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A baseline study of the metallothioneins content in digestive gland of the Norway lobster *Nephrops norvegicus* from Northern Adriatic Sea: Body size, season, gender and metal specific variability

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ABSTRACT

Metallothioneins content was investigated in digestive gland of two wild-caught Norway lobster *Nephrops norvegicus* populations from the Northern Adriatic Sea, in relation to body size, season and gender. Concomitant accumulation of cadmium, mercury, arsenic, lead, chromium and manganese, reactive oxygen species concentration and energy reserves in digestive gland were also assessed. While differences between genders were not recorded, metallothioneins content seasonal trends were affected by body size. Most of parameters displayed inconsistent trends across sampling sites. Significant correlation between metallothioneins content and cadmium, arsenic and mercury concentrations was recorded only for larger lobsters. A negative correlation of reactive oxygen species concentration and metallothioneins content was observed for small, but not large lobsters. Energy reserves, in particular lipids, could considerably influence biochemical and chemical parameters variations. The present results constitute the essential baseline for future studies aimed at evaluating the *N. norvegicus* health in relation to metal contamination of coastal sediments.

The Norway lobster *Nephrops norvegicus* (Linneaus, 1758) is among the most economically important crustacean species of the Mediterranean Sea and NE Atlantic. For the Adriatic Sea in particular, the overall population of *N. norvegicus* has been dramatically declining due to excessive exploitation of fishing grounds (Piccinetti et al., 2012). Besides, coastal zones may be subjected to intensive anthropogenic activities, such as increasing urbanization and industrialization. The resulting contamination with metals in particular represents a significant risk for aquatic biota, given their ubiquity, long-term persistence in the sediment and potential toxicity to benthic organisms. However, the assessment of contaminants based on chemical analyses in seawater and sediments is still predominant and little information is available regarding toxic effect and detoxification of metals for burrowing lifestyle crustaceans such as *N. norvegicus*.

Metallothioneins (MTs) represent a group of low molecular weight, cysteine-rich proteins essential for metal detoxification and homeostasis due to their ability of binding and sequestering several metals. Previous studies showed MTs responsiveness to elevated metal exposure in both invertebrates and vertebrates and prompted their application as biomarker of early metal stress in many marine organisms (Amiard et al., 2006). Even though the utility of MTs as biomarker of metal exposure was found to be uncertain or doubtful for some crustaceans (Legras et al., 2000; Ortega et al., 2017; Pedersen et al., 2014), evidence of increased expression and synthesis of MTs upon *in vivo* laboratory exposure to metals, primarily to cadmium, were reported for decapode crustaceans *Panularis argus*, *Charybdis japonica* and *N. norvegicus* (Canli et al., 1997; Moltó et al., 2005; Pan and Zhang, 2006). Essential metals such as Cu and Zn are also effective MTs inducers in crustaceans (Barka et al., 2001; Legras et al., 2000). Less information is available on MTs induction by other metals, but the increase of MTs content in crustaceans was recorded after laboratory exposure to mercury (Barka et al., 2001; 2007) and arsenic (Vellinger et al., 2013). Field surveys also revealed positive correlations of MTs content with As and Hg as well as with Pb, Cr and Mn (Faria et al., 2010; Lavadras et al., 2014; Martin-Díaz et al., 2009).

In addition to the metal-detoxifying role, crustaceans MTs are also involved in protection from reactive oxygen species (ROS) that can cause damage to cell macromolecules when produced in excessive quantities (Pan and Zhang, 2006; Moltó et al., 2007; Lobato et al., 2013; Felix-Portillo et al., 2014). Metals can be distributed into soluble cytosol, or prevail within insoluble fraction that include metal-rich granules, involved in the second important metal detoxification pathway in crustaceans (Barka, 2007; Legras et al., 2000; Mouneyrac et al., 2001; Nunez-Nogueira et al., 2010).

While controlled laboratory-based exposure studies provided substantial evidence on the direct link of toxic chemicals and biomarkers response, field studies of anthropogenic contamination effects still represent a considerable challenge for the scientific community due to limited knowledge on natural variability of biomarkers in sentinel marine organisms. Confounding factors such as intrinsic biotic and environmental parameters cause large amplitudes of biomarkers' response that could consequently be overestimated and incorrectly attributed to the toxic effect of contaminants. Thus, definition of natural biomarker variation range using data collected from reference sites has become a priority task of ecotoxicological studies as a foundation for realistic evaluation of contaminants effect in the field (Barrick et al., 2016; Davies and Vethaak, 2012 and references therein). Fluctuations of MTs content related to gender, season and reproductive status were already identified in the tissues of some decapode crustacean species (Chiodi Boudet et al., 2013; Giarratano et al., 2016; Lavadras et al., 2014; Maria et al., 2009; Mouneyrac et al., 2001). Consequently, in order to avoid possible misinterpretation of contaminants' effect in the natural habitat it is essential to establish the MTs baseline levels in relation to potentially confounding factors.

The aim of this study was to investigate the MTs content in digestive gland of *N. norvegicus* with respect to body size, season, gender and metals concentrations. In addition, ROS level and energy reserves were assessed as potential source of MTs content variability. Samples were obtained from two fishing areas of the Kvarner bay (Northern Adriatic Sea, Croatia). Since in general, the investigations of anthropogenic impact have been mostly concentrated along the coastal line, data concerning biological effect of potential contaminants in species from off-shore marine habitats is currently scarce and fragmented. The results of the present study will represent the necessary prerequisite for future studies oriented towards evaluation of the risk of metal contamination for benthic crustaceans.

Kvarner Bay is a semi - enclosed and relatively shallow coastal area on the North eastern part of Adriatic Sea (Croatia). Sampling site S1 wass located in the inner part of the bay, closer to the Rijeka harbour (~ 8 - 11 km) that is the major source of metals contamination. The second sampling site S2 was positioned approximately 70 km southern from Rijeka harbour and is more influenced by open sea circulation (Fig. 1). Data on the level of metals in the sediment and biota for sampling sites is currently unavailable. Although generally improving trends have been recently detected for coastal zones of the Kvarner Bay, elevated levels of metals were occasionally recorded in the sediments of various near shore locations, mostly within areas adjacent to urbanized areas and zones of intensive industrial activity, whereas sediments from sites furthest from the coast displayed substantially lower values (Cukrov et al., 2011, 2014). Sediment metal enrichment of various offshore areas of the Kvarner Bay corresponds to that of unpolluted sites within central and southern Adriatic far from any known anthropogenic sources (Ilijanić et al., 2014). Nevertheless, the possibility of accidental discharge cannot be ruled out since both sampling sites are intersected with important transport routes for cargo and touristic ships. In addition, the northern Adriatic is enriched with mercury predominantly originating from inland deposits and driven by sea currents towards the rest of the Adriatic Sea (Kotnik et al., 2015).

Specimens of *Nephrops norvegicus* were collected in autumn 2014 and spring 2015 by bottom trawl fishing gear. Trawling depths for sites S1 and S2 were at 55-62 and 70-78 m, respectively. Immediately following capture healthy and undamaged animals were selected and transported to the laboratory in thermally isolated containers. The gender of organisms was determined by checking the morphology of the first pair of abdominal swimmeret (pleopodes) that are thicker and rigid in males. The carapace length (CL; from eye socked to mid hind edge of carapace) was recorded for each lobster. For each site, season and gender the organisms were subsequently classified into two non-overlapping and relatively well-defined body-size group according to their CL (Table 1). Lobsters of CL below 36 mm were considered as "small" whereas the second body-size group, hereafter referred to as "large", comprised all lobsters samples of CL larger than 36 mm due to heterogeneity of CL values and difficulties in obtaining a balanced gender ratio. The total of 144 specimens of *N. norvegicus* were dissected to obtain digestive gland tissue samples that were immediately frozen in liquid nitrogen and stored at -80°C.

