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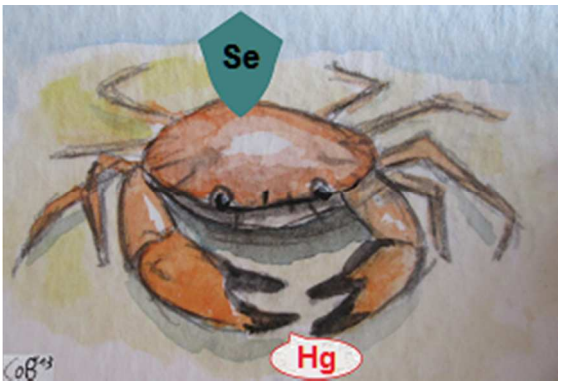

Selenium antagonism to mercury in crabs
58x39mm (120 x 120 DPI)

Evaluation of dietary exposure of crabs to inorganic mercury or methylmercury, with or without co-exposure to selenium

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Abstract

The present work aimed to evaluate the influence of dietary inorganic mercury or methylmercury, with or without selenate co-exposure, in order to examine dietary selenium's role in counteracting mercury species toxicity as well as the effect of the Se:Hg molar ratio in the diet and tissues of exposed crabs (*Carcinus maena*). From the dissection processes, it was possible to obtain for each crab muscle from legs and thorax, hepatopancreas, gills, stomach, hindgut, heart, testis/ovary and carapace. All samples were analyzed by CV-AFS for total mercury, and HR-ICP-MS for total selenium. For the crabs exposed to inorganic mercury, the accumulation was statistically confirmed (p-value < 0.05 by comparison of tissues from the exposed crabs to those from control crabs) in the hepatopancreas, muscle from thorax, gill and heart, whereas for the co-exposure to selenium, the accumulation of mercury was significant only in the heart. In contrast, when considering the methylmercury exposure, the Hg accumulation was statistically significant in the muscle from thorax and legs, hepatopancreas, gill, heart and testis/ovary. However, for the co-exposure to selenium, the Hg accumulation was only significant in the muscle from thorax, hepatopancreas and gills. Therefore, the accumulation of Hg and the Hg-Se interactions in the hepatopancreas, heart and muscle of the crabs were highly significant for the exposures to Hg species without co-exposure to Se. Thus, the dietary exposure to Se played a very important role concerning the uptake of the Hg species investigated in the present work, showing to influence the uptake and accumulation patterns.

Keywords: mercury, selenium, selenium antagonism to mercury, crab tissues

Introduction

The European shore crab *Carcinus maenas*, or green crab, is a widely distributed epibenthic portunid crab that inhabits hard and soft intertidal shallow habitats of European coasts and estuaries. It is an aggressive and voracious predator, feeding on a wide variety of prey items that is an important feature in structuring marine and estuarine benthic communities. It is now accepted that *C. maenas* is a species that can have significant impacts, both economically and ecologically [1,2].

In this context, the problem of mercury contamination is enhanced when it enters the food chain after biomethylation and bioaccumulation processes. Alkylmercury, formed in the bottom sediment of the ocean and in freshwater systems, is enriched to a high degree in the aquatic food chain, with the highest levels occurring in predatory fish. From the aquatic environment, methylmercury becomes incorporated in the terrestrial environment by species feeding on aquatic organisms [3]. Predator species, both vertebrate and invertebrate, will be subject to biomagnification processes (mainly of methylmercury) along the food web and are likely to have higher mercury body burdens, which is of health concern and justifies further research in the area [2]. Considering that the crabs are frequently consumed by local populations and also used as fish baits, this species may directly or indirectly represent a major pathway for human contamination. This species is one of the most important and exploited natural resources in temperate estuarine systems and therefore can represent a major pathway for human mercury consumption [4].

Interactions between mercury chemical forms and biological barriers are of major interest in understanding bioaccumulation mechanisms and combined toxicological effects. Whatever the level of complexity of living organisms, these barriers control the uptake and accessibility of contaminants to the internal compartments. Although composed of a complex arrangement of epithelial cells, each barrier is in fact based on the same fundamental structure: the plasma membrane and its phospholipidic bilayer, including proteins. Basic Hg uptake by the cells is by passive diffusion through the membranes [5]. However, it has long been observed that Se protects animals from the toxicity of both inorganic mercury and methylmercury [6]. It appears that both the absolute and relative amounts of methylmercury and selenium present in the diet contribute to the associated risks or benefits of its consumption [7]. Selenium is essential for the activity of 25–30 genetically unique enzymes (selenoenzymes). All forms of life that have nervous systems possess selenoenzymes to protect their brains from oxidative damage. Homeostatic mechanisms normally maintain optimal selenoenzyme activities in brain tissues, but high methylmercury (MeHg) exposure may sequester Se and irreversibly inhibit selenoenzyme activities [8].

Biochemically, an irreversible inhibitor is one that forms covalent bonds with components of the active site of an enzyme. As selenocysteine is the principal active site catalytic component of selenoenzymes, methylmercury is by definition a highly specific irreversible selenoenzyme inhibitor once it forms covalent bonds between its mercury moiety and the selenium of the enzyme's selenocysteine. But in this case the inhibitor–enzyme complex not only abolishes the activity of the inhibited selenoenzyme, it also restricts selenium release from the MeHg–SeCys complex, severely limiting the bioavailability of

that selenium for participation in future intracellular cycles of SeCys synthesis [7].

Thus, considering the toxic character of mercury species and the possible occurrence of an antagonistic effect for co-exposure to selenium, this study explores the features between dietary inorganic mercury or methylmercury, with or without selenium, in order to examine dietary selenium's role in counteracting mercury toxicity as well as the effect of Hg:Se molar ratio in the diets and tissues of exposed crabs.

