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Shell Organic Matrix (*Conchix*) of the Mediterranean Mussel *Mytilus galloprovincialis* L. as the Medium for Assessment of Trace Metals in the Boka Kotorska Bay

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Abstract: The content of trace metals, namely Zn, Cu, Fe, Mn, Pb, Cd, and Hg, in four types of media, i.e., soft tissues, shells, and the products of shell demineralization (organic matrix—*conchix* and extract) of the Mediterranean mussel *Mytilus galloprovincialis* L., at three sites in the Boka Kotorska Bay of the Adriatic Sea were determined. The main aim was to investigate the accumulation patterns of trace metals in *conchix* and their possible relationship with other tested media. *Conchix* weight within a group of mussels from Sv. Nedjelja was significantly higher in comparison with the IMB, while *conchix* % in the shell showed a negative correlation with dry shell weight. The highest metal pollution index (MPI) values found in the soft tissues of mussels from Sv. Nedjelja, Cogi, and the IMB were 2.319, 2.711, and 2.929 µg g^{-1}, respectively. PCA analysis showed similarities in trace metal accumulation in all media except *conchix*. According to CCA analysis, *conchixes* were grouped around Cu, Fe, and Hg, while Cd and Zn were in correlation with the soft tissues. Moreover, the shells were in correlation with Mn. Simple isolation with high yield, close contact to the environment in comparison with calcified shell layers, and susceptibility to possible pollution sources due to the accumulation of specific metals are the main reasons to consider *conchix* of *M. galloprovincialis* as a medium with potential in trace metal assessments of marine ecosystems.

Keywords: organic matrix; periostracum; trace metals; shell; Adriatic Sea

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1. Introduction

Bivalves are globally distributed along the coasts of many countries inhabited by more than the half of world's population. Several features, such as sessile nature, filter-feeding, and bioaccumulation of trace metals in much higher concentrations in comparison with the ambient environment make bivalves suitable bioindicators of trace metal contamination [1]. During the last decade, the Mediterranean mussel *Mytilus galloprovincialis* (L.) has been used in many trace metal studies [2–5]. Moreover, *M. galloprovincialis* L. was defined as a common indicator for trace/heavy metal pollution according to the United Nations' environmental program for the Mediterranean region [6].

The adverse effects of trace metals on marine organisms and indirectly on humans via the consumption of seafood are well known [7–9]. The anthropogenic impacts on many coastal waters are becoming increasingly pronounced due to large amounts of effluents enriched with toxic trace metals [10]. Physicochemical and biogeochemical processes may produce the heterogeneous distribution of trace metals in different environmental matrices of the Adriatic estuaries [11]. The Boka Kotorska Bay, southern Adriatic Sea, records constant growth of maritime transport with higher numbers of cruise ship visits per year [12]. These large ships contribute to increased sediment re-suspension, which can lead to the transition of trace metals from sediments to the water column. So far, the investigation of trace metal content in the Boka Kotorska Bay in seawater, sediments, sea grasses, and bivalves [13–15] has been conducted.

Bivalves' soft tissues, rather than shells, were more popular among researchers in metal bioaccumulation studies [16]. Despite less involvement, trace metal assessment in bivalves' shells was widely accepted and applied in aquatic ecosystem monitoring [17–19]. However, short-term laboratory treatments indicated that bivalve's shells could be less suitable for metal detection due to a slower accumulation rate in comparison with the soft tissues [20]. Although bivalves were recognized as good bioindicators, a more accurate methodology of using bivalves in trace metal biomonitoring should be developed [21]. To improve the methodology, trace metal assessment in different mediums/parts of bivalves, namely the calcite and aragonite shell layers separately, both calcareous shell layers without periostracum, and separated periostracum and byssus threads [22–26], was carried out.