Portions of 0.5 g of freeze dried digestive gland tissue samples were digested using Anton Paar Multiwave 3000 microwave system (Perkin Elmer, USA) equipped with pressurized vessels, using 5 mL of 65% nitric acid per sample (HNO_3 Suprapur, Merck, Germany), over a 20 minutes operation cycle at 200 °C. Digested samples were transferred to 25 ml volumetric flasks and added with ultrapure water (Siemens). The concentrations of Cd, As, Pb, Cr and Mn were determined using the inductively coupled plasma mass spectrometer, ICP MS NexION 300X, equipped with S10 autosampler (Perkin Elmer, USA). Multielement solution (NexION Setup Solution, Perkin Elmer, USA) was used as tuning solution, covering a wide range of masses of elements. Multielement standard solution (Perkin Elmer, USA) was used to prepare calibration curves. The calibration curves with R^2>0,999 were accepted for concentration calculation. For each experiment, a run included blank, certified reference material (CRM) and samples which were analysed in triplicate to eliminate any batch-specific error. The method for measurement of Cd, As, Pb, Cr and Mn was validated using IAEA-407 reference material (fish tissue) (International Atomic Energy Agency, Austria). The mean recovery values for Cd, As, Pb, Cr and Mn were 97%, 89%, 105%, 110% and 108%, respectively. For Hg determination, approx. 0.1 g of freeze dried digestive gland tissue was weighed, transferred directly into analyser's vessel and analysed using the atomic absorption spectrometer AMA 254 (Advanced Mercury Analyser, Leco, USA). Single element standard solution for Hg was used for instrument calibration (LGC Standards, USA). The method for Hg measurement was validated using NIST 2976 reference material (mussels tissue) (National Institute of Standards and Technology, USA). The mean recovery for Hg was 104%. The concentrations of all metals were expressed as μg per g of tissue dry weight (d.w.). Data for Cu and Zn concentrations were provided by Glad (personal communication).

Measurement of MTs content in digestive gland was performed in accordance to the method of Viarengo et al. (1997). MTs content was determined in a partially purified low molecular weight metalloproteins fraction following acidic ethanol/chloroform extraction of the homogenate, by spectrophotometric measurement at 412 nm. For the calculation of MTs content, serial dilutions of reduced glutathione (GSH) were used as reference standard, assuming the content of 18 Cys residues (Zhu et al., 1994). MTs concentration was expressed as nmoles of GSH per g of wet tissue weight (w.w.).

The OxiSelectTM *In Vitro* ROS/RNS Assay Kit (Cell Biolabs, inc. USA) was used for the measurement of total free radicals in the samples, according to the manufacturer instructions. The method is based on the measurement of 2', 7'- dichlorodihydrofluorescein (DCF) fluorescence that results from oxidation of highly reactive dichlorodihydrofluorescein (DCFH). The intensity of DCF fluorescence that is proportional to total free radicals level in the sample was measured using the fluorescence plate reader Fluoroscan Ascent^{TM} (Labsystem, Finland) at 480 nm excitation/530 nm emission. For calculation of the free radical content, serial dilutions of H_2O_2 were used, and the concentration of ROS/RNS was expressed as mmol/mg of tissue wet weight (w.w.).

Quantitative determination of total lipids in digestive gland tissue was performed by sulfo-phospho-vanillin colorimetric method (Glad, 2017). Briefly, lipids were extracted by vigorous mixing of freeze dried digestive gland tissue portions (10-20 mg) with chloroform/methanol 2:1 (V/V) solution, followed by incubation at +4ºC for 10 min and centrifugation at 4000 rpm for 10 min. Aliquots of each sample were evaporated to dryness under nitrogen stream in glass tubes, vigorously mixed with concentrated sulphuric acid and heated at 100ºC for 10 minutes. The total lipid content was determined in cooled samples using phospho-vanillin reagent and serial dilutions of cholesterol standards. The absorbance values of blank and samples were recorded at 490 nm and the concentration was expressed as % of lipids per g of tissue dry weight (d.w.). For determination of proteins in digestive gland tissue, portions of freeze dried tissue (10-20 mg) were mixed with 10% sodium hydroxide and incubated for 1 hour at 100ºC to digest proteins. Samples were then subjected to Bradford assay (Bradford, 1976) with bovine serum albumin as protein standard. The absorbance values of blank and samples were recorded at 595 nm. Concentration was expressed as % of proteins per g of tissue dry weight (d.w.).

Data are graphically presented as box and whisker plots (small, N=6; large, N=12). Since the Shapiro-Wilk and Levene's tests revealed that requirements of normality and homogeneity of variance were not met, the non-parametric Kruskal Wallis and Mann-Whitney's U-test were used for statistical analyses. The relationships between MTs content and metal concentrations, ROS and energy reserves level were determined using Spearman's rank correlation analysis. Differences were considered significant at $p<0.05$. Data for MTs content of all samples was used for frequency distribution histogram. Principal component analysis (PCA) was performed in order to investigate the overall data pattern. The background level was calculated as the mean value of MTs content of all 144 samples in total. All the analyses were performed using RStudio, version 0.98.1028 (RStudio Team, 2015).

For each body size group of *N. norvegicus*, data on season and gender specific MTs content, metals and ROS concentrations and energy reserves level in digestive gland tissue were presented separately for sites S1 and S2.

Content of MTs in digestive gland of small and large *Nephrops norvegicus* ranged from 1.1 to 44.4 and from 7.2 to 39.8 nmol/ g w.w., for sites S1 and S2, respectively (Fig. 2). Values for MTs content varied significantly between seasons at site S1, with a contrasting trend displayed between body size groups. At site S2, MTs content was significantly higher in spring only for small male lobsters. Differences between small and large lobsters were also found at site S1 (Table 2). Significant differences in MTs content were observed between sites, being higher at site S1 in autumn for small males of both genders and in spring for large females. The opposite pattern was observed at site S2 (Table 3). Concentration of Cd ranged from 3 to 28.1 µg/g d.w. and from 4.1 to 37.9 µg/g d.w. for lobsters from sites S1 and S2, respectively (Fig. 3). At site S1, Cd values displayed marked seasonality for both body size classes, being significantly higher predominantly in spring. Gender dependent differences were not recorded. At site S2, no seasonality could be observed. Concentrations of Cd did not show differences with respect to body size (Table 2). Values recorded at S2 were occasionally significantly higher than at site S1, predominantly in autumn (Table 3). Concentration of As ranged from 33.6 to 594.4 µg/g d.w. and from 61.1 to 1254.1 µg/g d.w., at sites S1 and S2, respectively (Fig. 3). Gender and season dependent As accumulation was detected for both body size groups and sites, mainly being significantly higher in spring and in males. Significantly higher As levels were found in smaller males, except in spring at site S2 (Table 2). Concentration of As was almost regularly significantly higher at site S2 (Table 3). Concentration of Hg ranged from 1 to 5.3 µg/g d.w. and from 0.9 to 5.6 µg/g d.w., at sites S1 and S2, respectively (Fig. 3). Significantly higher Hg concentration was recorded almost regularly in spring, predominantly in males. Differences related to body size, although sometimes significant, did not express a clear trend (Table 2). Concentrations of Hg were often significantly higher at site S1, particularly in spring (Table 3). Concentrations of Pb ranged from 0.04 to 3.6 µg/g d.w. and from 0.06 to 1.6 µg/g d.w. at sites S1 and S2, respectively (Fig. 3). At site S1, Pb levels displayed similar trends, but significantly higher concentrations were recorded predominantly in male lobsters and in spring. Opposite seasonal patterns with higher autumn values were observed at site S2 for both body size categories of females only. Concentration of Pb was significantly higher in small lobsters of both genders at site S1 in spring, and at site S2 in females in autumn (Table 2). Differences of Pb accumulation between sites were season-specific, that is, significantly higher at S1 and S2 in spring and autumn, respectively (Table 3). Concentration of Cr ranged from 1.1 to 8 μg/g d.w. and from 1.1 to 5.45 μg/g d.w. at sites S1 and S2, respectively (Fig. 3). The season related pattern was more consistent at site S2, where significantly higher Cr concentrations were recorded in autumn. There were no differences between genders. Significant differences were detected between body size groups only for males at site S1, displaying higher Cr concentrations in large and small lobsters in autumn and spring, respectively (Table 2). Concentration of Cr was significantly higher at S1 only in spring (Table 3).The range of Mn concentrations for lobsters from sites S1 and S2 was between 7 to 33.7 μg/g d.w. and from 7.6 to 42.2 μg/g d.w., respectively (Fig. 3). Seasonal differences were recorded only for females of both body size groups, displaying significantly higher levels in spring at site S1, and in autumn at site S2 (Table 2). Values for Mg concentration were significantly higher in small lobsters, but almost exclusively at site S1 (Table 2). Accumulation of Mn was significantly higher at site S2 for females only (Table 3).

