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INTERSPECIFIC AND GEOGRAPHICAL VARIATIONS OF TRACE METAL CONCENTRATIONS IN CEPHALOPODS FROM TUNISIAN WATERS

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ABSTRACT: The concentrations of 6 metals (Ag, Cd, Cu, Hg, Pb, and Zn) were investigated and compared in three tissues (arms, digestive gland and mantle) of three cephalopod species from the Tunisian waters: the common octopus (*Octopus vulgaris*), the common cuttlefish (*Sepia officinalis*) and the European squid (*Loligo vulgaris*). Whatever the species or the sites, the digestive gland displayed the highest concentrations of Ag, Cd, Cu, Pb and Zn highlighting its major role in their bioaccumulation and detoxification. This is also true for Hg but only for the digestive gland of *O.vulgaris*. Muscle from the arms and the mantle contained thus relatively low trace metal concentrations except for Hg in *L.vulgaris* and *S.officinalis*. Geographic comparison of metal concentrations in Tunisian cephalopods from 3 locations indicates that higher concentrations of Ag, Pb and Hg were observed in cephalopods from northern and eastern coasts, whereas the highest Cd levels was detected in the southeastern reflecting different conditions of exposure. Comparing the trace element concentrations between species, Ag, Cd, Cu, Hg and Zn concentrations were the highest in the digestive gland of octopuses. This may be related to the differences in ecological features and swimming behaviour among different cephalopod species. Effects of length and sex on metal levels were also considered, indicating a limited influence of sex on metal concentration.

KEYWORDS: Octopus, Cuttlefish, Squid, Trace elements, Bioaccumulation, Mediterranean sea

INTRODUCTION

Metals in cephalopods have received increasing attention in last decades as these molluscs play a major role both as predator and prey in marine ecosystems (Boyle and Rodhouse 2005). As cephalopods bioaccumulate large quantities of various metals, they represent an important source of contaminants for their predators (e.g. Bustamante et al., 1998a, Dorneles et al., 2007). Cephalopods also represent an increasing resource for humans (Piatkowski et al., 2001) and consequently cephalopod catches raised dramatically these last decades (Boyle and Rodhouse, 2005; FAO, 2007).

Cephalopods are generally short-lived species, most of them reproducing between 1 to 2 years old and then die. Because of their short-life span and their capacity to bioaccumulate very high metal concentrations in their tissues, cephalopods were proposed as reliable biomonitoring species to follow the variation of pollutants in the marine environment over time (Seixas et al. 2005; Bustamante et al. 2006; Miramand et al. 2006; Pierce et al. 2008; Kojadinovic et al. 2011).

In the Mediterranean Sea, trace metal concentrations have been reported in different cephalopod species (i.e., *Sepia officinalis, Octopus vulgaris* and *Eledone cirrhosa*) collected from the Northern and Northeastern region (Renzoni et al., 1973; Miramand and Guary, 1980; Ayas and Ozogul, 2011). On the contrary, there is still limited information available on cephalopods from African coasts of the Mediterranean Sea (Stoepler et al., 1979). To the best of our knowledge, no data on metal concentrations in cephalopod species from Tunisia have been reported while the fisheries of these molluscs have a very high economic importance in this country.

In this context, this study was undertaken (a) to investigate the geographical variations of trace metals in the different tissues of the European squid *Loligo vulgaris*, the common octopus *Octopus vulgaris* and the common cuttlefish *Sepia officinalis* in three locations of the Tunisian coast and (b) to establish comparison between species in every sampling sites (c) to examine the influence of size and the sex on metal concentration in cephalopods. For this purpose, Ag, Cd, Cu, Hg, Pb and Zn were determined in the digestive gland and the muscles (both mantle and arms) of *Loligo vulgaris*, *O. vulgaris* and *S. officinalis*.

MATERIALS AND METHODS

Biological material and sample preparation

Seventy one individuals belonging to three cephalopod species, the european squid *Loligo vulgaris* (n=24), the common octopus *Octopus vulgaris* (n=20) and the common cuttlefish *Sepia officinalis* (n=27) (see Table 1 for details), were sampled in April 2010 in three locations, all situated in the authorized fishing area of the Tunisian coast (Fig. 1).

The first location, Bizerte, lies in the north of Tunisia and characterized with many industrial activities especially plastic and iron industries (El Ati-Hellal et al., 2005). The second site, Monastir, is located in the middle of east coast of Tunisia; the coastal area of this city can be characterized as unpolluted, given the non apparent sources of contamination (Hamza-Chaffai et al., 1997; Banni et al., 2007; Jebali et al., 2007). The last sampling site, Sfax, is located in the Gulf of Gabés (southeastern coast of Tunisia) and is considered as a major hub for Tunisian aquatic resources, contributing to approx. 65% of the national production (CGP, 1996). Important industrial activities, mainly crude phosphate treatments, chemical industries, tannery and plastic plants, are being developed along this region, possibly affecting the local marine ecosystems (Hamza-Chaffai et al., 1995); (Boujelben, 1998); (Banni et al., 2005).

Cephalopods were collected by nets or trawling by professional fishermen. From the moment organisms were caught, they were placed on ice for the time they were brought to the laboratory and then rapidly frozen at -25° C in individual plastic bags until dissections. Each individual was then defrosted, weighed (total body weight) and measured (dorsal mantle length -DML-). The determination of the sex and maturity stage were also realized. The maturity stage was evaluated by direct observation of the reproductive structures colors. The origin, number of individuals and biological details of individuals for each species of cephalopods are given in Table 1. Subsequently, samples of arm muscle, mantle muscle and digestive gland were removed for trace metal analysis.

Metal analysis

After dissection, tissue samples were frozen at -20°C, freeze-dried during 3 days and then finely ground in a porcelain mortar with a pestle. One aliquot of approximately 300 mg of each homogenized dry sample was digested with 5 ml of 65% ultrapure HNO_3 (Merck ®) at 80°C until the solution was clear. Then acid was evaporated and the residue was dissolved in 0.3N ultrapure nitric acid. Concentrations of Ag, Cd, Cu, Pb and Zn were determined by flame and graphite furnace atomic absorption spectrophotometry with a Hitachi Z5000 with Zeeman correction. The total Hg concentrations in the powder obtained from the tissues were determined by analyzing Hg directly with an Advanced Mercury Analyzer (ALTEC AMA 254) on aliquots ranging from approx. 10 mg of dry sample weighed to the nearest 0.01 mg (Bustamante et al. 2006a). Certified reference materials, Dogfish Liver DOLT-4 (NRCC), Lobster Hepatopancreas Tort-2 (NRCC) and Dogfish Muscle DORM-2 (NRCC), were treated and analysed in the same way as the samples. The results for standard reference materials were in good agreement with certified values with recoveries ranging from 91% for Ag to

98% for Hg (Table 2). The detection limits (µg g^{-1}dw) were 0.03 for Ag, 0.034 for Cd, 0.5 for Cu, 0.005 for Hg, 3.2 for Pb and 3 for Zn.

Statistical analysis

All data were tested for normality by Shapiro-test and homogeneity of variances by Bartlett's test. Since data did not respect the former assumptions of parametric analysis, non-parametric tests were applied. Kruskal-Wallis test (K-W test) followed by a multiple comparison test with Holm adjustment method was performed to detect differences between geographical areas, and between tissues in species. The level of significance for statistical analyses was always set at α=0.05.

Principal Component Analysis (PCA) was used to investigate the distribution of metal concentrations in the selected tissues within the species from the 3 locations (for details on PCA used, see Metian et al. (2013).

Generalized Additive Models (GAMs) were used to identify size-related, spatial and sex trends in explaining variability in metals concentrations (Zuur 2007). GAMs were fitted to average log-transformed on metal concentrations in digestive gland, arms, and mantle for each species and the dorsal mantle (DML) was considered as a continuous explanatory variable, while the sex and the location were treated as categorical explanatory variables in the model. The assumption of Gaussian error distributions was finally checked through the residuals of the model (homogeneity, normality, and no obvious pattern in residuals in general). All statistical analyses were performed using the free software R (R Development Core Team, 2010).