The periostracum is a thin yellow to brownish organic layer at the shell surface that envelops calcified parts, protects bivalves against dissolution in acidic water, prevents fouling by microorganisms, and tends to accumulate trace metals from the environment [27]. The surfaces of periostracum and deposited crystals are covered by a proteinaceous organic matrix [28]. Recently, the isolation procedure of organic matrices (*conchixes*) from the Mediterranean mussel has been published [29]. During the process, an intact shell organic matrix, including the periostracum, was obtained. Considering the limitations of the soft tissues and shells to a certain degree, our idea was to examine *conchix* as a relatively novel medium to improve the methodology for trace metal assessment.

Keeping in mind the above, the main aim of this study was to investigate the accumulation patterns of certain trace metals in the products of shell demineralization (*conchix* and shell extract), soft tissues, and the shells of the Mediterranean mussel at three sites within the Boka Kotorska Bay, Montenegro for the marine ecosystem assessment.

2. Materials and Methods

2.1. Description of Sampling Sites

We collected the Mediterranean mussels *M. galloprovincialis* on the 2nd of November 2019 from the three mussel farms: Sv. Nedjelja (42°27′29.60″ N 18°40′20.90″ E), Cogi (42°29′8.20″ N 18°44′46.06″ E), and the Institute of Marine Biology Kotor (IMB) within the Boka Kotorska Bay, Adriatic Sea, Montenegro (Figure 1). The first site, Sv. Nedjelja, is located in the Tivat Bay, at the entrance to Kotor Bay with a sandy bottom and a higher level of seawater exchange during the year. The second site, Cogi, is a fish and mussel farm near Orahovac, in the smaller Kotor Bay, while the IMB is placed near the port of Kotor. The Cogi and IMB sites were muddy sediments, had a lower level of seawater exchange due to higher inner positioning within the bay, and had higher seasonal salinity oscillations caused by freshwater inflow from the land and underwater springs. All three sampling sites had the same level and sources of anthropogenic impact in the Bay area, such as land-based sources, air-based sources, and maritime transportation [30].

2.2. Sampling and Preparation

Mussels of a similar shell length (50–70 mm), 50 per site, were collected, placed into polyethylene bags, kept in a cooler box with ice, and transported to the laboratory. After cleaning and rinsing with Milli-Q water (Millipore SAS, Molsheim, France) the soft tissues were carefully separated from the shell using a ceramic knife while the byssus and the ligament were removed. The shells and the soft tissues (Figure 2b) of the individual mussels were analyzed. The shells were divided into two halves; one shell valve was used for the determination of trace metals, while the other shell valve (Figure 2a) was subjected to the demineralization process. The soft tissues were frozen at −18 °C, freeze-dried at −40 °C for 48 h (Alpha 2–4 LD plus, CHRIST, Hagen, Germany), reduced to powder, and homogenized.

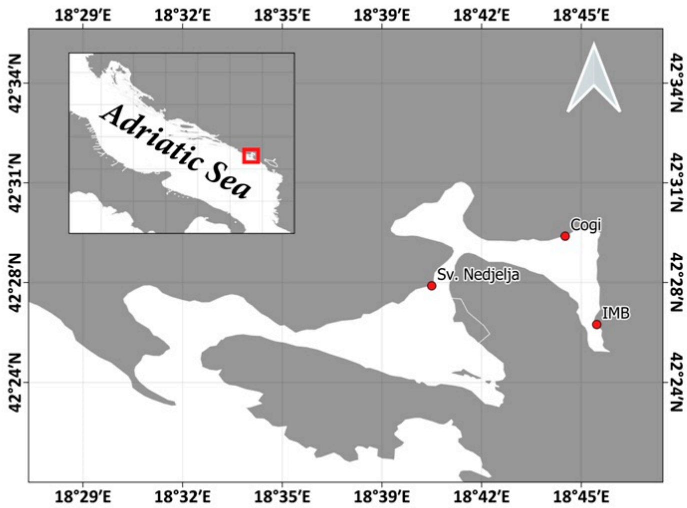

Figure 1. Sampling sites in the Boka Kotorska Bay, Montenegro. Scale: the sides of the large square grid represent a length c. 10 km.