Values for ROS varied between 0.8 and 16.9 mmol/ mg w.w. and 1.4 and 12.4 mmol/ mg w.w. at sites S1 and S2, respectively (Fig. 4). The pattern of ROS level was inconsistent, but gender and season dependent significant differences were observed at both sites. Significantly higher concentration of ROS in small lobsters was occasionally found (Table 2). Values for ROS were regularly significantly different between sites, being higher predominantly at S2, while the opposite trend was detected only for males in spring (Table 3).

The values for lipid content varied between 20 and 50 % d.w. and between 24 and 37 % d.w., at sites S1 and S2, respectively. Lipid content was elevated in autumn (Table 4). A gender dependent lipid content could be discerned only at site S1, being higher in female lobsters from both body size groups. Total protein content at sites S1 and S2 ranged from 18 to 31 % d.w. and 18 and 35 % d.w., respectively. No consistent pattern could be detected at either of the two sites.

A significant negative correlation was detected between MTs and metals (r_s= Mn, -0.4; Cd, -0.46, Cu, -0.46; Zn, -0.41, $p<0.05$) for small lobsters (Table 5). Large organisms displayed significant ($p<0.05$) positive correlation with Cd (r_s=0.25), Hg (r_s=0.31) and As (r_s=0.37). Content of MTs was negatively correlated to ROS (r_s=-0.45, $p<0.05$) in digestive gland of small lobsters only. Large, but not small lobsters, displayed significant negative correlation with lipids (r_s=-0.31, $p<0.05$). Significant correlations ($p<0.05$) were recorded between metal concentrations and lipids, namely for Hg, Pb, Mn and Cr (r_s=-0.38 to -0.67) and for Cd, As, Pb Hg, Mn and Cu (r_s= -0.3 to -0.56) in digestive gland of small and large lobsters, respectively.

A principal component analysis (PCA) was performed using data on MTs content, ROS level, metals accumulation and energy reserves, obtained from all individuals sampled of both body size groups, seasons, genders and sites. The first two principal components PC1 and PC2 accounted for 59.2% and 51.6% for small and large lobsters, respectively (Fig. 5, upper and lower panel). Generally large variation was observed for samples spread on both PCA ordination plots. The contribution of the variables in each of the first two PCs varied between two body size groups. With exception of site S1 in autumn, small lobsters were mostly grouped to the left part of the PC1, with contribution of Cd, As, Cu, Zn concentrations and ROS level that were negatively correlated to PC1, while MTs content was positively correlated to PC1. Small males and females from site S1 in autumn were separated along PC2 as a result of the differences in the accumulation of Mn, Cr, Pb and lipids content (negatively correlated to PC2). Large males and females from site S1 sampled in autumn were distributed at the left side of PC1 and had generally lower levels of metals and MTs content and higher lipids content. Large lobsters from site S2 were roughly separated along PC2, with spring samples being located in its positive side due to high accumulation of Hg and As, and autumn sample in the negative side due to association with higher ROS level and Cr and Zn concentrations.

Figure 6. represents the frequency distribution histogram for MTs content of all 144 samples in total. The preliminary threshold value was defined as mean + 1σ and expressed the value of 25.2 nmol/ g w.w.

Due to natural variability related to intrinsic and abiotic factors, the linkage of MTs to metal exposure is generally difficult to establish during field studies, even for samples from metal-polluted environments (Legras et al., 2000; Mouneyrac et al., 2001). Accordingly, in the present study, seasonal patterns of MTs content were generally inconsistent and differed between smaller and larger lobsters at both sites, while gender related differences were less pronounced. The seasonality of MTs digestive gland content was previously reported by Giarratano et al. (2016), for crabs that displayed higher MTs content in autumn than in spring. Besides, Chiodi Boudet et al. (2013) reported different patterns of seasonal variations for MTs content of white shrimp *Palaemonetes argentines* from polluted and unpolluted marine site. Individual metal concentrations and ROS in the digestive gland of *N. norvegicus* from both sampling sites generally displayed relatively large fluctuations between body size groups and with respect to seasons and genders. Furthermore, spatial variations were also found, suggesting different bioavailability of metals at two sites and possibly an influence of local environmental conditions.

Seasonal variations and gender dependent differences of Cd and other metals that exert high MTs binding affinity, were already reported for *N. norvegicus* digestive gland (Canli and Furness, 1993a). Nevertheless, MTs content for larger body size lobsters displayed weak positive correlation (r_s=0.25) to Cd that, according to previous reports, predominantly accumulates in digestive gland tissue of *N. norvegicus* (Canli and Furness, 1993b). The notion of elevated MTs content in response to Cd could be supported by laboratory studies demonstrating Cd as an effective inducer of MTs synthesis in *N. norvegicus* (Canli et al., 1997) and other crustaceans (Moltó et al., 2005; Pan and Zhang, 2006). However, considering the fairly weak correlation, it is difficult to speculate to what extent the level of Cd in *N. norvegicus* digestive gland might be related to MTs content fluctuations in this particular case. According to recent data on sediment metal concentrations (Cukrov et al., 2011, 2014; Ilijanić et al., 2014), there are no indications of particular sediment Cd enrichment at sampling sites in comparison to other offshore areas of unpolluted Adriatic and Mediterranean regions. Besides, concentrations of Cd for digestive gland were in line with data previously reported for *N. norvegicus* (Canli and Furness, 1993a) and lobsters *H. gammarus* and *H. americanus* (Barrento et al., 2009; Leblanc and Prince, 2012) from unpolluted sites of Atlantic coast. Similarly, low enrichment of off-shore sediments and generally decreasing contamination trend for Cu and Zn in Kvarner Bay (Cukrov et al., 2011, 2014) could explain the lack of correlation between these metals and MTs content in *N. norvegicus*. The essential metals like Cu and Zn also possess high MTs binding ability and MTs induction potential when accumulated in excess (Amiard et al., 2006), but it seems that a pool of MTs that bind these metals could be considered as storage and donor for metalloproteins, such as for apohemocyanin and carboanhydrase (Henry et al., 2012).

