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Comparative evaluation of trace element concentration in grey mullet (*Mugil cephalus*) caught in Black and Aegean Seas. Potential health risk assessment.

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ABSTRACT

The consumption of fish worldwide has increased speedily in recent years particularly with the awareness of its nutritional and therapeutic benefits. In addition to being important source of protein, fish are enriched with essential minerals, vitamins, and unsaturated fatty acids. However, fish normally accumulate heavy elements from the environment which they inhabit which makes them a good indicator of heavy element contamination.

This study evaluates heavy elements' levels (Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) in commercially important grey mullet (*Mugil cephalus*) caught from Black Sea (Bulgaria) and Aegean Sea (Greece). The samples of edible part of fish species were randomly collected, processed, and analyzed for toxic and essential elements using ICP-OES. Results revealed that studied microelements concentrations varied among the fish species. Human health risk is evaluated using different indexes. Estimated weekly intake (EWI) for the elements do not exceeded the provisional tolerable weekly intake (PTWI) for all studied fish species. The target hazard quotients (THQ) of all elements were below 1, showing an absence of health hazard for the population consuming these fish species. Also, target risk (TR) of Ni and Pb indicated that consumption over a long period of time may not result in a carcinogenic effect. Additionally, the results from this study show that consumption of the edible muscle tissues of those two fish species from both areas do not pose a risk for human consumption.

INTRODUCTION

A matter of concern over the last decades is the contamination of marine waters with a wide range of pollutants such as heavy elements. Metal contaminants are of particular concern due to their high toxicity, persistence, and ability to accumulate in the food chain and aquatic ecosystems (Kumar *et al*., 2012; Zeng *et al*., 2012). Metals such as iron, chromium, copper, zinc and manganese, are essential metals since they play an important role in biological systems, whereas nickel, lead and cadmium are non-essential metals, as they are toxic, even in traces (Tüzen, 2003). Toxic effects can also be observed if the essential metal's level is excessively elevated (Tüzen, 2003; Makedonski *et al*, 2017). For this reason, it is of great importance to determine the chemical composition of organisms inhabited the water bodies such as fish, regarding the content of heavy metal in order to evaluate the possible risks to human health (Cid *et al*, 2001).

Fish is widely consumed in many parts of the world because of their positive health aspects such as the high levels of polyunsaturated fatty acids, retinol and alpha-tocopherol (Stancheva *et al*., 2012). On the contrary the fish species are constantly exposed to chemicals in contaminated water bodies. This is the reason why fish has been found to be good indicators of heavy elements contamination in aquatic systems because they occupy different trophic levels and are of different sizes and ages (Burger *et al*, 2002).

The Black Sea is a closed water basin, very isolated from the other water bodies and the environmental pollution in the region is due mainly to the inputs of European rivers (such as the Danube, the Dniester, the Don and the Dnieper). Pollution, loss of biodiversity and coastal degradation have been identified as the major issues affecting the environmental state of the Black Sea. There are several serious problems for the Black Sea associated with various types of pollution. The eutrophication phenomenon of the sea by various nutrients, largely as a result of pollution from agricultural, domestic and industrial sources is a major transboundary pollution issue.

The Greek coast of the Aegean Sea is oligotrophic and has the physical characteristics of semi-closed sea which is more vulnerable to human pressure and anthropogenic pollution. Pollution problems in this region are of industrial, urban or shipping origin and have rather local character. Also the Aegean coastline is affected by erosion which threatens vulnerable coastal ecosystems and protected areas.

The purpose of this research is to determine the concentration of some essential and trace elements (Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn) in muscle tissue of grey mullet (*Mugil cephalus*) caught from the Black Sea (Bulgaria) and the Aegean Sea (Greece). It should be noted that this fish species is regarded to be an essential part of the diet in both geographic regions. In addition, these data were used to evaluate the provisional tolerable daily intake (PTDI) for the elements under study and to calculate the target hazard quotients (THQ) and target risk (TR) values for all microelements as developed by the Environmental Protection Agency (EPA) in the US for the estimation of potential health risks associated with long term exposure to chemical pollutants (USEPA, 1989).