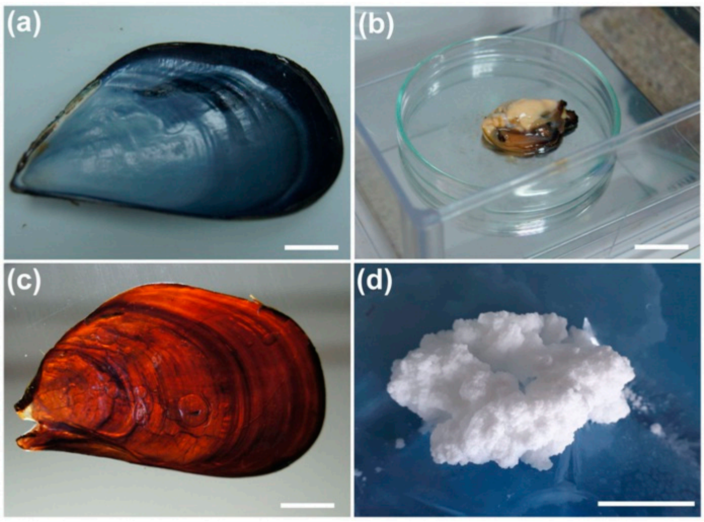

Figure 2. The samples of the Mediterranean mussel *M. galloprovincialis* L. used for trace metal analyses: (**a**) shell valve; (**b**) soft tissue, i.e., the products of shell demineralization; (**c**) *conchix*; and (**d**) shell extract. Scale bar = 1 cm.

2.3. Isolation of Conchix

Decalcification of three groups of the Mediterranean mussel was performed using Ethylenediaminetetraacetic (EDTA)-disodium-salt-based solution. Shell valves used for the isolation of *conchixes* were placed in a glass with a previously prepared EDTA-based solution (pH 7.25) and stored in an incubator at 39 °C. At every 24 h, shells were rinsed with dH_2O, and the solution was replaced. After the replacement, the used solution was deposited in the tank and dried for 3–4 weeks to obtain shell extract (Figure 2d). Isolation was completed after five days, and the obtained *conchixes* (Figure 2c) were carefully rinsed with dH_2O and dried in the air. The complete procedure of solution preparation used for demineralization and *conchix* isolation was thoroughly described in Ehrlich et al. [29].

2.4. Size and Weight Measurements

Allometric parameters, such as size and weight of the whole shell, shell valves, wet and dry tissues, and dried *conchixes* of the Mediterranean mussel, were measured. The length and width were measured using an electronic digital caliper (Maurer, Padova, Italy), while an electronic balance (Kern & Sohn GmbH, Balingen, Germany) was used for the weight measurement. According to values of *conchix* weight and dry shell valve weight, *conchix* % in the shell valve was calculated using Microsoft Office Excel 2007.

2.5. Condition Index

The condition index (CI) was calculated according to [31] as the ratio between dry soft tissue weight (DSTW) and dry shell weight (DSW): CI = DSTW (g)/DSW (g).

2.6. Trace Metal Determination

The mussel samples were analyzed for Fe, Mn, Zn, and Cu contents according to the International Organization for Standardization method [32] by atomic absorption spectrometry using a flame atomic absorption spectrometer (Varian SPECTRA AA-10, Varian Techtron Pty Limited, Mulgrave, VC, Australia), equipped with a flame furnace and operated with an air acetylene flame after mineralization by dry ashing. Dry ashing was conducted according to Tomovic et al. [33]. Briefly, after drying, the sample was charred on a hot plate and then incinerated in a muffle furnace at 450 °C overnight. After ash was obtained, it was moistened, treated with 10 mL of HCl/diH2O (1:1, *v*/*v*), and evaporated to dryness. The ash was redissolved with 10 mL of HCl/diH_2O (1:9, *v*/*v*), transferred to a 50 mL volumetric flask, and diluted to volume with diH_2O. Heavy metal analyses of Pb and Cd were performed by atomic absorption spectrometry after dry ashing according to the EN method [34], while Hg was determined using a direct mercury analyzer, namely the AMA 254 Advanced mercury analyzer (Altec, Prague, Czech Republic), based on the principles of sample thermal decomposition. The obtained results of the investigated elements in mussels were expressed in µg/g of sample dry weight. Each obtained value is the mean of five determinations. Procedural blanks were analyzed once for every five samples. To avoid possible contamination, all employed glassware and equipment were acid-washed.