The experimental evidences on MTs-inductive potential of other toxic metals such as Hg and As are generally scarce for crustaceans (Barka et al., 2001; Barka, 2007; Vellinger et al., 2013), but when these organisms were used for studying the adverse effect of anthropogenic contaminants in the field revealed correlations of both metals with MTs content (Faria et al., 2010, Martín-Díaz et al., 2009). A mild positive correlation of MTs was detected in the present study for Hg (r_s=0.31) and As (r_s=0.37) but again only for larger lobsters, indicating that for this body size class the level of Hg and As accumulated in digestive gland tissue could be sufficient to surpass the necessary threshold for MTs induction. This is consistent with the essential physiological role of MTs in detoxification and storage of metals in the form of insoluble MTs complex. Noteworthy, values for Hg concentrations exceeded those previously reported for digestive gland of *N. norvegicus* (Canli and Furness, 1993a) and other crustaceans from the Atlantic (Barrento et al., 2009) and the Pacific (Frías-Espericueta et al., 2016). Moreover, data reported herein are in agreement with consistently high Hg concentrations in the soft tissues of *N. norvegicus* and other organisms from Mediterranean waters that, as widely emphasised before, could be related to the large cinnabar deposits in the Mediterranean basin (up to 55% of total world reserves) and slow turnover of Mediterranean waters through the Gibraltar strait (Perugini et al., 2009; Renzoni et al., 1998). Besides, Hg enrichment of sediments and biota in the Northern Adriatic is predominantly linked to continuous discharge from nearby industrialized zones and large Hg mining sites (Kotnik et al., 2015). Levels of As were higher than in digestive gland of crustaceans from the Atlantic (Barrento et al., 2009; Leblanc and Prince, 2012) and the Pacific (Lewtas et al., 2014; Metian et al., 2010). A relatively high amount of total As was already reported for *N. norvegicus* digestive gland from the same Adriatic area (Sekulić et al., 1993). The findings of high As levels in comparison to crustaceans from other geographical areas are consistent with previous reports for temperate Mediterranean and tropical Caribbean seas (Fattorini et al., 2006) and could be linked to the influence of environmental conditions, particularly temperature and salinity fluctuations (Valentino-Álvarez et al., 2013; Vellinger et al., 2012).

In contrast to larger organisms, the lack or even inverse relationship of Cd, Hg and As accumulation and MTs content were found for small lobsters. The discrepancy between smaller and larger organisms could be explained by possibly major influence of body size on metal detoxification efficiency in *N. norvegicus*. This hypothesis needs further experimental verification.

Previous laboratory and field studies demonstrated positive correlation of Pb, Cr and Mn to MTs content in some crustaceans (Lavadras et al., 2014; Martín-Díaz et al., 2009). On the other hand, at exposure concentrations of Pb relevant for contaminated marine environment, the accumulation of Pb in digestive gland tissue of shrimp *L. vannamei* was not observed and could not be linked to MTs content increase (Nunez-Nogueira et al., 2010). Hence, the lack of correlation to MTs could be explained by relatively low concentrations of Pb that were two to tenfold lower than values previously reported by Canli and Furness (1993a) for digestive gland of *N. norvegicus*, and within values for other crustaceans from low to moderately contaminated marine areas (Canli et al., 2001; Leblanc and Prince, 2012; Lewtas et al., 2014; Yilmaz and Yilmaz, 2007). Similarly, concentrations of Cr reported herein were comparable to those reported for shrimps from unpolluted aquaculture site in the Pacific (Metian et al., 2010), although some studies reported lower levels for other decapode crustaceans collected from sites of varying contamination degree (Çiftçi et al 2011; Leblanc and Prince, 2012; Pereira et al 2009). It is also important to consider that both metals (Pb in particular) accumulate more effectively in other tissues of *N. norvegicus*, such as the gills (Cenov, 2017) further limiting the possibility for linking the observed fluctuations of these metals to MTs content in digestive gland. Accumulation of Mn was higher than previously found for digestive gland of lobsters *N. norvegicus* (Baden et al., 1999) and *H. americanus* from low to moderately contaminated areas in the Atlantic (Leblanc and Prince, 2012) but generally lower than in blue shrimp *Litopenaeus stylirostris* from the Pacific (Metian et al., 2010). The lack of correlation between Mn and MTs content is consistent with the tendency of slow Mn accumulation in the digestive gland with respect to gills or exoskeleton in particular (Cenov, 2017), and the variable pattern of accumulation recorded for *N. norvegicus* might reflect dietary intake Mn rather than its level in the surrounding environment (Ericsson and Baden, 1998).

As mentioned above, some metals accumulate more effectively in the gills than in the digestive gland. Thus, the response of MTs in the gills to metal accumulation should be also considered for investigation, taking also into account that relatively high MTs content detected in this particular tissue of *N. norvegicus* (Canli et al., 1993).

While Cd is mostly distributed in soluble cytosol (Pedersen et al., 2014) some metals are mainly detoxified as insoluble metal-rich granules, as reported for some crustaceans (Barka, 2007; Legras et al., 2000; Mouneyrac et al., 2001; Nunez – Noguiera et al., 2010). Since the total concentration of metals was taken into account here, their potentially toxic effect reflected in elevated MTs content might have remained obscured. Clearly, information on partitioning of metals between soluble and insoluble fraction is needed to further explain the MTs content fluctuations trends in relation to metal accumulation in *N. norvegicus*.

Levels of ROS displayed different seasonal pattern for small and large lobsters possibly in relation to fluctuations of environmental abiotic and biotic factors within the investigated areas, that were shown to influence the balance between pro-oxidant and antioxidant activity and maintenance of a steady-state ROS level in crustaceans (Liu et al., 2007; Schvezov et al., 2015). A protective role of MTs acting as scavengers of ROS arising from the action of metals was reported by Moltó et al (2007). Furthermore, Felix-Portillo et al (2014) reported the increased MTs mRNA expression following hypoxia exposure of white shrimps *L. vannamei* suggesting the possible role of MTs in the ROS – detoxifying mechanism. Another experimental study showed an increase of MTs level following exposure of *L. vannamei* to Cd, and a concomitant decrease of ROS production (Lobato et al., 2013). In this respect, a modest negative relationship between MTs and ROS accumulation ($r_s = -0.45$) for small but not for large organisms, indicates a body size dependent capacity of MTs to counteract the oxidative radicals. However, data on antioxidative system components and in particular on lipid peroxidation are needed for a more detailed picture on the capacity of *N. norvegicus* to cope with potential pro oxidants.

A gender-related specificity of MTs content in digestive gland of lobsters was not detected in the current study. Our findings are opposite to recent evidences of gender dependent MTs content variations reported for crabs *Neohelice granulata* (Buzzi and Marcovecchio, 2016) and *Callinectes* sp. (Lavradas et al., 2014). Moreover, the use of only one gender was suggested for investigations of metals accumulation and biochemical responses in the tissues of crustaceans (Giarratano et al., 2016; Martín-Díaz et al., 2009). The absence of gender dependent Cd concentrations clearly contrasted previous findings of significantly higher level of this metal in the digestive gland of *N. norvegicus* females (Canli and Furness, 1993a). Conversely, a notable and consistently higher accumulation in males was displayed for As and Hg for both body-size groups and in both seasons, in accordance to previous study on *N. norvegicus* (Canli and Furness, 1993a). The observed differences may be due to larger dimensions of males than females sampled within the frame of the present study. In fact, Barrento et al (2009) found higher As concentration in digestive gland of females that in that particular survey displayed faster growth rate.