MATERIALS AND METHODS

Fish handling and preservation

The fish samples of grey mullet (total number of 56) were acquired from local fishermen nets near port Varna, Black Sea (Bulgaria) and region of Kavala, Aegean Sea (Greece) (Figure 1). The sampling period was between February and November 2018. Total of 56 samples of grey mullet (*Mugil cephalus*) species were included in this study with the following biometric data which was determined before dissection: Black Sea grey mullet (N=30) with weight 513 ± 4.24 g and length 3.5 ± 0.7cm and Aegean Sea grey mullet (N=26) with weight 333.2 ± 61.1g and length 37.5 ± 5 cm.

The composite sample is constructed only of fillets of edible part of each individual. Approximately 1.0 ± 0.05 g sample of homogenized muscle from each composite sample were weighted, packed in polyethylene bags and stored at-18 °C until chemical analysis.

Reagents

For the purpose of this study the chemical reagents used were of analytical reagent grade. Ultrapure water with resistivity of 18 MΩ cm was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Before analysis the plastic and glassware were cleaned by soaking in 2M HNO_3 for 48 h, and rinsed ten times with deionised water. The calibration standard solutions were freshly prepared by dilution of Optima Family Multi-Element Standard, Matrix per Volume: 2% HNO_3 and Multi-Element Calibration Standard 3, Matrix per Volume: 5% HNO_3 stock solution (PerkinElmer®, USA). Certified reference materials DORM-2 (dogfish tissue) from National Research Council Canada (Ottawa, Canada) is used to validate the analytical method. Recoveries between 87.7 and 103.5 % were achieved to validate the calibration.

Sample treatment and instrumental parameters

Wet digestions were performed in triplicate by weighing around 1.0 g of the fish tissues with 10 ml HNO_3 (65% Merck, Suprapur) in a microwave digestion system MARS 6 (CEM Corporation, USA) subject to a program with the following parameters: step (1) 25-200 °C for 10 min at 1000 W; step (2) 200 for 10 min at 1000 W. The digested samples were diluted to 25 ml with Milli-Q water and stored in polyethylene bottles. A blank digest was performed in the same way. The concentrations of the elements Cd, Cr, Cu, Fe, Mn, Ni, Mn, Pb, Zn in the samples were determined using ICP-OES Spectrometer (Optima 8000, Perkin Elmer, USA) with the following operating condition: plasma gas flow, 8 L/min; Auxiliary gas flow, 0.4 L/min; Nebulizer gas flow, 0.6L/min; Nebulizer, Concentric Glass, MEINHARD® Type C.

Hazard risk estimation

Health-risk assessment of fish consumption

Human health risk assessment was performed by calculations of estimated daily intake (EDI), target hazard quotient (THQ) and hazard index (HI). Estimated daily intake (EDI) was estimated by

$$EDI = \frac{M_c x\ FIR}{BW_a}$$

where EDI is estimated daily intake (mg analyzed element/kg body weight/day), FIR – average daily consumption of fish (kg/person), M_C – average concentration of the essential elements in the samples (mg/kg) and BWa – average body weight (kg) (70 kg for an adult person).

According to the Ministry of Health of Bulgaria (EAFA, 2012), the average quantity of fish consumed per Bulgarian person (assuming average body weight of 65kg/females and 79 kg/males) per week in Bulgaria is 95.9 g (13.7 g per day). Eurostat (2014) declared that the seafood consumption in Greece was recorded as 376.9 g weekly (53.8 g per day). Multiplying these values by the average concentration of each heavy microelement in analyzed samples of grey mullet, the average weekly (daily) intake of elements per person can be estimated.

