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Heavy metal pollution in sediments and mussels: assessment by using pollution indices and metallothionein levels

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Abstract:	In the present work, concentration of eight metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) was determined in the sediments, transplanted and native mussels (Mytilus galloprovincialis). The study was conducted in Turkish marinas, shipyards and shipbreaking yards. The effect of metal pollution was evaluated by determining the levels of metallothionein (MT) in the mussels. The extent of contamination for each single metal was assessed by using geoaccumulation index (Igeo) and enrichment factor (EF). Whereas, to evaluate the overall metal pollution and effect, pollution load index (PLI), modified contamination degree (mCd), potential toxicity response index (RI), mean ERM quotient (m-ERM-Q) and mean PEL quotient (m-PEL-Q) were calculated. The influence of different background values on the calculations was discussed. The results indicated a significant metal pollution caused by Cu, Pb and Zn especially in shipyard and shipbreaking sites. Higher concentrations of MT were observed in the ship/breaking yard samples after the transplantation.
Response to Reviewers:	Materials and methods section: 1) Should be noted was mussels depurated before analysis or not;

	Yes, the mussels were left in filtered sea water for a week for depuration before the transplantation. The water was renewed daily during the depuration period. This information is now added to the relevant section (2.2. Sampling and Deployment) in the text (Lines 102-103). 2) Analysis of the SRM (standard reference materials) like BCSS-1 for sediments and NIST for mussel tissues is recommended for such research. Merck Certipur ICP multi-element standart solution was used as reference material for the validation of the metal analyses. However, the reference material was not particularly for mussels or sediments. Results and discussion section: 1) In the tables 1-6 the order of stations would be changed: M1A,...,SBY that is stations from Mediterranean area, and then M2,...S1, S2, S3, that is stations from Marmara area. The order of stations was changed as suggested (Tables 1-7). 2)Sentence on p.11, para 2, line 4 should be clarified. The sentence was changed as suggested (Lines 190-191). 3) Table 7 is very "heavy" and has to be modified, and may be presented as figure(s). We first tried to represent the data graphically. However, it was still too complicated. Therefore, the table was split into two separate tables as (a) and (b). The necessary change was indicated in the text and marked (Line 331). 4) The information on the mussels used for the transplantation should be added, and it could help to explain more clearly the observed changes of metals concentration in mussel tissues. The suggested additions were inserted for a more clear explanation about the changes of metal concentrations in mussel tissues (Lines 342-348; 351-354; 355-356). 5) The use of PCA need more detailed characteristic of the initial data. 6) PCA results are not reflected in the conclusions, and in the presented form look slightly excessive. Related to comments on 5 and 6 : PCA results were mentioned in the conclusions and details of the eigenvalues were removed from the text (Lines 380-382; 423-426).

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Dr. Jose Alexander Elvir

Associate Editor
Environmental Monitoring and Assessment

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Dear Dr. Elvir,

Attached please find the revised manuscript entitled “Heavy metal pollution in sediments and mussels: assessment by using pollution indices and metallothionein levels” to be considered for publication in the journal of “Environmental Monitoring and Assessment”.

I hope the manuscript is now acceptable for publication in the journal.

Best regards,

Oya S. OKAY

Heavy metal pollution in sediments and mussels: assessment by using pollution indices and metallothionein levels

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Abstract

In the present work, concentration of eight metals (Cd, Cr, Cu, Fe, Mn, Ni, Pb, Zn) was determined in the sediments, transplanted and native mussels (*Mytilus galloprovincialis*). The study was conducted in Turkish marinas, shipyards and shipbreaking yards. The effect of metal pollution was evaluated by determining the levels of metallothionein (MT) in the mussels. The extent of contamination for each single metal was assessed by using geoaccumulation index (I_{geo}) and enrichment factor (EF). Whereas, to evaluate the overall metal pollution and effect, pollution load index (PLI), modified contamination degree (mC_d), potential toxicity response index (RI), mean ERM quotient (m-ERM-Q) and mean PEL quotient (m-PEL-Q) were calculated. The influence of different background values on the calculations was discussed. The results indicated a significant metal pollution caused by Cu, Pb and Zn especially in shipyard and shipbreaking sites. Higher concentrations of MT were observed in the ship/breaking yard samples after the transplantation.

Keywords: sediments; mussels; metals; metallothionein; shipyards; marinas.

1. Introduction

Metals are one of the most hazardous pollutants in marine environment mainly due to their toxic and non-biodegradable features (Chakraborty et al. 2010). Although they naturally exist in the environment, the main source for metal pollution is anthropogenic. The atmosphere (Choi et al. 2012; Romic and Romic 2003) , wastewater discharges (Tang et al. 2010) and stormwater runoff (Herngren et al. 2006) play the most important roles in the deposition of metals in marine ecosystems. Once introduced into the aquatic environment, they accumulate in sediments, as well as in the organisms through the food chain (Buccolieri et al. 2006; Maceda-Veiga et al. 2013; L. Wang et al. 2011). It has been shown that metals have an impact on the structure and the functions of the marine ecosystems (Xu et al. 2014). Sediments are important matrices for monitoring purposes since they act as a sink for pollutants (Bai et al. 2011). Determination of metal concentrations in sediments provides comparison of anthropogenic and natural levels and assessment of pollution in aquatic environments (Eades et al. 2002). On the other hand, determining the metal concentrations in organisms is especially important due to the ecosystem and human health considerations (Devier et al. 2005). Mussels are the most commonly used indicator species in monitoring studies because of their wide geographical distribution and sessile life characteristics. They have the ability to accumulate organic and inorganic chemicals including metals at high concentrations. Therefore, native mussel populations and/or transplanted mussels are widely used in pollution monitoring purposes (Andral et al. 2011; Greenfield et al. 2014; Przytarska et al. 2010). The use of transplanted mussels as being in the same size/age and containing none/low/same levels of the contaminants are advantageous in pollution studies (Andral et al. 2011; Hunt and Slone 2010). In this study, the mussels were transplanted to the study sites and native mussels were also collected when available.

The effect of metals on mussels was investigated by determining metallothionein (MT) levels in the digestive glands. Metallothioneins are non-enzymatic proteins with a low molecular weight and high cysteine content. The thiol groups (–SH) of cysteine residues enable MTs to bind particular metals (Amiard et al. 2006). It is generally considered that these proteins play a role in the homeostatic control of essential metals (Cu, Zn) as they can act as essential metal stores ready to fulfill enzymatic and other metabolic demands (Amiard et al. 2006). The metals can show more specific forms of chemical attack through mimicry. In this regard the toxic metals may act as mimics of essential metals, binding to physiological sites that normally are reserved for an essential element. Essential metals are involved in various key metabolic and signalling functions (Kasprzak 2002). The MT synthesis is inducible by a wide variety of chemical and physical agents including metals, alcohols, urethane,

endotoxin, alkylating agents, hyper- or hypothermia and ionizing radiation (Rogers and Kavlock 2008). Cadmium induces the synthesis of MT in the liver, kidney, and other tissues (Klaassen et al. 1999). Zinc is an effective inducer of MT synthesis and when MT is saturated in intestinal cells, Zn absorption is decreased. Copper concentrations are maintained mainly through control of excretion, although copper binding to hepatic MT may act as a form of Cu storage.

The objectives of this study are: (1) to determine the distribution of eight heavy metals in the surface sediments and transplanted and, if exists, in native mussels, (2) to assess the metal contamination in the sediments by using several assessment methodologies used in the literature, (3) to evaluate the effect of metals on mussels by determining the metallothionein levels.

2. Materials and Methods

2.1. Study sites

Study sites in the coastal area of Turkey were shown in Figure 1. Sediment samples were collected from 8 stations. Two of the sampling stations (M1A-M1B and M1C) were located at a marina (Marina 1) in the Mediterranean coast and one station was selected as reference station (M1-Ref) for this site. At the coastal area of Marmara Sea, another marina (Marina 2) station (M2A) and three shipyard stations (S1, S2 and S3) were selected. SBY-s was the sediment station at the shipbreaking yard in Aliağa, İzmir. At some stations, sediment samples could not be collected due to the hard substrate at the bottom. The sediment sample collected from SBY reference station (SBY-Ref) was lost.

Fig 1 Sampling sites.

