introduction for this species is considered ballast water, being facilitated by a long lasting planktonic phase of rapana larvae [5]. More likely, rapana was introduced into the Black Sea as an associated species with Japanese oyster seeds [6], being recognised worldwide as a feared predator of mussels of economic interest. Within a decade since the first report, this species colonised the entire Black Sea, reaching the Caucasian and Crimean coasts and the Sea of Azov, and subsequently the North-West Black Sea populating the coastlines of Romania, Bulgaria and Turkey [7-9]. The extensive invasion of *Rapana venosa* in the Black Sea was possible due to its ecological ability, being tolerant to a wide range of salinities [5] and water pollution [9, 10], as well as due to the sufficient pray availability.

The external shell ornamentation includes smooth spiral ribs that end in regular knobs at both shoulder and periphery of the body whorl. In addition, fine spiral ridges are crossed by low vertical riblets. Older specimens can be eroded, but the colour is variable from grey to orange-brown and atypically blonde, with darker brown dashes on the spiral ribs. The aperture and columella vary from deep orange to yellow or off-white [11-13]. Generically, variations in shell appearance have been related to environmental effects and to genetic inheritance [14, 15], yet few advances were made for the fully comprehension of rapana shells' polymorphism.

Local variation may occur in morphometry and phenotypic traits depending on living marine substrate, thus hard bottom individuals develop thicker shell than individuals found on sandy substrate, which exhibit greater shell length [16-18]. Savini and Occhipinti report that rapana populations originating from hard rock substrate have predominantly dark-coloured shells, whereas populations on adjacent sand exhibit a higher frequency of white or pale brown shell [17].

Suspended solids, nutrients, heavy metals, detergents, hydrocarbons and organic micro pollutants originating from municipal/industrial wastewater treatment plants and other major industrial operators as well as the Danube River represent important pollution sources for the Romanian coast of the Black Sea [19-24]. According to annual reports of the Romanian Ministry for Environment, Water and Forests, toxic metals levels in the Black Sea waters are under the maximum admitted values except for the region of confluence with the Danube Delta [25, 26], as well as in the harbour area from Constanta and Mangalia, an identical trend being noticed by the heavy metals analysis in water sediments.

Bioaccumulation of minerals, metals and heavy metals in marine might be another factor which influences the variations of the rapana shell appearance. Some gastropods, including *Rapana venosa*, are commonly used as marine and / or freshwater pollution indicators [27] due to their capacity of bioaccumulation, high abundance and their broad geographical range [28-32]. The research performed by Jitar et al. during 2011-2012 identified as well that the concentrations of heavy metals in marine sediments, seawater and soft tissues of various marine species, including Rapana venosa as well as in case of freshwater molluscs [33], differ among regions of the Romanian coast of the Black Sea [34]. While accumulation of metals was thus proven in sediments and living tissue of rapana, few information regarding their presence in shells and their subsequent impact were further assessed.

This paper is part of an extensive study focusing on rapana sampled from the Romanian Coast of the Black Sea. The aim of the present work is to report differences displayed by shells of *Rapana venosa* in terms of aspect in tight connection with seawater pollution and marine benthic substrates. Morphological and phenotypical characteristics were registered for each shell to assess if there are significant differences among sample sites. Furthermore, a chemical analysis of the shell composition was performed for each study group in order to determine shell mineral concentrations, which can influence the shell

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coloration. In order to quantify the correlation between metal content and shell colour a statistical analysis has been performed providing a variability index able to assess the chromophore transition metals with relevant contribution on the colour.

Experimental part

Whole *Rapana venosa* individuals were collected in February 2013 from three representative sites influenced by different pollution sources and by different originating marine substrates (table 1). Midia Cape and Vadu - Corbu were chosen as exemplars for the rapana individuals found on sandy bottoms. These collecting sites are situated at Northern sector of the Romanian Black Sea Coast, thus the potential pollution source might be the Danube River being in accordance with the trend of the Black Sea marine currents (fig. 1) [10, 35].