Target hazard quotient (THQ)

The THQ is used to determine the non-carcinogenic risk level due to pollutant exposure. To assess the health risk from element contaminated fishes, the THQ was calculated as per USEPA Region III Risk Based Concentration Table (USEPA, 2019) by using the following equation:

$$THQ = \frac{(M_C\ .\ I_R\ .\ 10^{-3}.\ EF\ .\ ED)}{(RfD\ .\ BWa\ .\ ATn)}$$

where M_C is the toxic or essential element concentration in fish species (mg/kg ww), IR is the fish ingestion rate (13.7 g/kg dw) (EAFA, 2012), EF is the exposure frequency (365 days/year), ED is the exposure duration (30 years or 10950 days) for non-cancer risk as used by USEPA, RfD is the reference dose of individual element (0.001 µg/g day for Cd, 0.003 µg/g day for Cr, 0.04 µg/g day for Cu, 0.009 µg/g day for Fe, 0.14 µg/g for Mn, 0.02 µg/g day for Ni, 0.004 mg/kg for Pb, and 0.3 µg/g day for Zn) (USEPA, 2019), BW is an average adult body weight (65 kg for females and 79kg for males) and ATn is the average exposure time for non-carcinogens (10,950 days) (USEPA, 2019).

Hazard index (HI)

The hazard index from THQs is expressed as the total of the hazard quotients (USEPA, 2019):

$$HI = THQ_{Cd} + THQ_{Cr} + THQ_{Cu} + THQ_{Fe} + THQ_{Ni} + THQ_{Mn} + THQ_{Pb} + THQ_{Zn}$$

Target Risk (TR)

Target cancer risk (TR) indicates carcinogenic risks. The model for estimating TR was shown as follows:

$$TR = \frac{(M_C \, . \, I_R \, . \, 10^{-3} . \, CPSo . \, EF \, . \, ED)}{(BWa \, . \, ATc)}$$

where CPSo is the carcinogenic potency slope, oral (Ni= 1.7 mg/kg bw-day, Pb= 0.0085 mg/kg bw-day); ATc is the averaging time, carcinogens (day/years) and was calculated by multiplying exposure frequency in exposure duration over lifetime. TR value for intake of Ni and Pb was calculated to indicate the carcinogenic risk since Cd, Cr, Cu, Fe, Mn and Zn do not cause any carcinogenic effects.

Statistical analysis

The whole data were subjected to a statistical analysis. The descriptive statistics (mean, standard deviation, and range expressed in mg of element per kg (mg/kg), wet weight (ww)) of the results of the heavy elements contents were calculated using Microsoft Office Excel 2013 software (Seattle, WA, USA). Significance was established at $p < 0.05$.

RESULTS

Essential and toxic element concentration in grey mullet muscle tissues

Mean concentrations and standard deviations of the analyzed heavy elements of grey mullet from the Black Sea (Bulgaria) and the Aegean Sea (Greece) are presented in Table 1. The analytical concentrations of Cd and Ni in all samples were below the limit of detection (LOD).

Table 1: Heavy elements total concentration (mg/kg w.w) of grey mullet caught in the Black Sea and the Aegean Sea.

	Cd[a]	Cr	Cu	Fe	Mn	Ni	Pb	Zn[a]
AS	nd	0.04±0.001	1.11±0.009	4.52±0.055	0.14±0.0004	nd	0.23±0.009	3.89±0.029
BS	nd	0.07±0.002	1.10±0.053	10.67±0.071	0.31±0.0002	nd	0.26±0.004	4.47±0.0065

AS, Aegean Sea; BS, Black Sea

[a]The concentration of the elements is presented as average of three trials ± standard deviation. The means are not significantly different at (P > 0.05).

[b]nd - below LOD

Health-risk assessment for grey mullet consumption

The estimated daily intake of the elements was estimated and presented in Table 2.

Table 2: The estimated daily intakes (EDI) of heavy elements through grey mullet (Mugil cephalus) caught from the Black Sea (Bulgaria) and the Aegean Sea (Greece)[a].