Mussels were transplanted into three stations at each site. These stations were M1A, M1B and M1C, for Marina 1; M2A, M2B and M2C for Marina 2 and S1, S2 and S3 for the shipyard area. During the 30 days of exposure period in 2012, the mussels transplanted to M1-Ref and SBY stations were lost due to the unforeseen weather conditions. Thus, the deployment station at the shipbreaking site was relocated (indicated as SBY-m on the map) in the second transplantation period in 2013. On the other hand, all transplanted mussels to S3 station were killed in 30 days due to the extreme pollutant concentrations. Therefore, for the site study in 2013, they were transplanted to this station for only one week duration.

2.2. Sampling and Deployment

Sediments were sampled once in March 2012 and mussels were transplanted twice (2012 and 2013). Approximately 1000 grams of surface sediments (0-10 cm) were collected by free diving. Mediterranean mussels (4 to 5 cm in length) collected from relatively clean areas were deployed for 30 days (March of 2012) and 60 days (January of 2013). For depuration, the collected mussels were left in filtered and daily renewed sea water for a week prior to transplantation. The mussels in nets were deployed at a depth of approximately 1 meter from the water surface and 2 to 3 meters above the sediments.

After the exposure periods, the mussels were retrieved and separated for metal analyses and metallothionein assays. Mussels for the MT assays were cleaned using sea water, and each mussel was dissected under the field conditions using stainless steel disposable scalpel. The samples were then transferred to the laboratory in liquid nitrogen and stored at -80ºC. For the metal analyses, the soft tissues (except the foot) of the mussels were stored in plastic bags and kept in liquid nitrogen during the transportation. Sediment and mussel samples for metal analysis were stored at -20°*C.*

Local samples were collected only from M2A, S-Ref, SBY-m and SBY-Ref stations.

2.3. Metal Analysis of mussels and sediments

HNO_3/H_2O_2 based digestion test protocol (USEPA3052) was followed for the analysis of metals. The wet tissues of the mussel samples (0.5-1 g) were weighed and digested with 9 mL of HNO_3 (65%), and 1 mL of H_2O_2 (30%) for 48 h in the refrigerator in closed Teflon vessels. After then, samples were redigested using a microwave digestion system (CEM MARS-5). For the microwave digestion, samples were firstly exposed to 180 psi pressure at 180 ºC for 10 minutes, and then they were allowed to stand at 180°C for 15 minutes. This procedure was repeated three times. In the final stage, samples were allowed to cool to the ambient temperature. The solutions were then diluted to 25 mL with ultrapure Milli-Q water (Merck Millipore Corp, USA) and stored in polyethylene bottles, until analysis.

The sediment samples were sieved with a 2-mm mesh sized sieve, stored in desiccator and dried at 60°C for 24 hours prior to analysis. The dried and weighed samples (1 g) were placed in a Teflon cup and 6 mL of HNO_3 and 4 mL of HCl were added. Then the cups were capped and digested in microwave digestion system using the same procedure as described for the mussel samples. Cooled samples were diluted to 25 mL with deionized water and remaining particles were filtered and stored in polyethylene bottles until analysis. Blank digestions were also performed for both sediments and mussels.

The metal analyses were performed by using inductively coupled plasma-optically emission spectrometry (ICP-OES, Varian, model 725). Standard solutions were prepared with serial dilutions of the stock standard solutions (Merck Chemical Comp., Germany) of each metal.

2.4. Metallothionein Analysis

The measurement of MT concentration in mussel samples was conducted according to Viarengo et al., (1997) with minor modifications. The MT levels were determined spectrophotometrically using a microplate reader system (VersaMax, Molecular Devices Corp.) at 25°C. Assays were performed for all mussel samples in triplicate.

The mussel samples were thawed in an ice box, weighed, and homogenized at 2000 rpm for 30 seconds with a Teflon/glass homogenizer with 10 strokes (model RZR-2021, Heidolph, Germany) in ice-cold homogenization buffer. Three volumes of homogenization buffer (20 mM Tris-HCl pH 8.6 which contains 0.5 M sucrose, 0.5 mM PMSF and 0.01% β-mercaptoethanol) were used for each gram of mussels. The homogenates were centrifuged at 15,000 × g for 30 min at 4°C, and the supernatants were transferred into clean micro-centrifuge tubes. After first centrifugation step, 80 µL chloroform and 1.05 mL ice-cold ethanol were added to each mL of supernatant. These mixtures were centrifuged at 6000 × g for 10 minutes. After the second centrifugation step, three volumes of ethanol for each volume of supernatant were added and the mixture was maintained at -20°C for one hour. Then, these samples were re-centrifuged at 6000 × g for 10 minutes. The pellets were washed with homogenization buffer containing 87 % ethanol and 1 % chloroform mixture. These samples were centrifuged at 6000 × g for 10 minutes. After the last centrifugation step, the pellets were re-suspended in 300 µL of 5 mM Tris-HCl (pH 7) containing 1 mM EDTA.

For measurement of MT, 4.2 mL of 0.43 mM DTNB (buffered with 0.2 M Na-phosphate buffer, pH 8.0) were added to each re-suspended sample. These samples were incubated in room temperature for 30 minutes, and then centrifuged at 3000 × g for 5 minutes. After centrifugation, 250 µL supernatant were added to each flat-bottom microplate wells. Absorbance was read at 412 nm wavelength. MT concentrations were calculated from the calibration curve prepared by using standard GSH solutions (2.5-160 nmol GSH).

2.5. Statistical analysis

Statistical analyses of metal and metallothionein levels were performed using a statistical software (SPSS Inc., USA). Non-parametric one-way analysis of variance (Kruskal-Wallis) was used, followed by pairwise comparisons of groups using Mann-Whitney U test. Significance was designated at least $p<0.05$ for all data analysis. Pearson's correlation analysis and principal component analysis (PCA) were carried out using the software XLSTAT (Addinsoft Inc.).

2.6. Software for calculation of assessment values

A Python based software (Sedimet) was developed for the calculation and analysis of assessment indices. Background and measured metal concentrations are read from a MS Excel file in a matrix form. The output data from the software are written in a MS Excel file. The results of each assessment method are printed in different worksheets of the file. This software provides quick way to calculate indices using different background values, as applied in this study, and reduces the possibility of miscalculations. The software can be obtained from the authors upon request.

3. Results and Discussion

3.1. Metals in Sediments

The spatial pattern of grain size and total organic carbon (TOC) content, which are the most fundamental properties for classifying sediments, are shown in Table 1. Sediment samples used in this study exhibited a relatively broad range in both grain size and TOC content. For instance, the percentage of sand (> 63 µm) in the samples ranged from 43% to 97%, while the TOC content (%) was between 1.1 and 9.3. TOC in the marine environments is composed of both naturally-occurring forms derived from the decomposition of organisms and contaminants generated by the anthropogenic activities. The high organic production in the water column may result in high levels of organic carbon in sediments. On the other hand, the low percentages of organic carbon

may be related with oxidizing conditions in the surface sediments. Nevertheless, in general, the variation in organic carbon content of the sediment is primarily due to local hydrodynamic conditions which play an important role in the transport of particulate organic materials.

The concentrations of metals in the sediments of the study area were shown in Table 2. Iron, as expected, was found as the most abundant metal. This phenomenon may be related with the deposition of periodical Saharan dust episodes which contain high levels of iron (Heimbürger et al. 2011; Ternon et al. 2010). Iron concentration in sediment samples varied between 19 (Marina 1) and 80 g kg^{-1} (Shipyard 3).

The most polluted sediments by metals were found at the shipyard station (S3) situated at the inner part of the Tuzla bay where the main shipyard area of Turkey is located. The limited water circulation in the bay may be the main reason for these very/extremely high metal concentrations accumulated in the sediments. Among all sampling stations, Cu and Zn concentrations were the highest in shipyard and shipbreaking yard stations. Most probably, these high Cu and Zn concentrations in the sediments were due to the accumulated antifouling paint residues removed from the hulls of the boats and ships, during the re-painting/maintenance activities. It was found that metals in antifouling paint particles leach more rapidly than painted surfaces due to the greater surface area of pigments and additives exposed to the aqueous medium (Turner 2010).

The analysis results of Marina 1 reference station (M1-Ref) sediments show that the levels of heavy metals in this station are much lower than those measured in the other sites. Only, Mn concentration in M1-Ref is relatively higher than that of M2 sediments. Ni and Cr concentrations are lower in marina stations, however, comparatively higher concentrations were measured at the sediments of ship building/breaking yards area probably due to the results of blasting, removal of coating, painting, welding, cutting and metal grinding processes (Host 1996). The analysis results exceeding ERM values were indicated in boldface.