Experimental

Instrumental – CV-AFS

The mercury determination in the samples was performed by cold vapor atomic fluorescence spectrometry (CV-AFS) using a PSA model Merlin 10.025 (P S Analytical, UK), equipped with two peristaltic pumps for both reductant and sample solutions, a 17 mL liquid/gas separator, a permapure dryer unit, an AFS detector unit and a data acquisition system, employed for fluorescence measurements in continuous mode. The delay, analysis and memory times were, respectively, 28, 60 and 90 seconds. An autosampler ASX-510 (Cetac Technologies, USA) was used. Tubing of 0.8 mm inner diameter made of polytetrafluorethylene (PTFE) was used in this system. A 2.0% m/v stannous chloride solution dissolved in 10.0% v/v HCl was used as reducing agent and 5.0% v/v trace analysis nitric acid was used as carrier solution. The flow rates for the reductant and the sample solutions were 4-5 mL min^{-1} and 8-10 mL min^{-}

^{1}, respectively. Peak area was used for signal evaluation. Argon (99.998%, BOC, UK) was used as carrier gas for the mercury vapor at a pressure of 40 psi. Total mercury determinations in the tissues were accomplished through duplicate digestion with *aqua regia*. Each sample or standard solution was measured twice. Samples, standard aliquots, and reagents were weighed on an analytical balance AB 204-S/FACT (Mettler Toledo, Switzerland). The heating processes for the samples digestion and reductant preparation were done on a hot plate SCT1 (Stuart, UK). A digital ultrasonic bath XUB25 (Grant, UK) was used for the decontamination procedure of the materials.

Instrumental – HR-ICP-MS

The selenium determination in the samples digested in the microwave Mars-5 (CEM Instrument, UK) was carried out with an Element 2 HR-ICP-MS (Thermo Fisher Scientific, Germany) equipped with an autosampler ASX-260 (Cetac Technologies). The RF power adopted was 1350 W. All measurements (^{77}Se, ^{78}Se and ^{72}Ge) were carried out in high resolution mode (m/Δm ~ 9000) of the mass spectrometer in order to separate the analyte signals from serious spectral interferences. The instrument was optimized to the highest possible intensity (10^{7} cps on 1 µg L^{-1} In) and mass calibrated every day. Nickel cones and quartz torch were used. The Meinhard nebulizer was fed by a peristaltic pump at a sample flow rate of 1.0 mL/min. Germanium 10 µg L^{-1} was used as internal standard for all solutions. Samples, standard aliquots and reagents were weighed on an analytical balance AB 204-S/FACT (Mettler Toledo).

Reagents and Certified Reference Material

All chemicals used were of analytical grade or better, unless stated. The solutions were prepared using bi-distilled water (BDW), as well as the dilution of the samples, obtained from an A4000D Aquatron water still system (Stuart, UK). The reducing agent solution, daily prepared 2.0% m/v stannous chloride dihydrate (ACS reagent, ≥98%, Sigma-Aldrich), was dissolved in 10.0% v/v hydrochloric acid reagent grade 37% (Fisher Scientific, UK), heated to boiling for at least one minute before the analysis by CV-AFS. This hydrochloric acid was also used to prepare the HCl bath employed as part of the decontamination process of the materials used thoroughly this experiment for Hg measurements. Nitric acid reagent ACS (Fisher Scientific) was also used in one step for the decontamination process for Se, and in two steps for the materials intended for Hg measurements. For the a*qua regia* preparation, used for the digestion of the samples for mercury determination and also to prepare the working mercury solutions for the calibration curve, nitric acid (TraceMetal Grade, Fisher Chemical) and hydrochloric acid 37% analytical reagent (AnalaR NORMAPUR® ACS, VWS, UK) were used. The 5.0% v/v nitric acid solution used as carrier in the CV system was also prepared with the acid for trace analysis. Working solutions with concentrations of 0.05, 0.20, 0.50, 1.0 and 1.5 μg L^{-1} of mercury were prepared from a 10.0 μg L^{-1} mercury standard solution in 5.0% v/v nitric acid medium. This intermediate solution was prepared from sequential dilution of a 1000 mg L^{-1} mercury standard for ICP in 12% nitric acid (Fluka). However the working solutions were in a*qua regia* medium, which were submitted to the

same heating conditions as that applied for the samples digestion, before the addition of the standard.

For the measurements in the ICP-MS, hydrogen peroxide 32% (ACS, Fisher Scientific) and nitric acid trace analysis grade were used to digest the samples using a CEM Mars 5 Laboratory microwave digestion system (open vessels). Calibration standard solutions (blank, 0.1, 0.75, 5.0, 25.0, 60.0 and 100.0 μg L^{-1}) for selenium were prepared daily by appropriate dilution of a 10 mg L^{-1} multi element standard solution (Merck), also with nitric acid for trace analysis in order to match the acid concentration of the samples. Germanium standard solution, at a concentration of 10 μg L^{-1}, used as internal standard for all solutions, was prepared from a certified reference solution of germanium (10200 mg L^{-1}, Aldrich Chemical, USA).

A 100.0 mg L^{-1} mercury standard solution in BDW medium, used for spiking the servings (fish/squid) in order to perform the exposures to inorganic mercury, was also prepared from the 1000.0 mg L^{-1} mercury standard for ICP mentioned previously. For the exposures to methylmercury, a 100.0 mg kg^{-1} solution was used for spiking the servings, also in BDW medium, prepared from a stock solution of 9543 mg kg^{-1}, to which methylmercury (II) chloride salt (Aldrich, UK) was dissolved in methanol (VWR, BDH Prolabo, UK). Finally, for the exposures to selenate a 40.0 mg L^{-1} solution was prepared from the solid Na_2SeO_4 anhydrous (99.8%, Alfa Aesar, UK) in BDW. The certified reference materials from National Research Council of Canada, fish protein DORM-3 and dogfish liver DOLT-4, were added for validation purposes in all digestion runs for mercury and selenium determinations, respectively.

All materials used in the processing and analysis of samples for Hg determination were initially washed with tap water and left overnight in a 5-10% v/v liquid detergent bath (5545805, Arco LTD). Next, they were rinsed with tap water thoroughly for the complete detergent removal, followed by rinsing with BDW and then submitted to an extensive four steps decontamination procedure, being each of them a reagent bath prepared in BDW in which the materials were kept under ultrasound during one hour. These steps were: 10% v/v nitric acid (Bath 1), 10% v/v nitric acid (Bath 2), 10% v/v hydrochloric acid (Bath 3) and finally pure BDW (Bath 4). Between the steps, the materials were thoroughly rinsed with BDW. The boxes used for the exposures of the crabs were cleaned by rinsing with a 5-10% v/v liquid detergent solution in tap water and then a 10% v/v nitric acid solution in BDW.

Plastic containers (polypropylene, PP) intended to digestion and stock of samples previously to the selenium determination, were washed with tap water and then in a dishwasher at 75 °C (AA 15place megaload Nextra, Hoover, UK) with powder soap for dishwasher. Afterwards, the containers were thoroughly rinsed with BDW and immersed in a 10% v/v HNO_3 solution overnight, and rinsed thoroughly with BDW and air dried.

