Chemistry Research Journal, **2017, 2(6):236-250**

Available online www.chemrj.org

Research Article

ISSN: 2455-8990
CODEN(USA): CRJHA5

Trace Elements Concentrations in Black Sea Mussel (*Mytilus galloprovincialis*) and Rapa Whelks (*Rapana venosa*) from Bulgarian Black Sea Coast and Evaluation of Possible Health Risks to Consumers

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Abstract Wild and farmed Black Sea mussel (*M. galloprovincialis)* and rapa whelks (*R. venosa*) were collected during 2016 from six sites on the northern coast of Bulgarian Black Sea. The mussels and rapa whelks soft tissue was analyzed for ten elements (Cd, Cr, Cu, total Hg, Ni, Zn, Pb, Mn, Fe). Concentrations of these metals, in mg/kg dry weight, ranged from 0.73-3.45 for As, 0.005-0.640 for Cd, 0.040-0.382 for Cr, 0.86-7.70 for Cu, 4.2-112.9 for Fe, 0-0.121 for total Hg, 0.260-3.190 for Mn, 0.023-0.642 for Ni, 0-0.332 for Pb and 7.5-38.2 for Zn. The concentration of these elements does not exceed the maximum residual levels prescribed by different local and international regulation for seafood. The estimated daily intake (EDI) for average level molluscs (ALM) and high level molluscs (HLM) consumers was found to be lower than the ORD guidelines for Cd, Cr, Cu, total Hg, Ni, Zn, Pb, Mn, Fe. Furthermore, the target hazard quotient (THQ) and hazard index (HI) was found to be less than 1 for ALM and HLM consumers. Target risk due to Pb, As and Ni exposure through consumption of mussels and rapa whelks may not have the probability of contracting cancer over a long lifetime in future. Therefore, there were no potential human health risks to the ALM and HLM consumers of the mussels.

Keywords *M. galloprovincialis*; *R.venosa;* trace element; health risks; Bulgaria; Black Sea

1. Introduction

The Black Sea is the world's largest natural anoxic water basin below 180 m in depth. It is a closed sea with a very high degree of isolation from the world's oceans, but it receives freshwater inputs from some of the largest rivers in Europe; the Danube, the Dniester, and the Dnieper [1]. For this reason, Black Sea is considered one of the most polluted seas in the world, and the increasing concentration of nutrients in recent years have led to a higher degree of eutrophication. The fishery yield has declined dramatically, and the tourism industry in the region of Bulgarian coast of Black Sea also suffers from serious pollution of the Black Sea. Therefore, numerous studies have been carried out on metal accumulation in different marine species in the parts of Black Sea [2, 3].

Molluscs, such as mussels and veined rapa whelk, are an important source of some essential elements, vitamins such as niacin and thiamine, and are a good source of protein for humans. Even though they are an excellent source of nutrients, they are well known to accumulate a wide range of metals in their soft tissues and frequently are used as an indicator of marine pollution. A mussel species which has proved to be an important tool for the biomonitoring of environmental pollution in coastal areas is certainly the mussel *Mytilus galloprovincialis* [4]. The reliability of the mussel *M. galloprovincialis* as biomonitors of pollutant metal contamination has been demonstrated by a number of researchers [4,, 6,7,8,9,10]. Considering that mussels (*M. Galloprovincialis)* and veined rapa whelks (*R. venosa)* are edible and marketed commercially, the presence of metals could limit the quantity of molluscs that humans should consume as excessive consumption of metal-contaminated molluscs could result in toxicity to humans [11]. Since metals are non-biodegradable and cannot be metabolized into harmless forms, the measurement of metal levels in the soft tissue of mollucsc has become increasingly significant.

Heavy metals can be classified as potentially toxic (arsenic, cadmium, lead, mercury, nickel, etc.), probably essential (vanadium, cobalt) and essential (copper, zinc, iron, manganese, selenium) [12,13]. Mussels and veined rapa whelks are good indicators for the long term monitoring of metal accumulation in the marine environment.

The aims of this study were to determine the levels of As, Cd, Cr, Cu, total Hg, Ni, Zn, Pb, Mn, Fe in the soft tissue of mussels *M. galloprovincialis* and veined rapa whelks *R. venosa* from the northern part of Bulgarian Black sea coast and to evaluate the public health risks associated with the consumption of these marine species. The fact that people who live in the coastal areas hand-harvest and consume mussels from the seashore, wild mussels were also taken into consideration in addition to farmed one. The impact of each trace metal on consumer health was considered through: 1.) Estimated weekly intake (EWI) for average and highly level mussel and rapa whelks consumers in relation to the respective provisional tolerable weekly intake (PTWI) established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA); 2.) The target hazard quotient (THQ) for average and high level mussels and rapa whelks consumers in order to evaluate possible alert regarding adverse health effects that may be caused by trace metals individually; 3.) The total hazard index (HI) for risk assessment of multiple metals contained in mussels and rapa whelks; 4.) The target risk coefficient (TR) associated with cancerogenic human health risk trough consumption of Black sea mussels and rapa whelks.

2. Materials and Methods

2.1. Sampling, sample preparation and storage

Mussels (wild and farmed) and rapa whelks (wild) were collected manually from four different locations along the northern coastline of Bulgarian Black sea (Figure 1). Sampling was performed during spring and fall of the year 2016. In order to obtain a representative sample at the each location more than 2 kg of mussels and 4 kg of rapa whelks of similar length were collected, placed in plastic bags with seawater collected at the corresponding location and transported to the laboratory. The molluscs were cleaned, rinsed with seawater and dissected fresh. The soft tissue of the samples was rinsed with Milli-Q water to remove any remaining sand and/or other particles, freeze-dried and homogenized using a mill. About 25–30 mussels and 20 rapa whelks from each sampling site were selected, pulverized and analyzed for the trace elements. The average water content in the soft tissues varied between 80.1 % and 86.3 % depending on the season.

2.2. Chemical analysis

Wet digestions were performed in triplicate by weighing approximately 1.0 g of the mollusks tissues with a mixture of 10 ml HNO_3 (65% Merck, Suprapur) in a microwave digestion system MARS 6 (CEM Corporation, USA). The digested mussel and rapa whelks samples were diluted to 25 ml with Milli-Q water and stored in polyethylene bottles. A blank digest was performed in the same way.