2.7. Metal Pollution Index

To compare the total content of trace metals at the 3 sampling sites in different mediums, the metal pollution index (MPI) was used. The MPI was obtained using the following equation: $MPI = (M1 \times M2 \times M3 \times \ldots \times Mn)^{1/n}$, where M1 is the concentration value of the first metal; M2 is the concentration value of the second metal; and Mn is the concentration of the metal n expressed in $\mu g\ g^{-1}$. The number of metals was (n) = 7. Any MPI > 1 indicates that the investigated site is polluted, whereas any MPI < 1 indicates no pollution [35–37].

2.8. Statistical Analyses

Statistical analysis of the results was carried out using Statistica 14 Software Inc., (Palo Alto, CA, USA) [38]. The Kolmogorov–Smirnov test for normality of distribution was used prior to statistical analysis. Since the data were not in line with the requirements for the application of parametric tests, differences between each group were tested using the Mann–Whitney U test. Correlation analyses were carried out using the Spearman correlation test with a significance level of $p < 0.05$. Principal component analysis (PCA) was used to assess differentiation among the sites and mediums analyzed based on the trace metal level. The sites were compared through canonical correlation analysis (CCA) to determine their relationships relative to the levels of trace metal accumulation in the following four media: soft tissues; shells; shell organic matrix, i.e., *conchix*; and shell extracts of the Mediterranean mussel.

3. Results

The *conchix* weight within the group of mussels from Sv. Nedjelja was significantly higher in comparison with that from the IMB ($p = 0.043$), while significant differences between other groups, analyzed by the Mann–Whitney U test, were not observed ($p > 0.05$; Figure 3a). The weight of the dry shell valve in mussels from Cogi was significantly lower in comparison to that from the other two sites ($p < 0.05$), while the differences between Sv. Nedjelja and Cogi were not significant ($p > 0.05$).

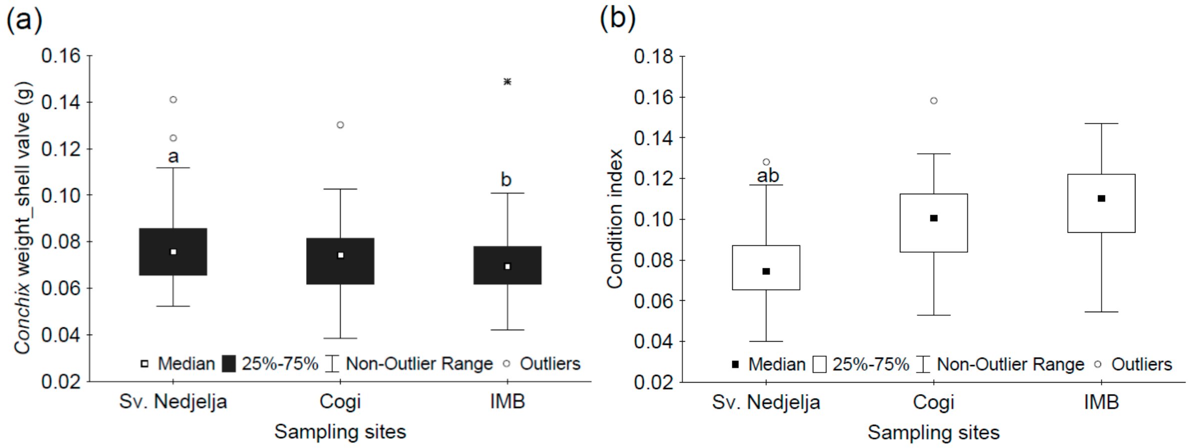

Figure 3. (**a**) *Conchix* weight in the shell valve; (**b**) condition index within the group of *M. galloprovincialis* L. specimens sampled from Sv. Nedjelja, Cogi, and the IMB; a and b—statistically significant difference ($p < 0.05$) in intercomparison; ab—statistically significant difference ($p < 0.05$) in comparison with the other sampling sites. *—extremes. Statistical significance was tested using the Mann–Whitney U test.