Experimental set-up

For the experiments, *Carcinus maenas* crabs, captured off the west coast in Scotland, of varying sizes, brown in appearance and mostly males, provided by University Marine Biological Station Millport, Isle of Cumbrae, Scotland, UK,

were used. Their body weight varied from 35 to 229 grams. The experimental designs always included a control group (to which no spiked fish/squid was served) to each of the five exposure groups, which were (I) selenate, (II) inorganic mercury, (III) inorganic mercury plus selenate, (IV) methylmercury and (V) methylmercury plus selenate. Each exposure was done as an independent experiment in which there were at least 4 exposed crabs and 2 control crabs.

In the Aquarium laboratory, crabs were first placed in big tanks with artificial seawater, air supply and water recirculation system, while waiting for the exposures, under temperatures of 15±4 °C. For the exposures, a certain number of crabs were transferred to individual small plastic boxes with approximate volume of 10.0 liters, containing about 5.0 liters of artificial seawater of salinity 33. As for the control crabs, they were always placed together in one of the plastic boxes. All boxes were continuously aerated. The set-up of individual boxes adopted in this experiment made possible to control the exposition level to mercury species and/or selenium of each crab.

The concentration of mercury species tested in this study was planned according to some considerations. The first of them was the maximum level for total mercury in crustaceans of 0.50 mg/kg wet weight, established by the Commission Regulation (EC) No 1881/2006. Then, making a body weight approximation of the smallest crabs used in this experiment, it was assumed that none of the crabs would weigh less than 30.0 grams. In this sense, by exposing the crabs to 15 µg of the respective mercury species, the maximum level established in the regulation would not be exceeded as well as the crabs would not be subjected to acute toxic conditions.

The spiking of mercury species and/or selenate in the 1.0-2.0 g servings of fish/squid was accomplished with the aid of a 100 µL syringe for gas chromatography, however the spiked volume of 50 µL was not exceeded for each compound, otherwise leaking of the solution from the serving happened, which would impair the experiment through loss of the compounds. The concentration of the mercury species and selenate solutions were 100 mg L^{-1} and 40 mg L^{-1}, respectively. The fish and squid used in the feeding sessions were low in mercury and selenium.

The exposures lasted 14±2 days, in which the crabs were fed three times. Therefore the crabs were individually contaminated with 5.0 µg of mercury (as inorganic Hg or methylmercury) and/or 2.0 µg of selenate in each one of the three feeding sessions, which summed at the end of the exposure 15.0 µg for mercury species and 6.0 µg for selenate. These amounts were chosen considering the 1:1 molar ratio for mercury-selenium, when both were present.

For the dissection process, specimens were transferred from their plastic boxes to another box with clean artificial seawater, where they individually underwent a clean-up step for approx. one hour. After that they were weighed, and then, in a plastic bowl, they were fully covered with liquid nitrogen. From this process it was possible to obtain for each crab, in general, ten samples, which were muscle from legs, muscle from thorax, hepatopancreas, gills, stomach, hindgut, heart, testis/ovary and carapace, besides faeces, which when available, were collected from the water in the plastic boxes used in the exposition step. All samples were homogenized, with the exception of faeces and carapace, and then transferred to a zip lock bag and stored at -20 °C until

analysis. Considering that for all exposures 34 crabs were involved, approximately 350 samples were generated at the end of the experiment.

Sample preparation

Firstly the samples were cut in small pieces using a ceramic knife, and further homogenised with the same knife, with the exception of carapace and faeces. A sample mass of around 100.0 mg of each tissue was transferred to a vial (PP for selenium determination and glass for mercury determination), which was previously properly decontaminated, according to prior description, and weighed. For the certified reference materials, a sample mass of 20.0 to 30.0 milligram was used Considering that the natural amount of water in fish is about 80% [9], this mass corresponds to a fish wet weight of approximately 100.0 mg, just like the mass adopted for the crabs samples in this study. All measurements refer to wet weight, and the digestion of the samples and CRMs was performed in duplicate. Analytical quality control was performed by including the CRMs DORM-3 or DOLT-4 in every digestion procedure.

For mercury measurements, the weighing of the samples were in glass vials, to which 5.0 mL of *aqua regia* were added and the samples were submitted to heating in a water bath for two hours using the hot plate. Therefore, the vials were gently closed with the lid. For the analysis, BDW was added to the samples to a final volume of approximately 25 mL, being the final mass of the digested and diluted sample accurately measured in the analytical balance. In this way, the final concentration of *aqua regia* in the samples was 20% v/v. The digests were a yellow to brown solution, often containing some

small white particles, especially for the certified reference material DORM-3. The recovery values obtained for mercury from DORM-3 analysis ranged from 76% to 112%, which were in good agreement with the 95% confidence level of the certified value, and the coefficient of variation between duplicates never exceeded 5.0%.

For selenium measurements, the weighing of the samples was in PP vials and the samples were microwave digested in these vials with concentrated HNO_3/H_2O_2 (1:2, v/v), being the nitric acid trace analysis grade and left to act overnight, and the hydrogen peroxide added shortly before the digestion, which followed the microwave digestion program: 5 minutes at 50 °C (1 minute temperature ramp), 5 minutes at 75 °C (1 minute temperature ramp), and 30 minutes at 95 °C (2 minutes temperature ramp) at 1600 W. For the analysis, BDW was added to the samples to a final volume of approximately 25 mL, being the final mass of the digested and diluted sample accurately measured in the analytical balance. In this way, the final concentration of nitric acid in the samples was 4% v/v. The sample obtained from digestions was a clear solution. The recovery values obtained for Se from DOLT-4 analysis ranged from 70% to 110%, which always agreed with the certified value at a 95% confidence level, and the coefficient of variation between duplicates was usually better than 12.0%.

General procedure – Hg quantification in the crab samples

For the determination of mercury by CV-AFS the digested samples or the standard solutions, both in *aqua regia* medium, were mixed with the carrier and

reductant solutions inside the mixing valve. The mixture was transported into the gas-liquid separator and the generated vapor was transported through a dryer tube to the detector by an argon flow. Once the instrument was used in the continuous mode, the carrier, which is also the blank solution, is recycled. For the samples which exceeded the adopted calibration range, a dilution step 1:30 with the 5.0% v/v nitric acid solution was applied. In order to check the validity of this dilution procedure, the CRM DORM-3 was submitted to this procedure and an analyte recovery of nearly 100% was obtained. The calibration solutions were prepared in *aqua regia* medium, exactly like the samples, being the *aqua regia* used for the standards preparation submitted to the same heating process as those employed for the samples digestion, in order to match the acidic condition between the samples and the standards. The mercury solutions concentrations used for the calibration curve were 0.05, 0.20, 0.50, 1.0 and 1.5 $\mu g\ L^{-1}$, besides the blank solution. The limit of quantification for Hg, defined as ten times the standard deviation of eight blank solutions, submitted to the heating process for digestion, divided by the slope of the calibration curve, was 43.1 ng L^{-1}, or 10.8 $\mu g\ kg^{-1}$, considering the sample mass of 0.1 g and the final mass of the diluted digest of 25 g. The blank solution presented an estimated mercury concentration of approximately 10 ng L^{-1}.

