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MONITORING OF HEAVY METAL RESIDUES, METAL-METAL INTERACTIONS AND THE EFFECT OF COOKING ON THE METAL LOAD IN SHELLFISH

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ABSTRACT

Heavy metal pollution is considered a major problem worldwide. Heavy metals find their way to human body mainly through diet, water and air. Shellfish represents a major source of animal-derived protein, vitamins and trace elements; however, it may contribute to human exposure to heavy metals. This study was undertaken to monitor the toxic metal (lead, cadmium, mercury and arsenic) residues in six shellfish namely, shrimp, crab, crayfish, clam, oyster and mussel collected from fish markets in Ismailia governorate, Egypt. Moreover, metal-metal interactions were also analyzed. Furthermore, human health risk assessment was estimated. Finally, the effect of different cooking methods on the toxic metal load in the shrimp was investigated. The results declared a clear and significant variation in the accumulation pattern of each metal in the examined shellfish. Positive correlations in the accumulation pattern of some metals were also observed in this study. High and continuous dietary intake of shellfish may constitute health hazards among consumers. Grilling and barbecuing of shellfish lead to an increase in the concentrations of toxic metals. However, boiling and frying significantly reduced the metal load in the shellfish. Public health implications of the examined metals were also discussed.

Keywords: Heavy metals, shellfish, cooking, health risk.

INTRODUCTION

Marine shellfish has important role in human diet, because they supply human with part of high quality protein and vitamins. They also contain several dietary minerals such as calcium and iron (Javaheri Baboli and Velayatzadeh, 2013). Heavy metal pollution of the aquatic system due to anthropogenic activities is a major problem in both developing and developed countries. Toxic metals like lead, cadmium, mercury and arsenic may interfere with the ecology of this aquatic system, and can find their way to human body through the food chain where causing potential public health hazards (Ismahene and El Hadi, 2012). However, few reports are published about the current situation of heavy metal pollution in the aquatic biota in Egypt.

Shellfish can accumulate higher concentrations of metals, as they are good biomarkers for metal pollution in the aquatic system. Thus, estimation of heavy metals in the tissues of these organisms reflect the current situation of such pollution and monitoring the potential risk to humans because a large population directly consumes them (Guerra-Garcia *et al*., 2010). It is well established, that excessive concentrations of heavy metals in food are linked to major adverse health effects especially cardiovascular, renal, neurological, and bone diseases. Moreover, heavy metals such as lead (Pb), cadmium (Cd), arsenic (As) and mercury (Hg), when ingested, produce severe adverse effects on human health. These metals are potent carcinogenic and mutagenic substances. Heavy metal toxicity can result in lower energy levels, and damage to blood composition, lungs, kidneys, liver and other vital organs (Chailapakul *et al*., 2008). Estimation of toxic metal residual concentrations in the shellfish is of a significant value for protection of consumers of such valuable animal-derived protein source.

Thus, this study was undertaken to estimate the residual concentrations of Pb, Cd, As and Hg in the edible tissue of six commercially important species of shellfish collected from Ismailia governorate, Egypt and to compare the results of the determined heavy metals with international regulatory standards. The inter-relationship between the determined heavy metals in the different shellfish species was also estimated. Furthermore, estimated daily intake (EDI) and health risk assessment for the examined metals were calculated and their public health significance was also discussed. Moreover, we investigated the effect of the most commonly used cooking methods in Egypt (barbecuing, grilling, boiling and frying) and water wash in reduction of the heavy metal load in the shrimp edible tissue.

MATERIALS AND METHODS

All experiments were conducted according to the regulations of Zagazig University, Egypt.

Sample collection & preparation: Ninety samples belonging to six commercial shellfish species were purchased from local anglers at Ismailia governorate, Egypt, in the period of May to July 2016. The chosen species were shrimp, crab, crayfish, clam, oyster and mussel (15 of each). Immediately after sampling all samples were preserved in icebox, labeled and transferred to the laboratory. Furthermore, another 30 shrimp samples were purchased from the same source and divided into 6 groups (n=5 each). These groups were used to investigate the effect of the commonly used cooking methods in Egypt (barbecuing, grilling, boiling and frying) (Darwish *et al*., 2015b) and water wash on the heavy metal load in the edible tissue of the shrimp. In brief, these groups were control group with no further treatment; the second group was immersed in water for wash for a period of 30 min. The third group was barbecued on wire net over charcoal, the distance between the fire and the samples was 50 cm; the fourth group was grilled in an electric oven, the temperature was set to be 180ºC. The fifth group was boiled in water, with the temperature of 100ºC; the sixth group was deep-fried in corn oil until browning of the samples (well-done).

Sample preparation: Inedible parts of the examined shellfish were removed upon arrival. Then, one gram of each sample was macerated in 5 ml of acid digestion mixture (3ml nitric acid (HNO_3) 65 % (Merk, Darmastadt, Germany): 2 ml perchloric acid ($HCLO_4$) 70 % (Merk, Darmastadt, Germany) (Darwish *et al*., 2015a). The content was left to stand overnight at room temperature in falcon tubes. Then, these tubes were incubated at 70ºC for 3 hours in water bath with swirling at 30 min intervals during the heating period. Tubes were left to cool at room temperature, diluted with 20 ml de-ionized water, and filtered by using filter paper (Watt man No.42). The filtrate was kept at room temperature until analysis for heavy metal contents**.**

Analytical procedures: Levels of As and Hg were measured using hydride generation/cold vapor atomic absorption spectroscopy and graphite furnace in case of Pb and Cd (Perkin Elmer® PinAAcle™ 900T atomic absorption spectrophotometer (Shelton, CT, USA). All the analyses were done at the central laboratory, Faculty of Veterinary Medicine, Zagazig University, Egypt.