Elements		Average concentration (mg/kg ww)	EDI (mg kg^{-1}_{bw} day^{-1})	RDA (mg/kg)	FDA RDI (mg/kg)	PTWI[b] (mg/week for 70kg adult)	PTDI[c] (mg/day for 70 kg adult)	% RDI
Essential								
Cr	AS	0.04	0.008	NA	0.12	35	5	6.52
	BS	0.07	0.054					44.83
Cu	AS	1.11	0.217	0.9	2.0	245	35	24.14
	BS	1.10	0.845					93.94
Zn	AS	3.89	0.761	8.0(F)/11(M)	15	490	70	9.52 (F)/6.92 (M)
	BS	4.47	3.436					42.94 (F)/31.23(M)
Mn	AS	0.14	0.002	NA	2	140-350	20-50	0.10
	BS	0.31	0.017					0.83
Fe	AS	4.52	0.885	8 (M and F > 51 yrs 18 (F 19-50 yrs)	18	392	56	4.92
	BS	10.67	8.200					45.56
Toxic								
Cd	AS	n.d	-	-	-	0.49	0.07	
	BS	n.d	-	-	-			
Pb	AS	0.23	0.045	-	-	1.75	0.25	
	BS	0.26	0.200	-	-			
Ni	AS	n.d.	-	-	-	2.45	0.35	
	BS	n.d.	-	-	-			

N.A, not available; AS, Aegean Sea; BS, Black Sea
[a]The values provided are for both males and females, unless (M) for males and (F) for females is noted
[b] PTWI: provisional permissible tolerable weekly intake (mg/kg body weight/week) for 70 kg adult cited in Türkmen *et al*.(2009) after FAO/WHO (2004)
[c]PTDI provisional permissible tolerable daily intake (mg/kg body weight/day) for 70 kg adult

The target hazard quotients for Cd, Cr, Cu, Fe, Mn, Ni, Pb and Zn, hazard index and target risk for Pb and Ni, separately for females and males, estimated through the consumption of the grey mullet fish species are shown in Table 3.

Table 3: Risk values (THQ and HI) of each microelements in muscle of grey mullet (*Mugil cephalus*) caught in Black and Aegean Sea (F-female; M-male).

	Target Hazard Quotient (THQ)																HI	
	Cd		**Cr**		**Cu**		**Fe**		**Mn**		**Ni**		**Pb**		**Zn**			
	F	M	F	M	F	M	F	M	F	M	F	M	F	M	F	M	M	F
AS	n.d	n.d	0.003	0.002	0.006	0.185	0.102	0.084	0.0002	0.0002	n.d	n.d	0.012	0.010	0.003	0.002	0.113	0.273
BS	n.d	n.d	0.005	0.004	0.006	0.183	0.240	0.197	0.0004	0.0004	n.d	n.d	0.013	0.011	0.003	0.002	0.266	0.397

AS, Aegean Sea; BS, Black Sea

TR was performed for Ni and Pb since only those elements from the analyzed ones are classified as cancerogenic and are presented in Table 4.

Table 4: Target risk of each metal contaminant in muscle of grey mullet (*Mugil cephalus*) caught in Black and Aegean Sea

	Target Risk			
	Females		***Males***	
	Ni	Pb	Ni	Pb
Aegean Sea	n.d	$5.06x10^{-6}$	n.d	$3.09x10^{-9}$
Black Sea	n.d	$5.72x10^{-5}$	n.d	$3.49x10^{-8}$

DISCUSSION

Toxic and essential element concentration in grey mullet muscle tissues

Chromium is an essential element. But its hexavalent form is toxic when ingested. The mean concentration of Cr measured in the grey mullet samples from the Black Sea was 0.07 mg/kg ww while those from the Aegean Sea - 0.04 mg/kg ww. In the current study, Cr levels in all samples were lower than the permissible limits set by Bulgarian legislation office (0.3 mg/kg ww) (Anonymous, 2004). Cr content was reported to be between 4.84 mg/kg ww up to 1.08 mg/kg ww in various samples from grey mullet caught from Iskenderun Bay, Turkey (Yilmaz, 2005), between 0.54 µg/g ww during sexual maturity of *Liza aurata* in southern part of the Caspian Sea and 0.61 µg/g ww during sexual rest (Norouzi *et al*, 2017). Chromium results were below those set by local food authorities and the one stated in the literature.

