

# Experimental study on copper uptake capacity in the Mediterranean mussel (*Mytilus galloprovincialis*)

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**Abstract** To investigate the effect of different sizes, sex, and exposure time on Cu uptake capacity, mussels *Mytilus galloprovincialis* of different shell sizes were exposed to different Cu concentrations in different aquariums. In another experiment, mussels were exposed to stable dissolved Cu for 6 days in the laboratory. All mussels tissue concentrations were analyzed using energy dispersive X-ray fluorescence (EDXRF) spectrometry. At the end of uptake, the rate of increase of Cu level in the soft tissues of mussels in different aquariums was 3.84–7.92 times higher than before exposure. While the results of Cu concentrations were negatively correlated with the shell sizes in the control and second groups ( $r_{\text{control}} = -0.862$ ,  $r_{\text{second}} = -0.851$   $p < 0.05$ ), this relation was not observed in the other groups ( $p > 0.05$ ). Also, results showed no significant difference between male and female ( $p > 0.05$ ). On the other hand, Cu concentration values in soft tissue were monitored daily and observed to be increasing up to the third day but afterwards to be descending, thus indicating a significant effect of the exposure time-related Cu uptake by mussels. Therefore, the exposure time to Cu metal of the mussel should be taken into account in the marine pollution investigations. In addition, by using the obtained Cu heavy

metal concentration results, the heavy metal intake by the human population was calculated by taking into account daily mussel consumption. The results were examined for potential human health risks and discussed. These results would be helpful to understand factors controlling Cu accumulation in mussels.

**Keywords** Mussel (*Mytilus galloprovincialis*) · Uptake · Exposure time · Copper · EDXRF analysis

## Introduction

The present state of knowledge on the use of indicator organisms to study trace metal pollution is reviewed, with particular reference to the use of mussels. Mussels are the most efficient and reliable indicators developed to the present time. The effects of sampling and environmental variables have been largely overlooked and that further study in the field and in the laboratory is necessary before the results of surveys using biological indicator organisms can be relied upon (Phillips 1970).

A wide variety of contaminants are well known to accumulate in mussel soft tissue. Thus, they are used widely to monitor metal pollution in the sea (Fatoki et al. 2012). Heavy metals are a major anthropogenic contaminant of estuarine and coastal waters (Sunlu 2006). Cu is the essential element to the metabolism of aquatic organisms and plays an important role in biochemical processes; however, it will be toxic to the living organisms when their concentrations exceed certain ranges (Fei and Tianxiang 2011). Cu enters marine and estuarine waters from municipal waste outfalls, mining and industrial effluents, sediment dredging, and of increasing importance, leaching from antifouling pans (Parry and Pipe 2004). Metal adsorption from the dissolved phase is a potentially significant source for the overall metal accumulation in

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mussels because these animals pump a substantial quantity of water through their gills (Chong and Wang 2001). A mussel species which has proved to be an important tool for the bio-monitoring of environmental pollution in coastal areas is the mussel *Mytilus galloprovincialis* (Jovic and Stankovic 2014). Mussels have different capacities of accumulation for different metals (Yap et al. 2003). There are many factors that affect them. Some of the most important factors are size, food acquisition capability, and weight (Saavedra et al. 2004). Size variation and heavy metal contents in the shell have been sometimes shown to be important variables (Cevik et al. 2008). The influence of shell length and sex on the Cu adsorption capacity of mussels has been widely studied and proposed as a useful means by which to evaluate the effect of contaminants on their ability to function under stressful conditions (Lobel et al. 1991; Widdows and Donkin 1992; Sze and Lee 2000; Mubiana et al. 2006; Zhong et al. 2013).