RESULTS

Metal concentrations and tissue distribution

Ag, Cd, Cu, Hg, Pb and Zn concentrations in the 3 selected tissues (digestive gland, arms, and mantle) of *L. vulgaris, O. vulgaris,* and *S. officinalis* are illustrated in Figure 2. Compared with the other tissues examined and regardless of the geographic location, the digestive gland contained the highest levels of Ag, Cd, Cu, Pb, and Zn (K-W test, $p < 0.001$) in all species. Concentration of Hg was also significantly higher (K-W, $p < 0.001$) in the digestive gland of *O. vulgaris* whereas its concentrations did not show significant differences among tissues of *L. vulgaris* and *S. officinalis* (K-W test, $p > 0.05$). In all species and with the exception of Hg, metal concentrations in the muscle were relatively low and were in the same range of concentration among the species.

Geographical variation

Metal concentrations in the tissues of cephalopods greatly differ both with species and sampling sites (Fig. 2). Hg concentrations were significantly higher (K-W, $p<0.05$) in *L. vulgaris* from Bizerte and Monastir compared to Sfax for all tissues. The same pattern was observed for Cd, Cu and Zn concentrations with significantly higher Cd and Zn concentrations in the arms and in the digestive gland in squids from Monastir (K-W, $p < 0.05$) and higher Cu concentrations in the mantle in squids from Bizerte (K-W, $p < 0.01$) compared to those from Sfax.

In *O. vulgaris*, specimens from Monastir and Bizerte showed also significantly higher Cu and Hg concentrations (K-W, $p < 0.05$) in the arms than those of Sfax. On the contrary, Cd concentrations in the digestive gland and the muscle tissues were higher (K-W, $p<0.05$) in Sfax than in the other sites. Similarly, the southeastern coast exhibited higher Zn concentrations (K-W, $p<0.05$) in the muscle tissues of the common octopus (Fig. 2).

Metal levels in the tissues of *S. officinalis* showed significant spatial variation as well. Concentrations were significantly higher in Bizerte and Monastir for Ag in the digestive gland

(K-W, $p<0.05$) and for Cu and Zn in both digestive gland and arms (K-W, $p<0.05$). In contrast, cuttlefish from Sfax showed higher concentrations for Cd in both digestive gland and mantle (K-W, $p<0.05$), and equal or higher for Hg according to the tissue (Fig. 2).

Interspecies comparison

Considering all sites together, Kruskal–Wallis one-way analysis of variance indicated that trace element concentrations between species are most of the time significantly different ($p<0.05$; comparison by tissues and trace elements). However, no difference was observed for Cd concentrations comparing mantles of the 3 species ($p=0.45$). Multiple comparisons were then performed and highlighted some specific differences between species. Considering the digestive gland for instance, *O. vulgaris* showed the highest concentrations of Ag, Cd and Hg among species (K-W, $p<0.05$); Cu and Zn concentrations were similar to *S. officinalis* and higher (K-W, $p<0.05$) than those of *L. vulgaris*. According to the multiple comparisons, Cu and Zn concentrations in the mantle were the highest in *O. vulgaris* compared to the other species (K-W, $p<0.05$) whereas Hg concentrations were significantly higher in squids than octopus (K-W, $p<0.05$). Finally, the common squid displayed the highest (K-W, $p<0.05$) levels for Cd and Hg in their arms compared to other investigated species whereas it presented the lowest Cu and Zn levels (K-W, $p<0.05$).

The Principal component analyses (PCA; integrating all the trace elements) were realized for each tissues (Figs. 3, 4, and 5). They showed a clear pattern of trace element concentrations for interspecies variation. Indeed, this analysis tore more or less species apart, especially for the digestive glands and arms (Figs. 3C and 4C). The first 2 principal components accounted for 74.44% of the variability in the metal concentrations in the digestive gland, with 52.54% on axis 1 and 21.90% on axis 2 (Fig. 3). Species were separated according to the axis 1 which clearly discriminated *O. vulgaris* and *L. vulgaris* relatively to the levels of Ag, Cd, Cu and Zn.

On the second axis, *O. vulgaris* and *L. vulgaris* were mainly distributed on the PCA according the relative levels of Hg (Fig. 3C).

In the arms, the PCA showed that the first two axes accounted 68.66% of variability, with 40.02% explained by axis 1 and 28.64% by axis 2 (Fig. 4). The first axis takes into account the relative levels of Cd and Zn, while the second axis is correlated with the Cu and Hg levels. Squids were clearly discriminated according to their high Hg and Cd levels whereas octopus and cuttlefish, thight on this PCA, were differentiated from squids according to their Cu and Zn levels (Fig. 4C).

Figure 5 shows the PCA realized for the mantle. Axis 1 and 2 of the PCA here accounted for 65.08% of the variability in metal levels in the mantle between individuals, with 36.69% on axis 1 and 28.38% on axis 2 (Fig. 5). Cu and Zn strongly influenced variability between species along axis 1 whereas Cd and Hg concentrations contributed to axis 2. Species were not clearly separated in comparison to the 2 previous PCA perfomed on digestive gland and arms (Fig. 5C vs. Figs. 3C and 4C). Nevertheless, *L. vulgaris* seems to be related to elevetated concentrations of Hg and Cd as in arms.

Although the aim of these PCAs was to illustrate the interspecies comparison, the regroupment of samples through sampling location (Figs. 3D, 4D and 5D) complement our first approach showing, when metals are integrated through 2 components, the superposition of Sfax and Monastir profile and therefore the different profile of Bizerte. For instance, Hg seems the main driver of Bizerte profile (Figs. 3D, 4D and 5D).

Effects of sex and size on metals accumulation

Results of Generalized additive models (GAMs) fitted to the average log-transformed metal concentrations in the three selected tissues of *L. vulgaris* did not show any significant differences for Ag, Cd and Cu concentrations considering sex and size. However, the GAMs established for Hg concentrations showed significant size effects, being negatively correlated with the body size (DML) in the digestive gland (P=0.015), mantle (P< 0.01) and arms (P=0.018) (Figs. 6A, 6B and 6C). In contrast, a strong positive linear effect of DML (P<0.01) on Hg concentrations was detected in the mantle (Fig. 6D).

In the case of *O. vulgaris*, the effect of size was detected for Cu, Hg and Pb concentrations: negative correlation was found between size and the digestive gland (P<0.01) and the mantle (P<0.001) for Hg (Figs. 7A and 7B), the digestive gland (P=0.049) for Pb (Fig. 7C) and in the arms (P=0.027) for Cu (Fig.7D). An effect of sex on which males showed significantly higher concentrations than females, was also found for Hg in the digestive gland (p=0.020), for Hg and Cu in the mantle (P=0.033 and P=0.023, respectively, and for Zn in the arms (P<0.01).

For *S. officinalis*, significant differences for size were included in the GAMs fitted for Hg and Cu concentrations. A linear positive correlation was detected between Hg levels and the body size in the digestive gland (P=0.021), the mantle (P<0.001) and the arms (P<0.01) (Figs. 8A, 8B and 8C) whereas Cu concentration decreased with size (P=0.011) (Fig. 8D). Considering the sex effect, males showed significantly lower Hg levels in the mantle (P=0.034) and the arms (P=0.035) than females.

DISCUSSION

Cephalopods are characterized by a strong ability to highly bioaccumulate trace elements in their tissues (Miramand and Bentley 1992; Bustamante et al., 1998b; Manso et al., 2007); the digestive gland appears to be the key organ, being deeply involved in the storage and/or detoxification of various essential and non essential metals of cephalopods (Bustamante et al., 2002a; Miramand et al., 2006; Raimundo et al., 2010). Since they are short-lived organisms, cephalopods are also interesting in reflecting the variation of metal concentrations in the environment where they live (Pierce et al., 2008). In the present study, the analysis of 6 metals (Ag, Cd, Cu, Hg, Pb and Zn) in edible tissues (muscles of both arms and mantle) and in the digestive gland of 3 major cephalopod species from Tunisia. It provides new dataset on an important seafood resources in the south Mediterranean Sea and it informs on the variation of contamination status along the entire tunisian coast.