During the first tests using *aqua regia* for the samples digestion, the use of 5.0 mL of fresh *aqua regia* topped up with BDW to 25 g in order to prepare the calibration standard solutions was tried. However with this procedure, recovery values for the CRM around 200% were generated, thus demonstrating the difference in the acidic condition of the *aqua regia* before and after the submission to heating process. In this sense, the problem was circumvented by

submitting aliquots of 5.0 mL of *aqua regia* in glass vials to the heating process for the digestion procedure simultaneously with the samples. After that, the standard solutions were prepared from the "boiled" *aqua regia* and the calibration curve obtained from these standards was successful, generating recovery values for the CRM of around 100%. Thus, the conditions for the determination of total mercury in the crab samples by CV-AFS were fully optimized. This finding highlights the extreme importance of having the same acidic condition for the samples and for the standards when dealing with CV-AFS in order to avoid interferences.

General procedure – Se quantification in the crab samples

For the determination of Se by HR-ICP-MS, the digested sample or the standard solution, both in nitric acid medium, was mixed with the internal standard solution inside the mixing valve with the aid of a peristaltic pump. The mixture was transported into the nebulizer for the formation of the fine aerosol by the action of argon flow and introduction in the torch plasma. The analytical signal for samples and standard solutions was normalized to that obtained for the internal standard, both in the high resolution mode. A solution of 1% (v/v) nitric acid trace analysis grade was aspirated into the plasma between samples for cleaning purposes.

Considering the broad calibration range, no dilution step was necessary. The standard solutions were prepared in nitric acid medium in order to match to the samples medium. However, once at the end of the microwave digestion procedure most of the reagents is volatilized, especially hydrogen peroxide, the

amount of acid used for the preparation of the standard solutions was half of that used initially for the digestion of samples, considering that the final volume of the digested sample was about 0.5 mL. The selenium standard solutions used for the calibration curve were 0.1, 0.75, 5.0, 25.0, 60.0 and 100.0 μg L^{-1}, besides the blank solution. The limit of quantification for Se was 100.0 μg kg^{-1}. This value was defined as ten times the standard deviation of the ratio of the signal for selenium and germanium, in high resolution mode, obtained for the reagent blank, then divided by the slope of the calibration curve, and finally taking into account the sample mass of 0.1 g and the final mass of the diluted digest of 25 g. The blank solution obtained for sample preparation presented an estimated selenium concentration of about 0,07 μg L^{-1}.

Results and Discussion

All samples obtained from the dissection sessions of the crabs were analyzed in duplicate, after proper procedures for digestion, by CV-AFS for total Hg and HR-ICP-MS for total Se. In the figures to be presented forward, the bars represent the amount of mercury in each of the investigated tissues, taking into account the total mass of each tissue, being “Control 1” and “Control 2” the control crabs, and “E” the exposed crabs to mercury/selenium compounds; “Muscle C” derived from carapace (or thorax) and “Muscle L” derived from legs. For the graphs, only the crabs which ate at least two out of the three spiked servings were included, and in order to keep the statistical meaning, only the crabs that ate all spiked servings were considered for the *t*-tests. Mortality

happened once in the exposure to methylmercury plus selenate, as well as in the exposure to inorganic mercury plus co-exposure to selenate. For that matter, it was chosen not to consider these subjects for the investigations.

Exposure to inorganic mercury with or without selenate

Figure 1A presents Hg measurement results for the inorganic mercury exposure. According to this figure, the tissues of the crabs from the control group contained a few micrograms of Hg, and for the exposed crabs, designated by the letter E, followed by their respective numbers, the amount of mercury is between 10.0 and 14.0 µg. Considering that the crabs were exposed to 15.0 µg of each mercury specie, it was possible to recover around 10.0 µg from the investigated tissues. For this exposure, apparently, mercury was basically accumulated in the hepatopancreas, muscle from thorax and legs, and heart, but mercury was also excreted. However, even though results from the Student *t*-test for inorganic mercury exposure, presented in Table 1, showed significant differences between the Hg amount in control and exposed crabs (p-value < 0.05) for hepatopancreas, muscle from thorax, gill and heart, this was not valid for muscle from legs, despite the very prominent visual difference in Hg amount for this tissue between control and exposed crabs, specially for the crabs E4 and E6.

Similar results as those found here for mercury accumulation in the gill and hindgut of crabs was reported by Laporte *et al.* [10], who stated that inorganic and organic mercury uptake across the gill and intestinal tissues of the crab *Callinectes sapidus* (blue crab) are rapid and of the same order of

magnitude, and that this is due to the relatively unspecific nature of the uptake, with the potential for accumulation of mercury by a variety of pathways.

For the co-exposure to selenate, Figure 1B, apparently the accumulation occurred mainly in the hepatopancreas and heart, being much less prominent in the muscle, especially when compared to the distribution pattern of the exposure to inorganic mercury only (Figure 1A). On the other hand, the *t*-test could only prove the accumulation of mercury in the heart of the exposed crabs. For the Hg accumulation in the hepatopancreas being proved in this exposure, we would then need to exceptionally adopt a more conservative probability for the *t*-test by applying a confidence level of 90% (α=0.1), which was not our choice. Additionally, a *t*-test for the total Hg amount measured from all investigated tissues attested that the Hg body retention was statistically similar (p-value = 0.7877) for both exposures to inorganic Hg. For this evaluation, equal variance was confirmed (F = 0.3534).

We emphasize that in both exposures to inorganic mercury with and without selenate co-exposure, the exposed crab number 3, E3, has eaten only two out of the three spiked servings, which justifies the lower amount of mercury found in its tissues, in comparison to those tissues from crabs which have eaten all three spiked servings. Considering this fact, this crab was not included in the *t*-test. To summarize, although the body retention of mercury for the co-exposure to selenium was similar to that for the inorganic mercury exposure, the distribution in the tissues presented a different pattern, which can be observed from Figures 1A and 1B as well as from the statistical figures presented on Table 1.