The concentrations of As, Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn in the samples were determined using ICP-OES Spectrometer (Optima 8000, Perkin Elmer, USA) with plasma gas flow -10L/min, auxiliary gas flow -0.7 L/min, nebulizer gas flow -0.2 L/min and axial plasma view. The analyses of total Hg was performed using continuous flow hydride generation inductively coupled plasma - optical emission spectrometry (CF-HG-ICP-OES, Optima 8000, Perkin Elmer, USA) with reducing agent 0.2% $NaBH_4$ prepared in 0.05% (w/v) NaOH. The accuracy of the applied analytical procedure for the determination of trace metals in mussels was tested using SRM 2976 (Mussel homogenate, NIST) certified reference material. The recovery ranges were 97 % for As, 97 % for Cd, 101% for Cr, 95.3 % for Cu, 98.8 % for Fe, 104.1 for Pb, 95.3 for Mn, 105.9% for Hg, 94.2% for Ni and 102.6 for Zn. Each measured and reported value is an average of five determinations.

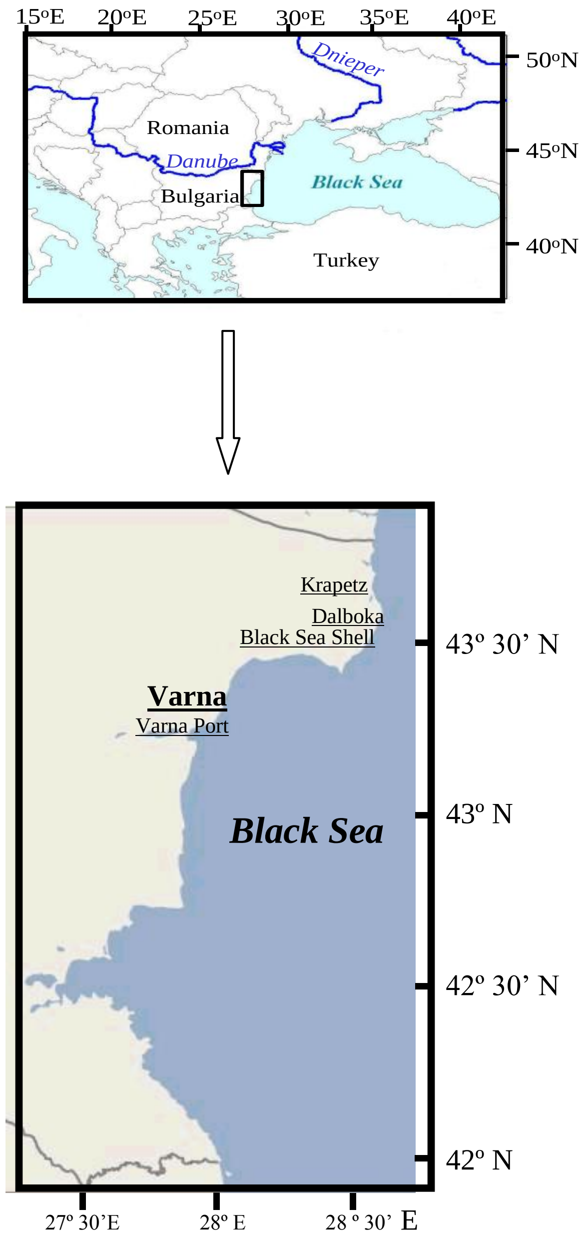

Figure 1: The map of sampling locations in the Bulgarian Black Sea coast

2.3. Data treatment for human health risk assessment

In this study, two levels of molluscan shellfish consumption values were considered namely 0.125 kg/week (one meal of molluscan shellfish every week for average level molluscan shellfish (ALM) consumers) and 0.250 kg/week (high level molluscan shellfish (HLM) consumers) [14]. To estimate the human health risk assessment derived from ingesting the molluscan shellfish, six assessments were made namely:

1.) Direct comparisons with seafood safety guidelines based on established maximum permissible limits (MPLs) set by the EC European Commission (2006), the USFDA for molluscan shellfish (FDA Guidance Document) [15], and Bulgarian Food Codex [16] although other agencies were also included for comparisons;

2.) The amount of molluscan shellfish that would need to be consumed per week by a 60-kg adult to reach the provisional tolerable weekly intake (PTWI) established by the Joint FAO/WHO Expert Committee on Food Additives (JECFA);

The PTWI is defined as the estimated amount of a substance in food or drinking water, expressed on a body weight basis (mg/kg of body weight), that can be ingested weekly over a lifetime without appreciable health risk [17]. Therefore, calculations were performed in order to determine the amount of molluscan shellfish from this study that exceed the PTWI limits. JECFA [18] established PTWIs for As, Cd, Cu, Hg, Pb , Fe, Ni, Cr and Zn as 0.015, 0.007, 3.50, 0.016, 0.025, 5.60, 0.035, 0.637 and 7.00 mg/week/kg b.w, respectively. Thus, the PTWIs for a 60 kg adult are equivalent to 0.9, 0.42, 210, 0.96, 1.5, 336, 2.10, 38.22 and 420 mg/week for As, Cd, Cu,Hg, Pb Fe, Ni, Cr and Zn, respectively.

3.) The estimated weekly intakes (EWI) for ALM and HLM consumers in order to evaluate a possible alert regarding adverse health effects that may be caused by an individual metal;

In order to evaluate a once- or long-term potential hazardous exposure to metals through consumption of mussels and rapa whelk by the population of Bulgaria, the EWI (μg/kg/day) values were calculated by using the following formula [19]:

$$EWI = \frac{M_c x Consumption\ rate}{BWa}$$

where : M_c - the metal concentration in molluscan shellfish ; *Consumption rate*- 0.125 kg/week and 0.250 kg/week, for ALM and HLM consumers, respectively [20]; BW_a(Average body weight)- 60 kg for adults.