The third sample site is the wild beach of Limanu, located in Southern sector of the Romanian Black Sea coast, established as natural protected area. Rapana individuals collected from Limanu originate from the adjacent mixed rocky-sandy bottom.

As it has been described in the previous contribution [36, 37], all samples were firstly cleaned and the soft tissue of the mollusc was removed. Each rapana individual was analysed from morphological and phenotypic point of view using a coding system presented in table 2, for evaluating morphologic traits like shell knobs (irregularities of the shell, usually occurring on the body whorl in fig. 2.1-2), marginal teeth (fig. 2.3), shell integrity (fig. 2.12-13) and phenotypic characters like outer shell colour (fig. 2.4-6),

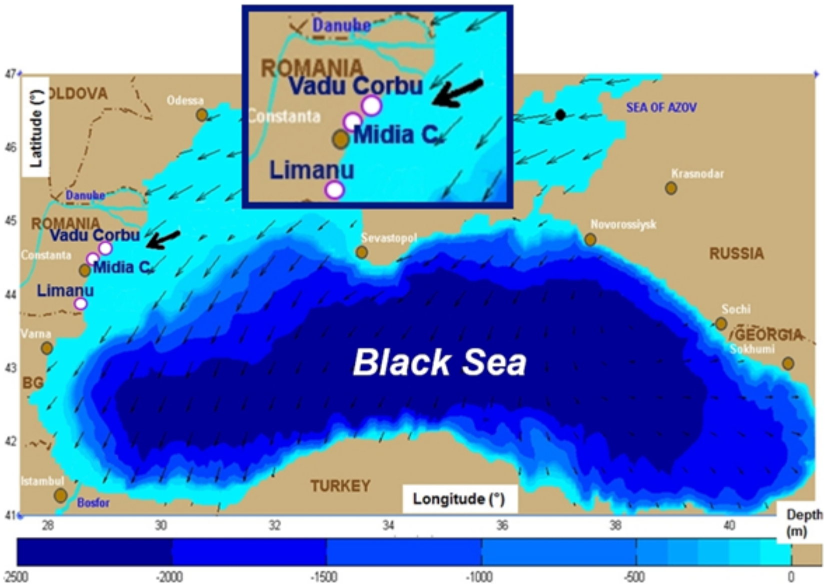

Fig. 1. *Rapana venosa* sampling sites: Vadu-Corbu and Midia Cape located in Northern sector of the Romanian Black Sea coast, which are under the influence of the Danube River in accordance with he trend of the Black Sea marine currents; Limanu, located in the Southern sector of the Romanian Black Sea coast, naturally protected area (adapted after Rusu [35])

Table 1
SAMPLING SITES CHARACTERISTICS

Sampling site	Pollution sources	Marine substrate
Vadu-Corbu	Danube River discharges	sandy
Midia Cape	Danube River, Rompetrol refinery	sandy
Limanu – 2 Mai	Mangalia municipal wastewater plant, harbour wastewater, tourist activities	rocky-sandy