Quality assurance and quality control: The reference material; DORM-3 (Fish protein, the National Research Council, Canada) was used to ensure the accuracy and validity of the analytical procedures of heavy metals. Recovery rates ranged from 80% to 115%. The detection limits (µg/g) for the analyzed metals were 0.1 for Pb; 0.005 for Cd; 0.02 for As and 0.2 for Hg. The registered values for Pb, Cd, As and Hg were expressed as µg/g wet weight (ppm).

Estimated daily intake (EDI): EDI (µg/kg/day) for heavy metals was obtained using the following equation described by the Human Health Evaluation Manual (US Environmental Protection Agency, EPA) (2010):

$$EDI = \frac{C_m x F_{IR}}{BW}$$

Where C_m is the concentration of the metal in the sample, (µg/g wet weight); F_{IR} is the food (fish) ingestion rate in Egypt, which was estimated at 48.57 g/day (FAO 2003); BW is the body weight of Egyptian adults, which was estimated at 70 kg.

Health risk assessment: Non-cancer risk of heavy metals for shellfish consumers in Egypt was evaluated. The risk assessment followed the guidelines recommended by the United States Environmental Protection Agency (US EPA), (1989). For non-carcinogenic effects, the EDI was compared with the recommended reference doses (RfD) (4E03, 1E03, 3E04, 5E04 mg/kg/d for Pb, Cd, As, Hg, respectively) (US EPA, 2010), as stated in the following equation:

$$\text{Hazard Ratio (HR)} = \frac{EDI}{RfD}$$

A hazard index (HI) to estimate the risk of mixed contaminants was also calculated by using the following equation:

$$HI = \sum HR_i$$

where i represents each metal

A HR and/or HI of >1 indicates that there is a potential risk to human health, whereas a result of ≤1 indicates no risk of adverse health effects.

Statistical analysis: Statistical significance was evaluated using either Tukey-Kramer honestly significant difference tests with $p< 0.05$ considered as significant. In case of reduction experiments, statistical significance with the control was done using Dennett's test. Correlation (Multivariate) analyses were performed using JMP program (SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Shellfish is considered a good indicator of environmental pollution with heavy metals, simply because it can accumulate high levels of these metals. However, these organisms are considered valuable source for animal derived protein, vitamins and essential trace elements, especially for people living nearby sea. Thus, estimating the residual levels of these metals, in particular the toxic ones, assessment of the possible human health risks and finding effective reduction strategies of the heavy metal load are major tasks of food and environmental hygienists.

Estimation of heavy metal concentrations and their correlations in the examined shellfish

Lead (Pb): The results obtained in figure (1A) showed the residual concentrations of Pb in the muscle tissue of different shellfish. It notes worthy, that oyster had significantly the highest Pb concentrations, while shrimp had the lowest Pb residues. Generally, the recorded mean ± SE concentrations of Pb in the examined shellfish were 1.01±0.21, 0.73±0.08, 0.63±0.06, 0.51±0.11, 0.45±0.09 and 0.35±0.05 ppm/wet weight in the examined oyster, crab, crayfish, clam, mussel and shrimp, respectively. Levels of Pb in the edible tissue of shellfish from our study were comparable with the levels reported by Olemedo *et al*. (2013) in fish and shellfish species in Andalusia (Southern Spain). However, Vázquez-Boucard *et al.* (2014) reported higher Pb concentrations (up to 7.2 ppm in dry weight) in the edible tissue of sentinel oysters from Sinaloa and Sonora, Mexico. Unlikely, Silva da Araújo*et al.* (2016) detected lower concentrations of Pb in the edible tissue of shrimp, crab and mussel collected from the Aratu Bay, Brazil.

Cadmium (Cd): The results recorded in Figure (1B) showed the mean residual concentrations of Cd (ppm/ww) in the edible tissue of the examined shellfish. Crayfish had significantly the highest mean concentrations of Cd (0.16±0.03), followed by crab (0.09±0.006); shrimp (0.06±0.006); mussel (0.04±0.002); oyster (0.03±0.003) and clam had the lowest Cd residues (0.02±0.001) (Figure 1B). The recorded mean concentrations of Cd in shellfish in this study were low when compared to the concentrations of Cd in the edible tissues of oyster and clams collected from the Nord Medoc salt marshes (Gironde estuary, France) (Baudrimont *et al.,* 2005). Lower concentrations were recorded by Silva da Araújo *et al.* (2016), who detected lower concentrations of Cd in the edible tissue of crustaceans collected from the Aratu Bay, Brazil.

Arsenic (As): The results obtained in figure (2A) showed the residual concentrations of As in the edible tissue of different shellfish. It is clear from the recorded results that crab had significantly the highest As residues (19.40±1.16 ppm/ww), followed by clam (16.72±1.23 ppm/ww), shrimp (15.91±1.60 ppm/ww), oyster (14.60±1.24 ppm/ww), crayfish (13.33±1.43 ppm/ww) and finally mussel (12.55±0.90 ppm/ww) (Fig. 2A). Levels of As in edible tissue of shellfish from our study were similar to those reported by Rattanachongkiat *et al.* (2004) in fish and crustacean species in Pak Pa-Nang Estuary and catchment, located in Southern Thailand. Unlikely, Kucuksezgin *et al.* (2014) detected lower concentrations of As in the marine biota collected from the Izmir Bay (Eastern Aegean sea). However, Krishnakumar *et al.* (2016) reported higher As concentrations (11-134 and 16-118 ppm) in the edible tissue of shrimp and bivalves (clams and oysters) harvested from the western Arabian Gulf.

