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Research Note

Concentration and distribution of macrominerals in tissues of Mediterranean mussel *Mytilus galloprovincialis* exposed to Cd and Cd-mixtures

Concentración y distribución de macrominerales en tejidos de mejillón del Mediterráneo *Mytilus galloprovincialis* expuesto a Cd y a mezclas de Cd

José A. García-Navarro[1], Miguel A. Vera-Sánchez[1] and Diego Romero[1]*

[1]Área de Toxicología, Facultad de Veterinaria, Universidad de Murcia, Campus Regional de Excelencia Internacional "Campus Mare Nostrum", Espinardo, E-30071, Murcia, España
*Corresponding author: diegorom@um.es

Abstract.- Marine ecosystems are under great pressure due to heavy metals pollution, and mussels remain an important knowledge source in this regard. In this study, Mediterranean mussels (*Mytilus galloprovincialis*) were exposed for 7 days to Cd and three Cd-mixtures (Cd+Pb, Cd+Cu and Cd+Pb+Cu). The accumulation and tissue distribution of inorganic elements (Ca, K, Mg, Na, P and S, macrominerals) that are usually not taken into consideration in field studies on in bioassays were evaluated. Regarding concentration, the element which differed the most with respect to the control group was K, while Mg was the only one which showed no significant statistical difference with the control group. The group exposed to ternary mixture was the one in which there were more alterations in concentration and distribution of these macrominerals with respect to control, whereas in the group exposed to single Cd no significant differences in the concentration of these inorganic elements were found. The concentration and distribution of macrominerals in the compartments studied (digestive gland, gill and remaining soft tissues) is susceptible to the presence of Cd and heavy metal Cd-mixtures, with percentages of changes until 28.7%. Study results suggest that macrominerals composition in mussel tissues could be useful in studies of heavy metal contamination in marine ecosystems.

Key words: Accumulation, distribution, heavy metals, macrominerals, mussels

Introduction

All living beings use inorganic elements (metals, no metals and metalloids) for the normal development of their biological processes, so they are commonly referred to as "essential minerals". They are necessary components both for the proper growth of the organism and its reproduction and, therefore, fundamental to the maintenance of a good health throughout life, regardless of the animal species concerned. Hence, many are the functions in which their participation has been described: they act as structural components in organs and tissues, serve as electrolytes in body fluids and act as catalysts in enzymatic and endocrine systems. Calcium (Ca), phosphorus (P), potassium (K), magnesium (Mg), sodium (Na) and sulfur (S) are considered as macrominerals, since the organisms need high amount of them (McDowell 2003, Fraga 2005).

In marine organisms, the corporal concentration of these macrominerals depends on multiple factors, as the feeding source, the quality of the environment and the species, as well as the development phase and physiological state (Lall 2002). Marine invertebrates have important amounts of elements such as Ca or K (Taboada *et al*. 2008), being important the participation of macrominerals in many functions, as the formation of body structures (shells and exoskeleton), the acid-base balance, muscular function and enzymatic activity, *inter alia* (Muneoka & Twarog 1983, Wilbur & Saleuddin 1983, Simkiss & Wilbur 1989, Rosenberg & Hughes 1991, Korchagin 1995, Klein *et al*. 1996, Lucu & Towle 2003, Li *et al*. 2004). In general, an excess or lack of these elements, although essentials, can cause biochemical defects, alteration of the physiological functions and structural disturbances (McDowell 2003, Pond *et al*. 2005). Therefore, knowledge concerning mineral concentrations in living organisms has vital importance for a better understanding of the relationship among chemical elements and living beings, as well as for understanding the link between health status and environmental exposure problems (Iyengar 1989).

Heavy metals cause numerous ecological effects on marine ecosystems, as well as a high public concern about food security (Wang *et al*. 2005, Rainbow 2007, Feng *et al*. 2008, Wang & Rainbow 2008, Lin *et al*. 2013), especially if they are present at levels above the toxicity thresholds (Macdonald *et al*. 1996, O'Connor 2004). But the environmental risk from pollution is the result of the toxicity of pollutant's mixtures, sometimes very complex. Considering that the effect of exposure to a metal on the accumulation of other inorganic elements has been scarcely documented (Regoli & Principato 1995, Serra *et al*. 1999), the aim of this study has been to know the influence of the exposure to heavy metals (Cd and Cd-mixtures, with Pb and Cu) on the accumulation and tissue distribution of macrominerals in the Mediterranean mussel *Mytilus galloprovincialis* (Lamarck, 1819) for digestive gland, gills and remaining soft tissues.