The concentrations of metals in sediments do not give direct information related to the degree of contamination as well as the effect on the benthic organisms. The degree of contamination may be evaluated by several methods indicated in the literature by using the geochemical background values of the metals. The background values were either measured in pre-industrialized sediments or “average shale” values were used directly. Therefore, several different background concentrations for single metals have been used in the previous studies.

The use of different background values eventually affects the assessment of metal sediment contamination and effect. It was suggested that using local uncontaminated sediment as a normalizing reference representing preindustrial conditions is a better approach (Carral et al. 1995; Christophoridis et al. 2009). On the other hand, background values for the Mediterranean area show big variations due to the different anthropogenic impacts (Buccolieri et al. 2006).

Metal associated risks were evaluated by using Effects Range Low/Effects Range Median (ERL/ERM) and Probable Effects Level (PEL) values indicated in Sediment Quality Guidelines (SQG) (Long and MacDonald 1998). The chemical concentrations corresponding to the 10^{th} and 50^{th} percentiles of adverse biological effects are called ERL and ERM, respectively. The PEL represents the concentration above which adverse effects are frequently expected. The comparison of the metal concentrations in sediment samples with those of ERM values shows that Cd at none of the stations, Cu at shipyard and shipbreaking yard stations may pose adverse biological effects (higher than ERM). Furthermore, ERM values for all metal concentrations (except Cd) were exceeded at station S3. Mn, Pb and Zn were the other notable metals which may cause risk for the aquatic organisms in some stations. When PEL values were considered, Cu, Pb and Zn were found the risk associated metals in most of the stations.

In this study, the degree of contamination for individual metals was assessed by using geoaccumulation index (I_{geo}) proposed by Müller (1981) and enrichment factor (EF) (Feng et al. 2004), to distinguish the anthropogenic sources from the natural ones, by using Equations (1) and (2), respectively. During the EF calculations, iron was used for geochemical normalization.

$$I_{geo} = \log_2(C_n / 1.5\, B_n) \qquad (1)$$

$$EF = \frac{(\text{Element}/\text{Fe})_{\text{sample}}}{(\text{Element}/\text{Fe})_{\text{reference}}} \qquad (2)$$

where C_n and B_n are the measured and the geochemical background concentrations of the target metal "n", respectively. I_{geo} value is directly related with the background values of the metals, therefore it has been calculated, for each metal, by using three geochemical background values (Table 3). In the Table, the first two I_{geo} values were calculated by using two different (relatively lower and higher) metal background values selected

245 from the literature (Buccolieri et al. 2006; Hakanson 1980; Taylor 1964; Turekian and Wedepohl 1961), to
246 estimate the probable ranges of I_{geo} values. The lower and higher background values (mg kg^{-1}) were 0.20 and
247 0.98 for Cd; 49 and 100 for Cr; 25 and 55 for Cu; 26313 and 56300 for Fe; 850 and 950 for Mn; 36 and 75 for
248 Ni; 12.5 and 70 for Pb; 70 and 175 for Zn. The third I_{geo} values were calculated by using the metal
249 concentrations determined in the M1-Ref sediment. The use of the reference station as background seems quite
250 reasonable, when the metal concentrations measured at that station were compared with the "average shale" or
251 metal concentrations presented for the different parts of the Mediterranean (Buccolieri et al. 2006). According to
252 the classification system, defined by (Müller 1981), negative I_{geo} values indicate the absence of metal
253 contamination. Those I_{geo} values calculated by using lower and higher background values showed that M1-Ref
254 station is unpolluted (Class 0) and regardless which background values were used, S3 station is
255 heavily/extremely polluted for most of the metals. The results pointed out that the most critical metals in terms
256 of metal pollution were Cu, Pb and Zn for all stations. For these metals, calculated I_{geo} values at S3 station were
257 between 8.6 (Class 6) and 9.3 (Class 6) for Cu; 3.6 (Class 4) and 6.1(Class 6) for Pb and 5.5 (Class 6) and 8.4
258 (Class 6) for Zn.
259
260 EF values for Cu, Pb and Zn for the study area were calculated by using two different Fe concentrations for
261 normalization and lower and higher background values of the single metals (Table 4). An EF value smaller than
262 1.5 implies a predominantly natural origin. The calculated values were generally higher than 1.5 at most of the
263 stations and lower at Mediterranean marina stations. As clearly seen from Table 4, EF values show big
264 variations depending on the choice of the background values. For example, EF values for SBY-s station ranged
265 between 9.5 and 45 for Cu, which is indicating "moderately severe enrichment" and "very severe enrichment"
266 respectively, according to the related classification system. Therefore, the consideration of the background
267 values from the same geographical region may produce more reliable results for the assessment of metal
268 pollution.
269
270 Besides of the single metal pollution evaluation methods, there have been also several methods to assess the
271 overall degree of heavy metal contamination in the literature. Some of these are pollution load index (PLI)
272 (Tomlinson et al. 1980) modified contamination degree (mC_d) (Abrahim and Parker 2008), potential toxicity
273 response index (RI) (El-Said et al. 2014), mean ERM quotient (*m-ERM-Q*) and mean PEL quotient (*m-PEL-Q*)
274 (Gao and Chen 2012; Long and MacDonald 1998). Similar to I_{geo} and EF calculations, lower and higher

background values of single metals were used during the PLI, mC_d and RI calculations. The calculated values of these indices for the study area were presented in Table 5a and 5b. Table 5a demonstrates the calculation results directly, whereas Table 5b shows the metal pollution and effect classes, according to the corresponding ranges given in the parentheses. The degree of metal pollution or effect increases from no/low pollution to high/extreme pollution as the index values increase. PLI and C_d values were calculated by using the contamination factor (CF); RI was calculated from the "Potential Ecological Risk Index (PERI)" results (Hakanson 1980). Originally, C_d given by (Hakanson 1980) was based on seven metals and one organic contaminant. Therefore, a modification of this method (mC_d) proposed by Abrahim and Parker (2008) to avoid the limited application was used in this study. The indices to evaluate the overall degree of heavy metal contamination are calculated as shown in the following equations:

$$PLI = \sqrt[n]{CF_1 \times CF_2 \times CF_3 \times \ldots \times CF_n} \qquad (3)$$

$$C_d = \sum_{i=1}^{n} CF^i \qquad (4)$$

$$mC_d = C_d / n \qquad (5)$$

$$RI = \sum_{i=1}^{n} PERI^i \qquad (6)$$

where n is the number of metals (Cd, Cr, Cu, Ni, Pb and Zn).

CF and PERI values used in the equations above were calculated by using the following formulas:

$$CF = M_x / M_b \qquad (7)$$

$$PERI = T_r \times \frac{C_i}{C_0} \qquad (8)$$

where M_x is the mean concentration of the target metal and M_b is the concentration of metal in the selected reference sediment. T_r is the response coefficient for the toxicity of each metal (Cd = 30, Cr = 2, Cu = Pb = Ni = 5, Zn = 1) (Hakanson 1980; Yi et al. 2011) and C_i and C_0 are the concentrations of individual and reference metals, respectively.

PLI results were found similar when lower and higher background concentrations were used during the calculations; however those results were considerably different than the PLI values calculated by use of M1-Ref background values. On the other hand, mC_d and RI values were more or less similar for all three cases. Therefore, RI values indicating the risk were more compatible with the mC_d results showing the contamination degree. As previously mentioned, RI values were calculated by using the toxic response factors of six metals (Cd, Cr, Cu, Pb, Ni and Zn). Among those metals, Cu and Pb for the first two cases (lower and higher background values) and Cu, Cd and Zn for the third case (M-Ref background values) were found as the most responsible metals for the biological effects of the sediments.

m-ERM-Q and m-PEL-Q values were calculated by using the following equations:

$$\text{m-ERM-Q} = \frac{\sum_{i=1}^{n} (C_i/ERM_i)}{n} \qquad (9)$$

$$\text{m-PEL-Q} = \frac{\sum_{i=1}^{n} (C_i/PEL_i)}{n} \qquad (10)$$

where C_i and n are the concentration of individual metals and number of metals, respectively. All metals were included for m-ERM-Q calculation, whereas Fe and Mn were excluded (no PEL values) during the m-PEL-Q calculation.

