Trace Elements In The Detoxifying And Accumulating Body Parts of *Mytilus Galloprovincialis* Lamark, 1819 (Crimea, Black Sea): Human Health Risks And Effect of The Sampling Site Location

Natalya S. Chelyadina (✉ chelyadina2007@mail.ru)

A.O. Kovalevsky Institute of Biology of the Southern Seas <https://orcid.org/0000-0003-2552-4073>

Sergey V. Kapranov

A.O. Kovalevsky Institute of Biology of the Southern Seas

Mark A. Popov

A.O. Kovalevsky Institute of Biology of the Southern Seas

Lyudmila L. Smirnova

Institute of Natural and Technical Systems, Russian Academy of Sciences

Nikolay I. Bobko

A.O. Kovalevsky Institute of Biology of the Southern Seas

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Abstract

The mussel *M. galloprovincialis* is one of the most popular species in world's mariculture and environment pollution bioindicators. Although this mollusk was in a focus of numerous publications, the detoxifying and accumulating role of some of its body parts was insufficiently studied. In the present work, using inductively coupled plasma mass spectrometry, the distribution of Cr, Co, Ni, Cu, Zn, As, Cd, Hg, Pb in soft tissues, byssus, and shell liquor of this bivalve was studied for the first time as a function of sampling location in the Black Sea off southwestern Crimea. Multivariate analysis showed significant differences in the overall distribution of the elements among the body parts and sampling sites under consideration. Human health risks from the consumption of this mollusk were shown to be unlikely. Byssus of *M. galloprovincialis* was characterized as an ideal indicator of marine environment pollution with Ni, Cu, Pb, Co, and Cr as it contained the highest contents of these elements among the body parts under study (Ni up to 91%, Cu and Pb up to 79%). For the first time, the concentrations of trace elements were determined in the shell liquor and its role as the trace element excretion system was demonstrated. In the shell liquor, the largest share among the body parts was that of Hg (up to 71%). In soft tissues, Zn, Cd and As were in largest proportions among the body parts (63, 62, and 54%, respectively). The trace element contents in soft tissues of *M. galloprovincialis* decreased in the following order: Zn > Cu > As > Ni > Pb > Cd > Cr > Co > Hg.

1. Introduction

The Mediterranean mussel *M. galloprovincialis* is an important source of animal protein with high nutritional value and one of the most popular target species in bivalve mariculture. This mollusk thrives in the coastal waters of the Mediterranean and Black Seas as well as the Atlantic of the Northern Hemisphere. It is also cultivated in countries of the southern Mediterranean, in South Africa and China (Atasara et al. 2015; FAO 2020; Gosling 2003; Kholodov et al. 2017; Lutz et al. 1991). In the Black Sea, *M. galloprovincialis* is extensively cultured and is one of the dominant mollusk species (Ivanov et al. 1989; Kholodov et al. 2017; Massa et al. 2017).

M. galloprovincialis is also widely used as environmental biomonitor. Substantial progress in this application has been achieved through the implementation of the "Mussel Watch" program (Cantillo, 1998; Rainbow and Phillips 1993; Stankovic and Jovic 2012). Mollusks are bioindicators of trace element pollution of environment (Casas et al. 2008; Gupta et al. 2011; Stankovic and Jovic 2012) because of their ability to accumulate trace elements in body parts to levels several orders of magnitude higher than in the surrounding water (Adams and Rowland 2003; Casas et al. 2008; Rainbow and Phillips 1993; Stankovic and Jovic 2012). Trace element contents in marine organisms depend on environmental factors, such as the trace element concentrations in seawater, temperature, salinity, dissolved oxygen concentration, pH value, and also on biological characteristics, such as aquatic species identity, sex and sexual ripening stage, type of tissue, organ, diet, etc. (Boening 1999; Sunlu 2006).

Many trace elements, including heavy metals, are biologically active and capable of disrupting human metabolic processes. Estimation of heavy metal contents in edible aquatic mollusk tissues is important for the assessment of human health risks from the mollusk consumption (Gupta et al. 2011). The most hazardous for human health are cadmium, copper, arsenic, nickel, mercury, lead, zinc, chromium, and cobalt (Briffa 2020; Gupta et al. 2011; Stankovic and Jovic 2012).

There exist a number of publications on the trace element levels in soft tissues and byssus of mytilids (Nicholson and Szefer, 2003; Szefer et al. 1999; Szefer et al. 2002; Yap et al. 2003a; Yap et al. 2003b). Mussel byssus is known to be a better bioindicator of coastal pollution with heavy metals as compared to other tissues (Szefer et al. 1999; Szefer et al. 2002). In *M. galloprovincialis* shell liquor, trace element levels were studied very poorly, and to our knowledge, there is only one publication on the content of Zn and Cu in this fluid (Chelyadina and Smirnova, 2018). At the same time, the need for the study of the mussel shell liquor element composition is quite obvious as shell liquor is an important mediator in the metabolism that occurs between tissue cells and the open circulatory system of the mussel and affects the liquor composition (Chelyadina et al. 2015).

The goals of this work are: to study the distribution of the above-mentioned hazardous elements (Cd, Cu, As, Ni, Hg, Pb, Zn, Cr, Co) in soft tissues, byssus, and shell liquor of the mussel *M. galloprovincialis* and its dependence on the sampling location in coastal waters of the southwestern Crimea (Black Sea); to reappraise the role of byssus as a bioindicator of water pollution with the trace elements under study; to assess the role of the shell liquor as the heavy metal detoxification medium; and to assess human health risks from the consumption of the cultivated mussel meat.