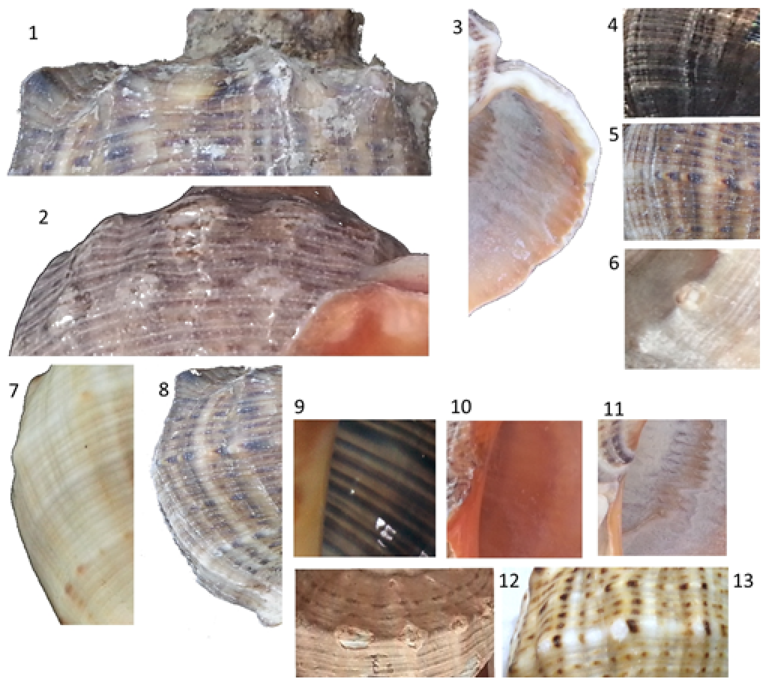

Fig. 2. Shell morphologic and phenotypic characteristics assessed by appearance coding: 1 - pronounced shell knobs; 2 - not pronounced shell knobs; 3 - not pronounced marginal teeth; 4 - dark outer shell colour; 5 – brown outer shell colour; 6 - light brown outer shell colour; 7 - scarcely evident shell stripes or veins; 8 - well evident shell stripes or veins; 9 - aperture colour, dark stripes on a whitish base; 10 - orange aperture colour; 11 – white aperture colour; 12 – eroded shell; 13 – whole shell.

Table 2
APPEARANCE CODING USED FOR EVALUATING MORPHOLOGIC AND PHENOTYPIC TRAITS OF RAPANA SHELL

Character	Code			
	0	1	2	3
Shell Integrity	entire	eroded	-	-
Shell Knobs	not pronounced	pronounced	-	-
Marginal Teeth	not pronounced	pronounced	-	-
Outer Shell Colour	dark	brown	light brown	-
Aperture Colour	orange	orange with dark stripes	dark stripes on a whitish base	white
Shell Stripes	well evident	scarcely evident	-	-

Table 3
FREQUENCY OF DETERMINED CODES FOR RAPANA SAMPLES COLLECTED FROM LIMANU, MIDIA CAPE AND VADU-CORBU WITH THE FOLLOWING MEANING: OUTER SHELL COLOR (0 = DARK, 1 = BROWN, 2 = LIGHT BROWN), SHELL STRIPES (0 = WELL EVIDENT, 1 = SCARCELY EVIDENT), APERTURE COLOUR (0 = ORANGE; 1 = ORANGE WITH DARK STRIPES; 2 = DARK STRIPES ON A WHITISH BASE; 3 = WHITE), SHELL KNOBS (0 = NOT PRONOUNCED; 1 = PRONOUNCED), MARGINAL TEETH (0 = NOT PRONOUNCED, 1 = PRONOUNCED), INTEGRITY (0 = ENTIRE, 1 = ERODED).

Code - Limanu	%	Code – Midia Cape	%	Code – Vadu-Corbu	%
200101	7.48	**201100**	8.82	**103100**	8.62
200001	7.48	**101100**	5.89	**002100**	6.25
201001	6.54	**001100**	5.89	**013100**	6.25
210001	6.54	**100110**	4.90	**100110**	4.55
210011	3.74	**200110**	3.92	**111100**	4.25
210101	3.74	**002100**	2.94	**001100**	3.16
101101	3.74	**012100**	2.94	**200100**	3.16
001001	3.74	**101110**	2.94	**101100**	2.75
100001	2.80	**110000**	2.94	**200100**	2.75
Other	***54.2***	***Other***	***58.82***	***Other***	***58.26***

shell stripes or veins (fig. 2.7-8), aperture colour (fig. 2.9-11).

The measurement results were processed with specific statistical methods in order to highlight useful information about rapana populations from the Romanian coast of the Black Sea. For mineral concentration analysis, dimensionless concentrations (C/C0) were calculated by dividing mineral measured concentration to the lowest registered value. Statistical analyses, including ANOVA for variance analysis and F-Test for distribution comparison, were performed using the software Excel Microsoft 2013.

The metallic elements were analysed by inductively coupled plasma optical emission spectrometry using Optima 5300 DV Perkin Elmer ICP-EOS Spectrometer with axial viewing plasma for metal analysis. For all analysed parameters standard methods have been used (ISO 11885:2007; EN 1484:1997), reference materials and certified reference materials.