According to the m-ERM-Q and m-PEL-Q results (Table 6), the most impacted areas are shipyard and shipbreaking yard stations. The probability of being toxic of S3 sediments is the highest (76%) and of M1-Ref is the lowest (9 %). Cu and Zn were found as the most responsible metals for the toxicity of sediments.

3.2. Metals and Metallothionein in mussels

The concentrations of metals and metallothionein responses determined for 2012 and 2013 were shown in Table 7a and Table 7b for Mediterranean and Marmara stations, respectively. Due to the differences in salinity between the two marine ecosystems, the mussels used for transplantation were collected from Mediterranean for Marina 1 and shipbreaking yard, and from the Marmara Sea for Marina 2 and shipyard area. Most of the mussels depurated the metals accumulated in their tissues in both sampling years at all stations. Cu, Fe, Mn and Zn concentrations increased at some sites significantly ($p<0.05$) in mussel tissues. Only the concentrations of two

metals (Cu and Zn) were higher in 2012 in Marina 1 when compared to the concentration of those metals in mussels before the transplantation. In Marmara sites, Cu, Zn and Fe were the most important metals similar to the results obtained for the Mediterranean sites. The concentrations of Cu in M1 stations were significantly higher in 2012 than the levels in 2013, although the exposure period in 2013 was longer. Therefore, it seems that the bioaccumulation of metals depends on the pollution levels of the study area rather than the duration of the transplantation. This was probably due to the more intensive hull maintenance activities in spring time. Additionally, the filtration rate of the mussels are higher in spring time which affects the bioaccumulation capacity. Although there have been numerous metal monitoring studies on bivalves, quantitative extrapolation of environmental exposures from tissue concentrations still have a lot of uncertainty (Rainbow 2002). In general, the processes responsible for metal uptake and accumulation in mussels are actively controlled by biological factors such as body size, sex, nutritional and reproductive status of the organisms and environmental factors such as salinity, temperature, organic matter concentration (Kumar et al. 2015; Lobel et al. 1991; Riget et al. 1996; Wang and Fisher 1999).

The concentrations of Cu and Zn in the local mussels collected from Marina 2 were the highest among all stations in 2012. The physicochemical characteristics of water affect the bioavailability of metals and therefore influence their bioaccumulation. The bioavailability of metals is higher at low salinity conditions (Kumar et al. 2015). It has been reported that especially Cd and Zn uptake by *Mytilus edulis* was maximum at low salinity (Ali and Taylor 2010; Jackim et al. 1977; Phillips 1976). The source of Cu and Zn, as in case of sediments, may be due to the release of antifouling paints accumulated in suspended solids and in the sediments. Fisher et al. (1996) and Wang and Fisher (1999) reported that the uptake from the dissolved phase is important for zinc. The relatively high Cu concentrations in the mussels were also associated with high GST activity in our studies (Okay et al. 2014), which was also compatible with other previous studies (Hoarau et al. 2001; Roméo et al. 2003). Fe concentrations were generally higher in the local mussels than that of the mussels transplanted to the same locations for both sampling years.

According to the results of MT, the levels significantly decreased in M1A in both exposure periods compared with mussels before the transplantation (Med-Bt) (p<0.05). This may be related with the depuration of most of the metals from the mussels, although the tissue concentrations of Cu and Zn in 2012 and Cu and Mn in 2013 were higher. Similarly, MT levels decreased in M1-Ref significantly while Fe and Mn concentrations increased

during the 2013 exposure period. On the other hand, both MT levels and Cu and Mn concentrations increased in SBY2 and SBY-Ref in the second exposure study. When the Marmara sites were investigated, the increase of MT at M2A and S2 stations in 2012 and at M2A, S1, S2 and S-Ref in 2013 were significant (p<0.05). All transplanted mussels died in S3 station in Tuzla region in 2012 sampling year, probably due to the extremely high pollutant concentrations. Thus, the mussels were transplanted in this location only for a week in 2013 and no significant change (p>0.05) was detected in MT levels. Those results point out that there is no simple/direct relation between MT levels and those metals at measured concentrations. Therefore, Pearson's correlation coefficients (PCC) and principal components analysis (PCA) were applied for eight selected metals and MT levels (Table 8).

The PCC results showed a correlation between metal pollution and MT levels. A positive correlation was determined between Cd, Cr, Pb and Ni for Mediterranean region. In addition, significant correlations were observed between the metals, except copper in the selected Marmara stations. The principal component analysis (PCA) was used to investigate the existing relationships between samples and variables and to deduce how many independent components were needed to explain the observed data variance (Rivetti et al. 2014). The three principal components with eigenvalues >1 were identified in the Mediterranean stations and two principal components (eigenvalues >1) were identified in the Marmara samples (Figure 2). The rotated component matrix showed that MT, Cd, Cr, Pb and Ni are essentially associated with PC1 for both Mediterranean and Marmara sites (Table 8). The plot of scores gives the positions of stations in the co-ordinates of principal components of PC1 and PC2. Generally, values for Marina 1 in year 2012 exhibited high score on the positive part of PC1 for Mediterranean sites and all values for 2012 sampling year exhibited high score on the positive part of PC1 in the Marmara sites.

Fig 2 The results of principal component analysis for the study sites.

The PCA results showed about 70% correlation between the effects and metal pollution on mussels. Metals can be classified according to their capacity of toxicity: toxic metals (Cr, Ni, Pb), essential metals with potential for toxicity (Cu, Fe and Mn) and Zn (Liu et al. 2008). Correlation matrix (Table 8) showed some correlations especially for toxic metals (Cr, Ni and Pb). This correlation was showing a probable common origin for these metals.

4. Conclusion

This study revealed that the copper, zinc and manganese were the most important metals in the sampling sites, in terms of level and effect. Especially, the sediments of the shipyard and shipbreaking yard sites contained a significant amount of heavy metals. The source of copper and zinc contamination is most probably due to antifouling paints. The highest metal concentrations were determined at S3 station located at the inner part of the shipyard area. This shows that the intensity of water circulation is an important factor on the distribution of pollutants in semi enclosed aquatic ecosystems.

The use of different background values in the calculations eventually resulted in different results. The results of this study showed that considering local background values in calculations generated more consistent results. On the other hand, the results of assessment methods were incompatible with each other in some cases. Among the overall degree of metal pollution assessment indices, pollution load index was found as insufficient to show the variations in metal contamination between the sites. Therefore, caution is required during the assessment of metal pollution and effect by using these evaluation indices.

Statistically significant differences in the metal levels were observed in the tissues of mussels before and after transplantation. As was seen in sediment samples, copper and zinc were the most accumulated metals in the tissues of the mussels during the exposure. An increase in the metal concentrations was determined in the polluted sites, whereas the metal levels decreased due to the depuration from the tissues in the unpolluted areas. These observations confirm that the transplantation of mussels in monitoring studies is an advantageous method for revealing the variations in pollution between the sites.

Although the observed induction in mussel metallothionein levels may be considered as a general stress response due to metal pollution in the surrounding ecosystem, no correlation was observed between metal concentrations and MT levels in mussel tissues in the presence of high metal concentrations in some sites.

The principal component and correlation analysis were used to examine the relations between the metal concentrations and the origin of the pollution, as well as to differentiate the polluted and unpolluted sites. The

results of these analyses suggested that the source of the toxic metals (Cr, Ni and Pb) may be the same and the metal concentrations in mussel samples varied according to the sampling years, not to sampling sites.

Surely, greater awareness by the managers of marinas and ship/shipbreaking yards during the removal and disposal of solid wastes especially antifouling paint residues and stricter legislation will help to improve the water and sediment quality and health status of the marine organisms.