For each of the exposures to inorganic mercury with or without selenate co-exposure, there was one crab which has not eaten any of the spiked servings. In these cases, the condition can be compared to those of a polluted environment, and then the mercury accumulation in the gills can be expected. In fact the concentration of mercury found in the gills of these crabs was around three times higher than those from the crabs which ate all the spiked servings, and in the same order as the measured concentration in their muscle. Usually the mercury concentration in the gills in the present study was about two to three times lower than that in the muscles. This finding matches with that from Coelho *et al.* 2008 [4], who observed that for an estuary area in Portugal, with high concentrations of mercury, the crabs *C. maenas* mainly accumulated mercury in the gills, enhancing with age.

Exposure to methylmercury with or without selenate

When the exposure to methylmercury is considered, Figure 2A, it is observed that most of the mercury seems to be accumulated in the muscles, which can also be observed for the co-exposure to selenium, Figure 2B. Though, in the last case, the excretion of mercury seems more prominent. However, by considering the *t*-test for these exposures, also in Table 1, the accumulation of mercury was significant for the methylmercury exposure not only in the muscles, but also in the hepatopancreas, heart, gill and testis/ovary. For the co-exposure to selenium, the accumulation of mercury was statistically confirmed only in the muscle from thorax, hepatopancreas and gill. Likewise, for the Hg accumulation in the muscle from legs to be statistically significant in this

exposure, it would be necessary to adopt a confidence level of 90% (α=0.1), which was not our choice. Finally, body retention of mercury for methylmercury exposure is lower than for selenium co-exposure, applying the *t*-test (p-value = 0.0428, F = 0.2354; Figures 2A and 2B).

In the work of Coelho *et al.* 2008 [4], it was also observed that, the highest accumulation of methylmercury occurred in the muscle (around 90% of total Hg), compared to muscle, hepatopancreas, gill and carapace. This is, at a certain point, in agreement with what was found in our work, considering that the muscles were the tissues that presented the most prominent mercury accumulation under exposition to methylmercury.

According to Boudou and Ribeyre [5], mercury uptake by the cells is by passive diffusion through the membranes. A high methylmercury bioaccumulation capacity is frequently attributed to the lipophilic character of this organic compound. Indeed, the predominance of methylmercury burdens in the muscle of fish rather than in the fatty tissues clearly shows that bioaccumulation is not governed solely by the liposolubility of this chemical form. In fact, neutral chemical species of inorganic Hg have octanol/water partition coefficients (K_{ow}) similar to, indeed higher than, those of methylmercury: $HgCl_2$, 3.3; HgOHCl, 1.2; $Hg(OH)_2$, 0.05; CH_3HgCl, 1.7; CH_3HgOH, 0.07. These facts agree with what was observed in the present experiment, namely, markedly preferential accumulation of mercury in muscle when dealing with exposure to methylmercury.

In this sense, it is noteworthy that the role of hepatopancreas can be absorbing the excess of heavy metals from the circulatory system, and consequently, keep the hemolymph heavy metals at fairly normal level. This

seems to be a way to prevent other organs from being affected, thus limiting the mercury distribution (in the present evaluation) to other internal target organs and, probably, its toxic effects to the organism [2]. This finding suggests that inorganic mercury represents a more toxic condition for the crabs than methylmercury itself, judging by the highest concentrations of mercury found in the hepatopancreas when dealing with the exposure to inorganic mercury. Accordingly, the mercury body retention for the methylmercury exposure from all tissues studied was the lowest of all exposures to mercury species, around 8 µg, which was confirmed by the *t*-test for the comparison to the co-exposure to selenium, as discussed previously, and also for the comparison to inorganic Hg exposure (p-value = 0.0046, F = 0.7281), and to inorganic Hg plus selenate exposure (p-value = 0.0202, F = 0.3838).

For the exposure to methylmercury, exposed crabs 1 and 4 (E1 and E4) have consumed only two out of the three spiked servings, which was also the case for crab E4 from the exposure to methylmercury plus selenate. Otherwise, for all the crabs that have not eaten all spiked servings, the sum of Hg amount from their tissues was higher than that of the crabs which ate all the spiked servings. This may be reflecting a relative high background mercury concentration in their tissues, which however is very hard to estimate accurately because the crabs were capture. Nevertheless, an idea of how much this is can be taken from the mercury amount in the control crabs. Then, the natural high variability among the crabs can explain why crabs that have not eaten all the spiked servings can present higher mercury amount than those that have eaten all the spiked servings.

Finally, an assessment of the total mercury concentration found in the tissues of control crabs from the present work (from all exposures) has been made in order to compare to those found by other references in *C. maena* captured in areas with low contamination [2,4]. Table 2 presents these data. The total mercury concentrations found in the present work are in the same order or lower than those shown in the cited references, which corroborates that a high variability can be expected on mercury concentration in the same tissue of different animals when dealing with capture crabs.

Exposure to selenate

Additionally, a group of crabs was also exposed to selenate, as shown in Figure 3. Surprisingly, also for this exposure, the amount of mercury was higher in the tissues of the exposed crabs in comparison to those of the control crabs, muscle being the most affected tissue. When we consider the *t*-test for this exposure, the accumulation of mercury was statistically significant in the muscle from legs, at a 95% confidence level. These results suggest that in the presence of selenium, the absorption of mercury from the environment or even from other tissues of the exposed crabs were rearranged and accumulated in the muscle. Nevertheless, in spite of the clear visual distinction between the amounts of mercury in the muscle from thorax in the control versus the exposed crabs, this difference was not statistically proven. However, the amount of Hg in muscle from thorax, when comparing exposed to control crabs, was almost two times higher than that for muscle from legs, to be exact 4.7 versus 2.6.

The crabs of selenate exposure were the ones with the smallest body weight, which made the collection of the smallest tissues (collection itself or of a significant amount of the tissue) very difficult, like testis/ovary, heart, hindgut and stomach, which justifies the occasional absence of these data for the evaluations. Additionally, the collection of faeces was very critical, and often at the end of the exposure period the remaining of it was only a suspension-like, largely sprayed in the seawater from the tank. Thus, it was not possible to take into account this sample for statistical evaluation of the exposures, notably for the lack of data for the control groups.

