

# 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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2 *Nephrops norvegicus* from Northern Adriatic Sea: Body size, season, gender and metal specific  
3 variability

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22 energy reserves, biomarker baseline

## 23 24 **ABSTRACT**

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

38  
39 The Norway lobster *Nephrops norvegicus* (Linnaeus, 1758) is among the most  
40 economically important crustacean species of the Mediterranean Sea and NE Atlantic. For the  
41 Adriatic Sea in particular, the overall population of *N. norvegicus* has been dramatically  
42 declining due to excessive exploitation of fishing grounds (Piccinetti et al., 2012). Besides,  
43 coastal zones may be subjected to intensive anthropogenic activities, such as increasing  
44 urbanization and industrialization. The resulting contamination with metals in particular

45 represents a significant risk for aquatic biota, given their ubiquity, long-term persistence in the  
46 sediment and potential toxicity to benthic organisms. However, the assessment of contaminants  
47 based on chemical analyses in seawater and sediments is still predominant and little information  
48 is available regarding toxic effect and detoxification of metals for burrowing lifestyle  
49 crustaceans such as *N. norvegicus*.

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

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

73 While controlled laboratory-based exposure studies provided substantial evidence on the  
74 direct link of toxic chemicals and biomarkers response, field studies of anthropogenic  
75 contamination effects still represent a considerable challenge for the scientific community due  
76 to limited knowledge on natural variability of biomarkers in sentinel marine organisms.  
77 Confounding factors such as intrinsic biotic and environmental parameters cause large  
78 amplitudes of biomarkers' response that could consequently be overestimated and incorrectly  
79 attributed to the toxic effect of contaminants. Thus, definition of natural biomarker variation  
80 range using data collected from reference sites has become a priority task of ecotoxicological  
81 studies as a foundation for realistic evaluation of contaminants effect in the field (Barrick et al.,  
82 2016; Davies and Vethaak, 2012 and references therein). Fluctuations of MTs content related  
83 to gender, season and reproductive status were already identified in the tissues of some  
84 decapode crustacean species (Chiodi Boudet et al., 2013; Giarratano et al., 2016; Lavadras et  
85 al., 2014; Maria et al., 2009; Mouneyrac et al., 2001). Consequently, in order to avoid possible  
86 misinterpretation of contaminants' effect in the natural habitat it is essential to establish the  
87 MTs baseline levels in relation to potentially confounding factors.

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

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

114 Specimens of *Nephrops norvegicus* were collected in autumn 2014 and spring 2015 by  
115 bottom trawl fishing gear. Trawling depths for sites S1 and S2 were at 55-62 and 70-78 m,  
116 respectively. Immediately following capture healthy and undamaged animals were selected and  
117 transported to the laboratory in thermally isolated containers. The gender of organisms was  
118 determined by checking the morphology of the first pair of abdominal swimmeret (pleopodes)  
119 that are thicker and rigid in males. The carapace length (CL; from eye socket to mid hind edge  
120 of carapace) was recorded for each lobster. For each site, season and gender the organisms were  
121 subsequently classified into two non-overlapping and relatively well-defined body-size group  
122 according to their CL (Table 1). Lobsters of CL below 36 mm were considered as “small”  
123 whereas the second body-size group, hereafter referred to as “large”, comprised all lobsters  
124 samples of CL larger than 36 mm due to heterogeneity of CL values and difficulties in obtaining  
125 a balanced gender ratio. The total of 144 specimens of *N. norvegicus* were dissected to obtain  
126 digestive gland tissue samples that were immediately frozen in liquid nitrogen and stored at -  
127 80°C.

128 Portions of 0.5 g of freeze dried digestive gland tissue samples were digested using Anton  
129 Paar Multiwave 3000 microwave system (Perkin Elmer, USA) equipped with pressurized  
130 vessels, using 5 mL of 65% nitric acid per sample (HNO<sub>3</sub> Suprapur, Merck, Germany), over a  
131 20 minutes operation cycle at 200 °C. Digested samples were transferred to 25 ml volumetric

132 flasks and added with ultrapure water (Siemens). The concentrations of Cd, As, Pb, Cr and Mn  
133 were determined using the inductively coupled plasma mass spectrometer, ICP MS NexION  
134 300X, equipped with S10 autosampler (Perkin Elmer, USA). Multielement solution (NexION  
135 Setup Solution, Perkin Elmer, USA) was used as tuning solution, covering a wide range of  
136 masses of elements. Multielement standard solution (Perkin Elmer, USA) was used to prepare  
137 calibration curves. The calibration curves with  $R^2 > 0,999$  were accepted for concentration  
138 calculation. For each experiment, a run included blank, certified reference material (CRM) and  
139 samples which were analysed in triplicate to eliminate any batch-specific error. The method for  
140 measurement of Cd, As, Pb, Cr and Mn was validated using IAEA-407 reference material (fish  
141 tissue) (International Atomic Energy Agency, Austria). The mean recovery values for Cd, As,  
142 Pb, Cr and Mn were 97%, 89%, 105%, 110% and 108%, respectively. For Hg determination,  
143 approx. 0.1 g of freeze dried digestive gland tissue was weighed, transferred directly into  
144 analyser's vessel and analysed using the atomic absorption spectrometer AMA 254 (Advanced  
145 Mercury Analyser, Leco, USA). Single element standard solution for Hg was used for  
146 instrument calibration (LGC Standards, USA). The method for Hg measurement was validated  
147 using NIST 2976 reference material (mussels tissue) (National Institute of Standards and  
148 Technology, USA). The mean recovery for Hg was 104%. The concentrations of all metals  
149 were expressed as  $\mu\text{g}$  per g of tissue dry weight (d.w.). Data for Cu and Zn concentrations were  
150 provided by Glad (personal communication).