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Figure 1

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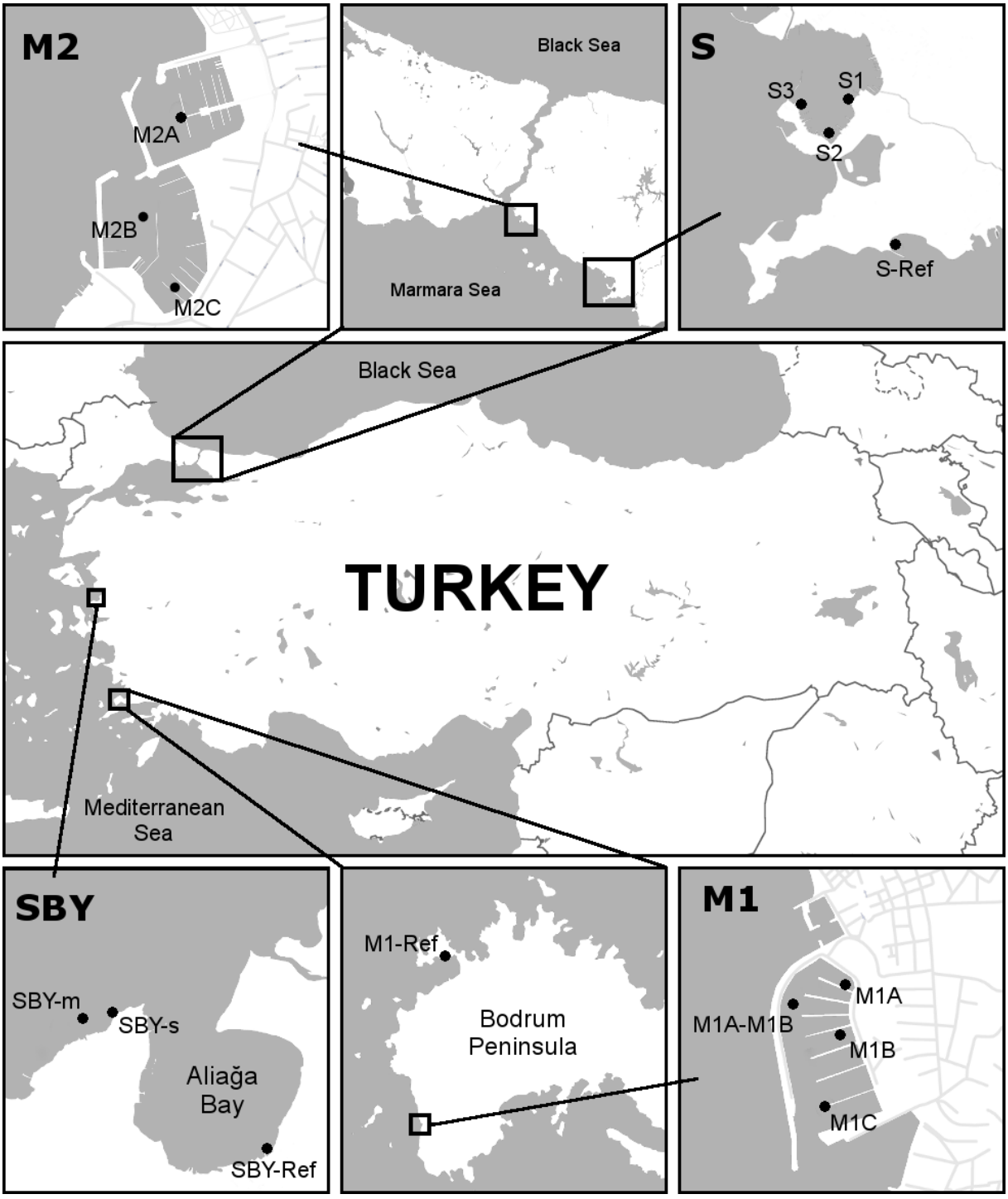

Fig 1

Figure 2

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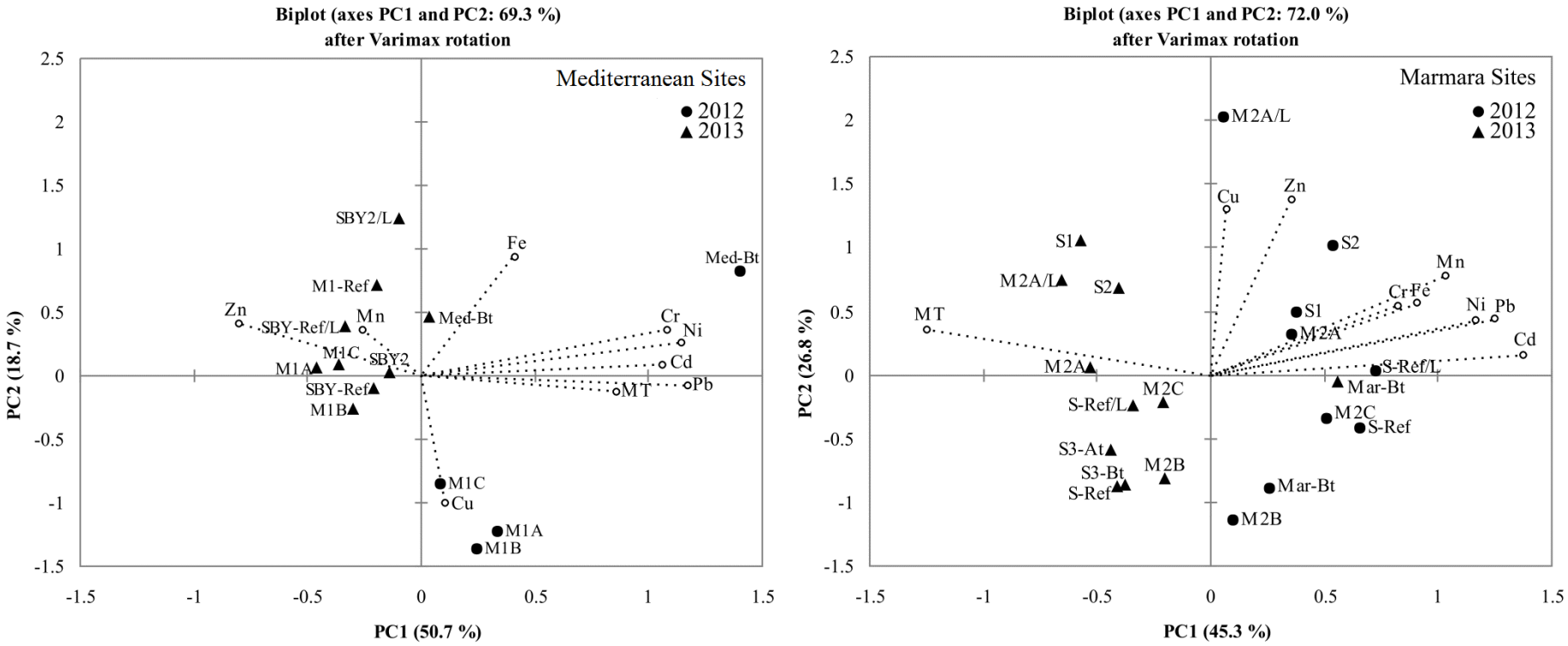

Fig 2

Table 1 Particle size distribution and total organic carbon (TOC) content of the sediment samples.

Stations	Particle size (%)				TOC (%)
	4.75 mm-500 µm	500 - 125µm	125 - 63µm	<63 µm	
M1A-M1B	2.0	35	37	26	1.5
M1C	17	13	13	57	4.6
M1-Ref	35	59	2.9	3.1	1.1
SBY-s	78	19	0.3	2.7	1.7
M2A	8.0	7.0	28	57	4.7
S1	53	10	8.0	29	3.1
S2	16	15	23	46	5.3
S3	5.0	24	21	50	9.3

Table 2 Concentrations (mg kg^{-1} dry weight) of heavy metals in sediments. The results exceeding ERM values were indicated in boldface.

Stations	Cd	Cr	Cu	Fe	Mn	Ni	Pb	Zn
M1A-M1B	0.010 ± 0.002	8.9 ± 1.0	36 ± 1.9	18526 ± 1030	**365 ± 26**	4.6 ± 0.5	**225 ± 15**	120 ± 7.0
M1C	0.011 ± 0.001	11 ± 1.4	55 ± 2.0	40714 ± 1850	**578 ± 24**	4.6 ± 0.5	**330 ± 18**	165 ± 5.6
M1-Ref	0.014 ± 0.004	9.9 ± 2.8	35 ± 1.8	25655 ± 3860	122 ± 18	4.6 ± 0.5	38 ± 5.7	23 ± 3.4
SBY-s	0.018 ± 0.005	35 ± 0.5	**609 ± 36**	30826 ± 1630	**347 ± 25**	29 ± 2.2	**235 ± 16**	**1148 ± 55**
M2	0.010 ± 0.002	19 ± 2.5	182 ± 11	19866 ± 1540	104 ± 7.6	5.1 ± 0.5	55 ± 4.2	275 ± 21
S1	0.013 ± 0.002	66 ± 2.1	**594 ± 22**	40002 ± 2140	**341 ± 16**	19 ± 1.6	110 ± 8.4	383 ± 20
S2	0.010 ± 0.003	76 ± 8.9	**697 ± 58**	42717 ± 2720	151 ± 8.2	20 ± 1.2	167 ± 7.3	**534 ± 25**
S3	0.036 ± 0.008	**996 ± 212**	**32195 ± 6582**	79701 ± 17760	**755 ± 164**	**214 ± 50**	**1316 ± 302**	**11916 ± 2446**
ERL-ERM[a]	1.2 - 9.6	81 - 370	34 - 270	NA[c]	NA - 260	20.9 - 51.6	46.7 - 218	150 - 410
PEL[b]	4.21	160.4	108.2	NA	NA	42.8	112	271

[a] Effects Range Low – Effects Range Median
[b] Probable Effects Level
[c] Not available

Table 3 I_{geo} values calculated by using lower[a] (L), higher[b] (H) background values for individual metals and M-Ref (R) metal results. Positive values were typed in boldface.