Comparison between species

The analysis of metals in the three species (*L. vulgaris*, *O. vulgaris* and *S. officinalis*) with different lifestyle highlighted differences in their capacity of metal accumulation, each species consistently exhibiting different metal concentrations along the Tunisian coasts. For instance, the highest concentrations of Ag, Cd, Cu and Zn were found in octopuses and the lowest in squids from each locality. This result is in accordance with previous observations for the two same species in the Portuguese waters (Lourenço et al., 2009). Furthermore, Bustamante et al. 2002) showed that benthic cephalopods displayed significantly higher Cd, Cu and Zn concentrations than pelagic ones, which could be explained by differences in their feeding behavior and their physiology. On the contrary, *L. vulgaris* showed the highest levels of Hg for all tissues in the northern coast, in both arms and mantle in the eastern coast and only in the arms in the southeastern coast. Food represents the main accumulation pathway for Hg in cephalopods (Lacoue-Labarthe et al., 2009b) but benthic cephalopods exhibit more complex diets than pelagic ones. Indeed, they feed on polychets, crustaceans, molluscs, echinoderms and fish (McQuaid, 1994) whereas squids mainly feed on fish and other cephalopod species (Pierce et al., 1994). In contrast to benthic prey, pelagic fish and cephalopods contain higher proportion of organic Hg (Bloom 1992; Bustamante et al., 2006b) which is more bioavailable than inorganic Hg. Such a pelagic diet would lead to higher Hg exposure and therefore to higher Hg bioaccumulation in pelagic cephalopods since their prey contain higher organic Hg loads than those of benthic ones (Cossa et al., 1990).

Metal distribution among tissues

Highest concentrations of Ag, Cd, Cu, Pb and Zn were found in the digestive gland of all species similarly to the same species from other locations (Miramand and Guary, 1980; Miramand and Bentley, 1992; Bustamante et al., 2002a; Bustamante et al., 2002b ; Nessim et al., 2003; Raimundo et al., 2004; Napoleão et al., 2005; Raimundo et al., 2005; Bustamante et al., 2008; Pereira et al., 2009). The intrinsic capacity of this organ to store these elements is likely related to its major role in the assimilation of trace metals and nutrients (Martin and Flegal 1975; Miramand and Bentley 1992 ; Bustamante et al., 2002a; Bustamante et al., 2006b; Lacoue-Labarthe et al., 2009a). Cephalopods are known for their high feeding rates and their elevated assimilation efficiencies to satisfy their fast growth rates. As a consequence of strong assimilation of essential and non essential metals, cephalopods evolved efficient detoxification processes in order to cope with their toxicity (Bustamante et al., 2002a). In this context, Hg generally appears to be an exception in the present study. Hg levels in the digestive gland of *L. vulgaris* and *S. officinalis* were generally similar than those measured in the muscle (both from arms and mantle), this result being consistent with previous works on squids from the Northern atlantic (Pierce et al., 2008) and from the Indian and Pacific oceans (Kojadinovic et al., 2011). In addition, Lacoue-Labarthe and coauthors (Lacoue-Labarthe et al., 2009b) reported that juvenile cuttlefish exposed to inorganic Hg via seawater accumulated mainly Hg in muscular parts (>70%). However, the proportion of the whole body Hg content associated with the digestive gland increased during exposure and depuration phases, suggesting that the metal was detoxified from the muscles towards this organ.

Our results confirmed that Hg organotropism in cephalopods differs according to the phylogeny (Sepiida and Teuthida vs Octopoda). Indeed, the digestive gland of the common octopus *O. vulgaris* displayed the highest Hg concentrations among the three tissues, which contrasts with *L. vulgaris* and *S. officinalis*. It is also consistent with previous works on different octopus species along the European coasts (Raimundo et al., 2004; Seixas et al., 2005; Bustamante et al., 2006a) that reported elevated Hg concentrations in ocopus. The preferential concentration of Hg in digestive gland of *O. vulgaris* might be related to different Hg uptake pathway in *O. vulgaris* compared to the other species and most probably a higher contribution of food pathway to global upake or a higher retention of Hg from octopuses than the others (Bustamante et al. 2006).

Accumulation differences between sites

As mentioned previously, cephalopods are short-lived species and thus they are supposed to reflect short-term ambient concentrations of metals in the surrounding environment (Bustamante et al., 2008; Pierce et al., 2008). Due to the low metal concentrations found in the muscle tissues and their low variability of all species, the digestive gland was used to compare the Ag, Cu, Cd, Pb and Zn concentrations between sampling sites. Geographical variation of metal concentrations was not similar for all the species: concentrations of Ag and Pb found in cuttelfish and octopus were significatly higher in the northern (Bizerte) and eastern (Monastir) coasts than in the southeastern (Sfax). Interestingly, Ag and Pb concentrations did not vary in squids, which can be related the difference in diet but aslo the different habitats. Squids are neritic and/or oceanic cephalopods eaten mainly on fish whereas octopuses and cuttlefishes are more benthic cephalopods (closer to the coast) feeding most of the time on bottom invertebrates such crustaceans, bivalves and polychetes (Boyle, 1990; McQuaid, 1994). A previous study conducted along the Tunisian coast on algae showed that the highest concentration of Pb was observed in Bizerte; important industrial releases, the sewage wastes, harbors and the ferry traffic in Bizerte could be the source (El Ati-Hellal et al., 2005). A similar geographical trend was observerd for the Cu concentrations in cuttlefish and Zn concentrations in both squids and cuttlefish. This variation of Cu and Zn in the digestive gland is difficult to confront with the environmental data due to the lack of reliable measurements. However, the uniform concentrations of Zn in octopuses and Cu in squids and octopuses captured along the Tunisian coast may be explained by metabolic functions such as homeostatic mechanisms that regulate these levels in the organism (Canli et al., 2001).

Cd behavior was distincted from the other metals, especially in the case Cd concentrations in octopus and cuttlefishes from southeastern coast: they were higher than the Cd concentrations from the northern and eastern coasts. This could be related to the higher level of Cd in the coastal waters of Sfax subjected to the continuous discharge of metals from local industrial activities and from the phosphogypsum stock (Hamza-Chaffai et al., 1997). Several reports indicated the contaminated status of the coast, especially by Cd (Hamza-Chaffai et al., 2003; Smaoui-Damak et al., 2003; Banni et al., 2005; Banni et al., 2007). Thus, high Cd concentrations have been previously measured in other marine organisms collected in this area such as the clam *Ruditapes decussates* (Hamza-Chaffai et al., 2003; Smaoui-Damak et al., 2003) and the scorpionfish *Scorpaena porcus* (Hamza-Chaffai et al., 1995). In addition, the higher Cd concentrations in the muscle from arms and mantle in Sfax indicate that the sequestration capacity of the digestive gland was exceeded and resulted in a transfer of the metal toward muscle tissues. This Cd transfer from digestive glands to edible tissues and the impact of increased Cd contamination coul lead to a food safety problem of the human consumer in this highly Cd contaminated area.

Geographical variations were also observed for Hg concentrations in the digestive gland and the muscle. Clearly, specimens captured in the northern and the eastern coasts exhibited higher levels of Hg in all tissue than those from the south. A similar pattern has been shown in small pelagic fish in this areas (Joiris et al., 1999). Eastern sites may be subjected to potential natural inputs from nearby Hg ferous belts and previous mining areas (Joiris et al., 1999). Additionally, there is a high industrial activities along the Italian coast, Italy facing Tunisia.

Influence of biological parameters

The potential impact of length (dorsal mantle length; DML) and sex have been preliminary considered here, the “preliminary” character need to be emphasized given the limited number of samples to draw a conclusion but at least it gave us a hint of potential biological parameters in these cephalopods caught in North Africa. Considering all tissues in all species, metal concentrations did not show significant differences between males and females except for few cases. In *O. vulgaris*, males showed always significant higher values than females for Hg concentrations in the digestive gland and mantle, Cu concentrations in the mantle and Zn concentrations in the arms. On contrary, females presented the highest Hg concentrations in the muscle (mantle and arms) of *S. officinalis*. This is probably due to the existence of physiological and/ or ecological inter-sex differences (Monteiro et al., 1992) such as different behaviour (Jozet-Alves et al., 2008), sexual dimorphism for some species (Jackson et al., 2007) or maternal transfer/elimination of metals through eggs (showed in cuttlefish; Lacoue-Labarthe et al., 2008). A previous study on cephalopods provides conflicting results in terms of metal concentrations and sex: thus *O. vulgaris* from the East Mediterranean coast showed higher Cd and Cu concentrations in mantle tissue in female than male (Nessim et al., 2003). Additionally, (Ayas and Ozogul, 2011) described such influence of gender for Cd, Cu, Pb and Zn levels in the mantle of *S. officinalis* from the Turkish waters. However, other works on cephalopods (Miramand and Bentley, 1992;; Barghigiani et al., 2000; Kojadinovic et al., 2011) reported the lack of relation between heavy metals concentrations and sex.