Materials and methods

Mussel collection and conditioning period

Mediterranean mussels (*M. galloprovincialis*), with 4-5 cm in length and 9-10 g in weight, were collected at Cabo Home (Galicia, NW Spain) (geographic reference 42°15.007; 08°52.333), an uncontaminated location without local assets of pollutants (Albentosa *et al.* 2012, Besada *et al.* 2014, González-Fernández *et al.* 2015). Mussels were adjusted for two weeks under laboratory-managed water pH (8.03 ± 0.07), osmolarity (1086.3 ± 28.39 mmol kg^{-1}), temperature (24 ºC), non-stop aeration and natural photoperiod conditions. After two days to acclimatization, mussels were fed with the microalgae *Isochrysis galbana*, clone t-ISO (0.1% of microalgal organic matter per mussel live weight).

Heavy metal exposure

Mussels were exposed to single (Cd), binary (Cd+Pb and Cd+Cu) and ternary (Cd+Pb+Cu) mixtures at entire doses of 100 µg L^{-1} (Cd and Cu) and 1000 µg L^{-1} (Pb). The test was carried in three tanks per treatment, with 12 mussels every (n= 36 mussels per exposure group) throughout seven days; then, a complete of nine mussels per treatment had been randomly selected for macrominerals analysis. A control group of n= 36 non-exposed mussels was additionally set up (three tanks with 12 mussels each). Heavy metal stock solutions [$Pb(NO_3)_2$, $CdCl_2$ and $CuSO_4$] were prepared in milliQ water. In the course of the exposure time, temperature, pH, osmolarity, photoperiod, aeration and feeding conditions have been controlled as defined above, and no mussels died.

The dose of the heavy metals was adjusted in artificial sea water and then, it was introduced every day to each tank. The real (final) concentrations of metals in water were 933.69 ± 84.66 µg L^{-1} (Pb), 83.39 ± 10.44 µg L^{-1} (Cd) and 82.40 ± 4.83 µg L^{-1} (Cu).

Sample collection and processing

Prior to sampling, mussels were slightly opened, and washed with double distilled water. Next, the mussels were kept on blotting paper to remove the internal water. After 5 min, gills, the digestive gland and the remaining of the soft tissues –labeled as RST– were cautiously separated from every mussel, weighed and frozen at -80 ºC until analysis. Overall soft tissue (OST) was computed using information from all the specific organs.

Metal analysis

Samples were analysed using inductively coupled plasma optical emission spectrometry (ICP-OES, ICAP 6500 Duo, Thermo Scientific, with One Fast System) to find out macrominerals (Ca, K, Mg, Na, P and S) contents. Gills, the digestive gland and RST were treated with trace mineral grade nitric acid (69% Suprapure, Merck®) and 33% H_2O_2 (Suprapure, Merck®) in special Teflon reaction tubes, heated in a microwave digestion system (UltraClave-Microwave Milestone®) for 20 min at 220 °C, and finally diluted to 25 mL with double deionised water (MilliQ). The detection limit for the analysed elements was 0.001 µg g^{-1}. Two readings for every sample were performed and concentration values were used as the mean of both readings. To check for possible contaminants, one blank sample for every 11 samples was also analysed. Multi-element calibration standards (SCP Science, in 4% nitric acid) were prepared with specific concentrations of inorganic elements, taking as a reference UNE-EN ISO 11885 (UNE 2021)[1] for the determination of elements by inductively coupled plasma atomic emission spectroscopy. Furthermore, intermediate patterns of all elements were prepared. The calibration device was established per batch with a minimum of three points for every single lot. Each run started out with the calibration standards, continued with samples and intermediate patterns, and finished with the series with intermediate patterns (10% variation coefficient). The wavelengths (nm) were 184.006/315.887 (Ca), 766.490 (K), 202.582/279.079 (Mg), 589.592 (Na), 185.942/213.618/214.914 (P) and 180.731/182.034 (S). The uncertainty percentages of the elements were 4.34 (Ca), 4.56 (K), 4.70 (Mg), 5.23 (Na), 3.45 (P) and 4.25 (S).