Results and discussions

A number of 306 specimens have been randomly sampled from Midia Cape beach, 269 specimens from Vadu - Corbu beach, and 298 specimens from Limanu. *Rapana venosa* shells showed 180 different code combinations of phenotypical and morphological characteristics. For all the samples dominant codes were difficult to establish. Table 3 presents the most frequent shell code combinations. In similar proportions for each sample site, meaning in about 60% from cases, a code combination appears just once or twice for the entire collected rapana population. Therefore, rapana shells exhibit a great variability regarding its aspect.

The dominant codes for Limanu were 200101 and 200001 in amount of 7.48% for each one, meaning that in this case, *Rapana venosa* population is characterized by a light brown outer shell colour with both scarcely evident shell stripes or with evident shell stripes, with an orange aperture and presenting an eroded shell with not pronounced knobs (fig. 3.3). For Vadu - Corbu the dominant code 103100 is represented by rapana individuals with brown shell with evident stripes and a whitish aperture, less eroded shell and with scarcely pronounced knobs (fig. 3.2). For Midia Cape, 201100 and 101100 were the dominant codes that represent individuals with either brown or light brown shell colour with evident shell stripes, presenting an orange aperture with dark stripes and being less eroded with not pronounced knobs (fig. 3.1). Since dominant codes represent less than 10% from rapana population, shells characteristics were further separately examined.

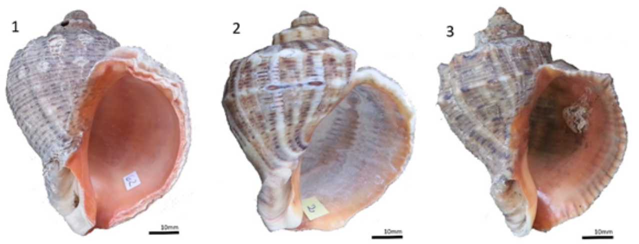

Fig. 3. *Rapana venosa* typical individuals from Midia Cape (1), Vadu-Corbu (2) and from Limanu (3)

The results presented in figure 4 highlight the observation that Limanu population have eroded shells compared with Vadu-Corbu and Midia Cape rapana populations. The differences may be due to the different originating marine benthic zones (rocky, respectively sandy bottoms). Shell knobs displayed in the histogram from figure 4 are less obvious on *Rapana venosa* samples collected from Limanu and pronounced for rapana populations from Midia Cape and Vadu-Corbu. This finding supports the previous