2. Material And Methods

2.1 Object of research

The object of this study is the bivalve *M. galloprovincialis* with the shell sizes of 54.01 ± 2.9 mm. The mussel *M. galloprovincialis* is a common species in the Black Sea shelf zone and in the fouling of various hydraulic structures. The animals form independent biocenoses and are part of other communities. *M. galloprovincialis* has a significant potential for acclimatization, which allows them to adapt to different living conditions and occur in nearly all biotopes of the region. At present, this species has spread throughout temperate shelf waters of almost all oceans. It can exist in wide ranges of salinity ($8 - 40 \text{ g} \cdot \text{L}^{-1}$) and water temperature ($1 - 28^\circ\text{C}$). The optimum temperature is $12 - 20^\circ\text{C}$ and salinity $12 - 25 \text{ g} \cdot \text{L}^{-1}$ (Ivanov et al. 1989; Kapranov et al. 2021b). The mussel *M. galloprovincialis*, being an active filter feeder, filters water at a rate of 0.5 to $7.5 \text{ L} \cdot \text{h}^{-1}$ and more and, as a result, accumulates trace elements from the incoming water and food, phytoplankton and detritus (Coombs and Keller, 1981; Temerdashev et al. 2017). Soft tissues of mussels accumulate trace elements and partly remove them into shell liquor, which is involved in the metabolism between tissue cells and circulating blood (hemolymph). Shell liquor, byssus, feces, pseudo-feces and mussel gametes during the spawning make up the mollusk's excretion system (Chelyadina et al. 2015; Kapranov et al. 2021; Pospelova 2008).

M. galloprovincialis is a sedentary organism that, after the larval stage, settles and attaches to the substratum (rock, sediments, rope collectors, etc.) with byssal threads, which are secreted from a byssal gland in the mussel foot and provide tight attachment. Byssal threads consist of collagen fibrils incorporated into matrix protein core. Byssus plays not only the role of an attachment organ, but also performs the function of extracting heavy metals from the mussel body (Nicholson and Szefer, 2003; Suhre et al. 2014; Szefer et al. 2006).

2.2 Characteristics of the sampling sites

In this study, mussels were sampled in June 2017, and the temperature of the seawater environment was measured at the sampling time using a meteorological thermometer TM-10 (*Termopribor*, Klin, Russia).

The sampling sites were selected so as to encompass environments with different physicochemical properties and different expected levels of trace element exposure. The first sampling site was the mollusk farm (Station 1) located at the outer roadstead of Sevastopol (44°37'13.4" N; 33°30'13.6" E, Fig. 1). The animals at Station 1 were harvested from rope collectors at a depth of 2 – 3 m, where the water temperature was 21.4°C. The second sampling site was the seafloor under the farm (Station 2). The depth at Station 2 was 16 m and the water temperature at the seafloor was 11.0°C. The third place for the mussel sampling (44°36'41.1" N; 37°33'0.3" E, Fig. 1) was the innermost part of Sevastopol Bay (Station 3). The animals at this station were collected from the quay wall at a depth of 2 – 3 m at the water temperature of 21.8°C.

The water in the mollusk farm area (Station 1) is ranked as mesotrophic; the trophic index (TRIX) value was 2.73. Hidden upwellings are typical for this area. The farm has sufficient water exchange and nutrient supply due to local currents even in the small wind-wave mixing regime (Kuftarkova et al. 2006). Food resources in this marine farm are favorable for the growth and development of mollusks as microalgae included in the diet of the cultivated mussels are permanently present in the phytoplankton (Ryabushko et al. 2017). The seasonal variation of salinity in the surface water is weak. The amplitude of the seasonal fluctuations of the long-term mean values did not exceed 0.4‰ over the last two decades, and interannual salinity fluctuations in any season were below 1‰, being in the range of 17.26 – 18.39‰. The pH values in the surface water on the farm varied in the range of 8.14 – 8.88, with the median being 8.35 (Kapranov et al. 2021b; Kuftarkova et al. 2006).

The seabed under the farm (Station 2) consists of highly silted sand with rare boulders. The salinity in the farm water gradually increases from the surface to the bottom. The median difference between the bottom and surface salinities is 0.1‰, and the median difference between the respective pH values is nearly 0.

The innermost part of Sevastopol Bay (Station 3) is a typical estuary, with the Chernaya River, a major source of heavy metals (Gruzinov et al. 2019), flowing into this area. Close to this sampling site, there are a shipbreaking plant and a city power plant, which are the additional sources of heavy metal pollution. In the inner part of the bay, the salinity of the upper layer varied in the range from 11.16 to 17.74‰. This area is characterized by an increase in the water salinity from the surface to the bottom and seawards, with a pronounced wedge of salt water penetrating relatively far upstream from the river mouth. The pH value at the sampling site was 8.42 (Boltachev et al. 2010), and the mean TRIX value in Sevastopol Bay was 4.01 (Slepchuk et al. 2017), which corresponds to the moderate trophic level.

2.3 Analytical sample preparation

The mussel shells were cleaned of epifauna, algae and mineral residue with a knife and wire brush and washed with seawater. Then, each mussel was dissected by cutting the adductor muscle using a plastic scalpel to isolate individual body parts (soft tissues, byssus, and shell liquor), which were further analyzed as separate samples. From the open shell, after cutting the mantle in the anterior part, the turbid shell liquor was poured in dry test tubes pre-cleaned with nitric acid and deionized water, and then, byssus and soft tissues were excised with the scalpel. The weight of the shell liquor in the mussels under study was up to 30% of their total weight. The soft tissues and byssus were blotted with filter paper and weighed. The dry weight of the soft tissues was determined after oven drying at 105°C. Weights of all body parts were measured on an analytical balance AXIS ANG200C (Gdańsk, Poland). Byssus and tissue samples were placed in digestion tubes made of PTFE using a plastic spatula. For the digestion, concentrated nitric acid of analytic grade was additionally purified by sub-boiling distillation in an acid purification system DST-1000 (*Saville*, USA) and added into the digestion tubes in a proportion of 4 mL per 100 mg sample. The PTFE-capped digestion tubes with the samples and nitric acid aliquots were kept in an autoclave at 120°C for around 1.5 hours. Deionized water was used to dilute the digested samples to about 1000 mL·g⁻¹ dry weight (d.w.).