Results from different correlations estimated between heavy metal concentrations in tissues of different species and size (viz. mantle length) showed significant negative relationships for Hg in all tissues of *L. vulgaris*, for Hg and Pb in the digestive gland, Hg in the mantle and Cu in the arms in *O. vulgaris* and for Cu in the mantle in *S. officinalis*. The negative relationships between heavy metal concntrations in the tissues and cephalopods sizes are consistent with previous studies: Cd concentrations decreased with length in the squid *L. forbesi* from the European waters (Pierce et al., 2008) as well as for *Octopus salutii* and *Eledone cirrhosa* from the Mediterranean Sea (Barghigiani et al., 1993; Storelli and Marcotrigiano, 1999). A plausible explanation for the negative correlations between these metals and cephalopods size could be attributed to the existence of a «dilution» effect linked to the rapid increase of tissue mass due to growth (that is very rapid in cephalopods) with regards to proportionally lower intake of trace elements (Pierce et al., 2008). On the contrary to the above results, Hg concentrations were positively correlated with size in all tissues of *S. officinalis*. This result is an agreement with observations described for the same metal in *Eledone cirrhosa* (Rossi etal. 1993), in loliginid squid (Bustamante et al. 2006; Pierce et al., 2008) and in cuttlefish (Chouvelon et al. 2011). The discrepancy of these observations resulted probably from the more prominent effect of different factors, such as food availability (i.e., quality and quantity of food) and growth rates (which may be affected by temperature) (Villanueva et al., 2002), on the metal accumulation in cephalopods.

CONCLUSION

The present work provides new information on bioaccumulation and tissue distribution of 6 heavy metals in three species (*O. vulgaris*, *L. vulgaris* and *S. officinalis*) from the Tunisian waters. Among the major information provided in the present work, which by the way represents a first investigation in North Africa, we confirmed the important role of digestive gland and the variation of Hg organotropisms among species. It also keep the debate ongoing concerning the effect of sex and lengths on metal concentrations in cephalopods. In addition, metal concentrations measured in the edible tissues indicated that for elevated consumption of flesh, toxic risk may be reached for Cd in all the species collected in contaminated areas and for Hg in squid and cuttlefish. Evaluation of this risk deserves further investigations.

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Table 1: Biological measurements obtained for the cephalopod samples. N: number of individuals; DML: Dorsal mantle length.

Species	**Site**	**N**	**Sex**	**Maturity stage**	**DML (mm)**		**Weight (g)**	
					Mean ± SD	**Range**	**Mean ± SD**	**Range**
L. vulgaris	**Bizerte**	4	4♀	III	196 ± 29	167 - 227	220 ± 81	145- 318
	Monastir	10	7♂ ; 3♀	III, II	188 ± 21	162 - 225	130 ± 23	97- 157
	Sfax	10	4♂ ; 6♀	III, II	213 ± 20	190 - 256	220 ± 36	184- 297
O. vulgaris	**Bizerte**	7	2♂ ; 5 ♀	III, II	116 ± 18	98 - 150	623 ± 348	343- 1267
	Monastir	5	3♂ ; 2♀	III, II	126 ± 20	112 - 160	746 ± 143	624- 983
	Sfax	8	2♂ ; 6♀	III, II	133 ± 16	113 - 155	851 ± 157	607- 1033
S. officinalis	**Bizerte**	7	1♂; 6♀	III	119 ± 16	97 - 140	208± 79	105- 328
	Monastir	10	8♂ ; 2♀	III	119 ± 11	103 - 135	186 ± 47	117 – 258
	Sfax	10	4♂ ; 6♀	III, II, I	99 ± 20	65 - 130	136 ± 66	41- 248

Table 2: Comparison of metal concentrations (µg.g^{-1} d.w) of certified reference materials from the NRCC determined in the present study with certified values.

Metal	**TORT-2 (n=6)**		**DOLT-4 (n=3)**		**DORM-2 (n=4)**	
	Certified values	Present study	Certified values	Present study	Certified values	Present study
Zn	180 ± 6	170 ± 12	-		25.3 ± 2.3	23. 5 ± 0.7
Cu	106 ± 10	98 ± 10	-	-	2.34 ± 0.16	2.29 ± 0.18
Cd	26.7 ± 0.6	25.62 ± 1.82	-	-	0.043 ± 0.008	0.042 ± 0.016
Pb	0.35 ± 0.13	0.43 ± 0.27	-	-	-	-
Ag	-	-	0.93 ± 0.07	0.85 ± 0.01	-	-
Hg	0.27 ± 0.06	0.26 ± 0.03	2.58 ± 0.22	2.54 ± 0.18	-	-

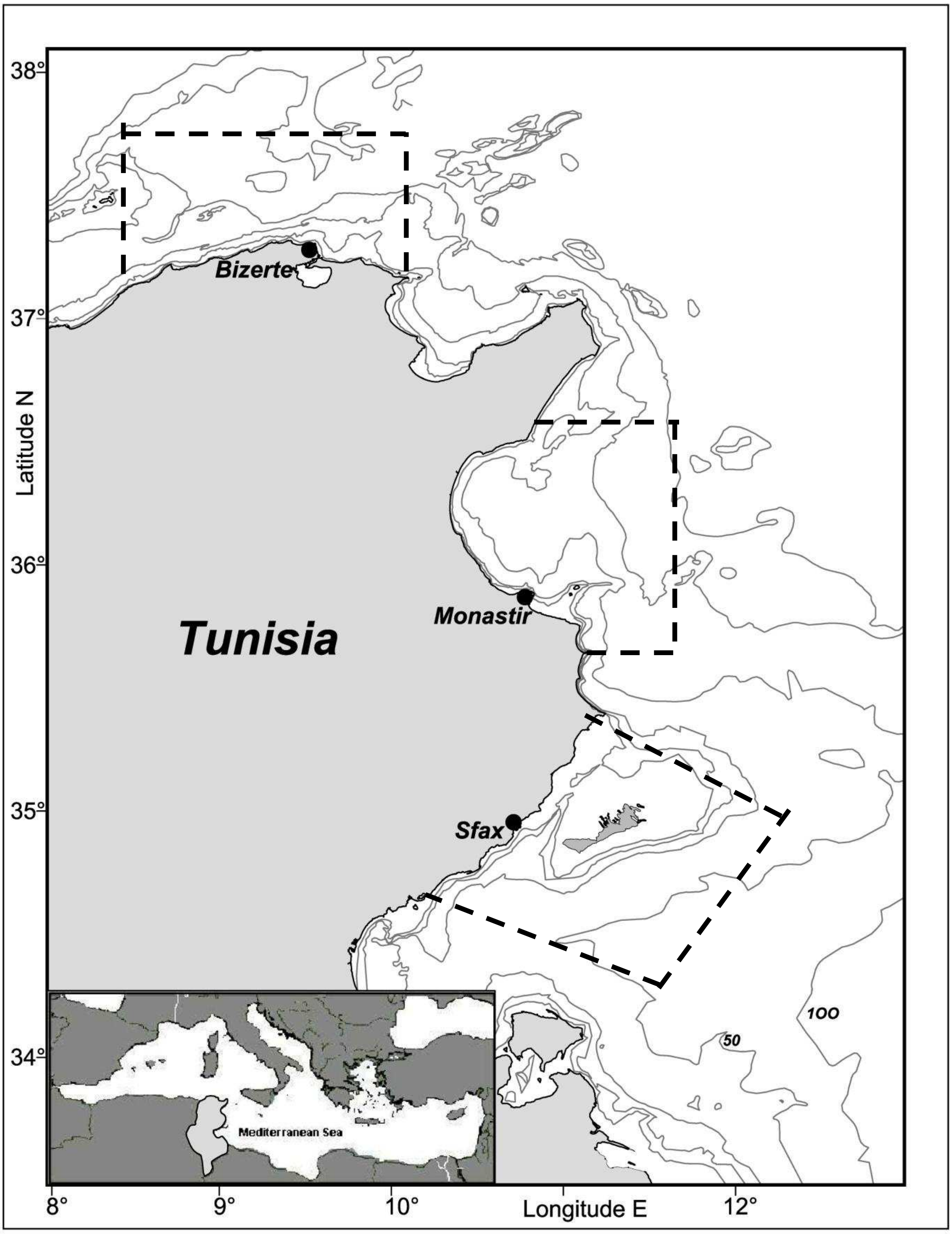

Figure 1: Sampling locations (Bizerte, Monastir and Sfax) along the Tunisian coast.