Variations in energy reserves, in particular lipids content, indicated that the physiology of *N. norvegicus* was influenced by season in both small and large organisms. Lipids content variation could be related to season dependent *N. norvegicus* feeding activity that in the Adriatic Sea tends to be higher in autumn (Cristo and Cartes, 1998). Generally lower level of lipids in spring could be a consequence of reduced feeding rhythm during the winter (Watts et al., 2016). More expressed consumption of energy reserves is displayed by males that commonly undergo moults more frequently than females (Sardà, 1995). As suggested by Rosa and Nunes (2002), lipids stored in digestive gland tissue could be more important for moulting activity than for oogenesis, which seems to depend on dietary intake of lipids. Mostly moderate negative correlations of MTs content and metals accumulation with lipids in particular, observed here for both body size categories, prompts for caution when interpreting these parameters in *N. norvegicus*, taking into consideration the expressed seasonal fluctuations on biochemical composition in digestive gland. For improved interpretation of chemical and biological data in relation to physiological conditions of *N. norvegicus*, the digestive gland glycogen level data would be also helpful, since it was shown to decline during starvation (Philp et al., 2015).

It is important to note that data interpretation could be impaired by confounding factors such as the reproductive status that affects metal accumulation and MTs content in some crustacean species (Mouneyrac et al., 2001). In the present study, the influence of reproductive cycle on fluctuations of MTs level and metal concentrations in *N. norvegicus* digestive gland observed could not be tackled, due to low proportion of females in trawl catch with respect to males. It is generally accepted that the reproductive season of *N. norvegicus* in the Adriatic Sea peaks in late spring and summer while the proportion of mature females declines in autumn, in accordance to changes in maturation stages of the ovaries (Orsi-Rellini et al., 1998). Nevertheless, the possible linkage of observed MTs content and metal accumulation seasonality and assumed differences in the reproductive stage between *N. norvegicus* sampled in spring and in autumn was not discerned in the present study, possibly due to expressed spatial and temporal heterogeneity of individuals in terms of gonad maturation stage even within the same body size group. This was suggested by occasional and unsynchronised occurrence of larger eggs-carrying females in both seasons at both sites, and only smaller eggs-carrying females in autumn at S1, but not at site S2. Thus, the important issue of reproductive cycle interference with MTs content and metal accumulation in *N. norvegicus* still remains unresolved.

An earlier study suggested that metal accumulation in the digestive gland tissue of *N. norvegicus* may vary considerably over different moulting stages (Canli and Furness, 1993a) that were not assessed in the present study. Similar observations were also reported for other crustaceans (Brouwer et al., 1992; Nørum et al., 2005). It is generally accepted that moulting frequency of adult *N. norvegicus* decreases with age and differs between females and males, being, as already mentioned, more frequent in the latter (Sardà, 1995). Whether factors such as moulting stages could be responsible for MTs content variation in the tissues of crustaceans is currently not sufficiently clear. Considering relatively heterogeneous carapace length of samples and obvious differences in the MTs trends between two body size groups, it is plausible that moulting frequency and concomitant, possibly gender-dependent size increments of *N. norvegicus* (Sardà, 1995) could represent an additional confounding factor for the interpretation of MTs content changes, in particular in relation to metals accumulation in the digestive gland tissue.

Finally, the comparison of MTs content between *N. norvegicus* digestive gland from the Kvarner Bay (present study) and that of other crustacean species from worldwide coastal and off-shore areas is impaired due to the well-known discrepancies between MTs content data related to differences in the method for MTs quantification (Pedersen et al., 2008). Considering the results previously obtained by spectrophotometric sulphydryl method (Viarengo et al., 1997), the values for MTs content presented herein are of the same order of magnitude as that of crab *Neohelice granulata* (Buzzi and Marcovecchio, 2016). Values higher by approximately one order of magnitude were reported for blue crab *Callinectes sapidus* from unpolluted sites (De Martinez Gaspar Martins and Bianchini, 2009).

A definition of threshold for the background is recommended to facilitate the interpretation of biological response, but requires the synthesis of field data both from uncontaminated and contaminated environments (Davies and Vethaak, 2012). The results of this study represent the only relatively comprehensive data available on the MTs content so far for field sampled *N. norvegicus* from the Mediterranean Sea. Furthermore, the significance of MTs content value deviation from the threshold value is currently not sufficiently understood for this benthic crustacean species. Thus, the threshold value for background level (mean + 1σ, 25.2 nmol/g w.w.) could be considered only as a tentative suggestion. Obviously, further investigation and collection of more data, particularly from metal enriched gradients are required to establish regionally specific threshold values crucial for discerning the natural variability of MTs content from potential adverse effect of toxic metals in *N. norvegicus.*

This study presents the first report on the metallothioneins content in the digestive gland of *N. norvegicus* taking into account the effect of body size, gender and spatio - temporal variations. Body size had significant influence on MTs content variations that displayed generally inconsistent seasonal patterns, raising questions whether and to what extent the effect of metals exposure could be masked and remain undetected. By contrast, differences between males and females were negligible. Despite seasonal fluctuations of both MTs content and metal accumulation, the observed mild positive relationship with Cd, Hg and As and was in accordance to the well-known metal-scavenging function of these proteins. A negative relationship with ROS reinforced the notion of possible MTs involvement in antioxidative response. This study also stressed that variations of biochemical and chemical parameters measured in *N. norvegicus* digestive gland tissue could be linked to energy reserves, particularly lipids. Thus, data presented here provide a solid starting point for future studies that should be aimed in particular to filling the knowledge gaps concerning MTs response to increased metal body burden.

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Figure captions

Fig 1. Map of sampling area in the Kvarner Bay, NE Adriatic Sea.