2.4 Trace element analysis

The concentrations of trace elements (Cr, Co, Ni, Cu, Zn, As, Cd, Hg, Pb) in the diluted samples were measured using inductively coupled plasma mass spectrometry (ICP-MS) on an instrument PlasmaQuant® MS Elite (*Analytik Jena*, Germany). The plasma flow was 9.0 L·min⁻¹, the sampling depth was 8 mm, and the RF power was 1.25 kW. The dwell time for each element was 10 ms, one point per peak in the peak-hopping mode. To make sure there are no significant polyatomic interferences, the measurements were carried out with the collision reaction interface (CRI) switched off and on. In the CRI mode on, gaseous hydrogen with the flow rate 40 mL·min⁻¹ was used as the skimmer gas. No internal standard was used since the undesirable matrix effects were not expected due to the high dilution of the samples. The signal drift was taken into account by measuring the element concentrations in the diluted standard IV-ICPMS-71A after each fifth sample and using an interpolating polynomial relationship to correct the apparent concentrations in time (Kapranov et al. 2021a).

The calibration curves were obtained using a multielement standard IV-ICPMS-71A (*Inorganic Ventures*, USA) and a standard solution of mercury (II) nitrate (*Supelco*, USA) diluted with deionized water. The R² coefficients for all linear calibration fits were greater than 0.999. The detection limits in this analysis range from 0.03 (Pb) to 10 (Ni) ng·L⁻¹ (Chemnitzer 2019). The accuracy and precision of the ICP-MS analysis was verified by the measurement of the element concentrations in the certified European Reference Material ERM®-CE278k (tissue of the mussel *Mytilus edulis* Linnaeus, 1758). Samples of the reference

material (0.1 g) were digested in the extra pure nitric acid and diluted with deionized water as described above. The certified and observed values are given in Table S1 (Supplementary Material).

The seawater sampling was performed in three replications at the mussel sampling sites. The seawater was filtered through a membrane filter with 0.45 µm pore size (Sartorius) and acidified with the extra pure nitric acid in a proportion of 0.1 mL per 100 mL seawater. For the ICP-MS analysis, the samples were diluted 10-fold, and the dwell time was increased to 1 s.

2.5 Statistical analysis

All results are presented as mean ± 95% confidence interval. Statistical comparisons were performed using two-way permutational multivariate analysis of variance (PERMANOVA) and permutational multivariate analysis of dispersion homogeneity (PERMDISP). The effects of the group factors (body part type and sampling location) on the overall accumulation of trace elements were analyzed and visualized by means of principal coordinate analysis (PCO) with the Euclidean distance as the similarity measure. All statistical procedures were realized in PRIMER 6.1.16 & PERMANOVA+ 1.0.6 (Clarke et al. 2014). The differences were considered significant at $P < 0.05$.

2.6 Human health risk assessment

It is common to assess the human health risk from the estimated daily intake of a pollutant, i.e. the rate of daily consumption of the pollutant per body weight unit (Zhelyazkov et al. 2018):

$$EDI = CR \times C / BW_a$$

1

where CR is the food consumption rate, i. e. the average weight of daily consumed food (in $\text{kg} \cdot \text{day}^{-1} \cdot \text{capita}^{-1}$), C is the pollutant content in the food (in $\text{mg} \cdot \text{kg}^{-1}$), and BW_a is the average human body weight, which is typically assumed to be 70 kg.

The calculated EDI values are compared with the reference data: provisional tolerable daily intake (PTDI) established by FAO/WHO (Bat and Öztekin, 2016; FAO/WHO 2011; Filippini et al. 2020), tolerable upper daily intake (UDI) set by European Food Safety Authority (EFSA 2006; Filippini et al. 2020), or oral reference dose (RfD_o) introduced by the United States Environmental Protection Agency (USEPA 2020).

Target hazard quotient (THQ) set by USEPA is the ratio of the estimated daily intake of the pollutant to its upper oral reference dose:

$$THQ = EDI / RfD_o$$

2

If $THQ < 1$, there are no likely toxic risks for a consumer in the long-term dietary exposure.

To assess the overall noncarcinogenic risks from the consumption of multiple contaminants, hazard index (HI) is used, which is the sum of the THQ values for each contaminant (USEPA 1989):

$$HI = \sum_i THQ_i$$

3

The values of $HI < 1$ indicate no likely toxic risks to human health from the prolonged consumption of contaminated food.

3. Results

3.1 Trace element contents in *M. galloprovincialis* and seawater

Table 1 shows the trace element contents in soft tissues, byssus, and shell liquor of *M. galloprovincialis* sampled at the three stations as well as the concentrations in seawater from Stations 1 and 3. The highest concentrations (above $10 \mu\text{g} \cdot \text{L}^{-1}$) at Station 1 are registered for As, Zn, and Cu and at Station 3 for Zn, As, Cu, Ni, Cd, and Cr. There are differences in the trace element contents in soft tissues, byssus, and shell liquor that are associated with the overall mean levels of each element at the sampling sites. In all samples, the zinc content is highest. The trace element contents in the animals from different biotopes decrease in the following order: Station 2 > Station 3 > Station 1.

The largest shares of the trace element contents in soft tissues among the three body parts are observed for Zn, As, and Cd (Fig. 2), which suggest that these elements are most tightly bound to organic components of soft tissues. The contents of many elements under consideration are highest in byssus. The greatest shares of the trace element contents in mussel byssus are found for Ni (81–90%), Cu (61–79%), Pb (57–79%), and Co (54–69%). The shares of the trace element contents in byssus among the three body parts (Fig. 2) decrease as follows:

(Station 1) Ni > Cu > Co > Pb > Cr > Cd > As > Zn > Hg;

(Station 2) Ni > Cu > Co > Pb > Cr > Cd > As > Zn > Hg;

(Station 3) Ni > Pb > Cu > Cr > Co > Zn > As > Cd > Hg.