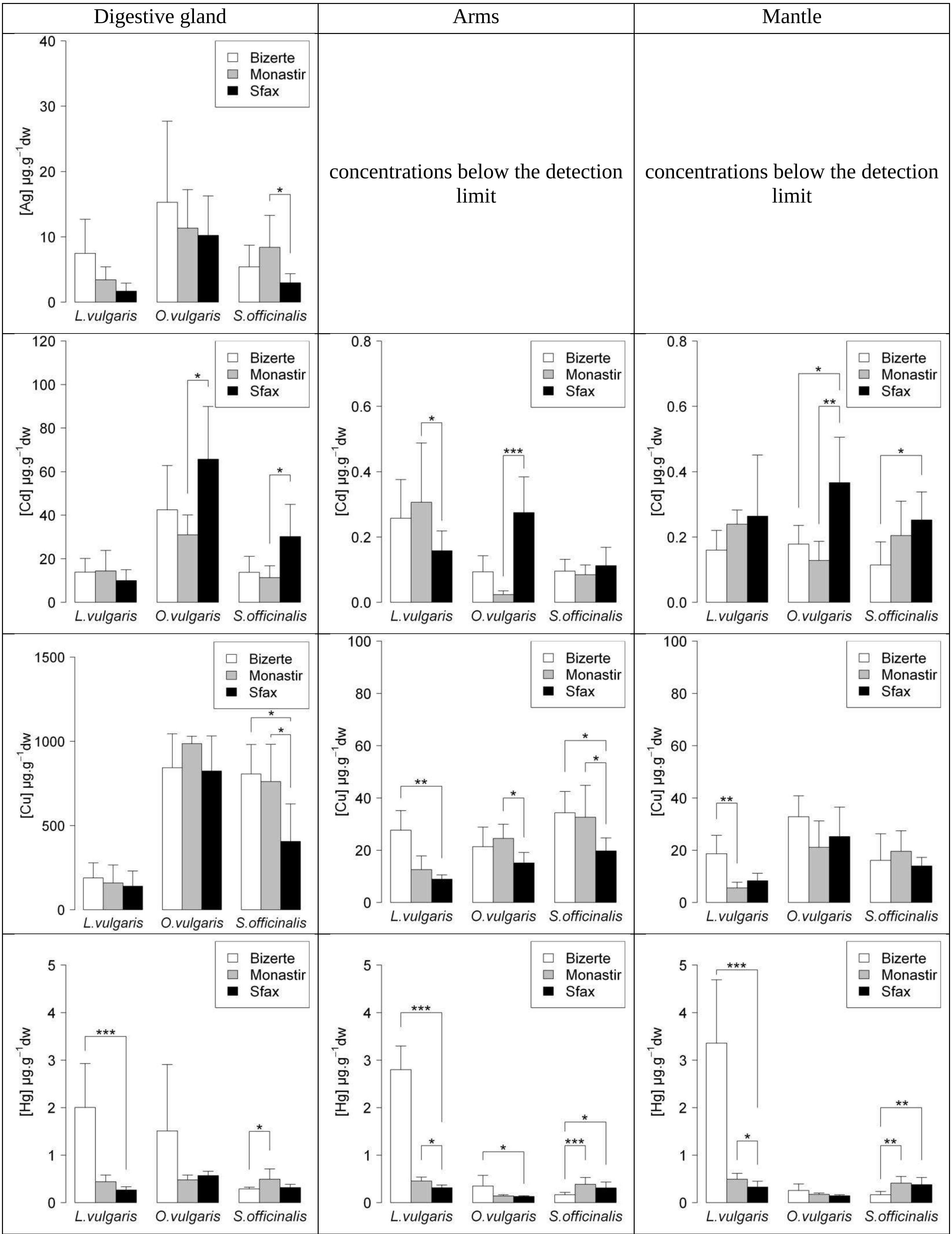

Figure 2: Concentrations of trace metals (µg.g^{-1} dw) in the digestive gland, arms and mantle (from left to right) of *L. vulgaris*, *O. vulgaris* and *S. officinalis* from Bizerte, Monastir and Sfax. To assess whether the differences between locations were significant Kruskal-Wallis test followed by a multiple comparison test with Holm adjustment method was performed. Significant differences: * for p<0.05; ** for p<0.01; *** for p<0.001.

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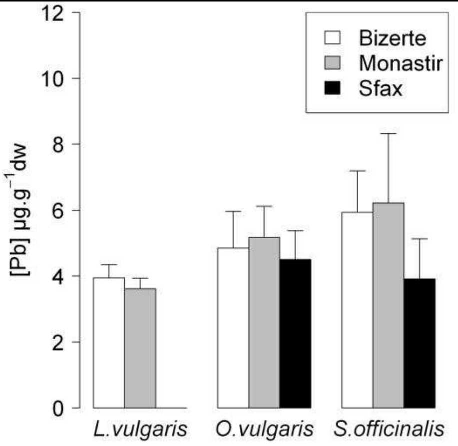
<tr><td>
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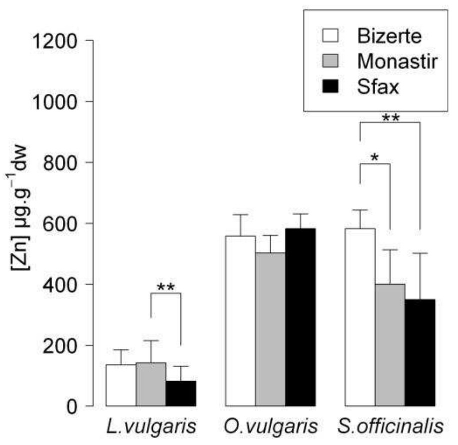
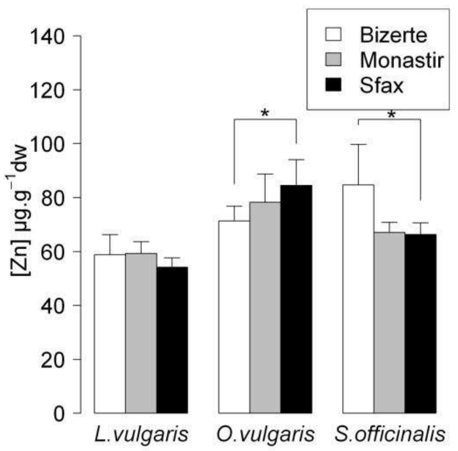
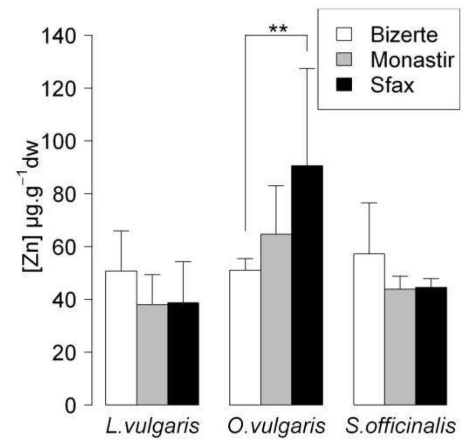
<tr><td>
</td><td>
</td><td>
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Figure 2 (continued)