4.) The target hazard quotient (THQ) of heavy metals for ALM and HLM consumers in all molluscan shellfish populations in order to evaluate a possible alert regarding adverse health effects that may be caused by an individual metal. The THQ value was determined with the following formula [21]:

$$THQ = \frac{(M_C \, . I_R \, . 10^{-3} . EF \, . ED)}{(RfD \, . BWa \, . ATn)}$$

where: *Mc* -metal concentration in mussels and rapa whelks (mg/kg ww); *IR*- consumption rate (17.86 and 35.7 g/day for ALM and HLM consumers, respectively); *EF*- exposure frequency (365 days/year); *ED* -exposure duration (72.5 years), equivalent to the average lifetime in Bulgaria; *RfD* -oral reference dose (As:0.3; Cd: 1.00; Cu: 40.0;Hg:0.3; Ni: 20.0; Pb:4; Mn:140; Fe: 700; Cr:3 and Zn:300 μg/kg/day provided by the USEPA's regional screening level (USEPA, 2015); *BWa*- average body weight (60 kg for adults); *ATn*- averaging exposure time for non-carcinogens (365 days/ year × ED); 10^{-3}- unit conversion factor.

The THQ values, developed by the USEPA [19], have been recognized as useful parameters for human health risk assessment of metals associated with the intake of marine products. The THQ is also a non-carcinogenic risk assessment which is a ratio between the estimated dose of metal exposure and the oral reference dose. A THQ > 1 signifies that the level of exposure is higher than the oral reference dose, which assumes that a daily exposure at this level is likely to cause negative health effects during a lifetime in a human population [22].

5) The total hazard index (HI) of multiple heavy metals for ALM and HLM consumers in all molluscan shellfish populations in order to evaluate possible health effects that may be caused by the combination of all metals studied. For the risk assessment of multiple metals found in the mussels and rapa whelks, the HI was calculated by summing all the calculated THQ_i values for the determined heavy metals [14], by using the following equation:

$$\sum_{i=1}^{n} THQ_i$$

where THQ_i the targeted hazard quotient of an individual metal and *n* in the present study is 10 (As, Cd, Cr, Cu, Hg, Ni, Zn, Pb, Mn, Fe).

6.) Target cancer risk (TR) indicates carcinogenic risks. The model for estimating TR was shown as follows:

$$TR = \frac{(M_C\,.\,I_R\,.\,10^{-3}.\,CPSo.\,EF\,.\,ED)}{(BWa\,.\,ATc)}$$

where *Mc* -metal concentration in mussels and rapa whelks (mg/kg ww);); *IR*- consumption rate (17.86 and 35.7 g/day for ALM and HLM consumers, respectively); *CPSo* is the carcinogenic potency slope, oral (As = 1.5; Ni= 1.7 and Pb= 0.0085 mg/kg bw-day); *EF*- exposure frequency (365 days/year); *ED* -exposure duration (72.5 years), equivalent to the average lifetime in Bulgaria; *BWa*- average body weight (60 kg for adults); *ATc* is the averaging time, carcinogens (day/years) and was calculated by multiplying exposure frequency in exposure duration over lifetime.

TR value for intake of As, Ni and Pb was calculated to indicate the carcinogenic risk since Cu, Hg and Zn do not cause any carcinogenic effects. The lower end of the range of acceptable risk distribution is defined by a single constraint on the 95th percentile of risk distribution that must be equal or lower than 10^{-6} for carcinogens (TR) and may be up to 10^{-4} in some circumstance [23].

3. Results and Discussion

The concentration levels of studied heavy metals (As, Cd, Cr, Cu, Hg, Ni, Zn, Pb, Mn, Fe) detected in bivalves samples from different stations of Bulgarian Black Sea coast are illustrated in Table 1. Among the different metals analyzed lead, cadmium, chromium and nickel are classified as chemical hazards [24,25, 26].

Table 1: Mean concentration and standard deviations of metals in molluscan shellfish (mg/kg dry weight) collected along Bulgarian Black Sea coast.

	Location		**As**	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Mussel	VP	W	0.73±0.06	0.14±0.01	0.15±0.02	0.86±0.03	19.93±1.89	0.02±0.19	2.73±0.29	0.41±0.01	<LOD	17.58±0.33
Mussel	BSS	F	1.58±0.05	0.33±0.01	0.38±0.01	2.26±0.17	103.84±5.30	< LOD	3.14±0.16	0.64±0.03	0.16±0.02	19.03±0.50
Mussel	B	W	1.94±0.05	0.342±0.005	0.28±0.02	2.12±0.10	78.41±4.97	< LOD	2.77±0.04	0.39±0.01	0.22±0.03	38.22±3.44
Mussel	D	F	2.24±0.04	0.64±0.01	0.34±0.004	2.01±0.05	112.8±4.9	0.12±0.01	3.19±0.09	0.58±0.03	0.33±0.05	29.86±0.24
R.Whelk	K	W	4.17±0.37	0.005±0.001	0.040±0.01	5.1±0.4	4.2±0.3	0.11±0.02	0.26±0.02	0.023±0.002	0.320±0.03	8.6±0.7
R.Whelk	V	W	2.2±0.2	0.008±0.002	0.050±0.01	7.7±0.5	9.4±0.6	0.08±0.01	0.48±0.04	0.045±0.004	0.120±0.01	7.5±0.6
BFC(2004)			**4.0**	**1.0**	**0.3**	**30**	**-**	**1.0**	**-**	**-**	**1.5**	**50.0**
EC (2006)			**-**	**1.0**	**-**	**-**	**-**	**0.5**	**-**	**-**	**1.5**	**-**
FDA (2007)			**86**	**4.0**	**13**	**-**	**-**	**1.0**	**-**	**80.0**	**1.7**	**-**
FAO (1983)			**-**	**0.5**	**-**	**30**	**-**	**-**	**-**	**-**	**0.5**	**40.0**

Legend: 1.) VP-Varna Port, BSS-Black Sea Shell, B-Balgarevo, D-Dalboka,K- Krapetz,V- Varna; 2.) w-wild, f-farmed

3.1. Comparison with food safety guideline and provisional tolerable weekly intake (PTWI)

3.1.1. As

Arsenic, a naturally occurring element, is a worldwide contaminant that is found in rock, soil, water, air and food. Arsenic is highly toxic and carcinogenic element for humans. Humans can be exposed to arsenic through the intake of food and drinking water, but for most people, the major exposure source is the diet, mainly fish and seafood [27]. Arsenic concentration in this study ranged between 0.73 mg/ kg w.w in wild mussels from Varna Port up 3.45 mg/ kg w.w in rapa whelk from Krapetz. According to Bulgarian Food Codex [16] and Montenegrin Food regulation [16], MPLs of As in shell fish should not be more than 4 mg/ kg. The Food and Drug Administration of the United States [15] guidelines 86 mg/kg w.w for As in shellfish. In the literature As concentration in the mollusks is found to be between 2.64 mg/kg d.w [28] up to 30 mg/kg d.w [29] (Table 2). As occurs naturally in the environment in organic and inorganic form as inorganic As is more toxic than organic form.