Table 1

Trace metal concentration in three body parts of *M. galloprovincialis* from three biotopes ($\mu\text{g}\cdot\text{g}^{-1}$ d.w.) and in the seawater environment ($\mu\text{g}\cdot\text{L}^{-1}$)

	Station 1				Station 2			Station 3				S
	Soft tissues	Byssus	Shell liquor	Seawater	Soft tissues	Byssus	Shell liquor	Soft tissues	Byssus	Shell liquor		
Zn	88.7±11.9	98.32±18.85	6.91±2.3	27.98±5.4	131.43±20.2	145.86±9.59	5.31±1.32	111.62±14.84	121.1±18.59	19.7±5.3		1
Cu	7.8±3.6	37.36±8.54	2.19±1.7	15.27±3.5	12.47±3.4	45.22±6.77	3.1±0.51	10.59±3.56	25.22±6.77	5.42±1.7		2
As	6.0±0.88	5.37±1.87	1.96±0.69	47.84±8.9	7.79±2.42	4.97±0.86	1.56±0.45	5.5±0.44	4.1±1.1	2.1±0.9		4
Ni	3.94±1.69	39.38±10.72	1.24±1.66	7.37±2.5	4.2±1.37	44.49±7.69	0.43±0.26	3.43±2.45	24.49±7.69	2.4±0.9		1
Pb	1.7±0.26	3.4±0.8	0.92±0.35	1.28±0.38	2.89±1.01	5.36±1.61	0.66±0.18	2.95±1.32	12.64±3.3	0.49±0.15		1
Cd	1.1±0.16	3.27±0.94	1.28±0.5	2.05±0.42	1.5±0.4	2.1±0.71	0.45±0.22	1.5±0.3	0.73±0.12	0.2±0.08		1
Cr	1.1±0.16	1.52±0.50	0.18±0.21	4.39±1.1	1.49±0.43	1.73±0.22	1.2±0.94	1.2±0.4	2.24±0.4	0.8±0.2		1
Co	0.35±0.07	0.98±0.39	0.22±0.17	2.20±0.46	0.45±0.07	1.39±0.2	0.15±0.01	0.23±0.16	0.39±0.1	0.1±0.008		2
Hg	0.09±0.01	0.1±0.04	0.18±0.04	0.04±0.02	0.19±0.07	0.11±0.02	0.22±0.06	0.05±0.009	0.06±0.02	0.27±0.08		0

The shell liquor has low trace element concentrations at all stations (Table 1). However, the level of Hg in it is higher than in the other body parts (up to 71% among the three ones), which indicates that the liquor makes a considerable contribution to the mercury excretion for the organism detoxification.

3.2 Multivariate analysis of element concentrations

The overall trace element distributions in all the three body parts of the animals are significantly different ($P < 0.05$, PERMANOVA) at all the three stations (Table S2, Supplementary Material). There are no significant differences in the element content dispersions when sampling location is used as group factor ($P = 0.5641$, PERMDISP), and it can be asserted that the overall element levels are significantly different at the three stations. However, the dispersions with the body part type as group factor (except for the byssus – shell liquor pair) are significantly heterogeneous ($P = 0.0102$, PERMDISP), and it cannot be unequivocally stated whether the differences in the distributions are due to the mean element levels or dispersions.

In soft tissues and byssus of *M. galloprovincialis*, there are significant differences in the overall element contents among the stations ($P = 0.0037$ and $P = 0.0003$, respectively) with no significant differences in the dispersions (Tables S3 and S4). The pairwise PERMANOVA for the soft tissues shows significant differences between Stations 1 and 2 and between Stations 1 and 3 (Table S3). The analogous test for byssus indicates significant differences between Stations 1 and 2 and between Stations 2 and 3 (Table S4). There are no significant differences in the shell liquor trace element levels among the stations ($P = 0.2092$, Table S5).

Principal component analysis (Fig. 3) applied to the square-root-transformed standardized element contents in soft tissues and byssus shows that the vectors of all elements are oriented in the positive direction of principal component 1 (PCO1), which characterizes the overall accumulation of the elements in the mussel parts. For both soft tissues and byssus, PCO1 accounts for >40% of the dispersion. Principal component 2 (PCO2) explains 16–22% of the total variation. The negative direction of PCO2 is associated with Zn, As, Cd, Pb, Hg, and in the positive direction of PCO2, there are vectors of Co, Ni, Cr for soft tissues and Co, Ni, Cu for byssus. It appears that the PCO2 is related to the station location: its positive half-plane is dominated mostly by the observations from Station 1, and in its negative half-plane are mainly the Station-2 points. The data for Station 3 are in between and largely close to PCO2 = 0. This illustrates the overall element accumulation in mussel tissues increasing from Station 1 through Station 3 to Station 2. Interestingly, sampling location, and thus, different element levels in the environment, was not the main factor determining the dispersion of the points. It is likely that the biological characteristics such as sexual differentiation and gonadal ripening played a major role in the different element accumulation in mussels collected at sampling sites not too far away from each other (about 7 km between Station 1 and Station 3).