Mercury (Hg): Total Hg was analyzed in this study and the results were recorded in figure 2B. In the examined shrimp, Hg concentrations ranged between 0.06 to 0.86 ppm/ww with a mean value of 0.43±0.05 ppm/ww. The mean concentrations of the total mercury were 0.31±0.08, 0.46±0.06, 0.43±0.06, 0.48±0.08 and 0.52±0.07 ppm/ww in crab, crayfish, clam, oyster and mussel, respectively (Figure 2B). Hg concentrations in our study were much lower than that recorded in clams and oysters from the Nord Medoc salt marshes, Gironde estuary, France (Baudrimont *et al*., 2005). The recorded concentrations of Hg in our study go in agreement with Páez-Osuna and Osuna-Martínez (2015), who recorded mean Hg concentration of 0.38±0.17 in oyster collected from subtropical coastal lagoons from the southeast Gulf of California, USA.

In Egypt, heavy industries were born since 1950s along the Nile Delta, Cairo, Tenth of Ramadan city, Suez Canal cities such as Ismailia, Suez and others. Undoubtedly, the impact of industrial pollution in Egypt appears in all environmental media: air, water, and land. The worst industrial waste liquids are those heavily laden with organic or heavy metals or with corrosive, toxic or microbially loaded substances. Such waters endanger public health through the direct use as well as through feeding with fish that live in the polluted streams (Abdel-Shafy and Aly, 2002). These anthropogenic activities might explain the high concentrations of the examined metals in the study area. The difference in the concentrations of the examined metals in our study with other reports might reflect the differences in the environmental pollution scenario among different localities. Furthermore, inter-species differences in their ability to accumulate the different metals might be attributed to the inter-species differences in their xenobiotic metabolizing enzymes and their ability in the detoxification of these toxic metals (Ikenaka *et al.,* 2015).

It must be indicated that the accumulation of certain elements may be affected by the elevated exposure to other elements. The clearest phenomenon is the elevation of the concentration of the essential elements by the high exposure levels for non-essential elements as a method of bio-adaptation. The accumulation of Se in tissues exposed to Hg (Cuvin-Aralar and Furness 1991; Yang *et al.,* 2008) is a well-known phenomenon. One of the explanations for this relation is that selenium (Se) has a protective action against Hg toxicity, and the formation of a stable and inert complex between selenite and Hg^{2+} has been described in mammals (Yang *et al.,* 2008). Although this study did not estimate the levels of essential elements, some correlations were observed between the measured non-essential elements. For instances, there was a positive correlation between Cd and Hg in shrimp (Fig. 3A) and Clam (Fig. 4B) (r = 0.353 and 0.355, respectively). Additionally, Pb had a positive correlation with Cd in the examined crab (Fig. 3B) (r = 0.315). Furthermore, As had a slight positive correlation with Pb in the crayfish (r = 0.244) (Fig. 4A), and with Cd in the oyster and mussel (r = 0.380 and 0.524) (Fig. 5A, B). Nearly similar findings were reported before between As-Hg (Maia *et al.,* 2017), and Cd-Pb (Darwish *et al.,* 2015a). Although the mechanisms behind these interactions are not clear, these correlations might be attributed to induction of metallothionein, an enzyme that is linked to metal-detoxification (Komsta-Szumska and Chmielnicka, 1983).

Most of studies performed on estimation of heavy metal residues in food subjects were done on raw samples, although most of foods are consumed cooked but not raw. Thus, a trial to investigate the effect of the commonly used methods of cooking in Egypt or water wash on the accumulation pattern of the tested metals was performed. The achieved results recorded in figure 6 showed that a water wash of samples for 30 min or boiling of samples in water achieved significant reductions in the metal load in muscle of shrimp compared with the control. In particular, a clear reduction in Hg concentrations was achieved. Interestingly, pan-frying achieved the highest reduction for all tested metals with percentages of 40%, 30%, 60% and 20% for Pb, Cd, Hg and As, respectively (Fig. 6). The reduction in pan-frying might be attributed to dissolving of the metals in the cooking oil, and evaporation of Hg (Gokoglu *et al*., 2004). Unlikely, grilling and barbecuing led to a significant increase in the metal concentrations, which may be attributed to the water evaporation and water loss leading to concentration of the metals in the tissue (Kalogeropoulos *et al*., 2012). It notes worthy, that the reduction percentages achieved in this study were corresponding to Atta *et al.* (1997) who found that Cu, Pb, Cd and Zn residual concentrations were significantly reduced in *Tilapia nilotica* on baking and steaming. Furthermore, Ersoy *et al.* (2006) observed a clear reduction in the Pb residual concentration after microwaving of sea bass fillets. Additionally, Kalogeropoulos *et al.* (2012) found that Pb and Cd concentrations increased upon grilling in squid and shrimp compared with the raw ones. The reduction was dependent on cooking conditions like time, temperature and method of cooking.

Health risk assessment: One major task of food and environment hygiene is to investigate the health risk assessment due to consumption of shellfish and other food subjects prior to introduction to consumers. Thus, health risk assessment for the examined metals in the shellfish was performed via comparison of the metal content in the offal with national and international maximum permissible limits (MPL), estimating estimated daily intake (EDI), hazard index (HI) and hazard ratio (HR).