[1]UNE-EN ISO 11885:2010. Calidad del agua. Determinación de elementos seleccionados por espectrometría de emisión óptica de plasma acoplado inductivamente (ICP-OES) (ISO 11885:2007). Asociación Española de Normalización (UNE), Madrid. <https://www.une.org/encuentra-tu-norma/busca-tu-norma/norma/?c=N0044517>

The recovery for reference materials was 94.34 (Ca), 94.56 (K), 94.70 (Mg), 95.23 (Na), 93.45 (P) and 104.25 (S). All concentrations are expressed in microgram per gram in wet weight (ww).

Data analysis

The statistics given for the macromineral concentrations and weight of soft tissue are geometric mean and standard error. Overall soft tissue concentrations were determined from the data (metal concentration and tissue weight) of gills, the digestive gland and RST. A Shapiro-Wilk test was used to check the data distribution. According to this distribution, Mann-Whitney U and t-Student tests were employed to compare Cd and Cd-mixture groups with respect to control group, and Kruskal-Wallis one-way ANOVA test to compare exposure groups between them. The weight of the tissues was checked with Kruskal-Wallis test. The significance level for each test was set as 0.05. All statistical analyses were accomplished with SPSS® v.24.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results and discussion

There are several reasons to study the possible variations in mineral concentrations in soft tissues from mussels exposed to heavy metals. On the one hand, a recent study on *Mytilus edulis* and *Perna viridis* show a great variability in macronutrients (C, N, P, S) and large cations (Na, Mg, K, Ca) in their tissues, as well as their potential relationship with the bioaccumulation of different trace elements, among which Pb, Cd and Cu are cited (Liu & Wang 2015). On the other hand, in many aquatic organisms have been described, among chemically similar cations, competitive effects by the sites of absorption and union (Hinkle *et al*. 1987, Blažka & Shaikh 1991, Roesijadi & Unger 1993, Di Toro *et al*. 2001), as well as the possibility of sharing similar metabolic pathways (Liu *et al*. 2012). Besides, some elements such as those used in this study are able to bind cell membranes and obstruct natural processes of transport across the cell wall (Boran & Altinok 2010). In their natural environment, marine organisms are simultaneously exposed to complex mixtures of metals. Although there is a lot of experimental evidence on interactions among elements (Popham & D'Auria 1982, Calabrese *et al*. 1984, Blackmore & Wang 2002, Fraysse *et al*. 2002), the physiological and biochemical mechanisms that underlie this interaction still remain not well explained (Shi & Wang 2004).

Control group

Regarding tissue weight, there were no statistical differences between exposure groups (Table 1). In all analysed tissues as well as in OST (Table 2), the element which was in a higher concentration was Na, followed by S and K. Except in the digestive gland, Ca was the element of control group which was in lower concentration, being similar the concentrations of P and Mg in RST and OST. Although some authors show some similarities with this order in the same species (*e.g.,* higher Na and lower Ca concentrations, Fuentes *et al.* 2009, Bongiorno *et al*. 2015; S>K>Ca, Jović *et al*. 2011, Santos *et al*. 2014), other studies show certain differences, associated to the bivalve species, geographical area and season of the year (*e.g.,* Ca>Mg concentration in *Mytilus coruscus* in autumn and winter, Li *et al.* 2010; P>Ca>Mg>K in *M. edulis* from China, Chi *et al*. 2012; Ca>K concentration in *M. galloprovincialis*, Fatoki *et al.* 2012). To compare our results with data reported by other authors, the percentage of moisture were determined (digestive gland= 82.7 ± 2.7; gills= 87.1 ± 1.2; RST= 83.3 ± 2.3; OST= 86.1 ± 2.9). Regarding the concentrations detected, our results were similar to those found in this species by several authors (K, Ca and S, Santos *et al*. 2014; Ca, Jović *et al*. 2011), but different to reported by others (K and Ca, Fatoki *et al*. 2012; Na, K, P, Ca and Mg, Bongiorno *et al*. 2015). In those studies, there were different techniques to the mineral analysis (flame atomic absorption spectrophotometry, spectroquant kits by spectrophotometry, energy dispersive X-ray fluorescence and inductively coupled plasma mass spectrometry), species (*M. galloprovincialis, M. coruscus, M. edulis*), seasons (January to December), and locations [local markets of Valencia -mussels from Ría de Vigo (Atlantic Sea), hatchery in Delta del Ebro and Valencia Harbour (Mediterranean Sea), Gulf of Trieste (North Adriatic Sea), Boka Kotorska Bay (Montenegro-southeastern Adriatic Sea), Tagus estuary in Lisbon (Portugal), Shengsi Islands (China), markets of China, Cape Town Harbour (Atlantic Ocean and Indian Ocean)]. According Telesca *et al*. (2019), there are compensatory mechanisms in *Mytilus* species as protective