Mussels are consumed by humans and have a wide geographical distribution (Fish and Fish 1996). In order to observe the health risk of any pollutant, it is very important to estimate the level of exposure, by detecting the routes of exposure to the target organisms (Arora et al. 2008). Considering that mussels *M. galloprovincialis* are edible and marketed commercially, the presence of metals could limit the quantity of mussels that humans should consume as excessive consumption of metal-contaminated mussels could result in toxicity to humans (Stanković et al. 2012). Heavy metal toxicity in aquatic organisms, in association with the long residence time within food chains and the potential risk of human exposure, makes it necessary to monitor the levels of these contaminants in marine organisms (Ferreira et al. 2005).

The main aim of the present study is to obtain information on the Cu uptake capacity of the soft tissue of mussels *M. galloprovincialis*, considering size, sex, and time effects, and to determine the daily intake of Cu by the human population. The results give important information about the consumption limits and the Cu pollution in the marine environment for human risks.

## Materials and methods

### Sampling and Cu uptake experiment

Mussels (*M. galloprovincialis*) were collected in March 2015 from Ardeşen beach in Rize province in Turkey (41°11'19" N–40°58'57" E). After transferring the collected samples to laboratory, the mussels were washed to remove encrusting organisms and pollution of sediment using seawater with the help of a brush and a scalpel. Three different sizes were selected as small (<50 mm), medium (50–70 mm), and large (>70 mm). The mussel samples were acclimated to laboratory conditions 5 days prior to start of the experiment. They were

stored in a 300-L capacity tank. Aeration was continuously provided by gently bubbling air through disposable Pasteur pipettes connected via plastic tubes to an air pump. During this acclimation and experiment period, mussels were not fed at any time.

The experiment procedure contains two main steps described below:

1. First, the effect of different concentrations on Cu uptake capacity by mussels was studied over a 5-day exposure period. The initial Cu solution concentrations were prepared at 4.8, 14.4, 24, 48.4, 145.2, and 242 mg L<sup>-1</sup> in 1 L seawater as stock solutions; the copper acetate salt (Cu(CH<sub>3</sub>COO)<sub>2</sub>·2H<sub>2</sub>O) was used. The seventy-five animals (15, 20, and 40 for large, medium, and small, respectively) were exposed in each aquarium of 40 L capacity containing 39 L seawater. While the first group was not given any Cu solutions, the second, third, fourth, fifth, sixth, and seventh groups were performed daily equivalently from concentrated 1 L stock solutions once every 24 h for 5 days. The final copper concentrations in each aquarium were 0.12, 0.36, 0.60, 1.21, 3.63, and 6.05 mg L<sup>-1</sup>, respectively. Each aquarium was examined daily. All dead mussels were removed and recorded in Table 1. Also, the laboratory conditions were monitored daily to ensure that these were similar in all experiments. Accumulation and toxic effects of heavy metals depend on the physical and chemical characteristics of water (Witeska and Jezierska 2003). Physicochemical water parameters measured by means of a multiparameter device during the experiments indicated that these parameters were consistent. Temperature was 14±2 °C. The dissolved oxygen levels were above 65 % of the saturation value in all aquariums during the period of the uptake experiments. Salinity ranged between 16 and 18 ‰, pH ranged between 7.5 and 8.4, and conductivity ranged between 21 and 23 μS/cm. After 5 days, the samples were taken from aquariums, separated into size-class based on shell length, and were transferred to the laboratory for analysis. All mussel samples were segregated according to their sex to study differences between male and female for analyses. The sexes of these different size samples were determined and recorded (Mikhailov et al. 1995).
2. Secondly, the effects of exposure over time on Cu uptake capacity were studied. Three different size mussels were taken from the stock to determine the Cu concentration before their exposure and put into plastic bags and labeled. An aquarium system was set up containing 40 L of seawater, 240 mussels (30, 60, and 150 for large, medium, and small, respectively) which were then exposed to a fixed Cu concentration (0.36 mg L<sup>-1</sup>) at the beginning of experiment. Forty mussel samples (5, 10, and 25 for large, medium, and small, respectively) were taken from

**Table 1** Cu uptake capacity results of the mussel soft tissues ( $\mu\text{g g}^{-1}$ ), the concentration values ( $\text{mg L}^{-1}$ ) in seawater, and the rate of mortality (%) of different size mussels exposed to Cu