Selenium versus mercury in the crab tissues

The statistical evaluation of the selenium amount in the tissues of the crabs from all exposures was also accomplished. In general, for all exposures the amount of selenium changed sharply in the various studied tissues. The summation of the selenium amount, estimated from the measured Se concentration for all available tissues of each crab as well as the total mass of each tissue, varied from 9 µg to 80 µg. Certainly, for the crabs with the highest amounts of selenium, the exposition to 6 µg of selenate, amount employed for the exposure in the present work, was not significant from the bioaccumulation point of view, and furthermore, the application of the Student *t*-test for these results did not attest the accumulation of selenium in any of the exposure with selenate, or its diminishment for any of the exposures, with the exception of the decreasing of selenium in the heart of the crabs exposed simultaneously to inorganic mercury and selenate, which was statistically significant. Beyond that,

the *t*-test confirmed the increasing of the selenium content in the gill of the crabs exposed to inorganic mercury. Accordingly, the accumulation of mercury was statistically significant in both cases, i.e., gill from inorganic mercury exposure and heart from inorganic mercury plus selenate exposure.

As a final point, by examining the number of significant differences between tissues of control and exposed crabs from all exposures as regards Hg accumulation, presented on Table 1, it turns out that selenium indeed had a very important role in the tissues protection against the accumulation of the mercury species. While for the exposures to inorganic mercury and methylmercury four and six tissues accumulated Hg, respectively, in the presence of selenate the accumulation diminished to one and three tissues, respectively, namely, heart (for Hg^{2+} + Se(VI)), and muscle from thorax, hepatopancreas and gill (for MeHg + Se(VI)), tissues which were also harmed when Se was not present. Also, the body retention of mercury species from the investigated tissues was statistically analogous among the exposures to inorganic Hg, inorganic Hg plus selenate and methylmercury plus selenate (Hg^{2+} versus MeHg + Se(VI): p-value = 0.7173, F = 0.2618, and Hg^{2+} + Se(VI) versus MeHg + Se(VI): p-value = 0.8911, F = 0.8045), however lower to methylmercury exposure in comparison to all other exposures to mercury species with or without selenium.

The statistical evaluation of the ratio between the amount of selenium and mercury in the tissues was also fulfilled, as presented in Table 3. For all statistical significant figures at a 95% confidence level, highlighted in bold, the diminishment of the Se:Hg ratio occurred. Furthermore, in agreement to what was observed from the statistical evaluation of the Hg amount in the tissues of

the crabs co-exposed to selenium, explicitly, the protective role the dietary selenium played against Hg accumulation in most of the tissues, the Se:Hg ratio was preserved for all investigated tissues under co-exposure to selenate as well, emphasizing the important role selenate played in order to protect the tissues. Only exception occurred with hepatopancreas for the exposure to methylmercury plus selenate, which had the ratio decreased. On the other hand, when selenate was not present, the tissues of the crabs exposed to mercury species had the Se:Hg ratio significantly harmed. Taking these facts into consideration, it is possible to state that selenium changes the mechanism of action of the investigated mercury species and reduces the tissues damage caused by them, factors which define a modification on the toxicodynamics of these species on crabs. Further, the co-exposure to selenium proved to alter the transport of the mercury species by blood to the various organs as well as to decrease their uptake and accumulation, which was also observed for the Se:Hg ratio evaluation, showing also a change in the toxicokinetics of mercury species in crabs tissues. According to Khan *et al.* [6], though the presence of selenium may presumably turn the mercury species less bioavailable because of the strong Hg-Se bonding, the formation of such compounds could also induce a selenium deficiency, specially when considering the large number of affected tissues for the exposures to mercury species without selenate in the evaluation of the Se:Hg ratio.

Conclusion

A study of Hg exposure at the organism level and at the main organs level gives a better understanding of the overall accumulation mechanisms involved during exposure. For all the performed exposures, the recovery of mercury from the analyzed tissues was very similar, except for the lower recovery for methylmercury exposure, as well as the profile for all control crabs, which presented low amounts of mercury in all investigated tissues.

Mercury amount and mercury–selenium interactions in hepatopancreas, heart and muscle were highly significant for the exposure to mercury species without selenium, supporting the concept that both elements should be monitored when evaluating potential health consequences from exposure to mercury species. In addition, the accumulation of mercury in the muscle of the crabs exposed to methylmercury, with or without selenate, was more prominent than that in the tissues with higher lipid content, namely, hepatopancreas and heart, which clearly supports the hypothesis that methylmercury bioaccumulation cannot be solely explained by the liposolubility of this specie. Furthermore, dietary selenium played a very important role against the toxicity of the mercury species investigated in this work, showing to change their toxicokinetics and toxicodynamics in crabs tissues, and in consequence, their uptake and accumulation patterns. This finding can be also supported by the evaluation of the Se:Hg ratio for the tissues, which was constant for all tissues from the co-exposures to selenate, with the exception of hepatopancreas for the exposure to methylmercury plus selenate.

Besides, considering that *C. maenas* is consumed by humans and is a prey to some fish and birds, it is one step in mercury accumulation through the food chain.

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References

1. Baeta, A., Cabral, H. N., Marques, J. C., Pardal, M. A. (2006). Feeding ecology of the green crab, *Carcinus maenas* (L., 1758) in a temperate estuary, Portugal. Crustaceana, 79(10), 1181-1193.
2. Costa, S., Viegas, I., Pereira, E., Duarte, A. C., Palmeira, C. M., Pardal, M. A. (2011). Differential sex, morphotype and tissue accumulation of mercury in the crab *Carcinus maenas*. Water Air Soil Pollut, 222, 65–75.
3. Nordberg, G. F., Fowler, B. A., Nordberg, M., Friberg, L. (2007). Handbook on the Toxicology of Metals (3th Edition ed.). Academic Press.
4. Coelho, J., Reis, A., Ventura, S., Pereira, M., Duarte, A., Pardal, M. (2008). Pattern and pathways for mercury lifespan bioaccumulation in *Carcinus maenas*. Marine Pollution Bulletin, 56, 1104–1110.