Table 2: The concentration ranges for trace elements (mg/kg wet or dry weight) in wild and farmed mussels and rapa whelk from different Black Sea coast regions

			As	Cd	Cr	Cu	Fe	Hg	Mn	Pb	Ni	Zn
M. galloprovincialis	Albanian sea coast SE Adriatic Sea[a]	dry	5.8-12.4	0.27–0.77	-	4.61–28.9	191.4-1579	0.083-0.421	12.3-81.3	1.39-5.69	2.19-6.18	59.8—244.6
	Croatian coast E Adriatic Sea[b]	dry	-	0.10-0.97	0.92-2.61	2.6-12.4	-	-	-	0.28-1.34	-	36.0-105.4
	Croatian coast E Adriatic Sea[c]	dry	4-30	-	1-29	3.7-11.1	53.4-719	-	2.0-13.0	2.0-7	0.8-5	59.1-273
	Venice, Italy W Adriatic Sea[d]	dry	12.0-18.0	-	2.0-2.4	15.8-35.5	260-458.6	-	14.1-18.6	2.5-3.9	1.6-2.6	100.2-242
	Turkish coast, SE Black sea[e]	wet	2.64-3.57	-	0.37-0.8	2.12-3.4	76.86-292.59	-	4.68-8.93	2.69-3.85	7.37-21.04	52.16-85.56
	Turkish coast E Black Sea[e]	wet	-	1-4	1.0-3.0	90-260	1150-4030	-	11-59	3-21	1.0-6.0	180-630
	Turkish coast S Black Sea[g]	wet	2.3	0.56	-	0.8	29.3	ND		0.7		49.1
R. venosa	Turkish coast S Black Sea[e]	dry	6.7	4.4	-	14.3	39.6	ND		ND		21.65
	Turkish coast NE Black Sea[e]	dry	-	0.1-1.6	0.1-0.2	-	-	0.4-0.7	-	0.1-0.7	-	-
	Bulgarian coast N Black Sea[f]	dry	1.9-6.8	0.5-1.0	9.0-19	16.5-44.0	0.2-1.2	0.06-0.3	245-283	15.3-33	1.8-18	6.4-17.2

[a] Jovíc & Stankovíc (2014)
[b] Kljakovic-Gaspic et al. (2002)
[c] Çullaj et al. (2006)
[d] Giusti L & Zhang H (2002)
[e] Mülayim, A and Balkıs, H (2015)
[f] Moncheva et al (2011)
[g] Bat & Öztekin (2016)

For inorganic As, the PTWI is 0.015 mg/kg [30] of b.w per week, or 0.9 mg/day for a subject of 60 kg. The percentage of inorganic As in mussels has been reported to be between 0.02 and 11% [12] in shellfish, so the inorganic forms usually only contribute a few percentage points [31, 32, 33]. The total As (inorganic and organic) is studied in this paper so it is hypothesized that maximum percentage is no more that 11 %. For the maximum As concentration of 3.45 for the *R.venosa*, ALM and HLM consumers would have an intake of 0.521 and 0.861 mg/week, which corresponds to 57.9 % and 95.8 % of the prescribed PTWI respectively (Table 3).Unfortunately there is a lack of information regarding weekly intake of Cr in mussels or other shellfish in the literature.

3.1.2. Cd

Cadmium is found in marine waters mostly in the dissolved form, distributed in the marine environment at low concentration and mussel accumulate Cd effectively and may act as poison to humans [10]. Cd levels in analyzed marine shells were between 0.005 mg/kg d.w. for rapa whelk from Krapez and 0.64 mg/kg d.w. for mussels species from region of Dalboka (Table 1). The maximum Cd level permitted for mollusks is 1.0 mg/kg w.w. according to the European Community [32] and Bulgarian Food Regulation [16]. The present Cd values did not exceed the MPLs set by those three health organization. Therefore, there was no apparent Cd risk of consuming molluscan shellfish. In the literature Cd levels were reported as 0.5-1.0 mg/kg d.w in *R. venosa* from Bulgarian Black sea region [34], between 0.06 and 0.53 mg/kg d.w for marine mussels collected from 20 geographical sites located in Peninsular Malaysia [35], and between 0.9-8.91mg/kg d.w in the sort tissues of wild and farmed *M.galloprovincialis* collected from Boka Kotorska Bay, Adriatic sea [36] (Table 2).

In 2010, the JECFA withdrew the $PTWI_{Cd}$ value of 7 µg/kg bw and established a provisional tolerable monthly intake ($PTMI_{Cd}$) of 25 µg/kg [18] considered that a monthly value was more appropriate. In the case of farmed mussels from the Dalboka region in which the highest Cd concentrations (0.64 mg/kg dw) were measured, ALM and HLM consumers by consuming of 0.125 and 0.250 kg molluscan shellfishes, respectively, would have an intake of 0.008 (0.64 mg/kg × 0.125 kg) and 0.16 (0.64 mg/kg ×0.250 kg) mg/week, which corresponds to 19% (0.08/0.42 × 100%) and 38.1 % (0.16/0.42 × 100%) of the prescribed PTWI (0.42 mg/week), respectively (Table 3).