In general, a significant correlation ($p < 0.05$; $r = 0.562$) between the *conchix* weight and the weight of the dry shell valve within the mussels' samples from all studied sites was determined using the Spearman correlation test.

The values of the condition index (CI) in mussels from Sv. Nedjelja were significantly lower in comparison with Cogi and the IMB ($p < 0.05$), while there were no statistically significant differences between Cogi and the IMB ($p > 0.05$; Figure 3b).

A statistically significant correlation between the *conchix* weight and CI within the mussels' samples from all studied sites was not observed ($p = 0.298$; $r = -0.085$).

Conchix % in the shell valve within the group of mussels from Cogi was significantly higher in comparison with the other two sites ($p < 0.05$), while the differences between Sv. Nedjelja and Cogi were not statistically significant ($p > 0.05$; Figure 4a).

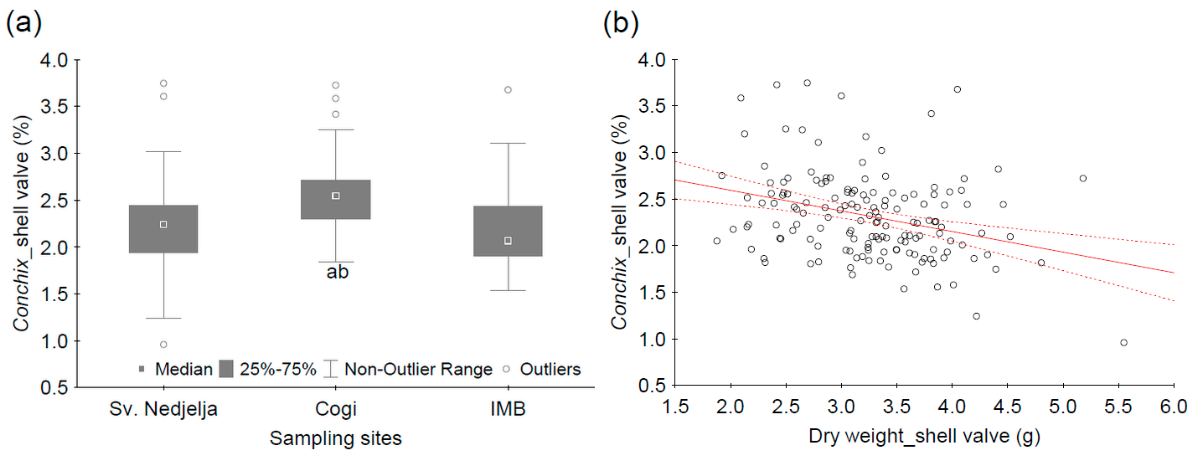

Figure 4. (**a**) *Conchix* % in the shell valve and (**b**) correlation of *conchix* % with a dry weight of the shell valve within the group of *M. galloprovincialis* L. specimens sampled from Sv. Nedjelja, Cogi, and the IMB. ab—statistically significant difference ($p < 0.05$) in comparison with the other sampling sites. Dashed lines represent confidence bands lines while the fitting line is in the middle. Statistical significance ($p < 0.05$) was tested using the Mann–Whitney U test, while the correlation was determined by the Spearman correlation test.

Data on *conchix* % in the shell valve from all studied groups showed a significant correlation with the CI ($p = 0.044$; $r = 0.163$) and a negative correlation ($p < 0.05$; $r = -0.316$) with the weight of the dry shell valve (Figure 4b).