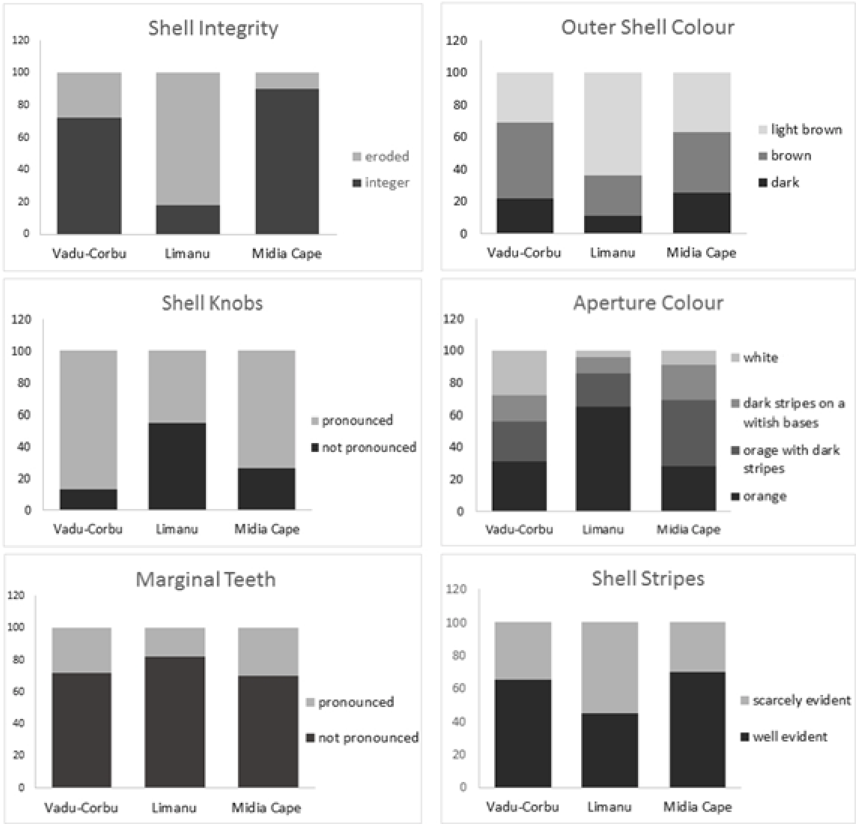

Fig. 4. Histograms of shell morphological characteristics (left): shell integrity, shell knobs and shell marginal teeth; histograms of shell phenotypical characteristics (right): shell outer colour, shell aperture colour and shell stripes for *Rapana venosa* sampled from Vadu – Corbu, Limanu, and Midia Cape, respectively

observation, whereby longer time underwater living on rocky substrate promotes their erosion degree [17]. In the present study, up to 55% of rock shells found at Limanu were eroded, whereas up to 75% of the sand shells from Midia Cape and 88% for those from Vadu-Corbu were complete. These results confirm the findings published by Savini and Occhipinti [38]. They observed that the presence of dams and sandy marine bottoms prevent shell erosion for rapana population sampled from Emilia Romagna regional coastline.

In contrast to shell knobs, in most examined rapana individuals from each sampling site, marginal teeth are less pronounced. This trait is encountered in similar extent for all sample groups, therefore sustaining the hypothesis that this characteristic is not influenced by environmental conditions, but are rather originating from the species genetics (fig. 4).

Determined standard deviation and variance data for phenotypic and morphologic characteristics of rapana shell (table 4) displayed greatest variance for aperture colour and outer shell colour for all samples. Great variability among sample groups (Midia Cape – Limanu – Vadu Corbu) was demonstrated by comparative analysis for determined variance of phenotypic and morphologic characteristic ($p<0.05$). Furthermore, by the pair-wise comparison for determined variance of phenotypic and morphologic characteristics depicted in table 4, differences between Vadu - Corbu and Midia Cape populations are small and statistically insignificant ($p>0.05$). Limanu samples differ statistically significant from the other sample groups, the greatest variability presented aperture colour and the outer shell colour ($p <0.05$).

Since great variability of phenotypic characteristics was noticed among collecting areas, the next purpose of this work was to evaluate the factors that may influence the appearance of rapana shells collected from the Romanian Black Sea Coast. Castellazzi et al. assume that the heterogeneity of shell characters could be the phenotypic expression of a higher genetic diversity, which can be explained by different events of introduction of larvae from different areas of the world [13]. Opposite to this theory, our hypothesis assumes that variations in the phenotypic expression appear due to various living conditions, mainly by bioaccumulation of minerals and metals into the living gastropod which may further influence shell calcification process. Shell mineralisation occurs at the extrapallial fluid, which is a jellified media supersaturated with respect to calcium carbonate relevant phases [39-41]. Biological and environmental factors such as food supply, water chemistry, salinity, pCO_2 and temperature can also affect the chemical and mineral composition of shells [42-44]. Our hypothesis is fortified by the findings of Chandler et al. [45] which found very high levels of genetic variation for *Rapana venosa* in its native range, while collections from all introduced populations (Black Sea, Mediterranean Sea, coasts of France and the Netherlands, in Chesapeake Bay, USA) showed a complete lack of genetic diversity by the investigation of two mitochondrial gene regions of *Rapana venosa*.