Table 3: Mean weekly intake (MWI, mg/kg) of metals for average (0.125 kg) and high (0.250 kg) level molluscs consumers (mg/week) and the percentage (PCT) of prescribed PTWI values

ALM																				
	As		***Cd***		***Cr***		***Cu***		***Fe***		***Hg***		***Mn***		***Ni***		***Pb***		***Zn***	
	MWI	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**
Min	0.091	10.114	0.001	0.149	0.048	0.125	0.107	0.051	0.525	0.156	0	0	0.03	0.4	0.00	0.1	0.00	0.0	0.94	0.2
Max	0.431	47.917	0.080	19.052	0.005	0.013	0.963	0.458	14.111	4.200	0.015	15.755	0.40	4.7	0.08	3.8	0.04	2.8	4.78	1.1
Mean	0.26	29.02	0.04	9.60	0.03	0.07	0.53	0.25	7.32	2.18	0.01	7.88	0.22	2.57	0.04	1.98	0.02	1.38	2.86	0.68

HLM																				
	As		***Cd***		***Cr***		***Cu***		***Fe***		***Hg***		***Mn***		***Ni***		***Pb***		***Zn***	
	MWI	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**	**MWI**	**PCT**
Min	0.182	20.227	0.001	0.298	0.096	0.250	0.215	0.102	1.050	0.313	0	0	0.1	0.8	0.0	0.3	0.0	0.0	1.9	0.4
Max	0.863	95.833	0.160	38.104	0.010	0.026	1.925	0.917	28.222	8.399	0.030	31.510	0.8	9.5	0.2	7.6	0.1	5.5	9.6	2.3
Mean	0.52	58.03	0.08	19.20	0.05	0.14	1.07	0.51	14.64	4.36	0.02	15.76	0.43	5.13	0.08	3.96	0.04	2.76	5.71	1.36

For comparison, Yap et al [35] reported that based on the measured Cd concentration in marine musses from Malaysia, the weekly intake of Cd was estimated from 0.01-0.07 to 0.01-0.13 mg/person/ week for ALM and HLM consumers, respectively; which accounts for 1.75-15.9 and 3.5-31.8% of the prescribed PTWI, respectively. In a study conducted in Adriatic Sea, Jovíc and Stankovic [14] found that, the weekly intake of Cd was 0.01–0.076 to 0.02–0.15 mg/person/week for ALM and HLM consumers, respectively; which accounted for 2.90–22.1 and 5.79–44.2% of the prescribed PTWI, respectively. With the Cd ranges lower than all the MPLs for Cd, the Cd levels found in the Black Sea *M. galloprovincialis* and *R. Venosa* in this study should not be a major public concern although Cd is a non-essential element for human organisms.

3.1.3. Cr

Chromium is an essential mineral in humans and has been related to carbohydrate, lipid, and protein metabolism. The recommended daily intake is 50-200 µg [37]. The amount of chromium in the diet is of great importance as Cr is involved in insulin function and lipid metabolism. The concentration level of Cr in the mussels and rapa whelks at the sampling sites ranged between 0.04(rapa whelks from Krapetz) and 0.382mg/kg w.w (farmed mussels from Black Sea Shell). The maximum Cr level permitted for shellfish is 0.3 mg/kg according to Bulgarian Food Codex [16] and 13.0 mg/kg according to FDA [26]. The maximum concentration of Cr exceeds the MPLs. An analysis of the chromium content in the sediments of the northern part of Bulgarian Black Sea where the Black Sea Shell Farm is situated indicated that the sea bed of the bay is rich to Cr and Mn and that their presence in the soft tissues of mussel and whelk rapa cannot be entirely anthropogenic[27]. The levels of Cr in the literature for mussels were between 0.74-2.40 mg/kg for samples from Adriatic Sea [14], 2.0-2.4 mg/kg, d.w for samples from Venetian Lagoon around the Island of Murano [38] and between 9.0-19 mg/kg for *R. Venosa* from Bulgarian Black Sea coast [34] (Table 2).

The PTWI value of Cr is 0.637 mg/kg that it equal to the minimum requirement of a person per kg of body weight. For the maximum Cr concentration of 0.382 for the farmed *M. galloprovincialis*, ALM and HLM consumers would have an intake of 0.048 and 0.096 mg/week, which corresponds to 0.13% and 0.25% of the prescribed PTWI (38.22 mg/week), respectively (Table 3).Unfortunately there is a lack of information regarding weekly intake of Cr in mussels or other shellfish in the literature.

3.1.4. Cu

The copper ranges between 0. 86 mg/kg ww (wild mussels from Varna Port) and 7.7 mg/kg ww (in rapa whelk from Varna) and were well below the MPLs suggested by FAO [39] and Bulgarian Food Regulation [16]. Thus, there should be no noticeable Cu risk of consuming the mussels and rapa whelk populations. The present Cu ranges were comparatively lower than those from Singapore [40], Malaysia [36] and Boka Kotorska Bay, Montenegro [4]. High levels of Cu in *M. galloprovincialis* had been reported in Eastern Black Sea, Turkey [41] and in Italy [10].

The JECFA established a PMTDI for Cu of 0.5 mg/kg bw/day which is equivalent to 3.5 mg/kg bw/week, i.e., 210 mg/week for a 60-kg adult [42]. From Table 3 it is visible that in the case for *R. Venosa* from station Varna which have the highest concentration of copper, the ALM and HLM consumers by consuming of 0.125 and 0.250 kg molluscs, respectively, would have an intake of 0.963 and 1.925 mg/week, which corresponds to 0.458% and 0.917 % of the prescribed PTWI, respectively. Yap et al [35] also reported that based on the mean Cu concentration in mussels from Malaysia, the weekly intake for ALM consumers and percentages of prescribed PTWI values are 0.06-2.19 mg/kg week and 0.03-1.04%, respectively while those for HML consumers are 0.12-4.38 mg/week and 0.06-2.08%, respectively. The measured concentrations of Cu in molluscs from the Bulgarian Black Sea indicate that it is necessary to consume a large amount of mussels in relation to the prescribed value.

3.1.5. Fe

Iron is essential nutrient metal required for human and in the form of hemoglobin transport oxygen in the blood. It was observed that the Fe concentration ranged widely from 4.2 mg/kg w.w to 103.84mg/kg w.w in different mollusks species from different coast of Bulgaria (Table 1). The highest value was observed in farmed *M. galloprovincialis* from Black Sea Shell, while the lowest value was observed in *R. venosa* from Krapetz region (North). There isn't set any MPLs of Fe in fish or other marine products according to Bulgarian Food Codex [16] or European Legislation [2] 5but in literature the iron values vary from 82.05 up to 450.1 mg/kg d.w for Adriatic Sea's mussel sample [13], from 90.63-292.59 mg/kg w.w for mussel from Giresun coast of the Black Sea, Turkey [28], from 260-458.6 mg/ kg d.w in soft tissue of *M. galloprovincialis* from four sites around Murano, Venice [39] and from 33.1-273.2 mg/kg for *M. galloprovincialis* from Albania coast of Adriatic Sea [7].