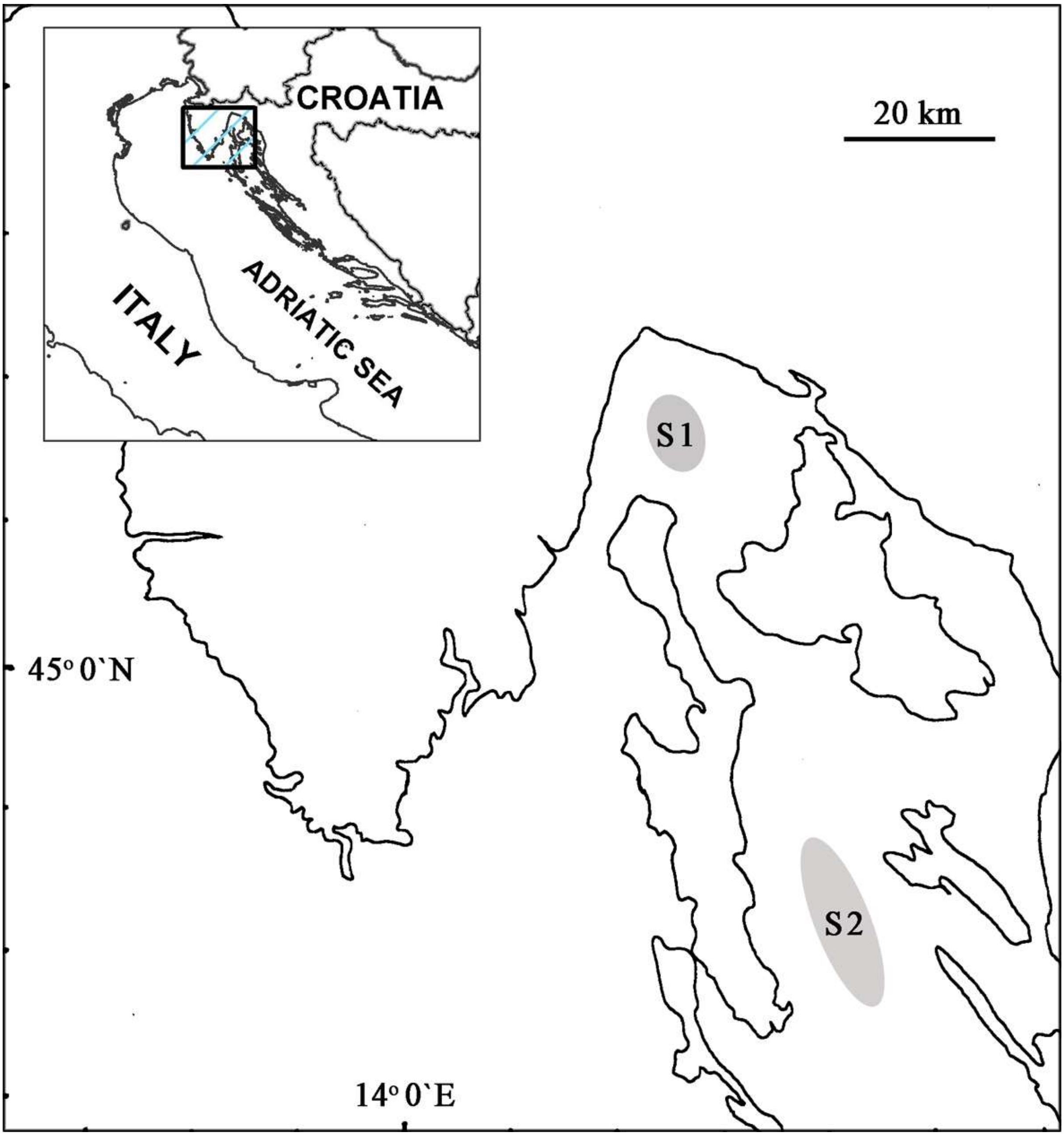
Fig 2. MTs content (nmol/g w.w.) in the digestive gland of small (<36mm) and large (>36 mm) *Nephrops norvegicus* from sites S1 and S2 in autumn (Aut) and spring (Spr). Square boxes indicate lower and upper quartile and whiskers represent minimum and maximum data values (1.5 interquartile range). Medians are depicted by solid line, and outliers as circles. □ – females; ■ – males; $^{\#}p<0.05$ - significant differences between seasons

Fig 3. Concentrations of Cd, As, Hg, Pb, Cr, Mn (µg/g d.w.) in the digestive gland of small (<36mm) and large (>36 mm) *Nephrops norvegicus* from sites S1 and S2 in autumn (Aut) and spring (Spr). Square boxes indicate lower and upper quartile and whiskers represent minimum and maximum data values (1.5 interquartile range). Medians are depicted by solid line, and outliers as circles. □ – females; ■ – males; $^{*}p<0.05$ - significant difference between males and females; $^{\#}p<0.05$ - significant differences between seasons

Fig 4. Content of ROS (mmol/mg w.w) in the digestive gland of small (<36mm) and large (>36 mm) *Nephrops norvegicus* from sites S1 and S2 in autumn (Aut) and spring (Spr). □ – females; Square boxes indicate lower and upper quartile and whiskers represent minimum and maximum data values (1.5 interquartile range). Medians are depicted by solid line, and outliers as circles. □ – females; ■ – males; $^{*}p<0.05$ - significant difference between males and females; $^{\#}p<0.05$ - significant differences between seasons

Fig 5. Score plots and variable loadings plots of principal component analysis (PCA) based on MTs content, concentrations of Cd, As, Hg, Pb, Cr, Mn, Cu and Zn, ROS content, lipids and proteins concentration in the digestive gland of small (<36 mm, upper panel) and large (>36 mm, lower panel) male and female lobsters from sites S1 and S2. Each point corresponds to one individual score. Data for Cu and Zn were provided by Glad (personal communication).