5. Boudou, A., Ribeyre, F. (1997). Aquatic ecotoxicology: From the ecosystem to the cellular and molecular levels. Environmental Health Perspectives, 105 (Suppl. 1), 21-35.
6. Khan, M. A., Wang, F. (2009). Mercury-selenium compounds and their toxicological significance: Toward a molecular understanding of the mercury-selenium antagonism. Environmental Toxicology and Chemistry, 28(8), 1567-1577.
7. Ralston, N. V., Ralston, C. R., Blackwell, J. L., Raymond, L. J. (2008). Dietary and tissue selenium in relation to methylmercury toxicity. NeuroToxicology, 29, 802–811.
8. Ralston, N. V. (2008). Selenium health benefit values as seafood safety criteria. EcoHealth, 5(4), 442-455.
9. The Composition of Fish. Access on 19th of October 2012, available at FAO: Food and Agriculture Organization of the United Nations: http://www.fao.org/wairdocs/tan/x5916e/x5916e01.htm
10. Laporte, J., Andres, S., Mason, R. (2002). Effect of ligands and other metals on the uptake of mercury and methylmercury across the gills and the intestine of the blue crab (*Callinectes sapidus*). Comparative Biochemistry and Physiology Part C, 131, 185-196.

Tables

Table 1 – Results of *t* and F tests applied to the amount of total mercury in the tissues (micrograms) to evaluate the exposures. Significant values in bold.

Exposure	**Hg^{2+}**		**Hg^{2+} + Se(VI)**		**MeHg**		**MeHg + Se(VI)**		**Se(VI)**	
Tissue	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F
Carapace	0.1461	0.1125	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Stomach	0.1135*	**0.0085**	0.1053	0.6511	0.0662	0.2953	<LOQ	<LOQ	N.A.	N.A.
Hepatopancreas	**0.0098**	0.0890	0.0717	0.0583	**0.0026**	0.2467	**0.0227**	0.0998	0.1979	0.3442
Muscle (Thorax)	**0.0143**	0.9515	0.1492	0.2046	**0.0319**	0.8857	**0.0053**	0.5144	0.1354*	**0.0430**
Muscle (Legs)	0.1684	0.5279	0.1266	0.0511	**0.0018**	0.1244	0.0872	0.7108	**0.0238**	0.2309
Hindgut	0.0763	0.1229	0.2400*	**0.0281**	0.0951	0.1196	0.0666	0.6724	N.A.	N.A.
Gill	**0.0141**	0.4609	0.4162*	**0.0188**	**0.0191**	0.2720	**0.0164**	0.5579	0.5680	0.8500
Heart	**0.0002**	0.6666	**0.0005**	0.7686	**0.0457**	0.9477	0.2313	0.2199	N.A.	N.A.
Testis/Ovary	<LOQ	<LOQ	<LOQ	<LOQ	**0.0047**	0.5903	<LOQ	<LOQ	N.A.	N.A.

* Student's *t*-test for unequal variance (heteroscedastic), applied when calculated F was lower than 0.05.

N.A.: Tissue not available.

< LOQ: Concentration of mercury measured in the tissue of the control crabs below the LOQ.

Table 2 – Comparison of an estimate of total mercury concentration, in mg kg^{-1}, found in tissues of control crabs (*C. maena*) from the present work to those of other authors.

Tissue / Source	Carapace	Hepatopanc.	Muscle[c]	Gill
Present work [a]	<LOQ[b]	<LOQ–0.077	<LOQ–0.150	<LOQ–0.033
Ref. [2]	0.010	0.010	0.025	0.020
Ref. [4]	N.A.	0.020–0.140	0.020–0.150	0.020–0.065

[a] Control crabs from all exposures; [b] LOQ = 0.011 mg kg^{-1}; [c] Estimated from muscle from legs and thorax (analyzed independently) for the crabs of the present work and as a pool from the other references; N.A.: Not available.

Table 3 – Results of *t* and F tests from the ratio Se/Hg in the tissues (micromols) to evaluate the different exposures. Significant values in bold.

Exposure	**Hg^{2+}**		**Hg^{2+} + Se(VI)**		**MeHg**		**MeHg + Se(VI)**		**Se(VI)**	
Tissue	p-value	F	p-value	F	p-value	F	p-value	F	p-value	F
Carapace	0.2912*	**0.0130**	I.D.	I.D.	I.D.	I.D.	I.D.	I.D.	I.D.	I.D.
Stomach	0.2279*	**0.0031**	0.4723	0.2012	**0.0004**	0.5564	I.D.	I.D.	I.D.	I.D.
Hepatopancreas	0.1071*	**0.0471**	0.2962*	**0.0213**	**0.0354**	0.3493	**0.0342**	0.0890	0.9322*	**0.0001**
Muscle (Thorax)	**0.0128**	0.1175	0.7880	0.1568	0.2405*	**0.0269**	0.0758	0.4449	**0.0049**	0.8626
Muscle (Legs)	**0.0033**	0.9870	0.5129	0.8180	0.0672	0.6421	0.0585	0.5547	0.4144*	**0.0268**
Hindgut	**0.0077**	0.4076	0.4502*	**0.0145**	**0.0042**	0.4002	0.2257	0.1674	I.D.	I.D.
Gill	0.3250*	**0.0091**	0.2283	0.2904	**0.0053**	0.1170	0.1492	0.0783	0.3339	0.9017
Heart	**0.0004**	0.1293	0.1970*	**0.0211**	0.1323*	**0.0259**	0.1975*	**0.0073**	I.D.	I.D.
Testis/Ovary	I.D.	I.D.	I.D.	I.D.	**0.0038***	**0.0105**	I.D.	I.D.	I.D.	I.D.

* Student's *t*-test for unequal variance (heteroscedastic), applied when calculated F was lower than 0.05.

I.D.: Insufficient data to calculate the ratio.