Table 1. Weight (geometric mean and standard error, grams) in mussel tissues after 7-day exposure of Cd-treatments / Peso (media geométrica y error estándar, en gramos) en tejidos de mejillón después de 7 días de exposición a tratamientos con Cd

Mussel tissues	Control	Cd	Cd+Pb	Cd+Cu	Cd+Pb+Cu
Digestive gland	0.15 ± 0.01	0.15 ± 0.02	0.15 ± 0.01	0.13 ± 0.01	0.11 ± 0.01
Gills	0.31 ± 0.01	0.29 ± 0.02	0.30 ± 0.01	0.31 ± 0.03	0.23 ± 0.03
RST	0.73 ± 0.02	0.73 ± 0.06	0.71 ± 0.03	0.69 ± 0.05	0.74 ± 0.03
OST	1.19 ± 0.04	1.17 ± 0.07	1.17 ± 0.04	1.14 ± 0.08	1.08 ± 0.07

RST= Remaining soft tissues; OST= Overall soft tissues

Table 2. Concentration of macrominerals (geometric mean and standard error, g 100 g^{-1}, wet weight) in mussel tissues after 7-day exposure of Cd-treatments / Concentración de macronutrientes (media geométrica y error estándar, g 100 g^{-1}, peso fresco) en tejidos de mejillón después de 7 días de exposición a tratamientos con Cd

Mussel tissues	Control	Cd	Cd+Pb	Cd+Cu	Cd+Pb+Cu
Digestive gland					
Ca	0.099 ± 0.028	0.099 ± 0.012	0.076 ± 0.014	0.100 ± 0.014	0.103 ± 0.017
K	0.271 ± 0.007	0.268 ± 0.007	0.260 ± 0.009	0.260 ± 0.008	0.243 ± 0.009*
Mg	0.075 ± 0.001	0.078 ± 0.002	0.070 ± 0.003	0.078 ± 0.003	0.079 ± 0.003
Na	0.542 ± 0.013	0.576 ± 0.017	0.518 ± 0.022	0.567 ± 0.025	0.578 ± 0.030
P	0.188 ± 0.004	0.186 ± 0.002	0.191 ± 0.004	0.176 ± 0.006	0.179 ± 0.006
S	0.401 ± 0.008	0.400 ± 0.006	0.441 ± 0.014*	0.395 ± 0.014	0.395 ± 0.014
Gills					
Ca	0.050 ± 0.002	0.051 ± 0.002	0.048 ± 0.005	0.057 ± 0.004	0.064 ± 0.005*
K	0.160 ± 0.004	0.168 ± 0.006	0.167 ± 0.003	0.158 ± 0.006	0.152 ± 0.005
Mg	0.109 ± 0.002	0.114 ± 0.002	0.110 ± 0.003	0.113 ± 0.002	0.111 ± 0.003
Na	0.837 ± 0.015	0.829 ± 0.023	0.810 ± 0.035	0.870 ± 0.026	0.893 ± 0.036
P	0.086 ± 0.003	0.093 ± 0.004	0.096 ± 0.003*a	0.084 ± 0.004	0.078 ± 0.003a
S	0.227 ± 0.004	0.237 ± 0.008	0.247 ± 0.009a	0.222 ± 0.006	0.216 ± 0.004a
RST					
Ca	0.054 ± 0.005	0.057 ± 0.004	0.068 ± 0.046	0.074 ± 0.028	0.056 ± 0.008
K	0.227 ± 0.008	0.221 ± 0.006	0.220 ± 0.005	0.204 ± 0.006*	0.206 ± 0.010
Mg	0.107 ± 0.002	0.106 ± 0.002	0.107 ± 0.002	0.108 ± 0.003	0.107 ± 0.003
Na	0.567 ± 0.013	0.579 ± 0.027	0.556 ± 0.025	0.642 ± 0.025*	0.623 ± 0.021*
P	0.097 ± 0.003	0.094 ± 0.003	0.092 ± 0.003	0.090 ± 0.003	0.087 ± 0.004*
S	0.284 ± 0.010	0.289 ± 0.011	0.279 ± 0.012	0.281 ± 0.009	0.297 ± 0.011
OST					
Ca	0.059 ± 0.006	0.061 ± 0.004	0.066 ± 0.027	0.075 ± 0.018	0.063 ± 0.005
K	0.215 ± 0.007	0.215 ± 0.006	0.211 ± 0.005	0.198 ± 0.006*	0.198 ± 0.009*
Mg	0.103 ± 0.002	0.106 ± 0.001	0.103 ± 0.002	0.106 ± 0.002	0.105 ± 0.002
Na	0.634 ± 0.010	0.644 ± 0.016	0.619 ± 0.023	0.695 ± 0.025*	0.676 ± 0.022
P	0.106 ± 0.003	0.105 ± 0.003	0.105 ± 0.003	0.100 ± 0.004	0.095 ± 0.004*
S	0.285 ± 0.009	0.290 ± 0.010	0.292 ± 0.008	0.277 ± 0.009	0.290 ± 0.009