Table 5 collects results for metal concentrations for each sample site, completed in last column by a statistical analysis able to quantify the variability of toxic metal content with relevant contribution on shell colour. Indeed, out of the set of elements analysed, the highest standard deviations calculated with adimensional concentration have been found for chromium (5.59) and nickel (5.9), while for the rest of metals these values were less than 1 (except for aluminium, for which was 1.15). Moreover, it can be noticed the preference of *Rapana venosa* regarding shell mineral accumulation, as same elements were registered for all sample sites in the same descending concentration order as follows: Cr>Ni>Al>Zn>Mn.

This is a very interesting result, as out of the series of transitional metals found: Cr, Fe, Mn, Ni, Mo that might be responsible for the shell colour, only Cr and Ni may actually act as major chromophore ions able to confer specific colouring characteristics for the species sampled from different marine coast sites. Thus, while Fe and Mn have

Table 4

VARIANCE (V) DETERMINED FOR PHENOTYPIC AND MORPHOLOGIC CHARACTERISTICS OF RAPANA SAMPLES COLLECTED FROM LIMANU, MIDIA CAPE AND VADU-CORBU; COMPARATIVE ANALYSIS AMONG SAMPLE GROUPS (MIDIA CAPE – LIMANU – VADU CORBU) AND PAIR-WISE COMPARISON BETWEEN SAMPLE GROUPS FOR DETERMINED VARIANCE OF PHENOTYPIC AND MORPHOLOGIC CHARACTERISTICS, BY REJECTING NULL HYPOTHESIS, H_0, CHARACTERS ARE EQUAL ($p < \alpha=0,05$).

	Limanu	Midia Cape	Vadu - Corbu	Midia C. – Limanu – Vadu C.	Midia C. - Limanu	Vadu C. - Limanu	Midia C. – Vadu C.
	V	*V*	*V*	*p value*	*p value*	*p value*	*p value*
Shell Integrity	0.15	0.10	0.11	0.002	0.001	0.001	0.09
Shell Knobs	0.25	0.20	0.20	0.002	0.006	0.007	0.13
Marginal Teeth	0.15	0.21	0.21	0.005	0.048	0.046	0.086
Outer Shell Colour	0.48	0.61	0.59	0.003	0.026	0.030	0.984
Aperture Colour	0.72	0.85	1.07	0.006	0.013	0.016	0.07
Shell Stripes	0.25	0.21	0.24	0.005	0.012	0.012	0.25

Table 5

MINERAL CONCENTRATION ANALYSIS AND STANDARD DEVIATION (SD) FOR ADIMENSIONAL CONCENTRATIONS (C/C_0) OF DETERMINED MINERALS OF *RAPANA VENOSA* SHELLS COLLECTED FROM MIDIA CAPE, VADU-CORBU AND LIMANU

	Midia Cape	Vadu – Corbu	Limanu	Midia Cape	Vadu – Corbu	Limanu	SD
	[mg/kg]	[mg/kg]	[mg/kg]	C/C_0	C/C_0	C/C_0	C/C_0
Cr	0.4	0.45	0.04	10.00	11.25	1.00	5.59
Fe	10.00	10.00	8.00	1.25	1.25	1.00	0.14
Mn	3.33	3.55	2.00	1.67	1.78	1.00	0.42
Ni	1.88	2.00	0.20	9.40	10.00	1.00	5.03
Mo	1.33	1.44	1.01	1.32	1.43	1.00	0.22
Zn	1.01	1.41	0.69	1.47	2.04	1.00	0.52
Al	6.34	7.31	2.31	2.74	3.16	1.00	1.15
Ag	1.84	1.70	1.63	1.13	1.04	1.00	0.06
Ca	360,523	351,866	376,850	0.98	0.96	1.00	0.03
Mg	469	562	440	1.07	1.07	1.00	0.04