Table 1 presents trace metal concentrations and the MPI in four different media of *M. galloprovincialis* L., namely shell (KS, KC, KI), soft tissue (TS, TC, TI), *conchix* (OS, OC, OI), and shell extract (ES, EC, EI) sampled from Sv. Nedjelja (S), Cogi (C), and the IMB (I). The highest contents of Zn, Mn, and Cd were found in the soft tissues, while the highest contents of Cu, Fe, and Hg were found in the *conchixes*. Lead contents were generally highest in soft tissues with a mean value of 0.92 μg g^{-1}, although the highest single concentration (1.22 μg g^{-1}) was found in *conchixes* from Sv. Nedjelja. The lowest concentrations were generally found in extracts, although Pb (<0.25), Cd (<0.03), and Hg (<0.001) contents were below the detection limits in both extracts and shells, except for Pb content in the shell sample from the IMB (0.31 μg g^{-1}). In addition, the lowest Fe contents were found in the shells as well. The highest MPI values were found in the soft tissues of mussels from Sv. Nedjelja, Cogi, and the IMB were 2.319, 2.711 and 2.929 μg g^{-1}, respectively (Table 1). The *conchixes* from Sv. Nedjelja had higher MPI values (2.158 μg g^{-1}) in comparison with Cogi and the IMB, which were 1.214 and 1.163 μg g^{-1}, respectively. MPI values of the shells and shell extracts at all three sites were lower than 1.

Table 1. Trace metal concentrations and metal pollution index (MPI) within the group of *M. galloprovincialis* L. specimens sampled from Sv. Nedjelja (S), Cogi (C), and the IMB (I) in the shell (KS, KC, KI), soft tissue (TS, TC, TI), *conchix* (OS, OC, OI), and shell extract (ES, EC, EI).

Trace Metals (μg g^{-1})	Medium/Site											
	KS	KC	KI	TS	TC	TI	OS	OC	OI	ES	EC	EI
Zn	11.6	11.6	11.0	125	135	104	7.85	13.6	7.59	5.3	12.7	4.15
Cu	2.09	3.91	2.74	2.93	3.39	3.53	15.8	11.0	12.6	2.02	1.05	2.14
Fe	0.69	0.81	0.64	21.0	11.0	35.4	68.3	21.6	40.8	3.92	4.82	3.86
Mn	2.44	2.57	2.31	2.78	3.63	3.62	2.07	1.60	2.80	1.51	1.48	1.62
Pb	<0.25	<0.25	0.31	0.54	1.16	1.06	1.22	<0.25	<0.25	<0.25	<0.25	<0.25
Cd	<0.03	<0.03	<0.03	0.69	0.73	0.41	0.06	0.067	<0.03	<0.03	<0.03	<0.03
Hg	<0.001	<0.001	<0.001	0.077	0.065	0.095	0.168	0.100	0.145	<0.001	<0.001	<0.001
MPI	0.258	0.291	0.297	2.319	2.711	2.929	2.158	1.214	1.163	0.275	0.291	0.269

Comparing location differences across the same sample groups, the highest contents of Zn (135 μg g^{-1}), Pb (1.16 μg g^{-1}), and Cd (0.73 μg g^{-1}) were found in the soft tissues of mussels from Cogi, while Cu (3.53 μg g^{-1}), Fe (35.4 μg g^{-1}) and Hg (0.095 μg g^{-1}) contents were the highest in IMB soft tissues. Manganese concentrations were almost identical for Cogi (3.63 μg g^{-1}) and IMB samples (3.62 μg g^{-1}), and both were higher than that in the soft tissues of Sv. Nedjelja mussel samples. Conversely, *conchixes* from Sv. Nedjelja mussels had the highest concentrations of Cu (15.8 μg g^{-1}), Fe (68.3 μg g^{-1}), Pb (1.22 μg g^{-1}), and Hg (0.168 μg g^{-1}). Shells from Cogi had the highest Cu (11.6 μg g^{-1}), Fe (0.81 μg g^{-1}), and Mn (2.57 μg g^{-1}) concentrations. Furthermore, the extracts from Cogi had the highest Zn (12.7 μg g^{-1}) and Fe (4.82 μg g^{-1}) concentrations, while Cu (2.14 μg g^{-1}) and Mn (1.62 μg g^{-1}) contents were the highest in extracts from the IMB.