RST= Remaining soft tissues; OST= Overall soft tissues; *$P < 0.05$ with respect to control group (t-Student or Mann-Whitney U test); For each element, the same small letter indicates statistically significant differences between heavy metal exposure groups (Kruskal-Wallis one-way ANOVA)

capacity to regional alterations of abiotic (like salinity and temperature) and biotic conditions (like food availability), which can be observed in shell composition. Bongiorno *et al*. (2015) reported fluctuations in ash contents of mussels according to season, accumulation and utilization of the energetic reserves, depending on the interaction among food availability, growth and reproduction. Thus, it is necessary a characterization of sampling location and environmental conditions for a better understanding of the biomonitoring data.

The highest concentrations of Ca, K, P and S were found in the digestive gland (Table 2). The gills had the highest concentration of Na, whereas Mg had similar concentrations in gills and RST (higher than those found in digestive gland). Regarding tissue distribution, all these macrominerals were mainly accumulated in the RST (Table 3). On the other hand, the proportion between digestive gland and gill was similar in percentage, except in the case of Mg and Na, where the percentage accumulated in gill was three times higher than the digestive gland one. There were no studies to compare this result, so future works could explain them.

Exposure groups

The element in which the most statistically significant differences were found was K, followed by Na and P, while Ca and S were the ones with the fewest differences (none in Mg). The group exposed to the ternary mixture was in which there were more macrominerals with statistically significant differences with respect to control group, followed by the group exposed to the mixture Cd+Cu and Cd+Pb, whereas in the group exposed to Cd no significant differences in macromineral concentrations were observed. At the tissue level, the digestive gland and the gills were where less significant differences with respect to control group were appreciated, being in RST and OST where more differences were observed.

Regarding Ca, its concentration increased slightly ($P > 0.05$) in the OST of all groups compared to the control group. In relation to the tissues, the concentration of this mineral just increased (28%, $P < 0.05$) in the gills of the group exposed to ternary mixture. Experimental studies in which the interference of a heavy metal on other essential elements are scarce, and even less so in the case of combined exposures. Thus, in the group exposed to Cd, the results obtained do not agree with previous reports (Sheir *et*

Table 3. Percentage of macrominerals (geometric mean and standard error) in mussel tissues after 7-day exposure of Cd-treatments / Porcentaje de macronuntrientes (media geométrica y error estándar) en tejidos de mejillón después de 7 días de exposición a tratamientos con Cd