In 1983, the JECFA established a PMTDI of 0.8 mg/kg of b.w, which corresponds to 5.6 mg/kg b.w/ week i.e 336 mg/kg for an adult weighting 60 kg. Based on the concentration of iron for the *M. galloprovincialis* and *R. venosa* in this study, it could be seen that it is necessary to consume large amounts of mussels and rapa whelk per week to exceed the estimated $PTWI_{Fe}$ (Table 3). If we take the maximum amount of iron (112.87 mg/kg w.w from mussels from the region of Dalboka) then the ALM and HLM consumers by consuming of 0.125 and 0.250 kg molluscs, respectively, would have an intake of 14.11 and 28.22 mg/week, which corresponds to 14.11 % and 8.40 % of the prescribed PTWI (336 mg/week), respectively. In the work of Jovíc et al. [14], the mean Fe concentrations indicate that the highest amount of mussels may be consumed from Croatia (10.5 kg per week) and the lowest from Albania (2.52 kg per week). In the case of mussels from the Albanian coast in which the highest iron concentrations were measured, average or high level mussels consumers by consuming of 125, i.e., 250 g mussels intake 16.7 and 33.4 mg Fe per week, which corresponds to 4.97% and 9.93% of the prescribed $PTWI_{Fe}$, respectively [14].

3.1.6. Hg

Hg is highly toxic elements and it is well known fact that marine organisms transfer the mercury into its highly toxic form-methyl mercury (MeHg). Diet is the main source of mercury to human exposure. The Hg levels in the analyzed species were up to 0.12 mg/kg w.w (mussels from the region of Dalboka) which is far below the MPLs set from Bulgarian Food Codex (1.0 mg/kg w/w) [16] and FDA (1.0 mg/kg w/w) [26]. In Montenegro, the concentration of Hg in *M. galloprovincialis* varied between 0.010 and 0.371 mg/kg w.w [10], between 0.1-0.81 mg/kg d.w for *M. galloprovincialis* collected from the Apulian coast, Southern Italy [43] and between 0.026-0.03 mg/kg d.w for the same shellfish species from Black Sea Romania [44].

In 1972, the JECFA established a PTWI value of 5 µg/kg of b.w for total Hg, of which no more than 3.3 lg/kg of b.w should be present as MeHg. The PTWI for MeHg was then reduced to 1.6 µg/kg bw, based on an assessment of the results from various epidemiological studies involving fish-eating populations and developmental neurotoxicity [45]. The average and high level consumers introduce weekly into their bodies through molluscan shellfish intake from 15.76% and 31.51% of the prescribed values for MeHg (which is around 36% of the total Hg) , respectively (Table 3).

3.1.7. Mn

This essential element occurs naturally in many food sources. Exposure to high levels of Mn may lead to adverse neurological effects. Therefore the amount of this element is controled and information related with recommended intake can be found: the United State Environmental Protection Agency (US EPA) report a non-cancerogenic TDI_{Mn} of 0.14 mg/kg b.w from diet, in Canada TDI values are 0.136 mg/kg Mn for infants and toddlers, 0.122 mg/kg for children, 0.142 mg/kg for teenagers and 0.156 mg/kg for adults. There isn't MPLs for Mn according to Bulgarian Food Codex [16]. For a 60 kg body weight of an adult, TDI_{Mn} was calculated to be 8.4 mg/kg bw/day. Based on consumption of 125, i.e 250 g w.w of molluscan shellfishes from Bulgarian Black Sea coastal area on a weekly basis, the human body receives 0.40 and 0.80 mg Mn/person/week, representing 4.7 and 9.5 % of the prescribed $PTWI_{Mn}$ for average and high level molluscan shellfish consumers, respectively (Table 3). These values are below or near the ones stated in the literature: Albanian coast -1.64 mg/week and 3.28 mg/week, respectively; Croatian coast- 0.13 mg/week and 0.26 mg/week [14].

3.1.8. Ni

The concentration of Ni varies between 0.023 mg/kg w.w for *R. venosa* from Krapetz and 0.642 mg/kg w.w for farmed *M. galloprovincialis* from Black Sea Shell. The MPLs for Ni are lacking in the literature but the only available Ni MPLs know as action level (Ni: 80 mg/kg w.w) was set by the US FDA/CFSAN [15]. Therefore, the Ni levels in all molluscan shellfish were well below the limit of Ni and those in literature -1.94-114 mg/kg for *Perna viridis* collected from the Straits of Mallaca and the Straits of Jahore, Malaysia [35]; 1-6 mg/kg d.w for *M. galloprovincialis* from Eastern Black Sea, Turkey [41]

Based on the maximum values found for Ni, by the consumption of 0.125 or 0.250 kg, of mussels from the present study, 0.08 or 0.2 mg/person/ week could enter into the human body, which is only 3.8 or 7.6% of the PTWI, respectively. For comparison, Jovíc and Stankovic [14] reported that based on the measured Ni concentrations in mussels from the Adriatic Sea, the weekly intake of Ni was estimated from 0.04 to 0.10 and from 0.09 to 0.21 mg/person/week for ALM and HLM consumers, respectively; which accounted for 2.08–4.94 and 4.17–9.88% of the prescribed PTWI (Table 3), respectively while Yap et al. [35] - from 0.40 or 0.80 mg/person/ week could enter into the human body, which is only 19.1 or 38.2% of the PTWI, respectively.

3.1.9. Pb

The International Agency for Research on Cancer (IARC) classified inorganic Pb as probably carcinogenic to humans [46]. This element is highly toxic and is accumulated in human body in soft tissues, bones and teeth. Its concentration in various food matrixes is strictly regulating. In current study the concentration of Pb was under LOD for some samples up to 0.332 mg/kg w.w for the farmed mussel from the northern region. The maximum concentration of Pb is within the MPLs suggested by the EC [32] (1.50 mg/kg ww) and USFDA/CFSAN [15] (1.70 mg/kg ww). Finally the present Pb ranges are within the legal limits of Pb (1.5 mg/kgww) as compiled by Bulgarian Food Codex [16]. Lead concentration in mussels and rapa whelks varies in the literature (Table 2).