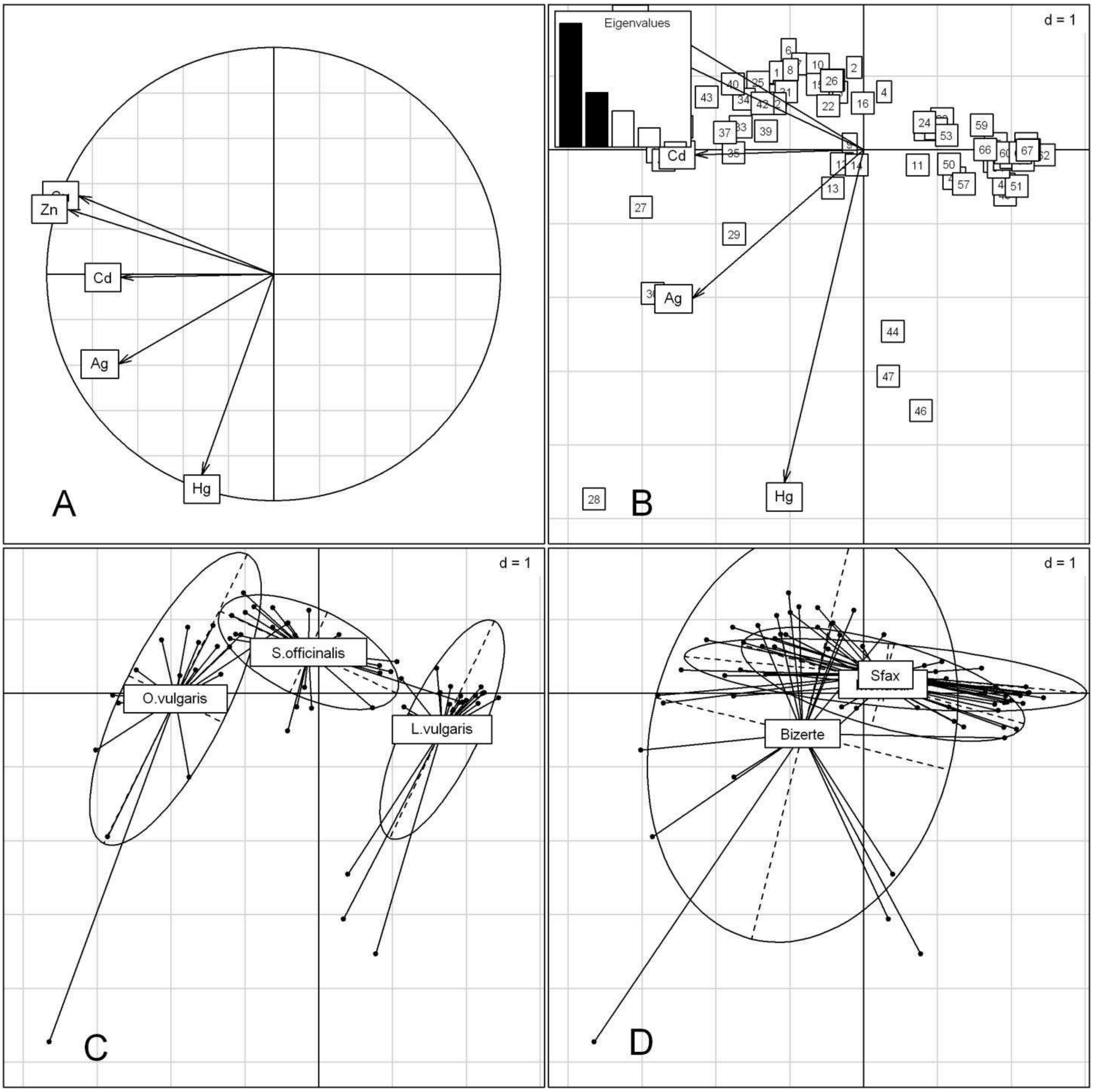

Figure 3: Principal component analysis (PCA) of cephalopods based on metal levels in the digestive gland. A) Correlation bi-plot showing the distribution of the variables. B) Projection of individuals on the correlation bi-plot with eigenvalue of the first two components. C) Grouping individuals by species. D) Grouping individuals by sampling sites.

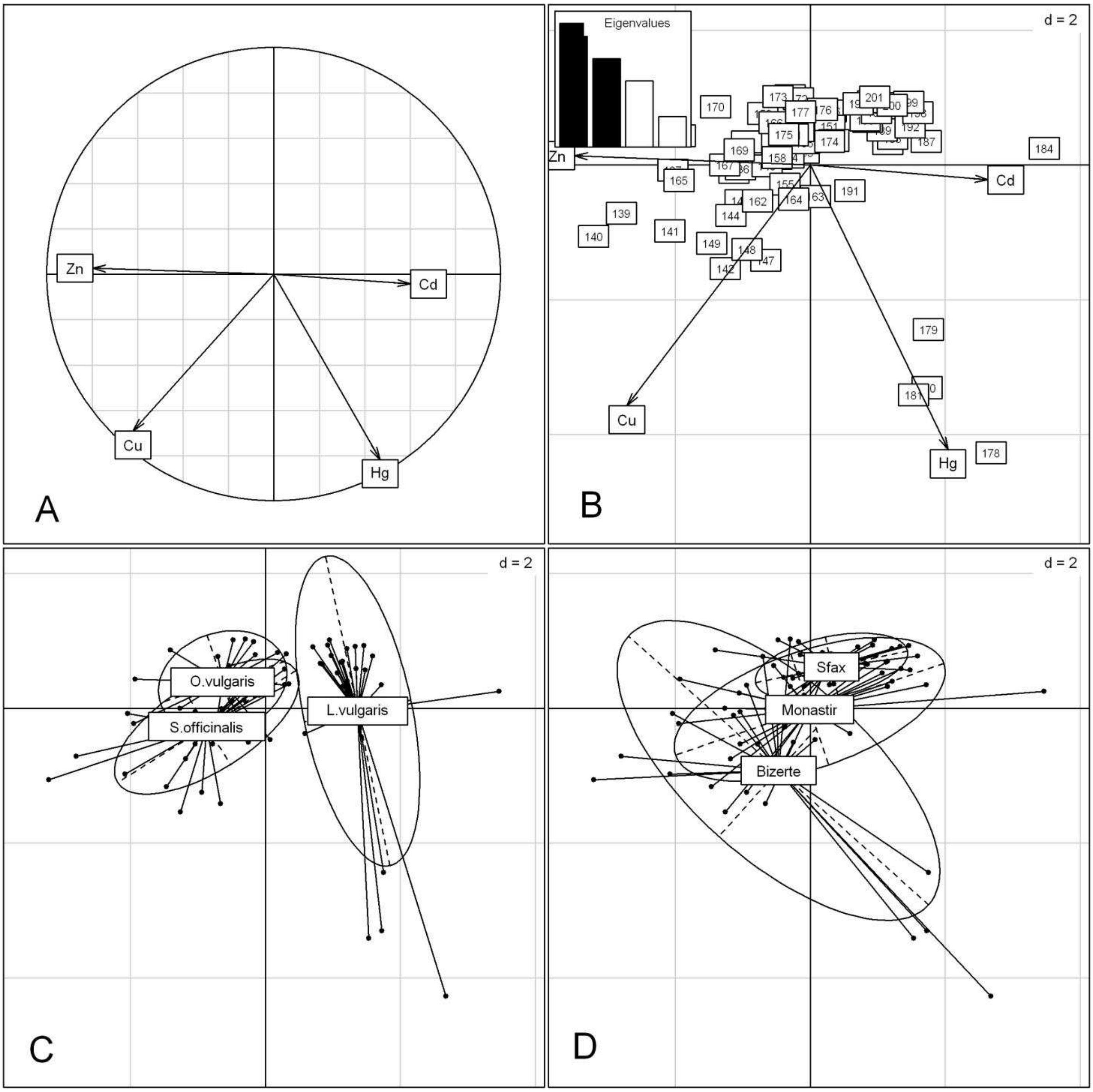

Figure 4: Principal component analysis (PCA) of cephalopods based on metal levels in the arms. A) Correlation bi-plot showing the distribution of the variables. B) Projection of individuals on the correlation bi-plot with eigenvalue of the first two components. C) Grouping individuals by species. D) Grouping individuals by sampling sites.