Figures

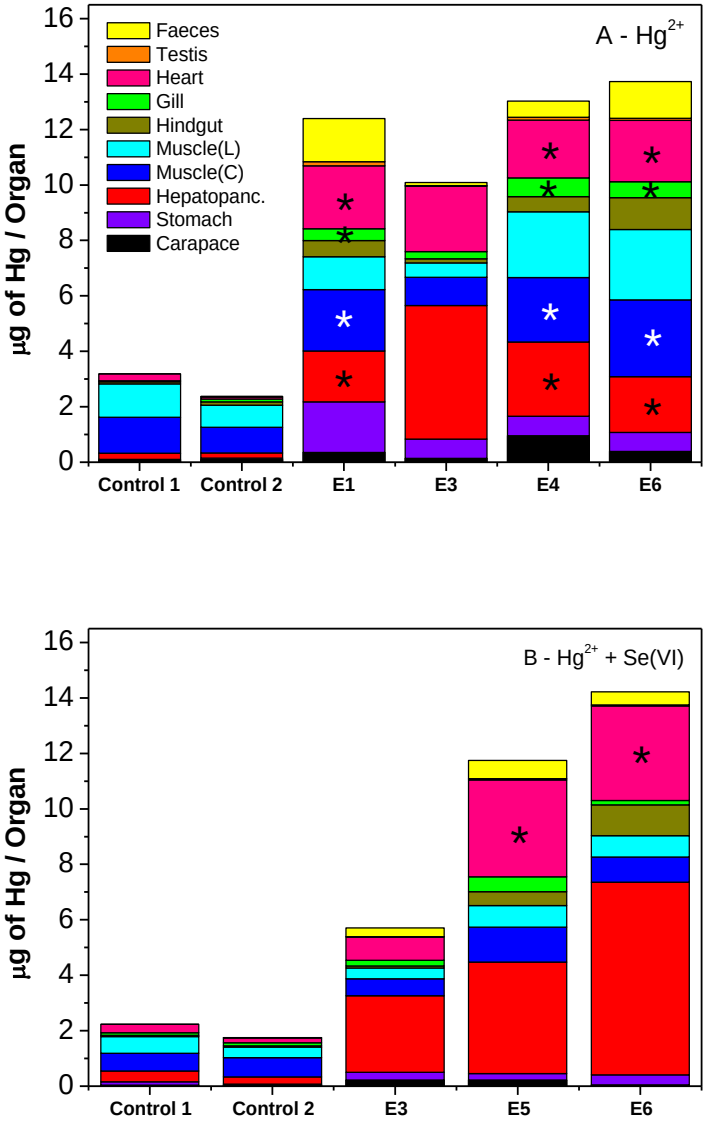

Figure 1 – Evaluation of the distribution of mercury in the tissues of crabs exposed to: A – Inorganic mercury; B – Inorganic mercury plus selenate. Legend: Control = control crabs; E = exposed crabs to inorganic mercury w/o selenate; * p-value < 0.05 = significant difference between the total mercury amount in the tissues comparing control and exposed crabs which have eaten all spiked servings.

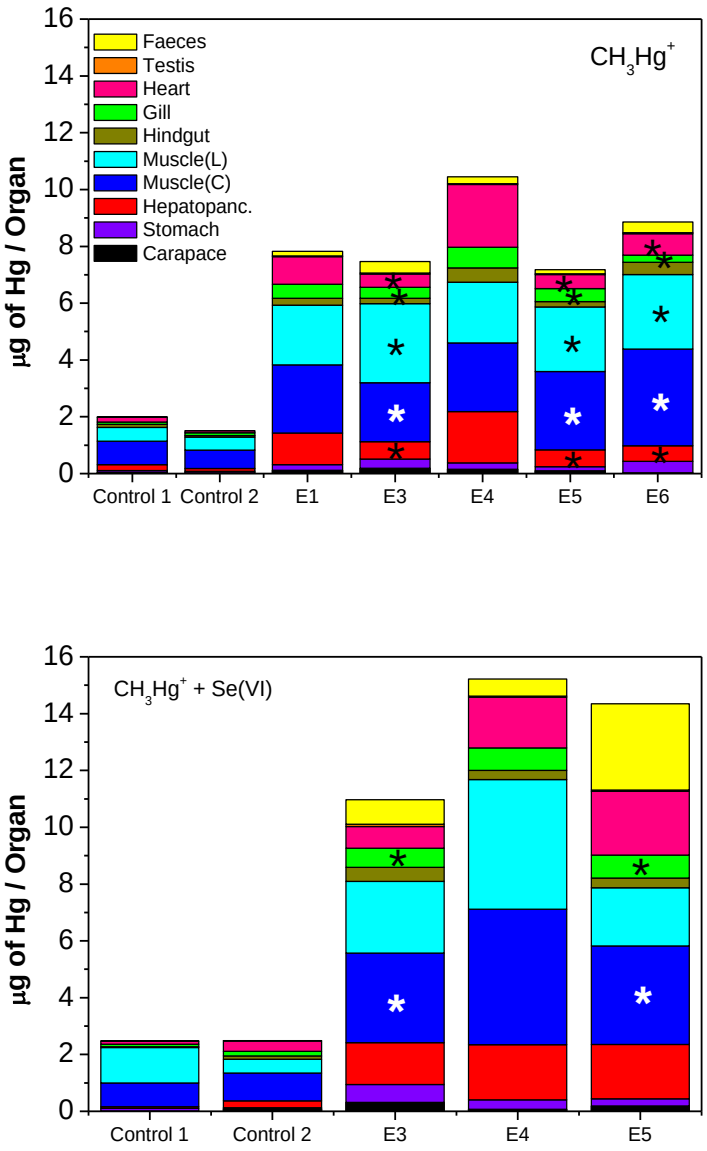

Figure 2 – Evaluation of the distribution of mercury in the tissues of crabs exposed to: A –Methylmercury; B – Methylmercury plus selenate. Legend: Control = control crabs; E = exposed crabs to methylmercury w/o selenate; * p-value < 0.05 = significant difference between the total mercury amount in the tissues comparing control and exposed crabs which have eaten all spiked servings.

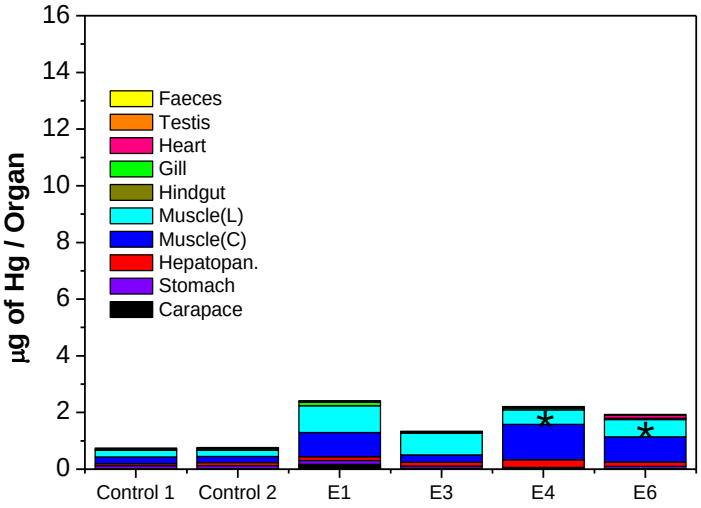

Figure 3 – Evaluation of the distribution of mercury in the tissues of crabs exposed to selenate. Legend: Control = control crabs; E = exposed crabs to selenate; * p-value < 0.05 = significant difference between the total mercury amount in the tissues comparing control and exposed crabs which have eaten all spiked servings.