Stations	Cd			Cr			Cu			Fe			Mn			Ni			Pb			Zn		
	L	**H**	**R**	**L**	**H**	**R**	**L**	**H**	**R**	**L**	**H**	**R**	**L**	**H**	**R**	**L**	**H**	**R**	**L**	**H**	**R**	**L**	**H**	**R**
M1A-M1B	-5.0	-7.2	-1.1	-3.0	-4.1	-0.7	-0.1	-1.2	-0.6	-1.5	-2.2	-1.1	-1.8	-2.0	**1.0**	-3.6	-4.6	-0.6	**3.6**	**1.1**	**2.0**	**0.2**	-1.1	**1.8**
M1C	-4.7	-7.0	-0.9	-2.7	-3.8	-0.4	**0.5**	-0.6	**0.1**	-0.4	-1,1	**0.1**	-1.1	-1.3	**1.7**	-3.6	-4.6	-0.6	**4.1**	**1.7**	**2.5**	**0.6**	-0.7	**2.2**
M1-Ref	-4.5	-6.7	-	-2.9	-3.9	-	-0.1	-1.2	-	-1.0	-1.7	-	-3.4	-3.5	-	-3.6	-4.6	-	**1.0**	-1.5	-	-2.2	-3.5	-
SBY-s	-4.0	-6.3	-0.2	-1.1	-2.1	**1.2**	**4.0**	**2.9**	**3.5**	-0.8	-1.5	-0.3	-1.9	-2.0	**0.9**	-0.9	-2.0	**2.1**	**3.7**	**1.2**	**2.0**	**3.5**	**2.1**	**5.0**
M2A	-5.1	-7.4	-1.2	-1.9	-3.0	**0.4**	**2.3**	**1.1**	**1.8**	-1.4	-2.1	-1.0	-3.6	-3.8	-0.8	-3.4	-4.5	-0.4	**1.5**	-0.9	-0.1	**1.4**	**0.1**	**3.0**
S1	-4.6	-6.8	-0.7	-0.2	-1.2	**2.1**	**4.0**	**2.8**	**3.5**	-0.4	-1.1	**0.1**	-1.9	-2.1	**0.9**	-1.5	-2.5	**1.5**	**2.5**	**0.1**	**0.9**	**1.9**	**0.5**	**3.5**
S2	-5.0	-7.2	-1.1	**0.0**	-1.0	**2.3**	**4.2**	**3.1**	**3.7**	-0.3	-1.0	**0.2**	-3.1	-3.2	-0.3	-1.4	-2.5	**1.5**	**3.2**	**0.7**	**1.6**	**2.3**	**1.0**	**3.9**
S3	-3.1	-5.3	**0.8**	**3.8**	**2.7**	**6.1**	**9.7**	**8.6**	**9.3**	**0.6**	-0.1	**1.1**	-0.8	-0.9	**2.0**	**2.0**	**0.9**	**5.0**	**6.1**	**3.6**	**4.5**	**6.8**	**5.5**	**8.4**

[a] Cd: 0.20; Cr:49; Cu: 25; Fe: 26313; Mn:850; Ni:36; Pb:12.5; Zn: 70 (mg kg^{-1})
[b] Cd: 0.98; Cr:100; Cu: 55; Fe: 56300; Mn:950; Ni:75; Pb:70; Zn: 175 (mg kg^{-1})

Table 4 EF values calculated for Cu, Pb and Zn by using lower[a] (L), higher[b] (H) and M1-Ref values as background of individual metals and three different background Fe values for normalization.

	Cu					Pb					Zn				
Stations	**Fe: 26313**		**Fe: 56300**		**Fe: M1-Ref**	**Fe: 26313**		**Fe: 56300**		**Fe: M-Ref**	**Fe: 26313**		**Fe: 56300**		**Fe: M-Ref**
	L	**H**	**L**	**H**		**L**	**H**	**L**	**H**		**L**	**H**	**L**	**H**	
M1A-M1B	2.0	0.9	4.3	2.0	1.4	26	4.6	55	9.8	8.2	2.4	1.0	5.2	2.1	7.2
M1C	1.4	0.6	3.0	1.4	1.0	17	3.1	37	6.5	5.5	1.5	0.6	3.3	1.3	4.5
M1-Ref	1.4	0.7	3.1	1.4	-	3.1	0.6	6.7	1.2	-	0.3	0.1	0.7	0.3	-
SBY-s	21	9.5	45	20	15	16	2.9	34	6.1	5.2	14	5.6	30	12	41
M2A	9.6	4.4	21	9.4	6.7	5.8	1.0	12	2.2	1.9	5.2	2.1	11	4.5	15
S1	16	7.1	33	15	11	5.8	1.0	12	2.2	1.9	3.6	1.4	7.7	3.1	11
S2	17	7.8	37	17	12	8.2	1.5	18	3.2	2.6	4.7	1.9	10	4.0	14
S3	425	193	910	414	296	35	6.2	74	13	11	56	23	120	48	166

[a] Cd: 0.20; Cr:49; Cu: 25; Fe: 26313; Mn:850; Ni:36; Pb:12.5; Zn: 70 (mg kg^{-1})
[b] Cd: 0.98; Cr:100; Cu: 55; Fe: 56300; Mn:950; Ni:75; Pb:70; Zn: 175 (mg kg^{-1})

Table 5 a) Calculated PLI, mCd and RI values by using lower[a], higher[b] and M-Ref values as background b) Corresponding overall degree of metal pollution (PLI and mC_d) and effect (RI) in the study site.

a)

	Lower			Higher			M-Ref		
Stations	**PLI**	**mC_d**	**RI**	**PLI**	**mC_d**	**RI**	**PLI**	**mC_d**	**RI**
M1A-M1B	0.6	2.8	101	0.2	0.7	21	1.6	2.3	68
M1C	0.8	4.2	148	0.3	1.0	30	2.3	3.3	90
M1-Ref	0.4	0.8	26	0.1	0.3	6.9	-	-	-
SBY-s	1.9	7.8	241	0.8	2.8	82	5.2	11	246
M2A	0.6	2.1	65	0.3	0.8	23	1.7	3.0	74
S1	1.5	5.7	175	0.6	2.1	67	4.2	6.6	178
S2	1.6	6.6	221	0.6	2.5	81	4.2	7.9	203
S3	14	199	7211	5.5	86	3124	38	204	5803

b)

	Lower			Higher			M-Ref		
Stations	**PLI (1-6)**	**mC_d (1-7)**	**RI (1-4)**	**PLI (1-6)**	**mC_d (1-7)**	**RI (1-4)**	**PLI (1-6)**	**mC_d (1-7)**	**RI (1-4)**
M1A-M1B	1	3	3	1	1	1	2	3	3
M1C	1	4	4	1	1	2	3	3	3
M1-Ref	1	1	1	1	1	1	-	-	-
SBY-s	2	4	4	1	3	3	6	5	4
M2A	1	3	3	1	1	1	2	3	3
S1	2	4	4	1	3	3	5	4	4
S2	2	4	4	1	3	3	5	4	4
S3	6	7	4	6	7	4	6	7	4

[a] Cd: 0.20; Cr:49; Cu: 25; Fe: 26313; Mn:850; Ni:36; Pb:12.5; Zn: 70 (mg kg^{-1})
[b] Cd: 0.98; Cr:100; Cu: 55; Fe: 56300; Mn:950; Ni:75; Pb:70; Zn: 175 (mg kg^{-1})

Table 6 Mean ERM quotient (m-ERM-Q) and mean PEL quotient (m-PEL-Q) values for the sediments.