A PTWI value of 0.025 mg/kg b.w for Pb was established by JECFA in 1986. With new available data that a PTWI of 25 µg of body weight could be responsible for a drop of 3 points in the IQ in children and an increase in systolic blood pressure in adults, the JECFA acknowledged that established PTWI value was not sufficiently protective but that they were unable to establish a new health-based guidance value [47]. The European Food Safety Authority (EFSA) concluded that Pb has the most harmful effect on the central nervous system of young children and on the cardiovascular system of adults, and identified three reference dietary intake values, 0.63 µg/kg bw/day for nephrotoxic effects in adults, 1.50 µg/kg bw/day for cardiovascular effects in adults and 0.50 µg/kg bw/day for neuro-developmental effects in children [48].

In the case of farmed mussels from the Dalboka region in which the highest Pb concentrations (0.33 mg/kg dw) were measured, ALM and HLM consumers by consuming of 0.125 and 0.250 kg molluscan shellfishes, respectively, would have an intake of 0.04 (0.33 mg/kg × 0.125 kg) and 0.10 (0.33 mg/kg ×0.250 kg) mg/week, which corresponds to 2.8% and 5.5 % of the prescribed PTWI (1.5 mg/week), respectively (Table 3). Yap et al [35] found the MWI for ALM consumers 0.03–1.30 mg Pb/ week and the percentages of prescribed PTWI values 12.7–489% for Pb-1 (chronical renal disease in adults, 0.63 µg/kg b.w/day), 5.40–206% for Pb-2 (effect on systolic blood pressure in adults, 1.50 µg/kg b.w/day and 16.1–618% for Pb-3 (neuro-developmental effects in children, 0.50 µg/kg b.w/day) and for HLM consumers 0.07–2.59 mg/week and the percentages of prescribed PTWI values are 25.5–979% for Pb-1, 10.7–412% for Pb-2 and 32.1–1236% for Pb-3.

3.1.10. Zn

Zn is an essential element in the nutrition for both animals and humans. But in high levels is harmful for the body. In this study Zn was the element present in the highest mean levels 7.5 to 38.22 mg/kg w.w. According to Bulgarian Food Codex, the MPLs is 50 mg/kg w.w. The present Zn ranges were below the MPLs. Therefore, there was no apparent Zn risk of consuming rapa whelks and mussels from Bulgarian Black Sea coast.

Based on the average, by consumption of 0.125 or 0.250 kg, of molluscan shellfish from the present study, 4.78 or 9.6 mg/person/week could enter into human body, which is only 1.1 or 2.3 % of the PTWI (7 mg/week/ kg b.w), respectively (Table 3).. Yap et al [35] reported that based on the measured Zn concentration in mussel from Malaysia, the values of 1.96 (ALM) or 3.91(HLM) mg/person/week could enter into the human body, which is only 0.47(ALM) or 0.93% (HLM), of the PTWI, respectively. On the other hand, Stankovic et al[11] estimated that the weekly intake of Zn was from 2.65 to 7.86 and 5.30–15.7 mg/person/week for ALM and HLM consumers, respectively; which accounted for 0.63–1.87 and 1.26–3.74% of the prescribed PTWI, respectively.

3.2. Estimated dietary intake

The EDI of heavy metals through molluscan shellfish by ALM and HLM consumers are presented in Table 4. The EDI (µg/kg ww/day) of heavy metals for ALM and HLM consumers are 0.002–0.0.009 and 0.03–0.017, respectively for As, 0.0.0001–0.00071 and 0.0002–0.0016, respectively for Cd, 0.0001–0.0008 and 0.0002–0.0016, respectively for Cr, 0.002–0.016 and 0.004–0.32, respectively for Cu, 0.01-0.24 and 0.02–0.47 respectively for Fe, 0.001-0.0007 and 0.001–0.013 respectively for Mn, 0.0001-0.0013 and 0.001–0.0024 respectively for Ni and 0.016–0.080 and 0.031-0.159, respectively for Zn. In comparison to the ORDs (µg/kg ww/day) for the EDI values for both ALM and HLM consumers, all populations were lower than the guidelines. Therefore, this strongly indicated that consumers would not experience significant health risks from the intake of these metals.

It is incomparable direct comparison of EDI with other reported values since there are differences in the parameters such as exposure time, consumption rate, average body weight and etc. Therefore it is of great importance to calculate the EDI based on similar equation/formulas with ALM and HLM consumers. Nicholson and Szefer [49] reported EDI values in marine mussels from Hong Kong for Cd 0.27 (for ALM) and 0.55 (for HLM), for Cu 0.75(for ALM) and 1.51 (for HLM), for Fe 4.92(for ALM) and 9.83 (for HLM), for Ni 0.14(for ALM) and 0.29 (for

HLM), and for Zn 5.47(for ALM) and 10.9 (for HLM). In a study of mussels conducted in Karnataka Coast, India [50] the EDI values were as follows: Cd- 0.60 for ALM and 1.20 for HLM; Cu-0.49 for ALM and 0.97 for HLM; Fe-23.9 for ALM and 47.9 for HLM; Ni-0.38 for ALM and 0.75 for HLM; and Zn-3.08 for ALM and 6.16 for HLM. Present EDI values of the elements under study for ALM and HLM consumers are common and/or close to those of marine molluscan shellfish reported in the literature.

Table 4: Estimated daily intake (EDI) of heavy metals for average (ALM) and high level mussel (HLM) consumers in all molluscan shellfish population

					EDI (ALM)					
	As	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Min	0.002	0.00001	0.0001	0.002	0.01	ND	0.001	0.0001	ND	0.016
Max	0.009	0.00071	0.0008	0.016	0.24	0.0003	0.007	0.0013	0.0007	0.080
Mean	0.004	0.00051	0.0004	0.007	0.11	0.0001	0.004	0.0007	0.0004	0.042
SD	0.002	0.00051	0.0003	0.005	0.10	0.0001	0.003	0.0005	0.0003	0.025
					EDI (HLM)					
	As	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Pb**	**Zn**
Min	0.003	0.00002	0.0002	0.004	0.02	ND	0.001	0.0001	ND	0.031
Max	0.017	0.00267	0.0016	0.032	0.47	0.0005	0.013	0.0024	0.0014	0.159
Mean	0.009	0.00102	0.0009	0.014	0.23	0.0002	0.009	0.0015	0.0008	0.084
SD	0.005	0.00101	0.0006	0.011	0.21	0.0002	0.006	0.0010	0.0005	0.050

3.3. Target hazard quotients (THQ), hazard Index (HI) and targer risk (TR)

Beside the PTWI values, the THQ, HI and TR values proposed by US EPA [19] have been recognized as useful parameters for risk assessment of metals associated with the intake of marine molluscan shellfish [51]. The mean metal concentration was used for calculating the THQ, HI and TR values.