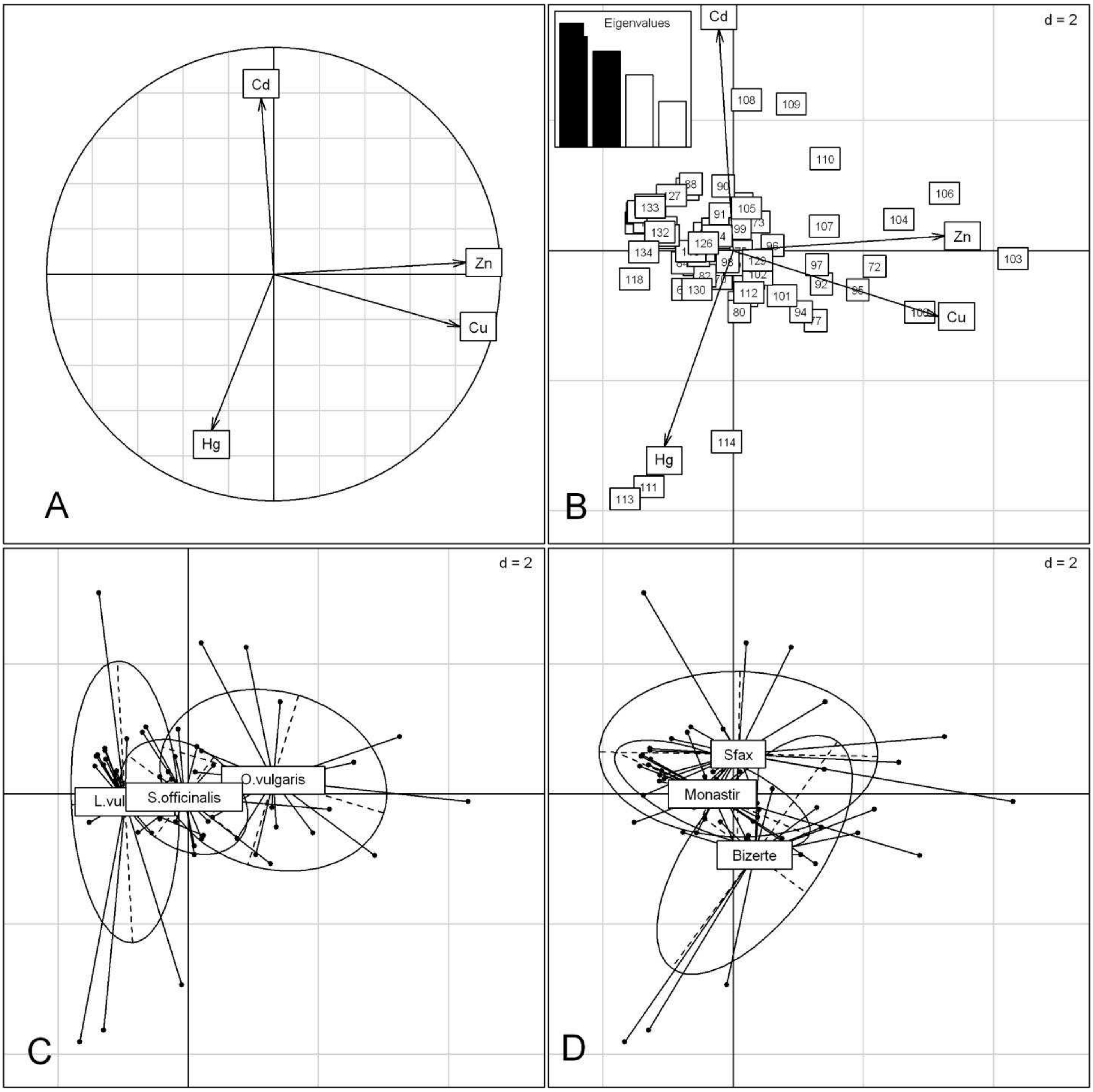

Figure 5: Principal component analysis (PCA) of cephalopods based on metal levels in the mantle. A) Correlation bi-plot showing the distribution of the variables. B) Projection of individuals on the correlation bi-plot with eigenvalue of the first two components. C) Grouping individuals by species. D) Grouping individuals by sampling sites.

Loligo vulgaris

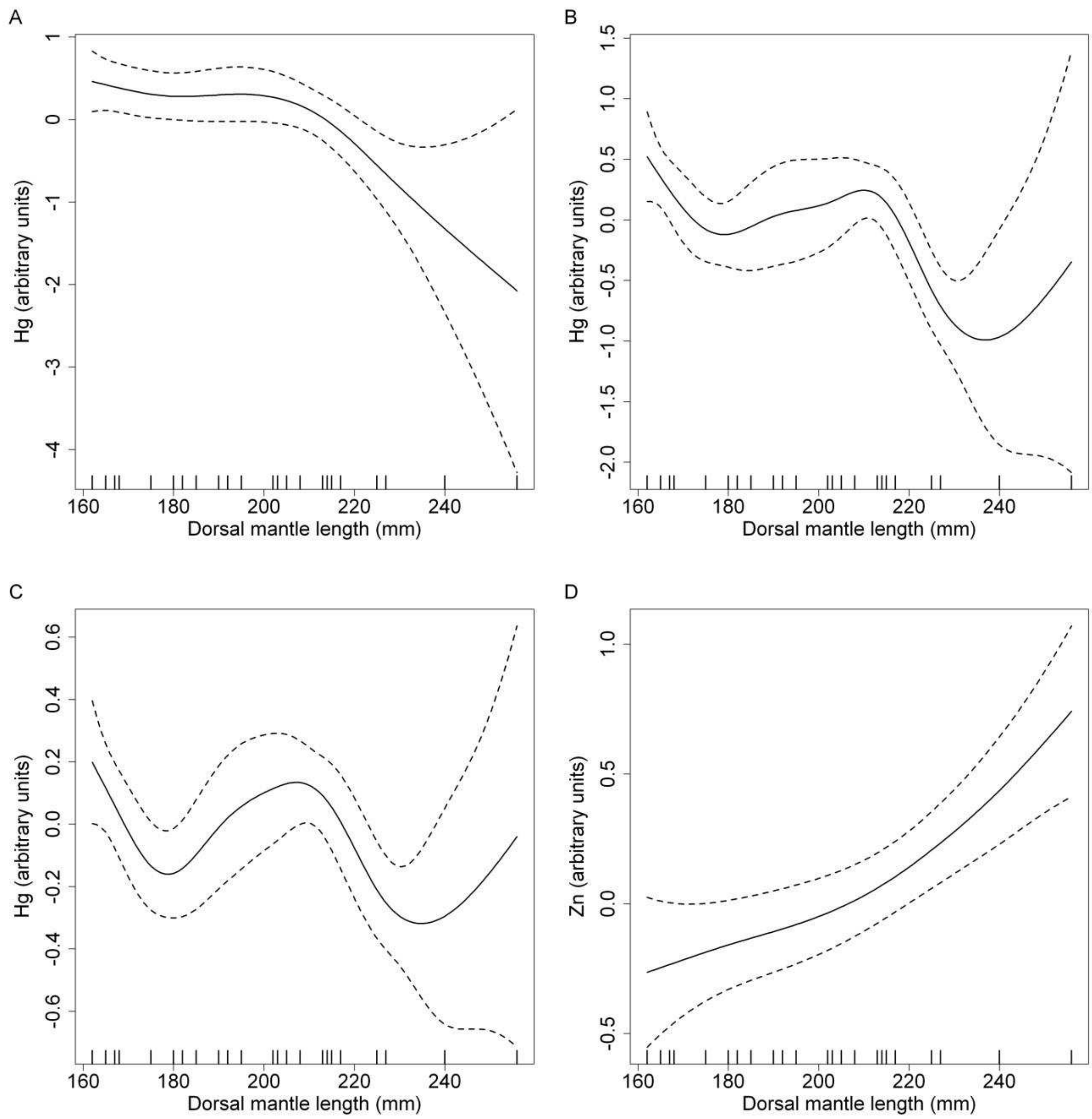

Figure 6: Smoothers for effects of dorsal mantle length on mercury (Hg) concentrations (µg.g^{-1} dw) in (A) the digestive gland, (B) the mantle and (C) the arms, and on zinc (Zn) concentrations (µg.g^{-1} dw) in (D) the mantle of *Loligo vulgaris*. The y-axis shows the contribution of the smoother to the predictor function (in arbitrary units). Smoothers illustrate the partial effect of DML, i.e. the effect of DML and once the effects of all other explanatory variables in the model have been taken into account. Dashed lines represent 95% confidence bands for the smoothers.