Stations	m-ERM-Q[a]	m-PEL-Q[b]
M1A-M1B	0.26	0.49
M1C	0.37	0.71
M1-Ref	0.08	0.15
SBY-s	1.13	2.14
M2A	0.29	0.57
S1	0.70	1.46
S2	0.87	1.81
S3	27	61

[a] Probability of being toxic (m-ERM-Q) (%) : < 0.1 : 9 ; 0.11–0.5 : 21 ; 0.51–1.5 : 49 ; >1.50: 76
[b] Impact degree (m-PEL-Q): < 0.1 : unimpacted ; 0.1-1.0 : moderate ; > 1 : high

Table 7a Metal and metallothionein levels (nmol mg^{-1} wet weight) in mussel samples of Mediterranean stations. Cr, Pb, Ni, Cu, Fe, Mn and Zn levels were expressed as mg kg^{-1} wet weight. Cd levels were expressed as µg $kg^{-}1$ wet weight. *n* is the number of mussel samples. Bold values show significantly higher values compared to before transplantation (Bt) values.

Year	Station	*n*	MT	*n*	Cd	Cr	Pb	Ni	Cu	Fe	Mn	Zn
2012	**Mediterranean-Bt**	15	5.32 ± 0.71	7	2.6 ± 0.2	3.1 ± 0.8	14 ± 3.9	4.3 ± 0.9	1.4 ± 0.5	60 ± 8.6	2.5 ± 0.1	16 ± 1.6
	M1A	15	2.70 ± 0.27^{a,b}	5	1.4 ± 0.2^{a,b}	0.6 ± 0.2^{a}	8.9 ± 1.0^{b}	1.8 ± 0.4^{b}	**11 ± 2.2**^{a,b}	19 ± 3.1^{a}	2.7 ± 0.2	19 ± 1.9
	M1B	14	3.53 ± 0.48	5	1.3 ± 0.1^{a,b}	0.4 ± 0.3^{a}	7.1 ± 0.6^{b}	1.5 ± 0.5^{a}	**12 ± 0.7**^{a,b}	17 ± 1.7^{a}	2.9 ± 0.2	20 ± 1.9^{b}
	M1C	13	3.84 ± 0.34^{b}	5	1.2 ± 0.1^{a,b}	1.1 ± 0.2^{a,b}	5.8 ± 1.2^{b}	1.0 ± 0.3^{a}	**14 ± 2.1**^{a,b}	27 ± 4.3^{a}	2.3 ± 0.1^{b}	34 ± 15
2013	**Mediterranean -Bt**	10	1.83 ± 0.23	5	2.1 ± 0.3	0.8 ± 0.3	5.8 ± 0.9	2.0 ± 0.5	1.1 ± 0.4	23 ± 3.1	1.8 ± 0.2	39 ± 9.2
	M1A	10	1.21 ± 0.09^{a,b}	5	0.5 ± 0.1^{a,b,c}	0.4 ± 0.2	3.4 ± 0.7^{a,b}	0.8 ± 0.1^{b}	**4.2 ± 0.9**^{a,b,c}	21 ± 2.9	**2.9 ± 0.1**^{a,c}	39 ± 8.6
	M1B	10	2.24 ± 0.29	5	0.6 ± 0.1^{a,b}	0.4 ± 0.2	4.0 ± 0.9^{a,b}	0.6 ± 0.1	**6.8 ± 1.6**^{a,b,c}	26 ± 4.4	**3.0 ± 0.4**^{a,c}	34 ± 5.2^{b}
	M1C	10	1.69 ± 0.30^{b}	5	0.5 ± 0.1^{a,b,c}	0.3 ± 0.1^{b}	2.8 ± 0.5^{a,b}	0.5 ± 0.1^{c}	1.9 ± 0.5^{b,c}	28 ± 3.8	**3.2 ± 0.3**^{a,b,c}	28 ± 5.7
	M1-Ref	10	0.74 ± 0.04^{a}	5	0.7 ± 0.1^{a}	0.5 ± 0.2	4.7 ± 1.0	1.1 ± 0.4	0.9 ± 0.2	**44 ± 9.0**^{a}	**8.2 ± 1.6**^{a}	25 ± 5.8
	SBY-m	5	4.07 ± 0.61^{a}	5	0.7 ± 0.1^{a,d}	0.4 ± 0.1	5.0 ± 0.5^{c}	0.8 ± 0.3	**2.8 ± 0.6**^{a}	28 ± 8.1^{d}	**2.9 ± 0.2**^{a}	32 ± 5.7
	SBY-m/L	20	3.14 ± 0.27	10	1.0 ± 0.1^{c,d}	0.7 ± 0.2	4.5 ± 0.6	1.3 ± 0.2	4.0 ± 0.8	71 ± 9.9^{c,d}	3.5 ± 0.2	39 ± 4.5
	SBY-Ref	10	2.98 ± 0.39^{a}	5	0.5 ± 0.1^{a}	0.5 ± 0.1	2.4 ± 0.8^{a}	0.9 ± 0.2	**3.3 ± 0.7**^{a}	30 ± 4.0	**2.5 ± 0.2**^{a}	25 ± 4.1
	SBY-Ref/L	10	2.78 ± 0.40	5	0.6 ± 0.1	0.4 ± 0.2	3.0 ± 0.8	0.9 ± 0.2	3.9 ± 0.4	36 ± 4.3	3.1 ± 0.3	38 ± 7.0

[a] Statistical differences were found between control groups (before transplantation) and transplanted samples, $p<0.05$;
[b] Statistical differences were found between 2012 and 2013 samples in same location, $p<0.05$;
[c] Statistical differences were found between reference site (Ref) and other sites, $p<0.05$;
[d] Statistical differences were found between transplanted samples and local samples (L) in same site, $p<0.05$

Table 7b Metal and metallothionein levels (nmol mg^{-1} wet weight) in mussel samples of Marmara stations. Cr, Pb, Ni, Cu, Fe, Mn and Zn levels were expressed as mg kg^{-1} wet weight. Cd levels were expressed as µg $kg^{-}1$ wet weight. *n* is the number of mussel samples. Bold values show significantly higher values compared to before transplantation (Bt) values.