Figure 5 presents PCA and CCA plots of trace metal concentrations in four different media (shell, soft tissue, *conchix*, and shell extract) of *M. galloprovincialis* L. sampled from Sv. Nedjelja (S), Cogi (C), and the IMB (I). PCA analysis showed similarities in trace metal accumulation in all media except *conchix*. However, the *conchix* sample from Cogi (OC) was slightly similar to extracts from the IMB and Sv. Nedjelja mussels. According to CCA analysis, *conchixes* are grouped around Cu, Fe, and Hg, while Cd and Zn are in correlation with soft tissues. Moreover, the shells are in correlation with Mn, although the highest Mn concentrations were found in the soft tissues (Table 1).

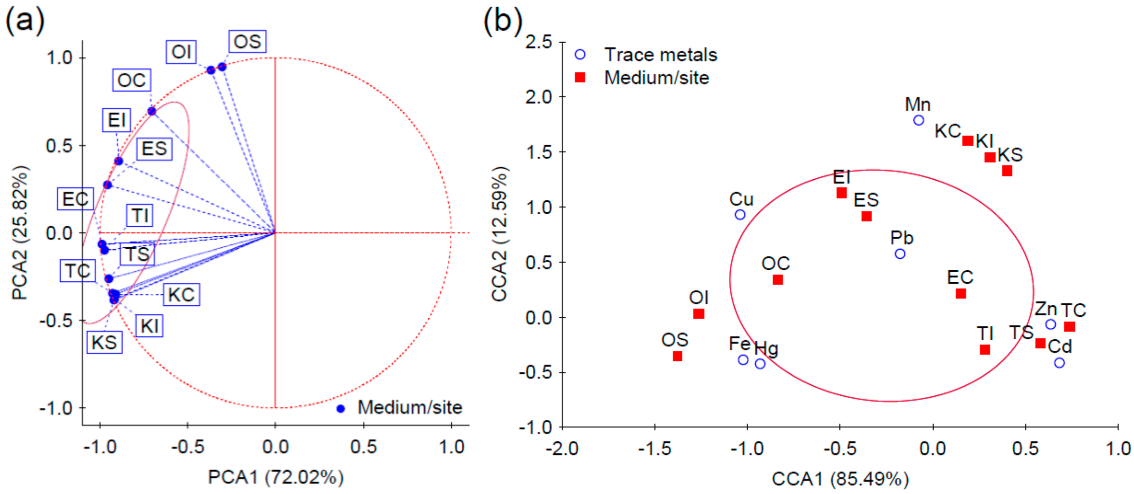

Figure 5. (**a**) The PCA and (**b**) CCA plots of trace metal concentrations within the group of *M. galloprovincialis* L. specimens sampled from Sv. Nedjelja (S), Cogi (C), and the IMB (I) in the shell (KS, KC, KI), soft tissue (TS, TC, TI), *conchix* (OS, OC, OI), and shell extract (ES, EC, EI).

4. Discussion

Despite a significant correlation between the *conchix* weight and dry shell weight, *conchix* % in the shell showed a negative correlation with dry shell weight. Thus, the values of *conchix* % in the shells of *M. galloprovincialis* L. were relatively stable due to their reduced contribution to the overall increase in shell weight during the mussels' growth in comparison with calcified shell layers. This could be explained by the much lower content of organic (*conchix*) than inorganic material in the shells from this study.

The concentrations of all metals analyzed were higher in the soft tissues and *conchixes* in comparison with the extracts and shells. Only Mn content in the shells from Sv. Nedjelja (2.44 $\mu g\ g^{-1}$) and Cogi (2.57 $\mu g\ g^{-1}$) were higher than in *conchixes* of mussels from the same locations (2.07 $\mu g\ g^{-1}$ and 1.6 $\mu g\ g^{-1}$, respectively). Mussels filter high amounts of water daily; therefore, they accumulate trace metals, which contribute to much higher content in the soft tissues than in ambient water [39]. However, despite different accumulation capacities, both soft tissues and shells can be used as bioindicators due to their ability to accumulate metals [40]. Trace metal concentration in the tissues is affected by the season, i.e., the reproductive cycle, as well as by environmental conditions, which can lead to higher concentrations of trace metals in winter and lower concentrations during the summer months [41]. It was explained by the dilution of metals during spring and summer due to the increased gonads' weight, which do not accumulate trace metals [42]. On the other hand, shells are considered more reliable indicators of contamination due to their lower trace metal variability in comparison with the soft tissues [43]. However, in our study, MPI values of organic mediums, such as soft tissues and *conchixes*, were >1, which indicated pollution, while the shells and shell extracts were unable to detect pollution (MPI < 1). The MPI values for the soft tissues at the investigated sites were in line with the data on the CI. A study by Andral et al. [44] showed that trace metals were inversely proportional to the CI, which was not the case in our study. Higher contents of most elements in mussels' soft tissues could be expected due to the sampling period in late autumn long after the spawning period.