The results reported in table 1 showed percentage of examined samples exceeding European Commission (EC) (2006) MPL, which were 1.0 ppm in Pb, 1.00 ppm in Cd, 10.0 ppm in As and 0.5 ppm in Hg. Pb content exceeded MPL with 20.00%, 13.33%, 46.66% and 13.33% in crab, clam, oyster and mussel, but none of shrimp and cray fish exceeded MPL (Table 1). None of the examined shellfish samples exceeded Cd MPL. Regarding As residues, 80.00%, 93.33%, 73.33%, 86.66%, 80.00% and 60.00% of the examined shrimp, crab, crayfish, clam, oyster and mussel samples, respectively exceeded the EC-MPL (2006) (Table 1). However, in case of Hg, these percentages were 33.30%, 33.30%, 39.96%, 46.62%, 59.94% and 59.94% in shrimp, crab, crayfish, clam, oyster and mussel samples, respectively (Table 1).

The recorded results in table 2 showed the EDI (µg/Kg Bwt/day), hazard ratio (HR) and hazard index (HI) of the examined toxic metals due to consumption of shellfish. EDI values of Pb in shellfish samples ranged from 0.24 to 0.70; 0.02 to 0.11 in cadmium. Whereas EDI values for As ranged from 0.63 to 1.05 and in Hg ranged from 2.49 to 4.31 (Table 2). It notes worthy, that HR values in case of Pb and Cd did not exceed one. However, it ranged between 2.09 to 3.50 in case of As and between 4.99 and 8.64 in Hg. HI values for the total four toxic metals examined (Pb, Cd, As and Hg) exceeded one in all shellfish examined in this study recording value range of 8.59 to 11.52 (Table 2). 0.002 to 0.176 in cadmium, whereas EDI values for Hg ranged from 0.025 to 0.296 (Table 2). Estimated daily intake, hazard ratio, and hazard index revealed higher dietary intake of some toxic metals like As and Hg identified potential risks and hazards; the greatest risks were identified for the end consumers of crustaceans and bivalve mollusks. These values were in agreement with that provided by Whyte *et al.* (2009), Jiang *et al.* (2016) and Raknuzzaman *et al.* (2016) in New Zealand, China and Bangladesh, respectively.

Lead toxicity is linked with anemia due to reduction in the haemoglobin synthesis, renal dysfunction, reproductive and cardiovascular systems and nervous symptoms (Ogwuegbu and Muhanga, 2005). Furthermore, Pb is reported to be the cause of many poisoning cases especially in children in many locations in Nigeria (Ajumobi *et al.* 2014); China (Xu *et al.* 2014) and Zambia (Yabe *et al.* 2015). Additionally, Pb led to a significant cytotoxicity in the human HepG2 cells (Darwish *et al.,* 2016).

Sever human exposure to Cd may result in pulmonary effects such as bronchitis and pneumonia. Renal effects may also result due to sub-chronic exposure to Cd (Young, 2005).

Acute or subacute arsenic exposure might induce gastrointestinal disturbances ranging from mild abdominal cramping and diarrhea to severe life-threatening hemorrhagic gastroenteritis associated with shock. Chronic exposure to arsenic is related to skin lesions, cancers, cardiovascular effects, diabetes, pulmonary malfunction, neurological symptoms, and developmental and reproductive toxicity (Feng *et al.*, 2013).

Mercury and its compounds are highly toxic. Acute health effects reported include kidney failure following exposure to high concentrations of inorganic mercury. Allergic skin reactions were also reported following contact with mercury. Mercury may lead to pulmonary effects. Nervous symptoms were also noted due to mercury exposure such as tremors or increased excitability. People at high risk for mercury exposure displays sever symptoms such as chest pain and pneumonia (FAO/WHO, 2002).

In conclusion, we highly recommend reduction of our daily consumption of shellfish and advice consumers with immersion water wash of shellfish followed by efficient cooking.

Table 1. Percentage of samples exceeding maximum permissible limits according to EC (2006)

	Pb	Cd	As	Hg
MPL (ppm)	**1.0**	**1.0**	**10.0**	**0.5**
Shrimp	0.00%	0.00%	80.00%	33.30%
Crab	20.00%	0.00%	93.33%	33.30%
Crayfish	0.00%	0.00%	73.33%	39.96%
Clam	13.33%	0.00%	86.66%	46.62%
Oyster	46.66%	0.00%	80.00%	59.94%
Mussel	13.33%	0.00%	60.00%	59.94%

MPL: Maximum Permissible Limits according to EC (2006)