Year	Station	*n*	MT	*n*	Cd	Cr	Pb	Ni	Cu	Fe	Mn	Zn
2012	**Marmara-Bt**	10	0.86 ± 0.11	7	1.6 ± 0.2	0.6 ± 0.2	4.8 ± 1.2	1.4 ± 0.2	1.5 ± 0.3	22 ± 3.8	1.6 ± 0.2	25 ± 6.1
	M2A	10	1.11 ± 0.07[a]	7	1.6 ± 0.1[b]	1.1 ± 0.3[b]	5.6 ± 1.5	2.1 ± 0.5	**6.6 ± 0.3**[a,b,d]	39 ± 6.4	1.8 ± 0.1	**40 ± 4.6**[a]
	M2A/L	10	1.17 ± 0.13	7	1.3 ± 0.1[b]	1.0 ± 0.2	5.8 ± 1.2	2.1 ± 0.3[b]	21 ± 4.3[b]	30 ± 57	2.2 ± 0.2[b]	60 ± 7.4
	M2B	10	0.88 ± 0.05	5	1.3 ± 0.2[b]	0.6 ± 0.4	4.9 ± 1.1	0.7 ± 0.3[a]	**2.3 ± 0.2**[a,b]	16 ± 2.6	1.4 ± 0.1	18 ± 2.5[b]
	M2C	10	1.02 ± 0.12	7	1.9 ± 0.1[b]	0.8 ± 0.2	8.4 ± 3.5	1.7 ± 0.4	**6.8 ± 1.6**[a]	**42 ± 3.0**[a,b]	1.6 ± 0.1	27 ± 4.5
	S1	10	0.79 ± 0.11	7	1.6 ± 0.2[b]	1.0 ± 0.4	6.3 ± 1.7	1.3 ± 0.5	**12 ± 3.6**[a,c]	**48 ± 10**[a]	2.0 ± 0.3	36 ± 3.9
	S2	10	1.31 ± 0.10[c]	7	1.9 ± 0.3[b]	0.5 ± 0.2	**10 ± 0.9**[a]	4.3 ± 1.6[b]	**19 ± 4.2**[a,c]	**48 ± 8.5**[a]	1.8 ± 0.1	40 ± 6.6
	S-Ref	10	0.66 ± 0.09	7	1.9 ± 0.3[b,d]	1.0 ± 0.2[b]	7.8 ± 1.0	1.6 ± 0.4	1.6 ± 0.3[b]	27 ± 4.5[d]	2.1 ± 0.2[b]	32 ± 3.5[b]
	S-Ref/L	10	0.53 ± 0.05	7	1.3 ± 0.2[b,d]	0.7 ± 0.2	6.1 ± 1.1	4.5 ± 1.7[b]	1.4 ± 0.3[b]	72 ± 13[b,d]	2.1 ± 0.1[b]	39 ± 7.4
2013	**Marmara-Bt**	10	1.05 ± 0.24	5	1.7 ± 0.1	1.3 ± 0.2	5.3 ± 1.5	2.5 ± 0.5	1.7 ± 0.2	61 ± 21	1.6 ± 0.2	37 ± 3.9
	M2A	10	3.16 ± 0.35[a,b]	5	0.8 ± 0.1[a,b,d]	0.3 ± 0.2[a,b]	3.3 ± 1.0	0.6 ± 0.1[a,d]	**3.4 ± 0.3**[a,b,d]	20 ± 3.2[a,d]	1.7 ± 0.3	29 ± 2.9
	M2A/L	10	2.92 ± 0.45	5	0.6 ± 0.1[b,d]	0.4 ± 0.2	2.7 ± 0.6[b]	0.3 ± 0.1[b,d]	8.5 ± 1.8[b,d]	35 ± 5.8[d]	1.5 ± 0.1[b]	39 ± 9.7
	M2B	10	0.89 ± 0.08	5	0.8 ± 0.1[a,b]	0.6 ± 0.2[a]	1.9 ± 0.3	0.7 ± 0.2[a]	1.2 ± 0.2[b]	18 ± 2.8[a]	1.2 ± 0.1[a]	27 ± 2.2[b]
	M2C	10	1.13 ± 0.12	5	0.9 ± 0.1[a,b]	0.8 ± 0.2	2.4 ± 0.4[b]	0.7 ± 0.2[a]	**5.9 ± 0.8**[a]	18 ± 1.8[a,b]	1.4 ± 0.1	32 ± 7.4
	S1	10	3.02 ± 0.27[a,b,c]	5	0.8 ± 0.1[a,b]	0.9 ± 0.1[c]	3.1 ± 0.4	0.6 ± 0.3[a]	**12 ± 1.7**[a,c]	30 ± 5.3[c]	1.4 ± 0.1	40 ± 6.1[c]
	S2	10	2.24 ± 0.30[a,b]	5	0.8 ± 0.1[a,b]	0.7 ± 0.1[a,c]	3.2 ± 0.2[b]	0.6 ± 0.2[a,b]	**12 ± 2.5**[a,c]	35 ± 3.7[c]	1.5 ± 0.1	35 ± 4.9[c]
	S-Ref	10	1.99 ± 0.36[a,b]	5	0.6 ± 0.1[a,b]	0.2 ± 0.1[a,b]	2.5 ± 0.3[b]	0.7 ± 0.2[a]	0.7 ± 0.2[a,b]	15 ± 1.6[a,d]	1.4 ± 0.1[b]	18 ± 2.7[a,b]
	S-Ref/L	10	2.41 ± 0.39[b]	5	0.7 ± 0.1[b]	0.8 ± 0.4	2.9 ± 0.7	0.8 ± 0.2[b]	0.7 ± 0.1[b]	29 ± 2.7[b,d]	1.4 ± 0.1[b]	28 ± 6.1
	S3-Bt	10	1.91 ± 0.15	5	0.8 ± 0.1	0.4 ± 0.2	1.7 ± 0.6	1.0 ± 0.4	1.8 ± 1.1	22 ± 4.6	1.1 ± 0.1	20 ± 4.4
	S3	10	1.89 ± 0.28	5	0.6 ± 0.1	0.1 ± 0.1	2.5 ± 1.1	0.5 ± 0.2	4.0 ± 0.7	32 ± 5.1	1.2 ± 0.1	22 ± 4.0

[a] Statistical differences were found between control groups (before transplantation) and transplanted samples, $p<0.05$;
[b] Statistical differences were found between 2012 and 2013 samples in same location, $p<0.05$;
[c] Statistical differences were found between reference site (Ref) and other sites, $p<0.05$;
[d] Statistical differences were found between transplanted samples and local samples (L) in same site, $p<0.05$

Table 8 Pearson's correlation coefficients (PCC) and principal components analysis (PCA), performed with 9 variables for Mediterranean and Marmara sites separately.PC1, PC2, PC3 are the factors obtained after Varimax rotation.

Region		PCC[a]								PCA[b]		
		MT	**Cd**	**Cr**	**Pb**	**Ni**	**Cu**	**Fe**	**Mn Zn**	**PC1**	**PC2**	**PC3**
Mediterranean	**MT**									**0.712**	-0.103	0.423
	Cd	0.506								**0.884**	0.072	0.157
	Cr	**0.632**	**0.804**							**0.900**	0.298	0.112
	Pb	**0.625**	**0.880**	**0.844**						**0.973**	-0.060	-0.056
	Ni	**0.573**	**0.921**	**0.913**	**0.924**					**0.950**	0.217	0.031
	Cu	0.264	0.019	-0.139	0.133	-0.140				0.086	**-0.837**	0.153
	Fe	0.297	0.239	0.510	0.261	0.416	-0.423			0.340	**0.785**	-0.057
	Mn	-0.502	-0.304	-0.196	-0.167	-0.191	-0.289	0.282		-0.218	0.304	**-0.876**
	Zn	-0.362	-0.384	-0.420	**-0.647**	-0.518	-0.159	0.001	-0.154	**-0.665**	0.343	0.550
Marmara	**MT**									**-0.837**	0.238	
	Cd	**-0.702**								**0.922**	0.106	
	Cr	-0.414	**0.572**							**0.551**	0.362	
	Pb	**-0.527**	**0.903**	0.409						**0.837**	0.299	
	Ni	**-0.528**	**0.645**	0.296	**0.738**					**0.782**	0.293	
	Cu	0.081	0.215	0.206	0.422	0.272				0.047	**0.875**	
	Fe	-0.295	**0.456**	0.439	**0.526**	**0.750**	0.210			**0.609**	0.378	
	Mn	**-0.455**	**0.680**	**0.514**	**0.718**	**0.620**	0.407	**0.509**		**0.691**	0.522	
	Zn	-0.049	0.272	**0.555**	0.368	**0.448**	**0.742**	**0.473**	**0.678**	0.237	**0.925**	

[a] Each value represents the calculated value of Pearson's correlation coefficient (r) for two variables. Significant r values (p<0.05) are bold type.
[b] Bold values indicate the significant correlations between variables and factors (at p<0.05).

Response to Reviewer's Comments

Materials and methods section:

1) Should be noted was mussels depurated before analysis or not;

   Yes, the mussels were left in filtered sea water for a week for depuration before the transplantation. The water was renewed daily during the depuration period. This information is now added to the relevant section (2.2. Sampling and Deployment) in the text (Lines 102-103).

2) Analysis of the SRM (standard reference materials) like BCSS-1 for sediments and NIST for mussel tissues is recommended for such research.

   Merck Certipur ICP multi-element standart solution was used as reference material for the validation of the metal analyses. However, the reference material was not particularly for mussels or sediments.

Results and discussion section:

1) In the tables 1-6 the order of stations would be changed: M1A,...,SBY that is stations from Mediterranean area, and then M2,...S1, S2, S3, that is stations from Marmara area.

   The order of stations was changed as suggested (Tables 1-7).

2) Sentence on p.11, para 2, line 4 should be clarified.

   The sentence was changed as suggested (Lines 190-191).

3) Table 7 is very "heavy" and has to be modified, and may be presented as figure(s).

   We first tried to represent the data graphically. However, it was still too complicated. Therefore, the table was split into two separate tables as (a) and (b). The necessary change was indicated in the text and marked (Line 331).

4) The information on the mussels used for the transplantation should be added, and it could help to explain more clearly the observed changes of metals concentration in mussel tissues.

   The suggested additions were inserted for a more clear explanation about the changes of metal concentrations in mussel tissues (Lines 342-348; 351-354; 355-356).

5) The use of PCA need more detailed characteristic of the initial data.

6) PCA results are not reflected in the conclusions, and in the presented form look slightly excessive.

Related to comments on 5 and 6 :

PCA results were mentioned in the conclusions and details of the eigenvalues were removed from the text (Lines 380-382; 423-426).