Fig 6. Frequency histogram and Gaussian distribution of values for MTs content in digestive gland of 144 *Nephrops norvegicus* samples in total.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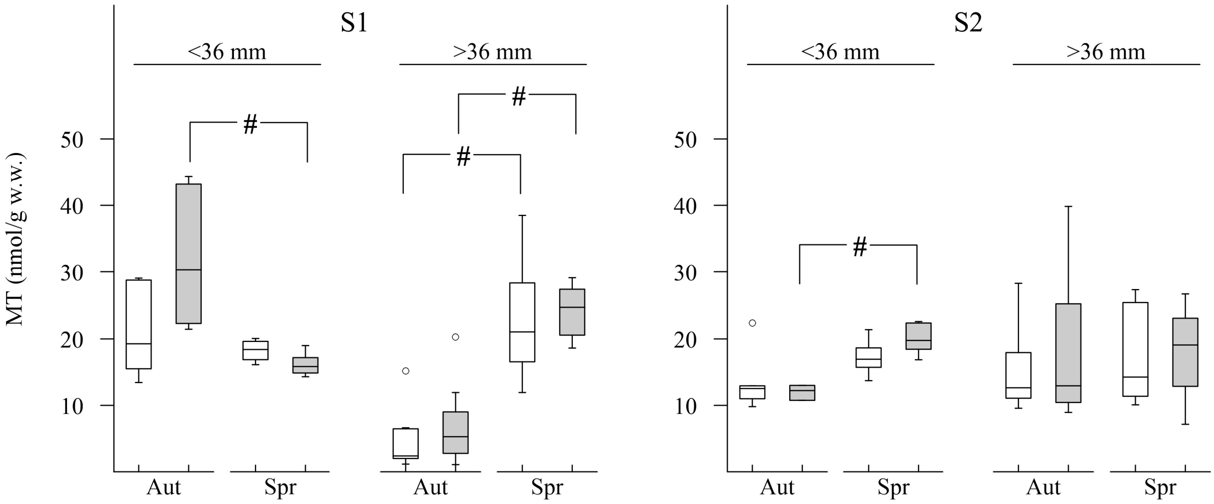
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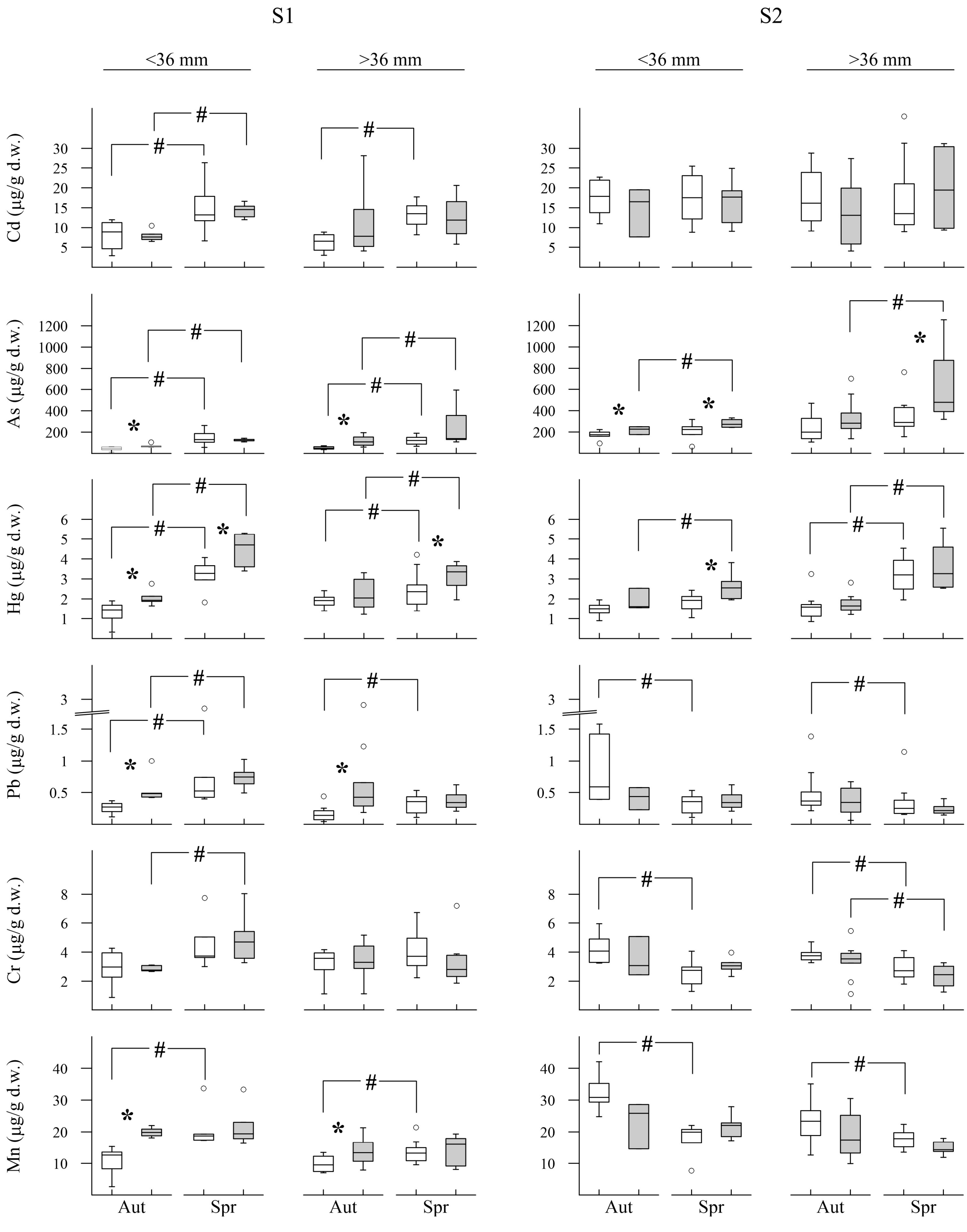
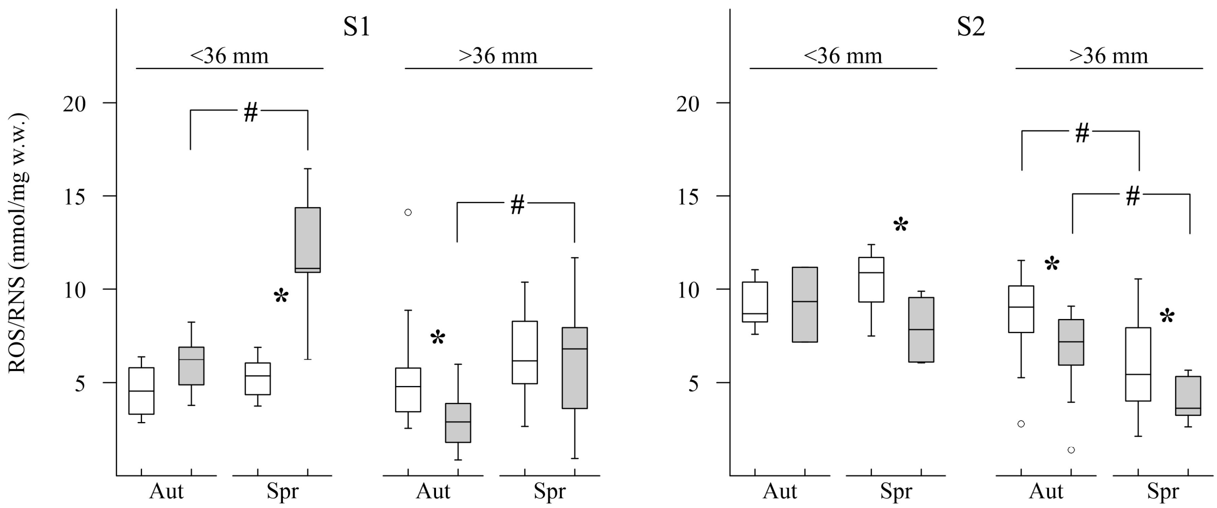
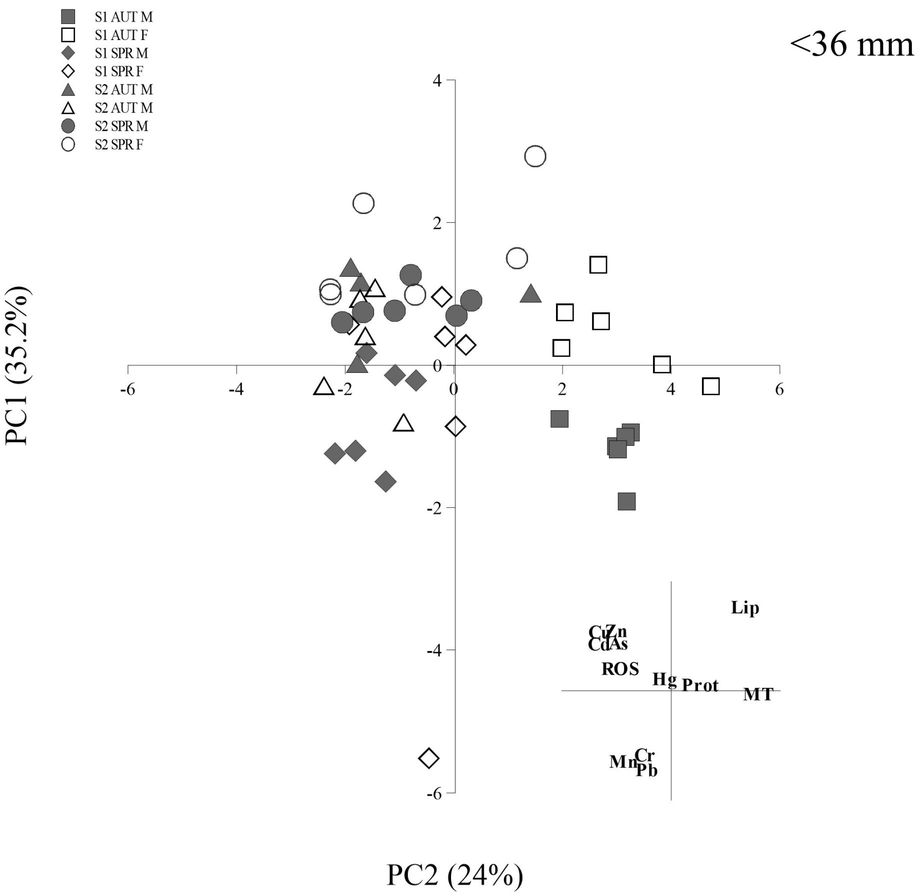
Aut
Spr
Aut
Spr