Fig. 4 demonstrates the results of principal component analysis of square-root-transformed standardized trace element data assorted according to the body part type, with both the element contents (Fig. 4a) and sampling location (Fig. 4b) used as variables. In both cases, most of observations related to a certain body part type are bunched in clusters separated from each other. This feature allows deciding which body part a particular suite of element contents originates from. By analogy with Fig. 3, PCO1 in Fig. 4a explaining 44.3% of the total variation is determined by the overall element accumulation (mainly associated with Pb, Cr, Cu, Ni, Co, whose vectors are close to the positive direction of PCO1) in mussel body parts, from shell liquor to byssus. The greatest contribution to PCO2 explaining 23.4% of the total variation is made by the chalcophilic elements Cd, As, Zn, Hg, which tend to be more concentrated in shell liquor and/or soft tissues. If the variable is sampling location (Fig. 4b), PCO1 explains 58.4% of the total variation and is related to all the three body parts, with shell liquor and byssus making the largest contribution, whereas PCO2 explaining 23.7% of the total variation is associated mainly with the element content dispersion in shell liquor. This indicates that it is the trace element composition in shell liquor that is most strongly affected by the station location. The vectors in Fig. 4b shifted to the origin point to the body parts in which the highest contents of the respective elements are observed: Cr, Co, Ni, Cu, Pb in byssus; Zn, As, Cd in soft tissues; and Hg in shell liquor.

3.3 Human health risk assessment

Given the potential hazards to human health from the trace element intake, trace elements in cultivated mussels were compared with maximum permissible trace element levels in edible mollusks from the European, Turkish and Russian regulations. The measured contents of Cu, As, Hg, Pb, and Zn in the mussels'

soft tissues did not exceed the maximum permissible levels, but the Cd content was above the level set in the Turkish regulations (Table 2).

It was found that EDI for all elements under consideration were lower than the corresponding reference values (RfD_o, PTDI, UDI). THQ and HI calculated from EDI according to Eqs. (1)–(3) were well below one, which indicate no likely health risk from consuming soft tissues of *M. galloprovincialis* harvested on the mollusk farm.

Table 2

Measured trace element contents and estimated daily intakes with soft tissues of *M. galloprovincialis* from the mollusk farm against upper tolerable levels and doses (RfD_o, PTDI, and UDI) established in different regulations. Target hazard quotients (THQ) and hazard index (HI) are given in the last column

	Contents, μg·g ⁻¹ w.w. ^a	European regulations, μg·g ⁻¹ w.w. (EC, 2006)	Russian regulations, μg·g ⁻¹ w.w. (SanPiN, 1992)	Turkish guidelines, μg·g ⁻¹ w.w. (Dural et al. 2007)	EDI ^b , mg·kg ⁻¹ b.w·day ⁻¹	RfD _o , mg·kg ⁻¹ b.w·day ⁻¹	PTDI, mg·kg ⁻¹ b.w·day ⁻¹	UDI, mg·kg ⁻¹ b.w·day ⁻¹	THQ
Zn	19.29	-	200	50	7.3·10 ⁻⁴	0.3	1	0.36	2.4·10 ⁻³
Cu	1.70	-	30	20	4.5·10 ⁻⁵	0.04	0.5	0.07	1.1·10 ⁻³
As	1.30	-	2	-	5.1·10 ⁻⁵	0.0003	0.002	-	1.5·10 ^{-2c}
Ni	0.74	-	-	-	3.1·10 ⁻⁵	0.002	0.003	0.0004	1.6·10 ⁻³
Pb	0.37	1.5	10	10	1.4·10 ⁻⁵	0.002	0.004	-	6.8·10 ⁻³
Cr	0.27	-	-	-	8.4·10 ⁻⁶	-	-	-	-
Cd	0.24	1.0	2	0.1	9.5·10 ⁻⁶	0.001	0.001	0.0004	9.5·10 ⁻³
Co	0.08	-	-	-	2.7·10 ⁻⁶	0.0003	-	0.0002	9.0·10 ⁻³
Hg	0.02	-	-	0.5	7.1·10 ⁻⁷	0.0003	0.0006	0.0006	2.4·10 ⁻³
HI									4.8·10 ⁻²
^a factor of conversion from dry to wet weight (w.w.) is 4.6									
^b factor of conversion from dry to cooked weight is 3.82 (Biandolino et al. 2021)									
^c converted from total to inorganic As using the factor 0.09 (Neff, 2002)									

4. Discussion

M. galloprovincialis can absorb trace elements from water, as well as ingest with phytoplankton and other suspended particles entering the digestive system (Haryono and Kilawati, 2017; Mikac et al. 1996). Trace element entering the food chain can be accumulated in the mollusk tissues to hazardous levels and be harmful to mollusk consumers' health (Manahan 2000). Cd, Pb, Hg and inorganic As have a negative impact on organisms and are harmful even in trace amounts. They cannot be metabolized into harmless forms and are accumulated in the human body over time causing chronic illness and other health problems (Myriam 2021; Stankovic and Jovic 2012). In the present study, the content of the most toxic elements in soft tissues of *M. galloprovincialis* from the Black Sea decreased in the following order: As > Pb > Cd > Hg. The same distribution was observed for *M. galloprovincialis* from the southeastern Adriatic, Montenegro (Stankovic et al. 2011).

As emphasized in (Horne 1969), the seawater of different oceans and seas contains all trace elements. Their concentrations increase in coastal waters, which are under impact of many anthropogenic factors. The mussel sampling sites under study (Fig. 1) are affected by numerous discharges, including untreated domestic wastewater, shipyard and dock effluents, storm water outlets, urban industrial emissions, farmland runoff (containing fertilizers and pesticides), and sewage of fleet stationed in city bays (Gruzinov et al. 2019; Smirnova and Riabinin, 2013). As mentioned in Materials and Methods, seawater at Station 3 is more heavily polluted than at Station 1, which accounts for the higher trace element concentrations at Station 3 (Table 1).

In benthic mussels collected from the seafloor under the farm (Station 2), the trace element contents in soft tissues were highest. It was noted that seafloor sediments are the main repository of trace elements, including potentially hazardous metals, in marine environment (Okoro et al. 2012; Sakai et al. 1986). Consequently, sediments can be considered indicators of trace element pollution of aquatic environment. Although many trace elements, being micronutrients, are necessary for the normal physiological activity of aquatic organisms, many of them can be concentrated in excess of physiological requirements in soft tissues and become toxic (Briffa et al. 2020; Rouane-Hacene et al. 2015; Stankovic and Jovic 2012).