Group	Concentration in seawater ( $\text{mg L}^{-1}$ )	The rate of mortality in the mussel (%)			Cu concentration in soft tissue of the mussels ( $\mu\text{g g}^{-1}$ )						
		Large	Medium	Small	Large		Medium		Small		Mean
					Male	Female	Male	Female	Male	Female	
Control	0	0	0	0	129±3.42	149±3.60	170±3.74	153±2.13	175±0.58	205±8.59	163.5±3.67
First	0.12	0	0	0	167±3.93	257±6.03	178±6.78	184±7.51	224±8.22	225±1.16	205.8±5.61
Second	0.36	2.5	3.3	0	593±11.12	506±12.57	684±16.12	681±17.93	702±8.02	736±7.81	650.5±31.18
Third	0.60	26.6	10	7.5	861±9.58	590±15.50	937±20.42	697±21.86	791±10.53	763±63.43	773.1±33.60
Fourth	1.21	33.3	30	10	1022±5.01	727±13.90	960±12.76	736±9.52	832±17.22	788±16.86	827.5±12.19
Fifth	3.63	100	100	100	NM	NM	NM	NM	NM	NM	NM
Sixth	6.05	100	100	100	NM	NM	NM	NM	NM	NM	NM

NM not measured

each mussel group every 24 h for 6 days and, then analyzed for Cu concentrations. All samples were washed with seawater for the two experiments before analysis.

After cleaning the shell surface, the average wet weight of the flesh per piece of the mussel were calculated as 5.3, 2.99, and 0.87 g for large, medium, and small size, respectively. Prior to analysis, all samples were dried in an incubator for 24 h at  $105 \pm 5$  °C. The average dry weight of soft tissue per piece of the mussel was calculated as 0.8, 0.5, and 0.15 g for large, medium, and small, respectively. The dried samples were ground in a spex mill, pressed with a hydraulic press applying a pressure of 7 tons during 20 s as pellet. The resulting pellets had a diameter of 40 mm and a uniform mass of between 2 and 3 g. Every analysis was replicated three times.

**Heavy metal analysis**

The EDXRF spectrometer (Epsilon5, PANalytical, Almelo, the Netherlands) was used for the Cu metal analysis of the mussel soft tissue samples (Yilmaz et al. 2011). Samples were irradiated by X-rays from a Gd tube under a vacuum equipped with a liquid nitrogen-cooled PAN-32 Ge X-ray detector having a Be window thickness of 8  $\mu\text{m}$ . The power, current, time, and high voltage of the instrument was 600 W, 6 mA, 600 s, and 100 kV, respectively.

The system’s software (Epsilon 5 software) automatically analyzed the sample spectrum and determined the net intensities of element peaks as soon as the measurement was completed. When elements overlap one another, accuracy is essential for trace element analysis. A set of secondary standards, available from PANalytical, was used for the calibration of this application. The prepared samples were again measured three times.

Detection limits (DL) obtained by EDXRF were calculated according to:

$$DL = \frac{3C_i}{N_p} \sqrt{\frac{N_b}{t}} \tag{1}$$

Where  $C_i$  is the concentration of the element  $i$ ,  $N_b$  is the counting rate for the background, and  $N_p$  is the counting peak (Van Grieken and Markowicz 1993). Detection limit was  $0.052 \mu\text{g g}^{-1}$  for Cu. The accuracy of the applied method and obtained calibration curves were checked by the measurement of a certified reference material mussel tissue from NIST-2976. The certified percentage of recovery for Cu metal was  $107 \pm 5$  %.