Octopus vulgaris

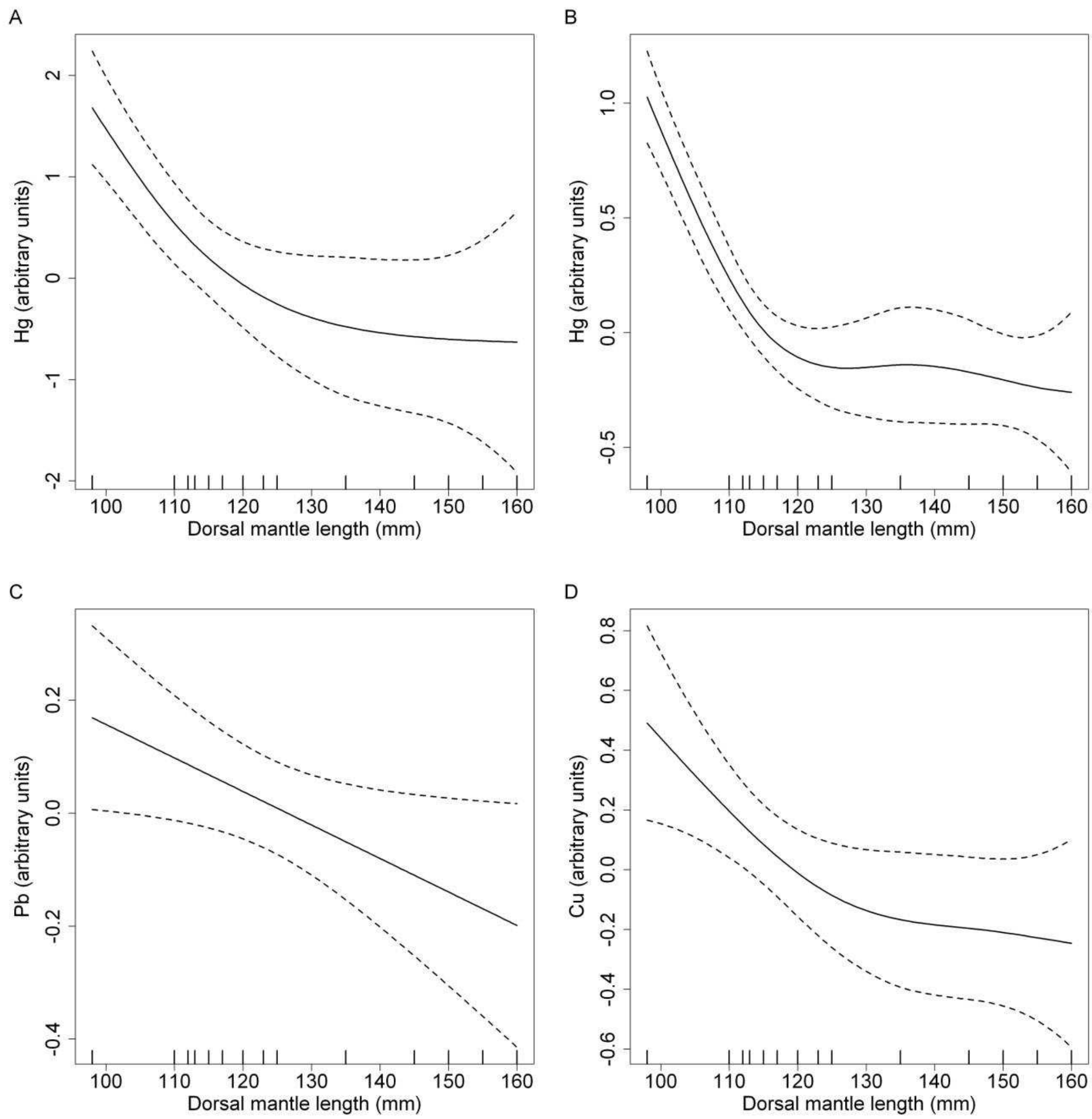

Figure 7: Smoothers for effects on mercury (Hg) concentrations (µg.g^{-1} dw) in (A) the digestive gland and in (B) the mantle, on lead (Pb) concentrations (µg.g^{-1} dw) in (C) the digestive gland and, on copper (Cu) concentrations (µg.g^{-1} dw) in (D) the arms of *Octopus vulgaris*. The y-axis shows the contribution of the smoother to the predictor function (in arbitrary units). Smoothers illustrate the partial effect of DML, i.e. the effect of DML once the effects of all other explanatory variables in the model have been taken into account. Dashed lines represent 95% confidence bands for the smoothers.

Sepia officinalis

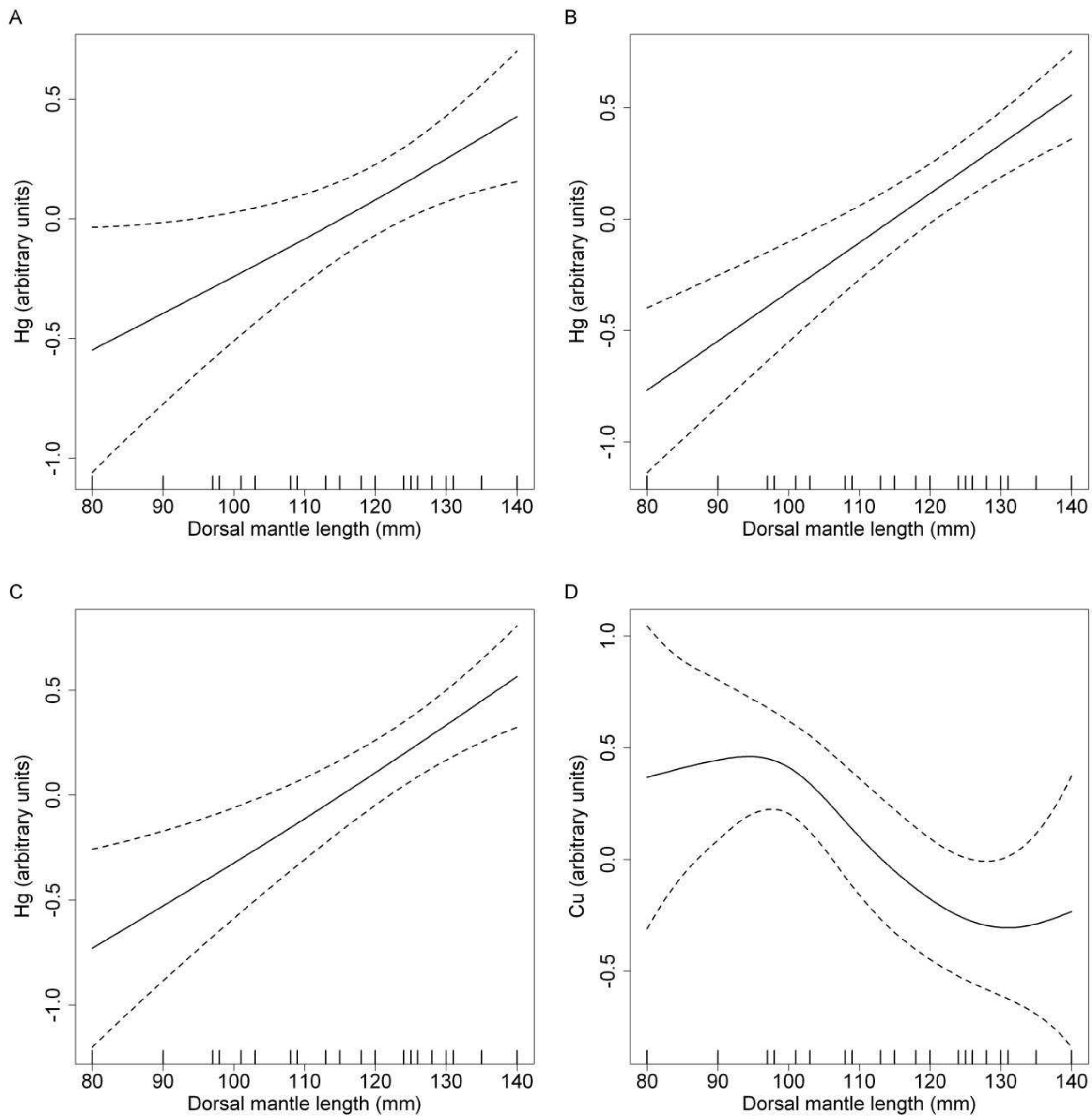

Figure 8: Smoothers for effects on mercury (Hg) concentrations (µg.g^{-1} dw) in (A) the digestive gland, (B) the mantle, and (C) the arms, and on copper (Cu) concentrations (µg.g^{-1} dw) in (D) the mantle of *Sepia officinalis*. The y-axis shows the contribution of the smoother to the predictor function (in arbitrary units). Smoothers illustrate the partial effect of DML, i.e. the effect of DML once the effects of all other explanatory variables in the model have been taken into account. Dashed lines represent 95% confidence bands for the smoothers.