Growing mussels assimilate essential elements, which can be divided into two groups based on their levels in tissues (Horne 1969). The first group is the structural elements that are most abundant in mussel tissues and form organic and inorganic compounds in the mollusk tissues: shells, soft tissues, and byssal threads. The second group includes catalytic elements (micronutrients) that are present in small amounts, mainly as components of compounds that catalyze biochemical processes. Most of the trace elements under study (Cr, Co, Ni, Cu, Zn, As) have low atomic numbers (from 24 to 33) and play a role in the

functioning of marine organism cells, being minor constituents of proteins, carbohydrates, and lipids. Ions of catalytic elements Cu, Zn, Cd, Co, Ni are enzyme activators (cofactors) (Horne 1969).

As shown in Fig. 2, considerable levels of Zn, As, Cd were observed in soft tissues and byssus of mussels from the biotopes under consideration. Byssus is known to not only adsorb trace elements from seawater, but also to participate in the extraction of some elements from soft tissues (Koide et al. 1982; Szefer et al. 1999; Yap et al. 2003b). High abundances of some elements (Ni, Cu, Pb, Co) in byssus are associated with histidine and lysine residues, which are the components of byssal proteins. These elements form strong coordination bonds with histidine and lysine (Leberman and Rabin 1959; Naik et al. 2012; Yamauchi and Odani, 1996), which strengthen byssus (Babarro and Reiriz 2010; Lucas et al. 2002; Reinecke et al. 2017).

The content of **Zn** in soft tissues or byssus reached 40–45% of the total Zn accumulated in the studied body parts and varied in the range from 88.7 to 145.6 $\mu\text{g}\cdot\text{g}^{-1}$ d.w. (Table 1). The observed high levels of Zn in soft tissue are related to the essentiality of this trace element for mussels. Zn serves as a catalyst in many physiological processes; it regulates growth, development, reproduction and metabolic processes in mollusks (Franca et al. 2005; Viarengo et al. 1990). The content of Zn in byssus depends on its concentration in seawater and also in soft tissues as it can be extracted from the mussel body into byssus. Yap et al. (2005) noticed that if large amounts of heavy metals are accumulated in soft tissues of *M. edulis*, then the metals are transferred to byssus in almost equal proportions, and the same pattern was noted in the present research with respect to *M. galloprovincialis* (Table 1).

Cu was concentrated mostly in soft tissues and byssus. The low proportions of Cu in shell liquor (5–13%) indicated little or no copper excretion through this fluid. The results showed that the Cu content in byssus reached 61–79% (Fig. 2). It is likely that this mussel body part can both concentrate copper from seawater and withdraw this metal from the mollusk tissues. Previously, *Mytilus edulis* was also shown to contain higher copper content in byssus than in soft tissue (Szefer et al. 1997). It is known that Cu ions, like Zn, in mollusks act as cofactors of some enzymes, playing a key role in their functioning (Horne 1969; Khristoforova et al. 1994). In particular, they stimulate polyphenol oxidase, ascorbate oxidase, and other enzymatic systems. The combined effect of excess zinc and copper leads to the destruction of mollusk mitochondria and suppresses sperm motility (Earnshaw 1986; Lyngby and Brix 1987). The major sources of Cu in the water body in question are wastewater, transport, copper-containing fertilizers and pesticides, welding and galvanization processes, and combustion of hydrocarbon fuels.

As is a metalloid that is rarely found in nature as a free element, but its compounds are found in air, water, soil and all living tissues (Kaur et al. 2011; Mayer et al. 1993). In aquatic organisms, arsenic occurs in organic and inorganic forms. Inorganic As compounds are highly toxic, whereas natural organoarsenicals are non-toxic (Neff 2002). The most important anthropogenic source of arsenic pollution off the southwestern coast of Crimea is chemical warfare burial sites with the warfare containing lewisite (Smirnova et al. 2005). Inorganic As is strongly carcinogenic. Depending on oxidation state, cell type, concentration and exposure, it can induce excessive apoptosis (Chiarelli and Roccheri 2014). In our study, the arsenic content in mussels from the mollusk farm did not exceed $1.3 \mu\text{g}\cdot\text{g}^{-1}$ w.w., which is below the maximum permissible level according to the Russian regulations (Table 2). In soft tissues of mussels, organic arsenic accounts for 91% of the total arsenic (Neff 2002). There is evidence that in the mollusk farm area, the As concentration in seawater exceeded the maximum permissible level; however, *M. galloprovincialis* did not accumulate this element in soft tissues in large amounts (Ryabushko et al. 2017), in agreement with our results.

In our studies, the highest As content was in soft tissues of benthic mussels (Station 2), which fact was noted also by Wu et al. (2014) for species sampled in the East China Sea: the closer the organism's habitat to the sediments, the higher the arsenic content in it. Despite the fact that the As content share in byssus reached as much as 40%, its content in soft tissues was significantly higher than in byssus and shell liquor ($P = 2\cdot 10^{-4}$ and $P = 6\cdot 10^{-5}$, respectively, Table S6). Ünlü and Fowler (1979) noted that active secretion of arsenic in byssus of the mussel *M. galloprovincialis* contributes to the elimination of arsenic from the mussel body.

Ni is discharged into the aquatic environment mainly with municipal and industrial wastewaters. For some invertebrates, it is a trace element with essential biological role since they produce enzymes that contain Ni in active sites. However, in high concentrations, Ni is toxic (Chalkiadakis et al. 2013). It is known that nickel, along with cadmium and arsenic, inhibits DNA repair mechanisms (Lucas et al. 2002). In byssus, the Ni content percentage was highest among all the elements under study (Fig. 2), and it was 8–10 times higher than in soft tissues (Table 1). Earlier, Szefer et al. (2002) noted that among all metals (Hg, Cd, Pb, Ag, Cu, Zn, Cr, Ni, Co, Mn и Fe), Ni was deposited in greatest proportions in byssus of *Mytilus edulis trossulus* (southern Baltic) as compared with its soft tissues.