**Estimated daily intake of heavy metals**

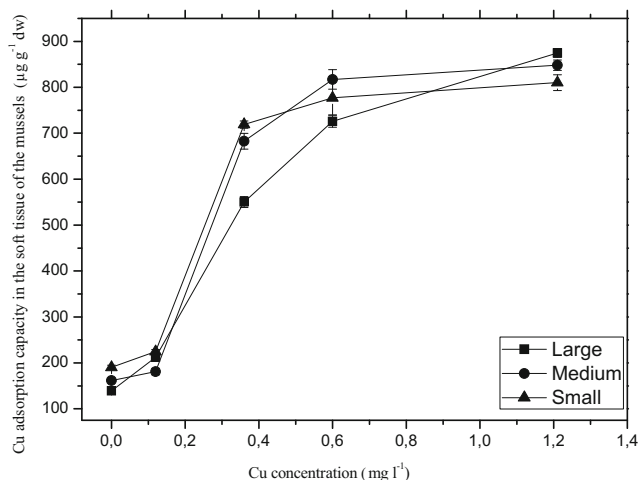
The estimated daily intake (EDI) of Cu metal depends on both the metal concentration level and the amount of consumption of mussel soft tissue. The EDI of metals for adults was determined using the following equation (Zhuang et al. 2008):

$$EDI = \frac{C_{\text{metal}} \times W}{m} \tag{2}$$

where  $C_{\text{metal}}$  is the concentration of heavy metals in the mussel;  $W$  represents the daily average consumption of mussels; and  $m$  is the body weight. Calculations were made assuming body weight of 70 kg for adults and a 0.06865 g/day (EPA 2002) average daily consumption.

**Statistical analysis**

The Kruskal-Wallis ANOVA and Pearson correlation tests were performed using a statistical package program (SPSS ver. 18) for the significant level of 0.05.



**Fig. 1** Variation of Cu uptake capacity ( $\mu\text{g g}^{-1} \text{dw}$ ) in the soft tissue of the mussels exposed to different concentrations ( $\text{mg L}^{-1}$ ) for 5 days

## Results and discussion

Cu uptake capacity results of the mussel soft tissues and the concentration values in seawater and the rate of mortality of different size mussels exposed to Cu are shown in Table 1. Table 1 shows that no mortality was observed in the control and first groups during the first 6 days of exposure. It can be seen from the results that the rates of mortality in mussels increased with increasing Cu metal concentration in the aquariums. As shown in Table 1, the rate of mortality was 100 % in the last two groups. This can be attributed to the following reason: the lethal concentration ( $\text{LC}_{50}$ ) value affects the rate of mortality in the mussels. The rates of mortality in the second, third, and fourth groups were lower than 50 %. It is evident that these Cu concentration values were below the  $\text{LC}_{50}$  values determined for the mussels. In addition, the results showed a lower rate of mortality for small mussels. This is consistent with observations made by Bat et al. (Bat et al. 2013) for Cu measured in *M. galloprovincialis*. They calculated the  $\text{LC}_{50}$  value for different sizes of the mussels experimentally. They found that the  $\text{LC}_{50}$  value for the larger mussels was lower than that of the smaller ones. This shows that smaller mussels have greater physiological vibrancy making them less susceptible to stress in general, and the upper limit of compensatory response was probably lower for the larger individuals leading to a faster failure of the detoxification systems (Daka and Ekweozor 2004).

As shown in Table 1 and Fig. 1, the uptake capacity in the soft tissues of the mussels exposed to different concentrations of Cu (0.12, 0.36, 0.60, and 1.21  $\text{mg L}^{-1}$ ) increased significantly with increasing Cu concentrations and reached a mean value of  $827.5 \mu\text{g g}^{-1} (\text{d.w.})$  in animals exposed for 5 days to 1.21  $\text{mg L}^{-1}$  Cu against a background level of  $163.5 \mu\text{g g}^{-1}$ . The corresponding values in mussels after exposure to 0.12, 0.36, and 0.60  $\text{mg L}^{-1}$  Cu were 205.8, 650.5, and