Copper is essential elements though it supports the formation of red blood cells, healthy bones and play an important role in the blood vessel system and the immune system. Very high concentrations in the body may lead to severe intoxication, liver and kidney damage (Ikem and Egiebor, 2005). The mean concentration of copper in this study was in the range of 1.10 mg/kg ww for the samples from the Black Sea and 1.11 mg/kg ww for the samples from the Aegean Sea. Copper in the literature ranges from 0.23 to 9.49 mg kg^{-1} for muscle of fish from Marmara Sea (Keskin *et al*., 2007), 0.32-6.48 mg kg^{-1} for muscle of various fish species from Marmara, Aegean and Mediterranean seas in Turkey (Türkmen *et al*., 2008) and 0.34-7.05 mg kg^{-1} ww for fish muscle from the central Aegean and Mediterranean Sea (Türkmen *et al*., 2009). The minimum and maximum copper levels in fish species from the Black Sea, Turkey were found as 0.65 µg/g in *T.trachurus* and 2.78 µg/g in *P.saltor* (Tuzen, 2009). The maximum copper level permitted for marine fish is 10 mg/kg according to Bulgarian Food Authority (Anonymous, 2004). In our study, the copper content in fish muscle did not exceed the upper limit allowed by Codex Alimentarius and Bulgarin Food Codex (10.0 mg/kg ww) (Anonymous, 2004).

Iron is essential for the proper human blood system function since it is found in haemoglobin and as ferritin and hemosiderin in fish. The maximum permitted iron level established by WHO (FAO/WHO, 1989) is 100 mg/g. The mean Fe levels in muscle ranged from 4.52 mg/kg ww for Aegean Sea fish sample up to 10.67 mg/kg ww for the samples from the Black Sea. There is no maximum permitted level for Fe in fish samples according to EU (2011) but in the literature the data related with iron concentration are within or higher than the values from this study (Yılmaz *et al*, 2005; Tuzen, 2003; Tuzen, 2009; Uluozlu *et al*, 2007).

The average Mn levels in the samples were between 0.14 mg/kg ww (Aegean Sea) and 0.31 mg/kg ww (Black Sea). In the muscles of *Merluccius hubbsi, Micropogonias furnieri, Pangasius hypothalamus, Oreochromis niloticus, Sparus aurata* and *Mugil cephalus* available in Gaza Strip markets, the manganese concentration varies between 0.376–0.834 μg/g ww (Kamal *et al*., 2013); around 0.22mg/kg in muscle tissues of Aegean chub from Tersakan River, Turkey (Şaşi *et al*, 2018) and between 0.093 to 2.023 mg/kg in ten different fish species (wild, farmed, freshwater, and marine) frequently consumed in Turkey (Varol *et al*, 2019). There is no data about the maximum Mn level permitted according to Bulgarian Food Codex but as it is obvious the data from current study are within those found in the literature.

According to European Commission Regulation (1881/2006/EC) (EC, 2011), the maximum acceptable concentrations (MAC) for Pb in fish meat is 0.3 mg/kg ww. National regulation of Republic of Bulgaria prescribed a value of 0.3 mg/kg ww in fresh fish meat (Anonymous, 2004). The mean Pb concentrations in this study were between 0.23 mg/kg ww for samples of grey mullet from the Aegean Sea and 0.26 mg/kg ww for samples from the Black Sea. Lead levels in the literature have been reported in the range of 0.22-0.85 mg/kg for muscle of fish from the middle Black Sea (Tuzen, 2003), 0.28 μg/g in *P.maxima* and 0.87 μg/g in *P.saltor* from the Black Sea, Turkey (Tuzen, 2009), 0.33-0.93 μg/kg for muscle of fish from the Black and Aegean seas (Uluozlu *et al*., 2007) and between 0.14 and 1.28 μg/kg for fish muscle from the Aegean and Mediterranean Sea (Türkmen *et al*., 2009). The values obtained from the analyzed samples showed good agreement with values reported in the literature and are below the level set by various health organizations.