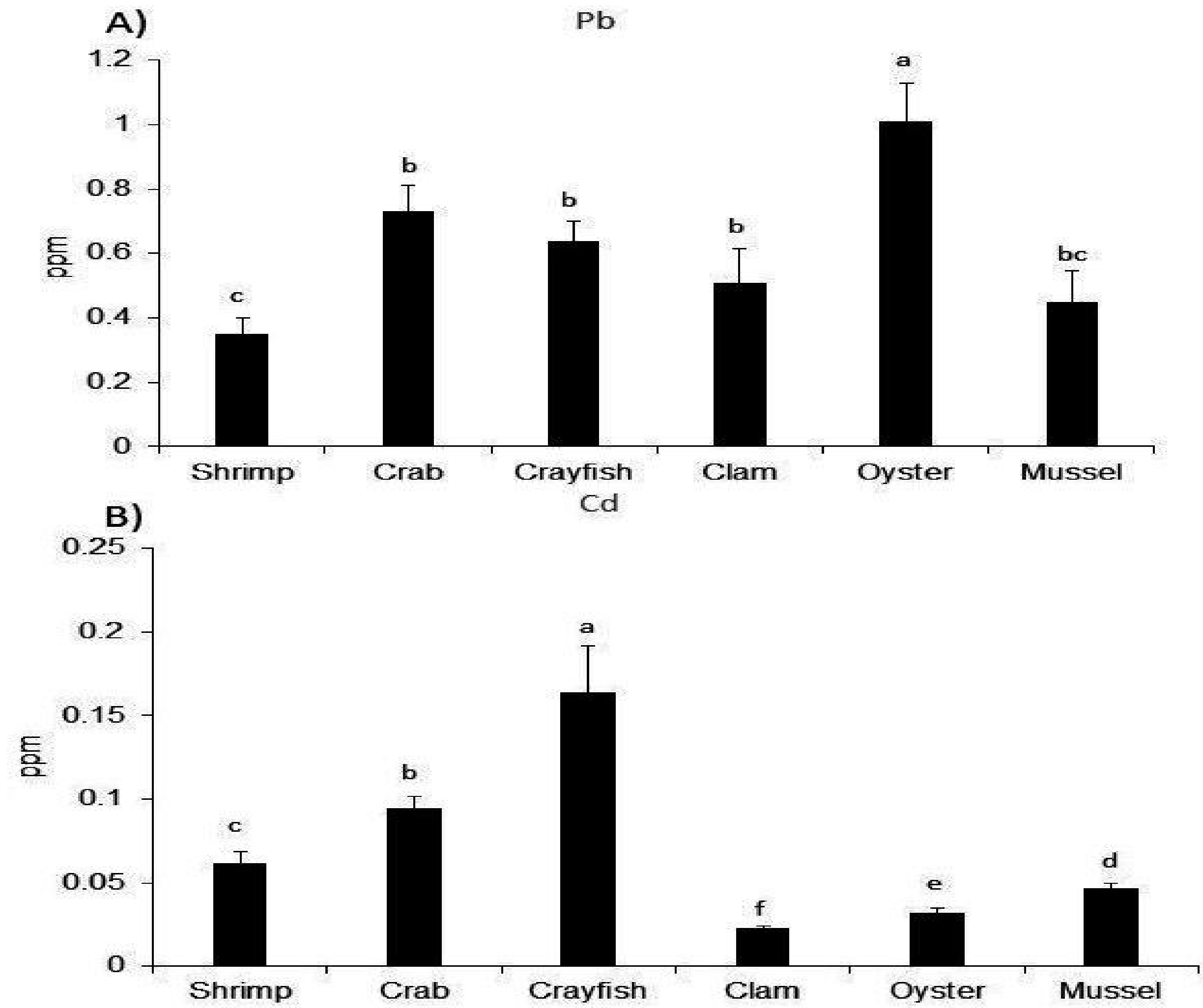

Fig. 1.Lead and cadmium residual concentrations in some shellfish
A) Lead (Pb) B) Cadmium (Cd) residual concentrations mg/kg wet weight (ppm) in the examined crustacea (n=15 for each species). Measurements were done in duplicate for each sample. Columns carrying different superscript letter is significantly different at $p<0.05$.