Mussel tissues	Control	Cd	Cd+Pb	Cd+Cu	Cd+Pb+Cu
Digestive gland					
Ca	21.11 ± 1.39	20.29 ± 1.75	14.34 ± 2.51	15.53 ± 3.24	16.48 ± 1.29*
K	15.85 ± 0.70	15.52 ± 1.17	15.44 ± 0.74	15.48 ± 1.05	12.25 ± 1.03*
Mg	9.11 ± 0.60	9.19 ± 0.76	8.52 ± 0.58	8.67 ± 0.47	7.50 ± 0.60
Na	10.75 ± 0.69	11.05 ± 0.79	10.52 ± 0.82	9.62 ± 0.59	8.52 ± 0.69*
P	22.30 ± 0.72	21.88 ± 1.35	22.67 ± 0.98	21.03 ± 1.20	18.97 ± 1.16*
S	17.78 ± 0.73	17.10 ± 1.10	18.91 ± 1.35	16.73 ± 1.06	13.63 ± 1.22*
Gills					
Ca	22.07 ± 1.35	20.84 ± 1.28	18.68 ± 2.36	20.39 ± 2.28	21.65 ± 1.60
K	19.38 ± 0.61	19.65 ± 0.99	20.52 ± 1.00	21.62 ± 1.27a	16.09 ± 1.15a
Mg	27.35 ± 0.47	27.22 ± 1.54	27.61 ± 1.11	28.97 ± 1.52a	22.16 ± 1.67*a
Na	34.22 ± 1.15	32.09 ± 2.17	34.00 ± 1.09	34.06 ± 1.56	27.61 ± 2.05*
P	21.19 ± 0.71	22.13 ± 1.01	23.47 ± 0.93a	23.17 ± 1.29b	17.43 ± 1.03*a,b
S	20.74 ± 0.82	20.46 ± 1.12	21.94 ± 0.95a	21.67 ± 1.41b	15.65 ± 0.92*a,b
RST					
Ca	55.84 ± 1.72	57.72 ± 2.06	62.56 ± 4.24	58.51 ± 5.02	60.68 ± 1.98
K	64.52 ± 0.80	64.11 ± 1.59	63.59 ± 1.35	62.15 ± 1.28a	70.78 ± 1.97*a
Mg	63.31 ± 0.82	62.76 ± 1.97	63.42 ± 1.42	61.78 ± 1.37a	69.38 ± 2.07*a
Na	54.54 ± 1.50	55.59 ± 2.50	54.92 ± 1.61	55.70 ± 1.47	62.66 ± 2.45*
P	56.24 ± 1.08	55.27 ± 1.64	53.33 ± 1.66a	55.02 ± 1.31	62.82 ± 2.01*a
S	61.14 ± 1.12	61.75 ± 1.47	58.30 ± 2.09a	60.77 ± 1.43b	69.86 ± 1.94*a,b

RST= Remaining soft tissues; $^{*}P < 0.05$ with respect to control group; For each element, the same small letter indicates statistically significant differences between heavy metal exposure groups

al. 2013) on mussels of the species *Mytilus edulis* exposed to this metal for 8 days at a dose 5 times lower than those used in this study, which indicated that there was a decrease in Ca concentrations in both gill and digestive gland due to different tissue lesions. On the other hand, Cd and Pb are two analogue metals to Ca, competing with it for the binding sites in the gill of the fish (Macdonald *et al*. 2002, Rogers *et al*. 2003, Playle 2004). Several studies show that this competition can carry a reduction of the Ca absortion (Niyogi & Wood 2004, Rogers & Wood 2004, Rainbow & Black 2005), leading to ionic and osmotic disturbances that contribute to the toxicity of these metals (Playle 2004). In oysters, Huanxin *et al*. (2000) describe that Ca is easily substituted for Cd, even producing cellular necrosis, whereas different studies in fishes and bivalve molluscs show several alterations associated to the homeostasis and physiology of Ca (Neff *et al*. 1987, Sunila 1988, Orrenius *et al*. 1989, Schoenmakers *et al*. 1992, Da Ros *et al*. 1995). In this study, a decrease in Ca concentration were observed only in the group exposed to Cd+Pb in digestive gland and gills, but it was not statistically significant. Regarding the differences between forms of exposure (individual *vs* mixtures), some studies indicate that the ionic imbalances produced by binary mixtures of Cd and Pb are greater than those caused by simple exposures (Kara 2010, Clemow *et al*. 2015, Van Ginneken *et al*. 2015), which could justify the significant imbalance of Ca found in gill of the groups exposed to the more complex Cd-mixture, although it would be necessary to perform new tests with different binary and ternary heavy metal mixtures to check whether it is a modulated effect by the complexity of the mixture.