$773.1 \mu\text{g g}^{-1}$ , respectively. Also, in cases of low concentration, the rate of increase of Cu uptake capacity in the soft tissue was observed to be higher than the former one. Whenever mussels encounter a mismatch in Cu adsorption, use, and excretion, they may accumulate high levels of this metal in their soft tissue (Vosloo et al. 2012). They have different capacities of adsorption for different Cu concentrations. For example, at the end of uptake, Cu levels in the soft tissues of the mussels were 5.06 times higher than before exposure. Another important point was that the rate of increase of uptake capacity in the soft tissues of the large size was higher compared with medium and small size. While the results of Cu concentrations were negatively correlated with the shell sizes in the control and second groups ( $r_{\text{control}} = -0.862$ ,  $r_{\text{second}} = -0.851$ ,  $p < 0.05$ ), this relation was not observed in the other groups ( $p > 0.05$ ). Some metal influx rate in mussels was relatively independent of shell size, whereas the influx rate was weakly (but significantly,  $p < 0.05$ ) correlated with the shell size of mussels (Chong and Wang 2001). This finding is in agreement with Fatoki et al. (Fatoki et al. 2012) and Richir and Gobert (Richir and Gobert 2014). However, Saavedra et al. (Saavedra et al. 2004) modeled relationships between trace element concentrations and the shell length for raft *M. galloprovincialis* measuring from 52 to 87 mm, and separated into four size classes; they observed no differences among Cd, Pb, Cr, Ni, As, Cu, and Zn concentrations at different shell lengths, as shown by the lack of significance ( $p > 0.05$ ) of corresponding linear regressions.

In contrast to the size effect, sex showed no statistically significant differences ( $p > 0.05$ ,  $U = 96$ ,  $Z = -0.684$ ). Many differences were observed between sexes in previous studies. Richir and Gobert found that mean trace metal concentrations in female and male *M. galloprovincialis* dry flesh differed significantly for most of the 19 studied trace elements ( $p < 0.05$ ) and were higher in females (Richir and Gobert 2014). After the main breeding period, no difference was found between the sexes (Suarez et al. 2005). Orren et al. (Orren et al. 1980) showed that the contribution of reproductive tissues to the total body weight of raft *M. galloprovincialis* was systematically higher in males all year round. So, further detailed investigations are required to understand Cu uptake capacity according to variance by sex of the mussels.

Table 2 show the process of Cu uptake capacity of different size mussels based on duration of exposure. Mussels were exposed to stable 0.36  $\text{mg L}^{-1}$  of Cu and uptake was followed each 24 h for 6 days, and Cu concentration in the soft tissues of the mussels in different shell sizes were analyzed independently. The results showed that Cu concentrations were higher than the concentrations before exposure for all days. The rates of uptake in all the tissues were higher at day 3 than at other days. After 3 days, decrease of Cu concentration in all tissue was observed. This is probably that mucus secretions in

**Table 2** Cu concentration ( $\mu\text{g g}^{-1}$ ) in different sizes exposed to stable Cu ( $0.36 \text{ mg L}^{-1}$ )

Size	Control	Day 1	Day 3	Day 4	Day 5	Day 6
Large	90 ± 3.83	224 ± 9.74	266 ± 7.79	265 ± 5.23	244 ± 1.02	158 ± 4.54
Medium	91 ± 2.73	301 ± 3.10	375 ± 7.45	354 ± 4.92	318 ± 5.31	258 ± 20.18
Small	104 ± 2.70	438 ± 9.05	463 ± 0.92	399 ± 3.59	332 ± 8.15	334 ± 26.99