The highest concentrations of Cu, Fe, and Hg were found in *conchixes*. The essential elements, including Fe and Cu, were more actively accumulated during the early ontogeny of the bivalve shells [45], indicating higher concentrations of these elements in the environment in the period of mussels' early life stages. Another study showed Fe distribution through the shell layers of *Arctica islandica*, with accumulation mainly in the periostracum [46]. Higher content of Fe in periostracum could contribute against dissolution by an acidic environment [47]. Moreover, higher Cu and Fe contents in *conchixes* compared to tissues, as well as slightly higher Hg contents, could be attributed to the redistribution of these elements from soft tissues during detoxification processes [27]. Hg content in *conchix* might be increased due to Hg variations during mussels' growth. The same processes can also explain site-related differences, i.e., highest Cu, Fe, Pb, and Hg concentrations in *conchixes* from Sv. Nedjelja samples, while soft tissues from the same mussels had the lowest concentrations of Cu, Mn, and Pb. The contents of the rest of the analyzed elements were also higher in the soft tissues from one of the other sites, Cogi or the IMB. Moreover, none of the analyzed metals in shells and extracts were found in the highest contents in Sv. Nedjelja samples, with the exception of Zn in shells (11.6 μg g^{-1} in both Sv. Nedjelja and Cogi samples).

PCA and CCA analysis showed previously explained differences between *conchixes* and other media in trace metal contents. Clear separation of accumulation patterns in *conchixes* in comparison with all other media at PCA plot could be explained by the higher affinity and specificity of certain metals, such as Cu, Fe, and Hg, to accumulate in the *conchix*. Furthermore, similarities in trace metal accumulation of shells and shell extracts could be expected since both contain inorganic components. However, trace metal accumulation in the shells and shell extracts of *M. galloprovincialis* was much lower in comparison with organic mediums, indicating a lower potential of inorganic mediums for the detection of trace metals in this study. In addition to Cu, Fe, and Hg correlations with *conchixes*, the correlation of Zn and Cd with the soft tissues can be observed by CCA plots. Higher concentrations of Cu in the periostracum and Zn in the soft tissues in many populations of green-lipped mussel were found [25], which is in compliance with Cu and Zn distribution in the tissues of the Mediterranean mussel in our study. Furthermore, CCA indicated Mn correlation with the shells. Different studies found that Mn can be incorporated into aragonite bivalve shells, substituting Ca in the crystal lattice of $CaCO_3$ [48,49].

In accordance with the above, the *conchix* of the Mediterranean mussel as an organic material in close contact with the environment, in comparison with calcified shell layers and relatively independent from the shell weight changes, could detect differences in the accumulation of certain metals in shorter periods than the whole shell. Moreover, it was found that metals accumulated in the periostracum were all assimilated from the soft tissues, and it is less likely to be affected by the bivalve's reproductive cycle [50]. This could be the advantage of *conchix* over the soft tissues in trace metal assessment. Great importance could be given to the method of *conchix* isolation suggested by our team [29] since it provides a high yield of shell organic matrix and could contribute to higher detection levels in trace metal assessments. Particularly, other authors indicated difficulties in periostracum separation due to peeling, which led to incomplete isolation [51]. However, more studies on trace metal accumulation patterns in the *conchix* of *M. galloprovincialis* in highly polluted environments and in different seasons need to be conducted.

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