Pb is a heavy metal that is toxic to animals and humans. It damages nervous system and causes various disorders (Nava-Ruiz et al. 2012). The main source of lead in the environment is anthropogenic activity (in particular, this heavy metal is released from anti-fouling paints and acid batteries). Lead toxicity in marine invertebrates depends on species identity and its life stage (Chiarelli and Roccheri 2014). In the present research, at all stations, the Pb content in byssus of *M. galloprovincialis* was significantly higher than in its soft tissues ($P < 10^{-5}$, Table S7), and we can suggest that this element is actively excreted through byssus. The highest percentage of Pb among the body parts was in byssus of *M. galloprovincialis* from Station 3, up to 79% (Fig. 2c). The high Pb concentration in this water area was apparently due to the shipbreaking plant activity and the inflow of the Chernaya River that can transport large amounts of Pb (Gruzinov et al. 2019). Thus, the results obtained support the idea that *M. galloprovincialis* byssus is an excellent indicator of the seawater pollution with lead. The Pb contents in soft tissues were comparable with other researchers' results obtained using mussels from the southwestern Black Sea (Bat et al. 2018; Belivermiş et al. 2016; Çulha et al. 2017).

Cd is a very toxic environmental pollutant and cell poison that causes different types of damage including cell death. A high percentage of its content was observed in soft tissues, up to 62% (Fig. 2c). Significant differences were found for the Cd content between soft tissues and byssus ($P = 4\cdot 10^{-4}$, Table S8). Also, significant differences in the Cd content were registered between soft tissues and shell liquor ($P = 7\cdot 10^{-4}$), which suggest that shell liquor is not used for the removal of this toxicant. Li et al. (2006) noted that bivalves do not regulate Cd levels and usually accumulate this element. Cd is accumulated in cells by

interacting with cellular components and molecular targets (Chiarelli and Roccheri 2014; Kingsley and Frazier 1979). In invertebrates, it stimulates the expression of antioxidant enzymes, metallothioneins and heat shock proteins. It inhibits the expression of digestive enzymes, esterases and phospholipases. Cd also affects tissue organization, immune responses, and cell cycles by inducing apoptosis (Sokolova et al. 2004). The relatively low levels of Cd as compared to higher levels of Pb in byssus may be a result of the more efficient transfer of Pb from soft tissues to byssus, as opposed to Cd, which is strongly accumulated in soft tissues in hepatopancreas (Szefer et al. 2006). The high content of cadmium in soft tissues of mussels appears to be related to its high levels in sediments in the adjacent bays (Gubanov et al. 2010).

Sources of **Cr** in the environment can be both anthropogenic and natural. Natural chromium occurs mainly in trivalent state, whereas hexavalent Cr (VI) in the environment originates almost entirely from human activities (Liang et al. 2021; Sacchi et al. 2021). This metal, especially in the hexavalent state, is a very toxic trace element posing certain threats to coastal ecosystems. Coastal chromium pollution is mainly due to the discharge of untreated or poorly treated industrial wastes. Cr (VI) is 30 times as toxic as Cr (III), and it is both mutagenic and carcinogenic (Natale et al. 2000). In the southwestern Crimea, the anthropogenic source of chromium can be shipyards and the shipbreaking plant, and the natural source of chromium is volcanic rocks of the Crimean peninsula.

The Cr content in our studies in soft tissues and byssus of *M. galloprovincialis* was more than twice as low as in *Mytilus edulis trossulus* from polluted waters of the Gulf of Gdańsk, Poland (Szefer et al. 2002). Our results showed that the content of Cr in byssus was significantly higher than that in soft tissues ($P < 1 \cdot 10^{-5}$, Table S9). Earlier, the same pattern was noted for *M. edulis trossulus* from the Gulf of Gdańsk, and byssus was recommended for use in chromium pollution biomonitoring (Szefer et al. 2002). It is known that Cr (VI) significantly affects the functional and structural parameters of mussel gills, and this indicates that this tissue is the main target of the Cr (VI) exposure (Ciacci et al. 2012). There is evidence of the effect of Cr (VI) in vitro on immune system of *M. galloprovincialis* (Barmo et al. 2011). In *M. edulis*, the DNA chain breaks in the gill cells under the influence of Cr (VI) (Emmanouil et al. 2007). An increase in the total content of Cr (VI) in tissues and destabilization of lysosomal membranes were observed in digestive gland of *M. galloprovincialis* treated with a high concentration of this heavy metal ($100 \mu\text{g} \cdot \text{L}^{-1}$), and oxidative stress occurred in this organ (Barmo et al. 2011).

Co is an important component of vitamin B₁₂ and a cofactor for several enzymes (Lehninger 1976; Nolan and Dahlgaard 1991). In all the water areas under study, cobalt was slightly more accumulated in soft tissues (up to 32% among the three body parts). In relatively low concentrations, this trace element becomes toxic. In mammals, it can induce apoptosis, necrosis, or inflammatory response in the body and is genotoxic (Briffa et al. 2020).

Co was accumulated and extracted mainly by byssus and, to a slight extent, by shell liquor (Table 1, Fig. 2). Significant differences in the Co content were found between soft tissues and byssus ($P = 2 \cdot 10^{-5}$, Table S10). As shown by Szefer et al. (2006), mytilids from industrialized regions also showed higher concentrations of Co in byssus than in soft tissues. Byssus of *M. edulis* was proposed to be used in Co pollution biomonitoring (Szefer et al. 1999).