Table 5a: Risk values (THQ, HI and TR) of each metal contaminant in marine molluscan shellfish from Bulgaria Black Sea coast (ALM)

Samples	**Location**	**THQ**										**HI**	**TR**		
		As	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Zn**	**Pb**		**As**	**Ni**	**Pb**
M. galloprovincialis	Varna Port (w)	0.0007	0.000043	0.00002	0.00001	0.000008	0.00002	0.000006	0.000006	0.00002	0.00000	0.001	3.37 x10^{-4}	2.14 x10^{-4}	0.00
	Bl.Sea Shell(f)	0.0016	0.000097	0.00004	0.00002	0.000044	0.00000	0.000007	0.000010	0.00002	0.00001	0.002	7.28 x10^{-4}	1.98 x10^{-4}	4.91 x10^{-5}
	Balgarevo (w)	0.0019	0.000102	0.00003	0.00002	0.000033	0.00000	0.000006	0.000006	0.00004	0.00002	0.002	8.98 x10^{-4}	1.22 x10^{-4}	6.66 x10^{-5}
	Dalboka (f)	0.0022	0.000191	0.00003	0.00001	0.000048	0.00012	0.000007	0.000009	0.00003	0.00002	0.003	10.4 x10^{-4}	1.78 x10^{-4}	1.02 x10^{-4}
R. venosa	Krapetz	0.0041	0.000001	0.00000	0.00004	0.000002	0.00011	0.000001	0.000000	0.00001	0.00002	0.004	19.3 x10^{-4}	7.09 x10^{-6}	9.87 x10^{-5}
	Varna	0.0022	0.000002	0.00000	0.00006	0.000004	0.00008	0.000001	0.000001	0.00001	0.00001	0.002	10.2 x10^{-4}	1.39 x10^{-5}	3.70 x10^{-5}

Table 5b: Risk values (THQ, HI and TR) of each metal contaminant in marine molluscan shellfish from Bulgaria Black Sea coast (HLM)

Samples	**Location**	**THQ**										**HI**	**TR**		
		As	**Cd**	**Cr**	**Cu**	**Fe**	**Hg**	**Mn**	**Ni**	**Zn**	**Pb**		**As**	**Ni**	**Pb**
M. galloprovincialis	Varna Port (w)	0.001	0.000075	0.00002	0.00001	0.000013	0.00004	0.000009	0.000011	0.00003	0.00000	0.002	6.73 x10^{-4}	4.29 x10^{-4}	0.00
	Bl.Sea Shell (f)	0.003	0.000171	0.00006	0.00003	0.000067	0.00000	0.000010	0.000017	0.00003	0.00002	0.004	14.6 x10^{-4}	6.72 x10^{-4}	2.34 x10^{-8}
	Balgarevo (w)	0.004	0.000179	0.00004	0.00003	0.000051	0.00000	0.000009	0.000010	0.00006	0.00002	0.004	18.0 x10^{-4}	4.16 x10^{-4}	3.17 x10^{-8}
	Dalboka	0.004	0.000336	0.00005	0.00003	0.000073	0.00021	0.000010	0.000015	0.00004	0.00004	0.005	20.8	6.06	4.87

	(f)												$x10^{-4}$	$x10^{-4}$	$x10^{-8}$
R. venosa	Krapetz	0.008	0.000003	0.00001	0.00007	0.000003	0.00019	0.000001	0.000001	0.00001	0.00004	0.009	38.6 $x10^{-4}$	2.41 $x10^{-5}$	4.70 $x10^{-8}$
	Varna	0.004	0.000004	0.00001	0.00010	0.000006	0.00014	0.000002	0.000001	0.00001	0.00001	0.005	17.9 x10-4	4.16 $x10^{-5}$	1.76 $x10^{-8}$

As shown in Table 5, for ALM and HLM consumers, the THQ values for all individual metals are found to be <1 . Therefore, THQ values for all heavy metals in all population for both ALM and HLM consumers indicated no health risk. This means that the ALM and HLM consumers may not experience health effects or would not present a potential risk on human health regarding the ten elements.

HI values for each molluscan shellfish are also illustrated in table 5. The calculated values for the HI were below the safety level of 1with highest contribution of As. The results suggested that the multiple trace metals contain in mussels and wherk rapa from Bulgarian Black Sea coast posed no risk for the consumers.

Calculated average value of carcinogenic risk (TR) of the species under analyzes for both ALM and HLM are within the limits of the 95^{th} percentile of risk distribution that must be equal or lower than 10^{-6} for carcinogens (TR) and may be up to 10^{-4} (Table 5) suggesting that the intakes of metals by consumption of these molluscan shellfish would not result in appreciable hazard risk on the human body.

4. Conclusion

The trace metal concentration in wild and farmed *M. galloproviancialis* and *R. venosa* from the Bulgarian Black Sea coast did not exceed the European Union and Bulgarian Food Codex requirements.

Calculation based on As, Cd, Cr, Cu, total Hg, Ni, Zn, Pb, Mn, Fe in the soft tissue of mussels *M. galloprovincialis* and veined rapa whelks *R. venosa* from the northern part of Bulgarian Black sea coast suggest that a large amount of molluscan shellfish would have to be consumed to exceed the prescribed PTWI values for adults.

The values for non carcinogenic risk (THQs) showed that adverse health effects might not occur when considering different molluscs consumption patterns. HI of each trace element were lower than one suggesting that these pollutants perhaps pose no hazard to local residents. Target risk (TR) due to Pb, As and Ni exposure through mussels and rapa whelks consumption may not have the probability of contracting cancer over a long lifetime in future. Overall, based in the human health risk assessment, all results for the heavy metals may not pose a potential health risk to consumers. Further studies are necessary in order to determine the metals inmolluscan shellfish from this area of Black Sea Bulgaria

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