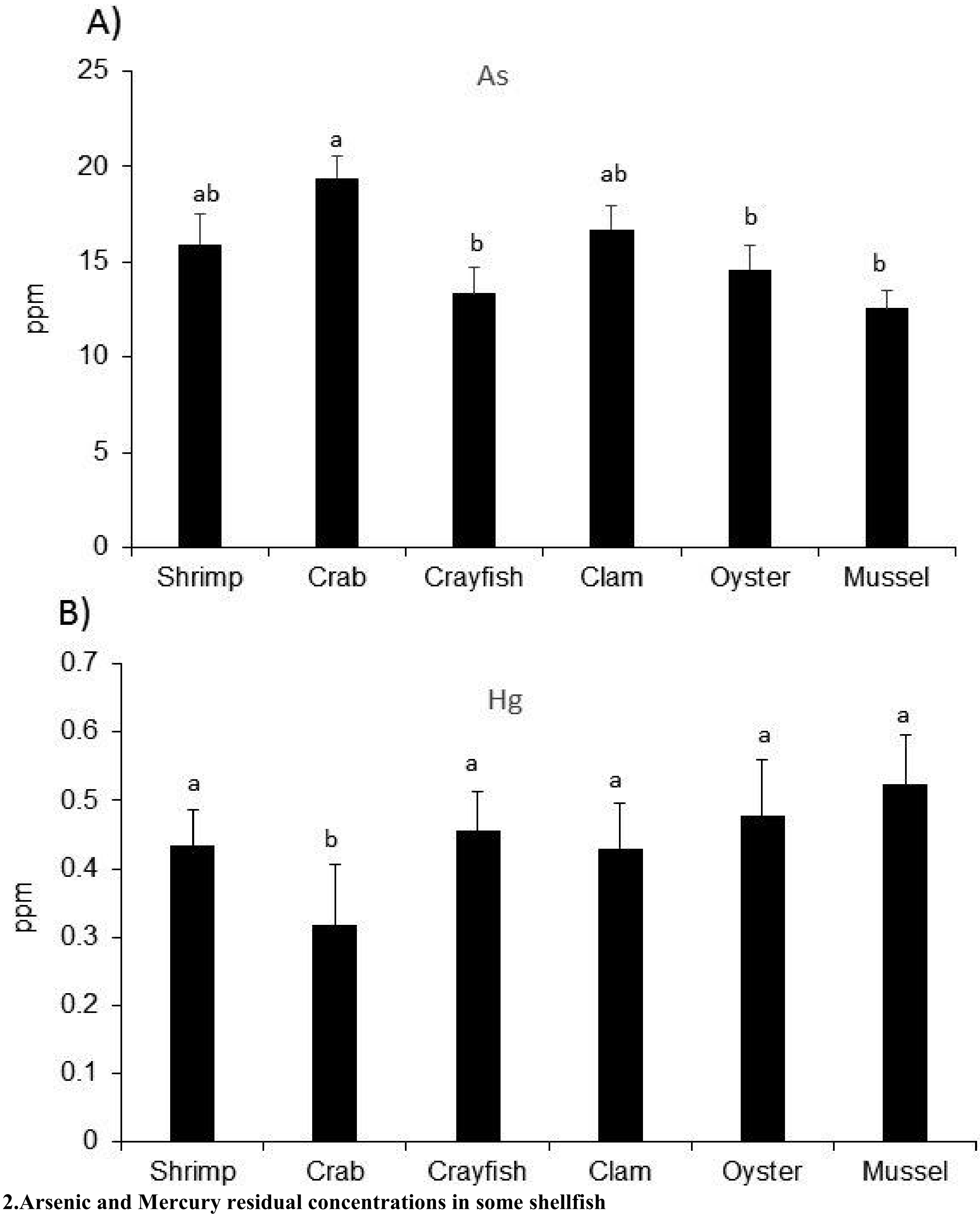

Fig. 2.Arsenic and Mercury residual concentrations in some shellfish
A) Arsenic (As) B) Mercury (Hg) residual concentrations mg/kg wet weight (ppm) in the examined crustacea (n=15 for each species). Measurements were done in duplicate for each sample. Columns carrying different superscript letter is significantly different at $p<0.05$.

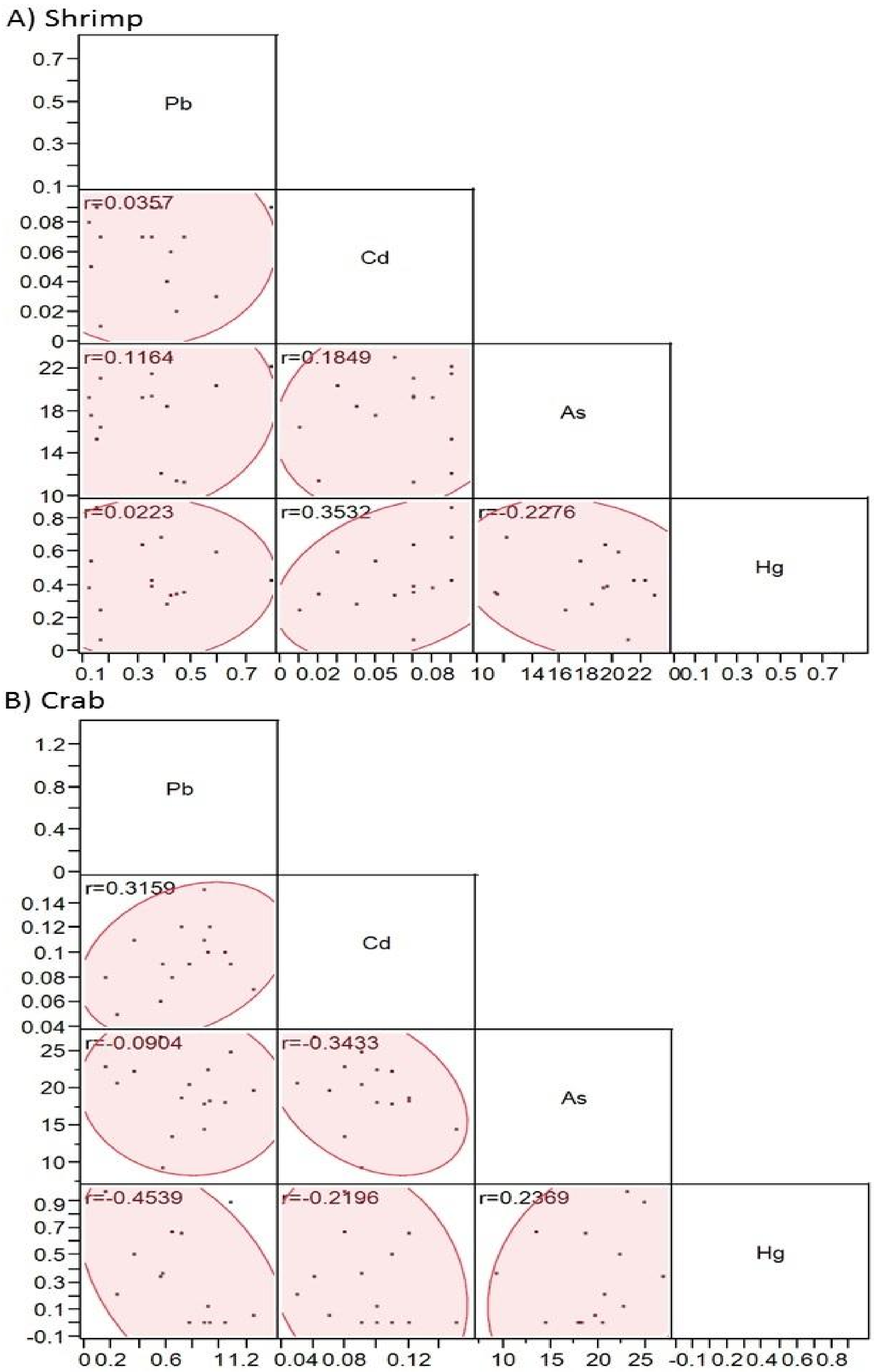

Fig. 3.Metal-metal interactions in shrimp and crab

Scatterplots between different metal concentrations in (A) shrimp and (B) Crab (n=15), r means Pearson correlation. This analysis was done using JMP software (SAS Institute, Cary, NC, USA).