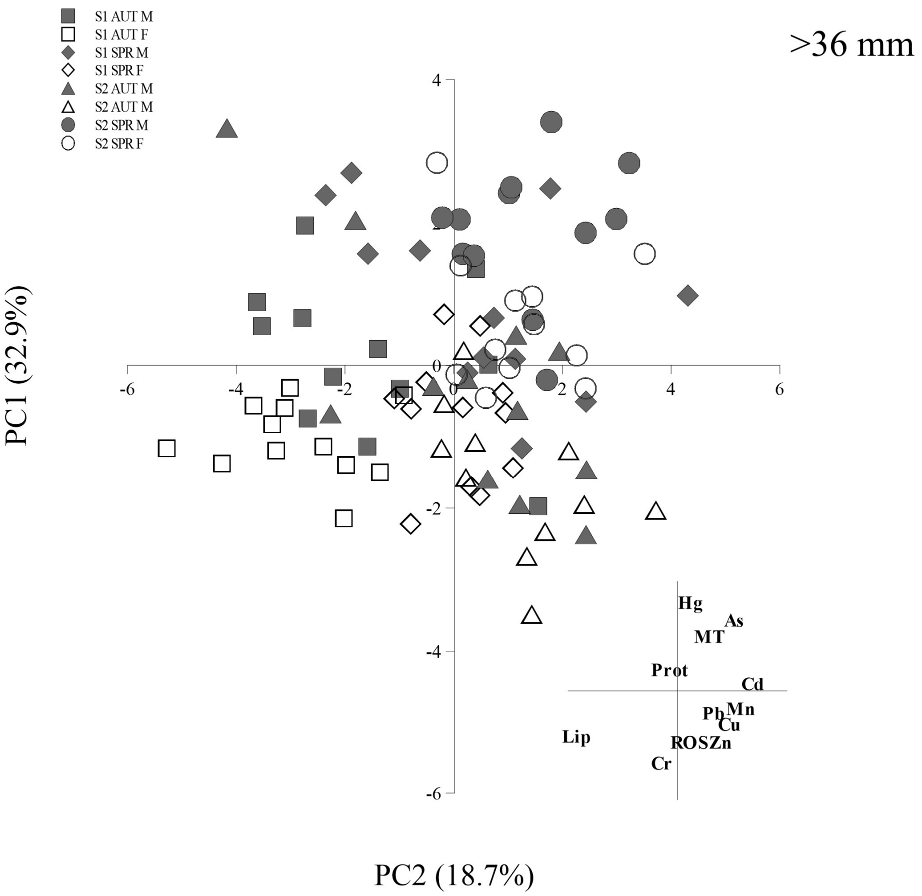
S1
S2
<36 mm
>36 mm
<36 mm
>36 mm
Cd (µg/g d.w.)
As (µg/g d.w.)
Hg (µg/g d.w.)
Pb (µg/g d.w.)
Cr (µg/g d.w.)
Mn (µg/g d.w.)
Aut
Spr
Aut
Spr
Aut
Spr
Aut
Spr



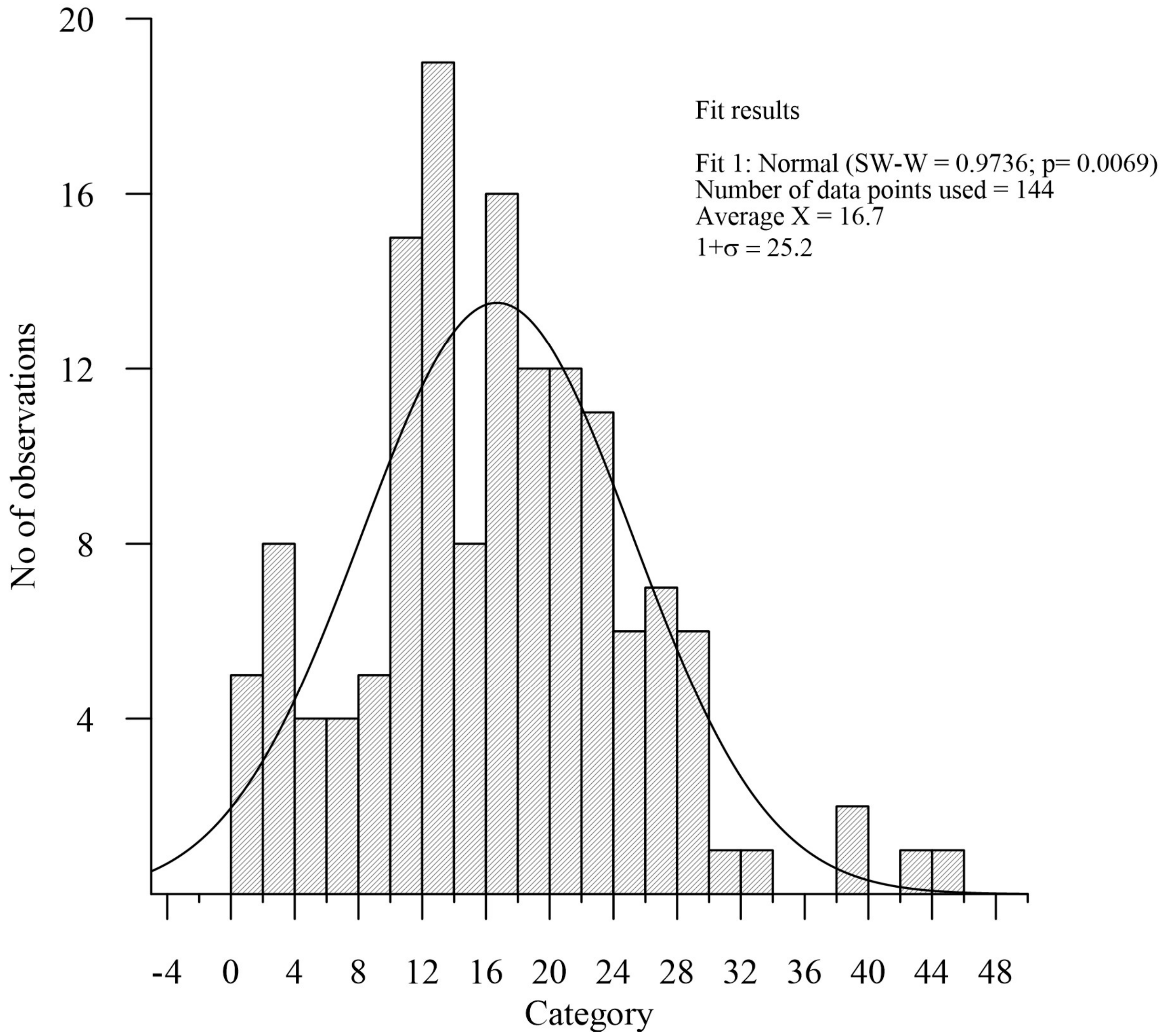
S1
S2
<36 mm
>36 mm
ROS/RNS (mmol/mg w.w.)
20
15
10
5
#
*
Aut
Spr



S1 AUT M
S1 AUT F
S1 SPR M
S1 SPR F
S2 AUT M
S2 AUT M
S2 SPR M
S2 SPR F
<36 mm
PC1 (35.2%)
PC2 (24%)
Lip
CuZn
CdAs
ROS
Hg
Prot
MT
MnCr
Pb



S1 AUT M
S1 AUT F
S1 SPR M
S1 SPR F
S2 AUT M
S2 AUT M
S2 SPR M
S2 SPR F
>36 mm
PC1 (32.9%)
PC2 (18.7%)
Hg
As
MT
Prot
Cd
PbMn
Cu
Lip
ROSZn
Cr


Fit results
Fit 1: Normal (SW-W = 0.9736; p= 0.0069)
Number of data points used = 144
Average X = 16.7
1+σ = 25.2
No of observations
20
16
12
8
4
-4
0
4
8
12
16
20
24
28
32
36
40
44
48
Category