In regard to the tissue distribution of Ca (Table 3), it can be observed that the treatments did not significantly altered the distribution of this element among tissues, with the exception of the digestive gland of the ternary mixture (decrease by 21.9%, $P < 0.05$), and the RST of this exposure group (increase by 8.7%, $P = 0.06$). Although the concentration of Ca in gills in this exposure group was higher than those found in the control group ($P < 0.05$), the percentage of Ca accumulated in this tissue over the total body was not higher than the control group. Sheir *et al*. (2013) reported an increase of necrosis in the digestive gland and gills after experimental exposure of Cd, so it could cause a decrease in the weight of these tissues. Then, histological studies are necessary to understand our result.

Regarding Mg, the concentration was very similar in all treatments, both in the OST and in each one of the analyzed tissues (Table 2). Only one study was found about Mg concentration in mussels exposed to heavy metal (specifically Cu). In this work, Jorge *et al*. (2013) reported (in juvenile mussels of *Lampsilis siliquoidea*) no variations in tissue Mg concentration. About the distribution of Mg, significant differences in tissues of the mussels exposed to the ternary mixture (compared to the control group and Cd+Cu exposure group) were found, decreasing the percentage in gill (19.0 and 23.5% respectively) and increasing in the RST (9.6 and 12.3% respectively). It could be a consequence of the decrease in gills weight (Table 1). The Mg is an essential element which takes part in different organic and structural functions (Rosenberg & Hughes 1991, Klein *et al*. 1996, Stecher *et al*. 1996, Aranda

et al. 2000), and one of the most important is related to the smooth muscle of the molluscs (Hooper *et al*. 2008). In situations of exposure to complex mixtures such as ternary, it could be possible a redistribution of Mg to the compartment called RST, where the musculature of the mussel is takes place.

The concentration of K in the OST decreased ($P < 0.05$) in the groups exposed to Cd+Cu (7.9%) and to the ternary mixture (7.9%), whereas the concentration of Na increased in the group exposed to Cd+Cu (9.6%, $P < 0.05$) and marginally in the group exposed to the ternary mixture (6.6%, $P = 0.09$). In the tissues of these same exposure groups, although the trend was the same as that observed in OST, only statistically significant differences were observed in the digestive gland (K concentration in mussels exposed to the ternary mixture, decreased by 10.3%) and in the RST (concentration of Na and K in mussels exposed to Cd+Cu, increased by 13.2%, and decreased by 10.1%, respectively; concentration of Na in mussels exposed to the ternary mixture, increased by 9.9%, and marginally significant, $P = 0.06$, in the concentration of K, decreased by 9.3%). Other relevant observations were the no significant differences in both K and Na concentrations in gills (any exposure group), and the tendency to decrease of both K and Na concentrations in the group exposure to Cd+Pb binary mixture (except K in gills). As seen, there seems to be a different behavior depending on the simultaneous presence of Cu and Pb. On the other hand, the Na-K pump is an integral membrane protein, fundamental in the physiology of the cell. Its function is the transport of the most important ions in biology, Na and K, and regulates therefore, the ionic balance. Exposure to heavy metals causes ionic imbalances that can alter this equilibrium. According to some authors, metals such as Cd in clams (*Anodonta cynegea*) and fishes (*Platichthys flesus*) may decrease K concentrations (Larsson *et al.* 1981, Hemelraad *et al.* 1990), and metals such as Cu and Pb can inhibit the uptake of Na in other marine species such as *Oncorhynchus mykiss* and *Daphnia magna* (De Schamphelaere & Janssen 2002, Grosell & Wood 2002, Rogers *et al.* 2005). This could partially explain our results, because in the OST and RST of the groups exposed to the Cd+Cu binary mixture and to the ternary mixture, the significant decrease in K concentration was clearly seen, while Na concentration did not decrease, but increased significantly or marginally, which could also be interpreted as an ionic imbalance. In this respect, it should be mentioned that what metals such as Cd and Cu make are to affect enzymes such as Na+/K+-ATPase and carbonic anhydrase, both of which are involved in the uptake of Na in the gill (Postel *et al.* 1998, Atli & Canli 2007, Birceanu *et al.* 2008, Jorge *et al.* 2013, Nogueira *et al.* 2013). However, in this study the concentrations of these elements in the gills of the groups exposed to Cd+Cu and to the ternary mixture were not significantly altered, which could justify *a priori* an adequate functioning of these enzymes at this level and therefore, that did not decrease Na concentration. However, when the percentage distribution of these elements was evaluated (to respect to control group), a decrease in the percentage of Na ($P < 0.05$) and K ($P = 0.06$) in gill (19.3 and 17.0%, respectively) and digestive gland (20.7 and 22.7%, respectively) in the group exposed to the ternary mixture, and an increase ($P < 0.05$) in the RST (14.9 and 9.7%, respectively) was observed; but an increase of the percentage of K in gills (11.6%, $P = 0.08$) in Cd+Cu binary exposure group was found. In addition, the percentage of K decreased by 25.6% ($P < 0.05$) in gills of mussels exposed to ternary group to respect Cd+Cu exposure group, and an increase (13.9%, $P < 0.05$) in RST were observed. Although it is not possible to explain these results, it could be assumed that certain alterations are occurring in gill, at least in the exposure to the more complex mixture. This lead us to think of the usefulness of determining not only tissue concentrations, but also distribution of the elements in the different compartments.