mussels have various functions, including trapping food and feces during ingestion and egestion, respectively, and protection against desiccation (Vosloo et al. 2012). Sze and Lee (Sze and Lee 1995) also concluded that mucus secretions play an important role in depuration of metals in mussels. They concluded that increased mucus secretions during metal exposure aid in protecting mussels from toxicity by trapping metals both before they are absorbed into the soft body tissues of the animals (Brown et al. 2004) and by trapping the metals that have been excreted by the animal, thus preventing re-exposure. When compared in Tables 1 and 2 for the Cu concentration of  $0.36 \text{ mg L}^{-1}$ , Cu uptake to organism at acute metal exposure was lower than chronic, since the acute Cu concentration resulted in reduced filtration activity and consequently in lower accumulation of metal. Kraak et al. (Kraak et al. 1993) examined similar findings for the zebra mussel. In their experiment, they argued that the filtration rate of mussels in acute metal concentrations was reduced because of metal detection in the water and not because of accumulation in the tissue. Also, the Cu concentrations showed a significantly negative correlation with the shell sizes ( $p < 0.05$ ). In addition, as shown from Table 2, Cu concentration values in the soft tissues of the small mussels were observed to be higher than in other sizes. The difference in concentration between small and large animals is well known and has been reported by many authors. Mubiana et al. (Mubiana et al. 2006) determined that for metals, tissue concentrations decreased with increase in body sizes. It is commonly thought that decreasing metal concentration in bigger individuals is caused partly by dilution effect due to unequal growth rate compared to metal accumulation (Newman 1995). It could also be due to differences in metabolic activity and thus metal metabolism or due to sexual maturity, since maturation is accompanied by changes in physiology and in the affinity of biochemical substances for metals (Swaileh and Adelung 1995). White and Rainbow (White and Rainbow 1987), however, reported that the decrease in the concentration of some metals with increasing body size of crustaceans may indicate that a significant proportion of these metals may be surface adsorbed, since smaller specimens have high surface area to volume ratios than larger ones.

The daily intake of Cu metal was estimated according to the average mussel consumption for adults in Table 3. According to data from Table 3, the daily intake of Cu increases with increasing Cu concentration. All values are

below the daily intake recommended by JECFA for Cu of  $500 \mu\text{g/kg bw/day}$  (JECFA 1982). Although Cu is an essential trace element, high levels of intake can cause harmful health effects (Gorell et al. 1997), it is not, however, carcinogenic to humans and animals.

### Conclusion

The present study confirms the ability of the mussel *M. galloprovincialis* to effectively reflect the environmental concentrations of Cu in its soft tissues. Mussels may be used for the monitoring of chronic or acute pollution, as metal uptake is lower in acute metal concentration. The shell size, solution concentration, and exposure time of the mussel *M. galloprovincialis* greatly affected the uptake capacity process. However, sex showed no similar influence. Also, the results indicated that exposure time is an important factor in the uptake process. It was observed that the Cu concentration reached  $266\text{--}463 \mu\text{g g}^{-1}$  at  $0.36 \text{ mg L}^{-1}$  for first 3 days, while it decreased to  $158\text{--}334 \mu\text{g g}^{-1}$  for the last day. This reveals that the most Cu was absorbed to the mussel tissues within the first 3 days and Cu concentration subsequently declined. Evidently, copper is more easily absorbed at the beginning of exposure. The measured Cu concentration values were below the daily intake recommended by JECFA. The health risk

**Table 3** Daily intake ( $\mu\text{g/kg/day}$ ) for Cu in the soft tissue of the mussel sample

Size	Sex	Group				
		Control	First	Second	Third	Fourth
Large	Male	0.13	0.16	0.58	0.84	1.00
	Female	0.15	0.25	0.50	0.58	0.71
	Mean	0.14	0.21	0.54	0.71	0.86
Medium	Male	0.17	0.17	0.67	0.92	0.94
	Female	0.15	0.18	0.67	0.68	0.72
	Mean	0.16	0.18	0.67	0.80	0.83
Small	Male	0.17	0.22	0.69	0.78	0.82
	Female	0.20	0.22	0.72	0.75	0.77
	Mean	0.19	0.22	0.71	0.76	0.75
Mean		0.16	0.16	0.20	0.64	0.76

assessment indicates that there is no health risk related to the consumption of mussels in marine environments.

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#### Compliance with ethical standards

**Conflict of interest** The authors declared that they have no conflicts of interest.

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