very low absorbance in visible domain, speciation of Cr and Ni ions can bring important contribution to colour tones: Cr^{6+} - orange, Cr^{3+} - green, Ni^{2+} - yellow, Ni^{3+} - green. Therefore, the major contribution on colour diversity will be supplied by Cr^{6+}, as the surface marine waters will favour the dominance of ions in the highest oxidative valence state. This is the origin of various orange nuances evidenced particularly on the aperture of rapana shells sampled at Limanu and Midia Cape sites. However, the dark stripes on orange aperture noticed at Midia Cape can be assessed to the supplementary contribution of the other chromophore metals found at this site as compared to Limanu. A different appearance is observed at Vadu - Corbu, where the aperture colour is rather whitish. In this case, even though the chromophore ions are in the highest content, the total contribution of the colourless ions, mainly Ca, Mg, and in a lower extent of Al, Zn, is maximum at Vadu - Corbu (> 900 mg/kg) as compared to Midia Cape and Limanu (approx. 800 mg/kg). Finally, one may conclude that the pollution with heavy metals may have a much more diversified influence on the aperture than on the outer shell colour. Therefore, slightly lower metals concentrations for Limanu samples, in particular Cr and Ni, as well as slightly higher concentrations of Ca justify their paler and lighter shell colour (light brown), as well as scarcely evident shell stripes. This advance differs from the observations made by Savini and Occhipinti for the Adriatic Sea, which have correlated shell colour with the nature of the marine substrate and concluded that populations found on hard rock substrates have predominantly dark-coloured shells, whereas populations on adjacent sand exhibit a higher frequency of white or pale brown shell [38].

The slight higher concentrations of toxic metals in shells originating from Vadu -Corbu compared with those from Midia Cape (Table 5), as well as corresponding appearance of these shells displaying a brown or dark shell colour with well evident veins seem to support the fact that the polluted water discharged in Danube has a great impact on rapana shell heavy metals bioaccumulation.

An accurate analogy with other rapana populations described in similar articles is rather difficult to achieve since these studies do not include a chemical analysis of rapana shell [1, 13, 46]. Considering these, the present research is an original approach of analysing morphological and phenotypical characteristics in corroboration with chemical analysis of *Rapana venosa* shells' minerals, revealing that bioaccumulation of some heavy metals (Ni, Cr) affects as well the gastropod's shell.

The chemical analysis of shells mineral composition represents a further step for the elucidation of general phenotypic characteristics of a specific area, which is under certain environmental influences, still cannot explain the particular differences found among individuals from the same collecting site. Further explanations in this regard can be the hereditary variation, the migration capacity of the species, but also the capacity of *Rapana venosa* for assimilating different minerals under constantly changing underwater conditions.

Conclusions

Morphologic traits like shell knobs and integrity are rather under the influence of benthic marine substrate. Rapana shells originating from a mixed rocky-sandy substrate appeared more eroded and presented less pronounced shell knobs than sandy individuals did. Moreover, exemplars collected from mixed sandy-rocky substrate exhibited paler shell colour, whereas individuals originating from a sandy substrates were dark coloured.

Rapana venosa found along the Romanian Black Sea coast is characterized by less pronounced marginal teeth, this feature being rather a genetic inborn characteristic.

The preference of *Rapana venosa* regarding shell mineral accumulation, is in descending concentration order as follows Cr>Ni>Al>Zn>Mn for all sample sites.

Differences among phenotypic traits and mineral composition of rapana shell among sampling areas were identified and are in accordance with industrial activities and uptake of Danube River pollution. Rapana population near to the Danube Delta displayed a brown or dark outer shell with well evident stripes and with an orange or orange with dark stripes aperture, in contrast to more distant rapana individuals which exhibited a light brown outer shell with scarcely evident shell stripes and an orange aperture. Thus, the greatest aspect variability was observed on the aperture colour and less on the outer shell for all studied groups. Cr, Ni represent the main chromophore ions, with major contribution on colour diversity supplied by Cr^{6+}, mainly responsible for orange tones, thus having a much more diversified influence on the aperture than on the outer shell colour.

Concluding, we can affirm that variations in rapana shell appearance are influenced by genetic factors, by marine benthic substrate and, respectively, by environmental conditions. Furthermore, chemical analysis of shells mineral composition represents a further step for the elucidation of general phenotypic characteristics, still cannot explain the particular differences found among individuals from the same collecting site. Deeper inquiry is still needed for better understanding of the role of environmental conditions and genetic mechanisms.

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