Phosphorus and sulfur are two important elements in heavy metal detoxification systems in some bivalves (George & Pirie 1979, Giambérini & Pihan 1996, Marigómez *et al.* 2002). In this study, the concentration of P found on OST of the metal-exposed groups were similar to those of the control group, except in the mussels exposed to the ternary mixture, where they decreased by 10.4% ($P < 0.05$). In the tissues, a decrease in P concentration in RST (10.3%, $P = 0.05$) and gills (9.3%, $P = 0.08$) of the mussels exposed to ternary mixture and an increase in gills (11.6%) of the mussels exposed to Cd+Pb ($P = 0.05$) with respect to control were found. In addition, a decrease of P concentration ($P < 0.05$) was found in gills from mussels exposed to ternary mixture with respect to Cd+Pb (18.8%) exposure group. Regarding S, no significant differences were found (with respect to control group) in the concentrations of this element on the OST nor on the tissues of the exposure groups, except in the digestive gland of the group treated with Cd+Pb were increased by 10.0% its concentration (in the gills also there was an increase of 8.8%, but marginally significant, $P = 0.06$). Furthermore, S concentration in gills decreased ($P < 0.05$) in mussels exposed to ternary mixture with respect to Cd+Pb exposure group (12.6%). Therefore, in the group exposed to the binary mixture Cd+Pb there was an increase in the concentration of P and S in gill and digestive gland. In this respect, in an experimental study on pond snail (*Lymnaea stagnalis*), Desouky (2006) shows an increase in the amount of P and S-based ligands in granules of various tissues for the sequestration of metals such as Cd, so it can also be thought that *M. galloprovincialis* may be producing a stimulus in the synthesis of lysosomal granules based on P and S for the metal sequestration, although perhaps the

single exposure of Cd was not sufficient and in the ternary mixture it occurs the failure of this process. Although this is mere speculation, it could find a possible tissue speciation in the generation of such granules: P in gill and S in gland. At the bibliographical level some publications were found that refer to the amounts of P in mussels as a nutritional aspect (Rivero 2006), but none about biomonitoring studies or experimental exposures in which the tissue concentration of these elements is determined. Based on this nutritional composition, the P concentration found in this study was within the normal range.

With respect to the distribution of P and S in the different tissue compartments, there were statistically significant differences in all the tissues of the group exposed to the ternary mixture, decreasing the percentage in gland (14.9 and 23.3% for P and S respectively) and gill (17.7 and 24.5% for P and S respectively), and increasing in the RST (11.7 and 14.3% for P and S respectively). In addition, P and S percentage decreased ($P < 0.05$) in gills from mussels exposed to ternary mixture to respect Cd+Pb (25.7 and 28.7% respectively) and Cd+Cu (24.8 and 27.8% respectively) exposure groups, with an increase in RST to respect Cd+Pb (P= 17.8%, and S= 19.8%) and Cd+Cu (S= 15.0%). Again, it was the more complex mixture that marked important differences, similar to response by other macrominerals.

In conclusion, it can be affirmed that the tissue composition of macrominerals is susceptible to the presence of Cd and heavy metal Cd-mixtures, with percentages of changes until 28.7%. This fact, together with the relative ease of obtaining this information through current analysis techniques (simultaneous detection of essential and non-essential elements), could be taken into account in all types of studies of heavy metal contamination in marine ecosystems. The study of the concentration of these elements in each of the different tissues, besides providing the information on the kinetics of them, allows to know the total amount of each element in each compartment, contributing with data that is usually not taken into consideration in field studies or in bioassays, but that have shown to be related with the presence of these pollutants.

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