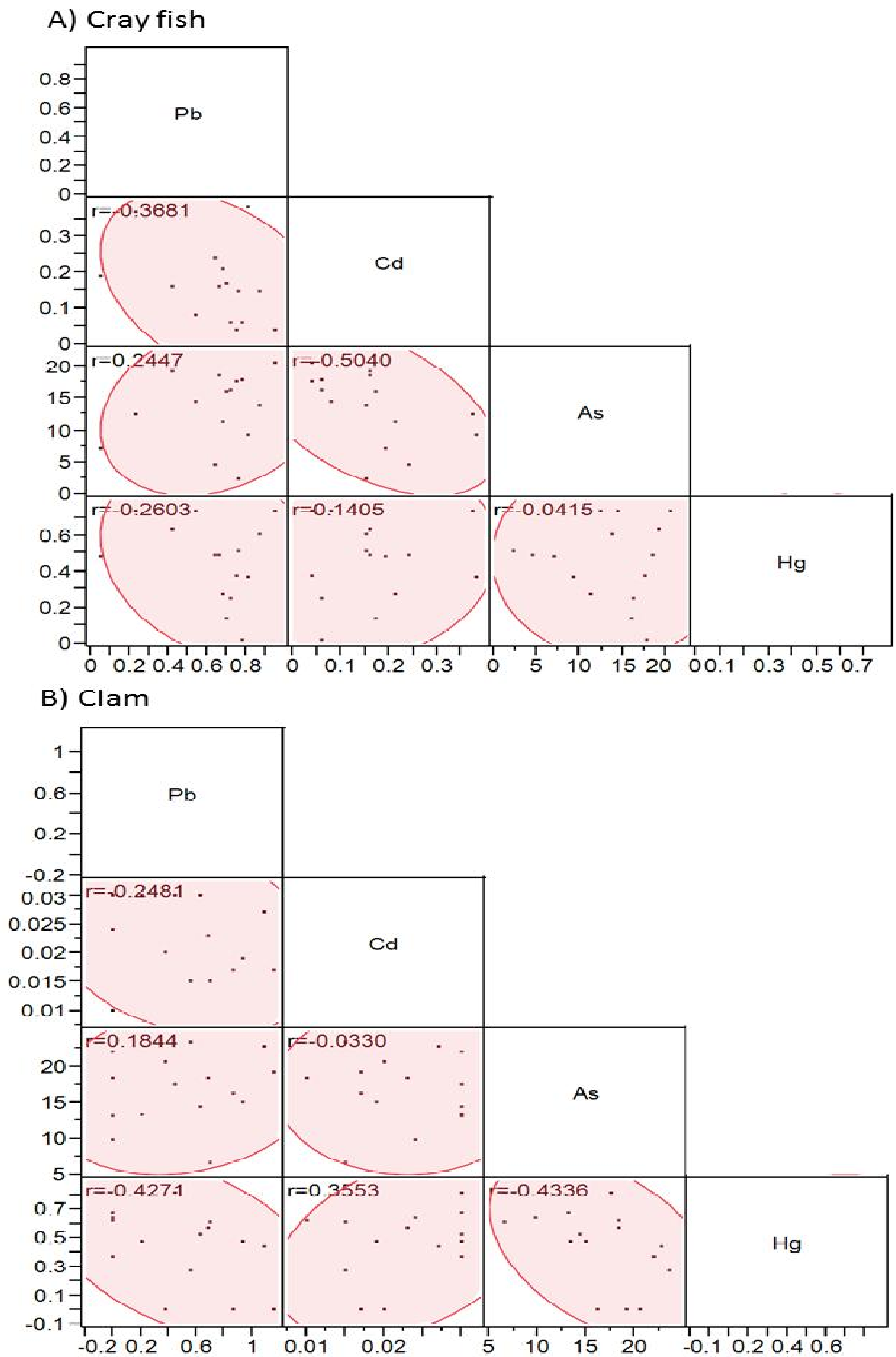

Fig. 4.Metal-metal interactions in crayfish and clam
Scatterplots between different metal concentrations in (A) crayfish and (B) clam (n=15), r means Pearson correlation. This analysis was done using JMP software (SAS Institute, Cary, NC, USA).