There are three chemical forms of **Hg** in the environment: elemental, organic, and inorganic. These forms are mutually transformable into each other, and they all can cause systemic toxicity (Graeme and Pollack 1998). Hg can occur naturally in the environment or come from anthropogenic sources. A significant fraction of Hg is volatilized and returns to the atmosphere, but much of this metal entering the coastal areas precipitates due to very low solubility of its compounds (Stankovic and Jovic 2012). Anthropogenic sources of Hg in the water areas under consideration can be ballast discharge from submarines and domestic wastewater. Natural sources of mercury include numerous methane seeps off coasts of southwestern Crimea and in bays of Sevastopol (Egorov et al. 2011). This element is accumulated in sediments, which are its main sink. It was noted that most of the inorganic and organic Hg compounds in the aquatic environment are adsorbed on suspended matter and settle to the bottom (Schiff 2000). For this reason, the highest content of mercury in soft tissue was found in mussels sampled from the seafloor under the farm. In sediments, bacteria can convert inorganic mercury to methylmercury, the most toxic Hg compound (Harada et al. 1998).

In our study, the mercury contents in soft tissues and byssus were below $0.2 \mu\text{g} \cdot \text{g}^{-1}$ (Table 1). The same pattern was observed for *M. galloprovincialis* from the Cantabrian coast (Bartolome 2010). It should be noted that in *M. galloprovincialis*, Hg was removed from soft tissues mainly through shell liquor (up to 71%). Our results show that the function of byssus and shell liquor in *M. galloprovincialis*, among others, is to protect its vitals by extracting and excreting toxic trace elements.

5. Conclusions

In the present study, the distribution of trace elements (Cd, Cu, As, Ni, Hg, Pb, Zn, Cr, Co) in soft tissues, byssus, and shell liquor of the mussel *M. galloprovincialis* living in the coastal waters of the Black Sea off southwestern Crimea has been studied. At all the sampling stations, the highest content has been noted for Zn (up to $131.4 \pm 20.2 \mu\text{g} \cdot \text{g}^{-1}$ d.w.). The trace element contents in soft tissues of *M. galloprovincialis* at all stations have been found to decrease in the following order: $\text{Zn} > \text{Cu} > \text{As} > \text{Ni} > \text{Pb} > \text{Cd} > \text{Cr} > \text{Co} > \text{Hg}$. The contents of Cd, Cu, As, Hg, Pb, and Zn in soft tissues of the cultured mussels did not exceed the permissible levels according to different regulations, except for Cd that exceeded the maximum level established in Turkish guidelines. When comparing the estimated daily intakes (EDI) of these elements with tolerable values set by international authorities (WHO, European Commission, USEPA), it has been found that EDI for all the elements were below the threshold values, indicating no likely risks of consuming the mussels cultivated on the mollusk farm.

The results have shown that the contents of the trace elements in question in soft tissues, byssus, and shell liquor of the mussels from different biotopes decreased in the following order: Station 2 (seafloor under the mollusk farm) > Station 3 (innermost part of Sevastopol Bay) > Station 1 (mollusk farm). The proximity of the sediments appears to be the most important factor affecting the element accumulation. Significant differences have been registered in the overall element contents in *M. galloprovincialis* from the three stations under study. Principal component analysis has shown a tendency of the data points to clustering with the location as the grouping factor.

A similar clustering tendency has been observed when the grouping factor is body part type. However, in this case the analysis of variance has not allowed judging about the differences in the overall element levels in the three body parts (except for the byssus – shell liquor pair, in which byssus has significantly higher element contents) due to the significantly inhomogeneous dispersions.

The contents of individual elements vary significantly in different mussel body parts. For example, Zn, Cd, and As have been found to have the largest shares in soft tissues: 44-63%, 34-62%, and 45-54%, respectively. Byssus has been characterized with the highest percentages of many trace element contents: Ni (81-91%), Cu (61-79%), Pb (57-79%) and Co (54-69%). This organ performs the function of not only attachment to the substrate, but also extracts heavy metals from soft tissues. Byssus of *M. galloprovincialis* is a better bioindicator of the environment pollution with Ni, Cu, Pb, Co, and Cr than its soft tissues.

For the first time, trace elements have been quantified in shell liquor and the role of shell liquor as a detoxifying system has been demonstrated in this study. The highest content of mercury in shell liquor (up to 71% among the body parts) suggests that this contaminant is efficiently accumulated and removed from the organism through this fluid.

The data analysis has shown the need for the further study of the role of byssus in the distribution of trace elements in mussel tissues. In particular, the question about the ratio of the accumulating (from the external environment) and eliminating (from the mussel body) functions of byssus remains open.

Declarations

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Authors' contributions CNS and PMA took part in collecting material, setting up and conducting the experiment, processing the data obtained and discussing the materials. KSV edited the final version of the manuscript, and contributed to the ICP-MS and data analysis. SLL contributed to the data analysis. BNI prepared analytical samples and contributed to the ICP-MS analysis.

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Data Availability The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Compliance with ethical standards

Ethics approval Ethical approval were observed during the experiment. All sampling was conducted in accordance with the Russia Federation law on harvest of biological resources in natural waters.

Consent to participate All authors consent to participate

Consent for publication All authors consent to publication

Competing interests The authors declare that they have no competing interests

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Figures

Figure 1

Location of the sampling stations (St. 1–3).

Figure 2

Proportions of the trace element contents in soft tissues, byssus and shell liquor of *M. galloprovincialis* sampled at (a) Station 1, (b) Station 2, and (c) Station 3

Figure 3

Principal component analysis of trace element contents by location factor in (a) soft tissues and (b) byssus of *M. galloprovincialis*. S1 = Station 1, S2 = Station 2, S3 = Station 3. Vectors: projections of the Pearson correlation of variables onto the PCO1 – PCO2 plane.

Figure 4

Principal component analysis of trace element contents in mussel body parts with (a) element contents and (b) locations as variables. Vectors: projections of the Pearson correlation of square-root-transformed standardized element contents onto the PCO1 – PCO2 plane.

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