Zn is an essential element and its concentration in this study varies between 3.89 mg/kg ww and 4.47 mg/kg ww. The concentration for zinc reported in the literature range of 16.1-31.4 mg kg^{-1} for muscles of fish from the Mediterranean Sea (Kalay *et al*., 1999), 3.51-53.5 mg kg^{-1} for species from the Aegean and Mediterranean Sea (Türkmen *et al*., 2009), 38.8 μg/g - 93.4 μg/g for different types of fishes from the Black Sea, Turkey (Tuzen, 2009) and 9.50-22.94 μg/g dw for fish muscle from the middle Black Sea (Tuzen, 2003). According to European Commission Regulation (1881/2006/EC)(EC, 2011), there is no value set for MAC for Zn in fish meat but Bulgarian Food Codex (Anonymous, 2004) prescribed a value of 50 mg/kg ww. Limit recommended by Food and Agriculture Organization for Zn is 30 mg/kg ww.(FAO, 1983). Results from the current study are in good agreement with the literature data and those set by various health organizations.

Health-risk assessment for grey mullet consumption

As can be seen in Table 2, the values of estimated daily intakes (EDI) of Fe, Zn, Cu, Mn, Pb, Cr, Cd and Ni in muscles of grey mullet fish in this study are well below their corresponding permissible tolerable daily intake. The result shown in Table 2 showed that from toxic elements Pb contributed the highest daily intake, which agreed well with the other results from the literature (Saha *et al*, 2013). An overall assessment of the % RDA for trace elements shows that grey mullet from the Aegean

Sea is a good Cu source, with Cu levels in the corresponding meal reaching or surpassing of 93.94.0% of the RDA. Additionally, this fish species may provide a higher percentage of Zn (42.94 and 31.23 % of the RDA) and Fe (45.56 % of the RDA). The results of EDI revealed that the EDI values for the examined fish samples were below the recommended values, indicated no risk to people’s health associated with the intake studied heavy elements through the consumption of these two fish samples.

THQ is an integrated risk index that compares the ingested amount of a contaminant with a standard reference dose (USEPA, 2000). The assessment of health risk is done based on assumptions. The acceptable value for THQ is equal to 1 according to USEPA (2019). In our study (Table 3), the THQ and HI for both males and females were less than one for all elements. Therefore, there is no non-carcinogenic health risk from ingestion of these elements individually and collectively through the consumption of these fish species. Males exhibit a significantly higher HI value than females. The grey mullet from the Black Sea exhibit a higher HI values from the samples from the Aegean Sea. The highest THQ was calculated for Fe for the sample of grey mullet caught from the Black Sea.

TR was performed for Ni and Pb since only those elements are classifiied as cancerogenic (plus As) (Table 4). The values of TR must be equal or lower than 10^{-6} for carcinogens and may be up to 10^{-4} .The calculated values in this study are within this range suggesting that the intake of Ni and Pb by consumption of grey mullet species caught from the Black and Aegean Sea would not result in appreciable hazard risk on the human body (Table 4).

CONCLUSION

The current study showed that the concentration of Cd, Cu, Cr, Ni, Mn, Fe and Zn in grey mullet samples from the Black and Aegean Seas were into the limits compared to the data obtained from the literature and do not exceed the maximum permissible levels for those elements set by Bulgarian Food Codex or EU. The grey mullet from the Aegean Sea is a good copper, zinc and iron source. No elements were found to be considered as potential health hazard for consumers according to the data for EDI. The THQ and HI values of the elements are lower than the standard values; therefore, continuous consumption of those fish species from these two areas may not create health risk in the long period. The target risk values for the toxic Pb and Ni were below 10^{-6}, indicating no carcinogenic risk. According to these results, the consumption of grey mullet samples from the Black Sea and the Aegean Sea is completely safe for human health.

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