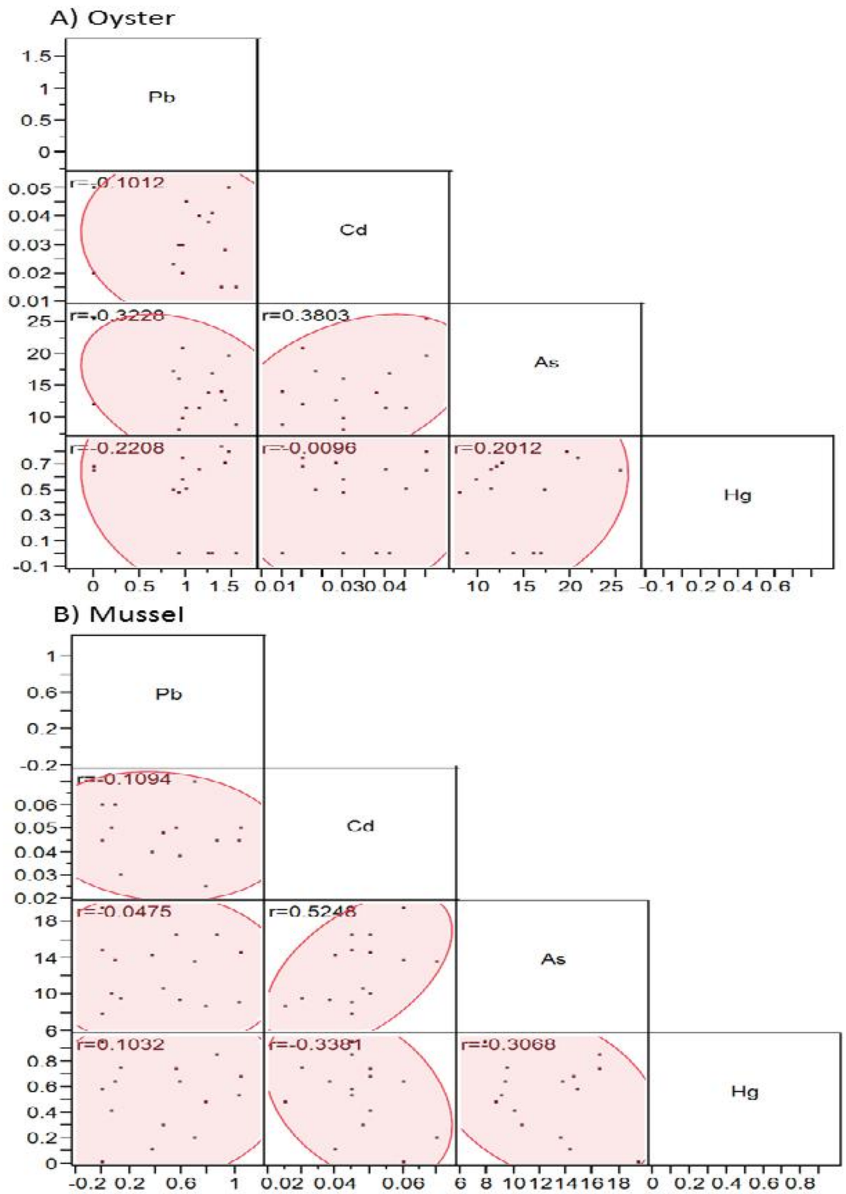

Fig. 5.Metal-metal interactions in oyster and mussel
Scatterplots between different metal concentrations in (A) oyster and (B) mussel (n=15), r means Pearson correlation. This analysis was done using JMP software (SAS Institute, Cary, NC, USA).

Table 2. Estimated Daily intake (EDI), Hazard ratio (HR) and Hazard index (HI) of different heavy metals due to consumption of different shellfish.

	Pb		Cd		As		Hg		
	EDI	**HR**	**EDI**	**HR**	**EDI**	**HR**	**EDI**	**HR**	**HI**
Shrimp	0.24	0.06	0.04	0.04	1.05	**3.50**	2.49	**4.99**	**8.59**
Crab	0.51	0.13	0.07	0.07	0.81	**2.69**	4.31	**8.64**	**11.52**
Crayfish	0.44	0.11	0.11	0.11	0.99	**3.31**	2.77	**5.53**	**9.07**
Clam	0.35	0.09	0.02	0.02	0.86	**2.86**	3.23	**6.47**	**9.43**
Oyster	0.70	0.17	0.02	0.02	0.86	**2.87**	3.96	**7.91**	**10.98**
Mussel	0.31	0.08	0.03	0.03	0.63	**2.09**	3.43	**6.86**	**9.06**

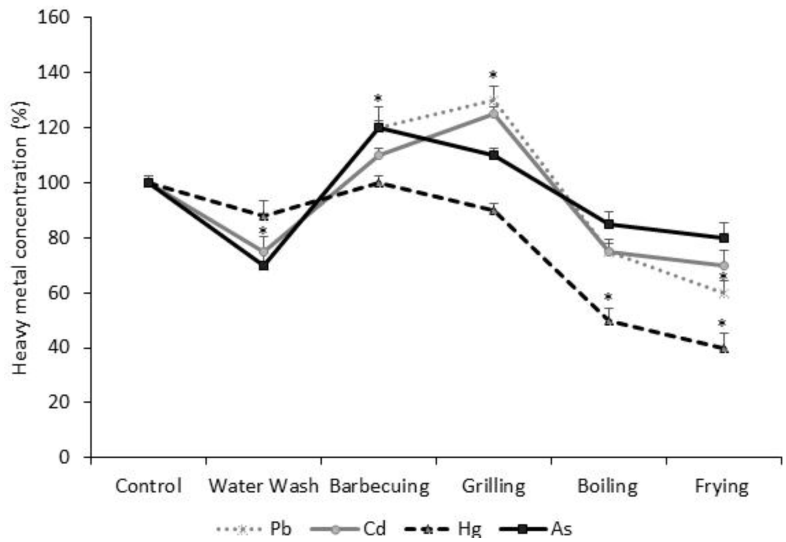

Fig. 6. Effect of water wash and different cooking methods on heavy metals load in shrimp

The reduction trials were performed on 6 groups (n=5 each), these groups were control group with no further treatment; the second group was immersed in water for wash for a period of 30 min. The third group was barbecued on wire net over charcoal, the fourth group was grilled in an electric oven. The fifth group was boiled in water. The sixth group was deep-fried in corn oil. The results were recorded as mean ± SD percentage of metal load in treated samples and in the control samples, the percentage was set to be 100%. Means carrying star marks are statistically significant with the control at P<0.05.

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Conflict of interest: The authors declare that there is no conflict of interest.

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