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

158 The OxiSelect™ *In Vitro* ROS/RNS Assay Kit (Cell Biolabs, inc. USA) was used for the  
159 measurement of total free radicals in the samples, according to the manufacturer instructions.  
160 The method is based on the measurement of 2', 7'- dichlorodihydrofluorescein (DCF)  
161 fluorescence that results from oxidation of highly reactive dichlorodihydrofluorescein (DCFH).  
162 The intensity of DCF fluorescence that is proportional to total free radicals level in the sample  
163 was measured using the fluorescence plate reader Fluoroscan Ascent™ (Labsystem, Finland)  
164 at 480 nm excitation/530 nm emission. For calculation of the free radical content, serial  
165 dilutions of  $\text{H}_2\text{O}_2$  were used, and the concentration of ROS/RNS was expressed as mmol/mg of  
166 tissue wet weight (w.w.).

167 Quantitative determination of total lipids in digestive gland tissue was performed by sulfo-  
168 phospho-vanillin colorimetric method (Glad, 2017). Briefly, lipids were extracted by vigorous  
169 mixing of freeze dried digestive gland tissue portions (10-20 mg) with chloroform/methanol  
170 2:1 (V/V) solution, followed by incubation at  $+4^\circ\text{C}$  for 10 min and centrifugation at 4000 rpm  
171 for 10 min. Aliquots of each sample were evaporated to dryness under nitrogen stream in glass  
172 tubes, vigorously mixed with concentrated sulphuric acid and heated at  $100^\circ\text{C}$  for 10 minutes.  
173 The total lipid content was determined in cooled samples using phospho-vanillin reagent and  
174 serial dilutions of cholesterol standards. The absorbance values of blank and samples were  
175 recorded at 490 nm and the concentration was expressed as % of lipids per g of tissue dry weight

176 (d.w.). For determination of proteins in digestive gland tissue, portions of freeze dried tissue  
177 (10-20 mg) were mixed with 10% sodium hydroxide and incubated for 1 hour at 100°C to digest  
178 proteins. Samples were then subjected to Bradford assay (Bradford, 1976) with bovine serum  
179 albumin as protein standard. The absorbance values of blank and samples were recorded at 595  
180 nm. Concentration was expressed as % of proteins per g of tissue dry weight (d.w.).

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

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

194 Content of MTs in digestive gland of small and large *Nephrops norvegicus* ranged from  
195 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  
196 for MTs content varied significantly between seasons at site S1, with a contrasting trend  
197 displayed between body size groups. At site S2, MTs content was significantly higher in spring  
198 only for small male lobsters. Differences between small and large lobsters were also found at  
199 site S1 (Table 2). Significant differences in MTs content were observed between sites, being  
200 higher at site S1 in autumn for small males of both genders and in spring for large females. The  
201 opposite pattern was observed at site S2 (Table 3). Concentration of Cd ranged from 3 to 28.1  
202  $\mu\text{g/g}$  d.w. and from 4.1 to 37.9  $\mu\text{g/g}$  d.w. for lobsters from sites S1 and S2, respectively (Fig.  
203 3). At site S1, Cd values displayed marked seasonality for both body size classes, being  
204 significantly higher predominantly in spring. Gender dependent differences were not recorded.  
205 At site S2, no seasonality could be observed. Concentrations of Cd did not show differences  
206 with respect to body size (Table 2). Values recorded at S2 were occasionally significantly  
207 higher than at site S1, predominantly in autumn (Table 3). Concentration of As ranged from  
208 33.6 to 594.4  $\mu\text{g/g}$  d.w. and from 61.1 to 1254.1  $\mu\text{g/g}$  d.w., at sites S1 and S2, respectively (Fig.  
209 3). Gender and season dependent As accumulation was detected for both body size groups and  
210 sites, mainly being significantly higher in spring and in males. Significantly higher As levels  
211 were found in smaller males, except in spring at site S2 (Table 2). Concentration of As was  
212 almost regularly significantly higher at site S2 (Table 3). Concentration of Hg ranged from 1 to  
213 5.3  $\mu\text{g/g}$  d.w. and from 0.9 to 5.6  $\mu\text{g/g}$  d.w., at sites S1 and S2, respectively (Fig. 3).  
214 Significantly higher Hg concentration was recorded almost regularly in spring, predominantly  
215 in males. Differences related to body size, although sometimes significant, did not express a  
216 clear trend (Table 2). Concentrations of Hg were often significantly higher at site S1,  
217 particularly in spring (Table 3). Concentrations of Pb ranged from 0.04 to 3.6  $\mu\text{g/g}$  d.w. and  
218 from 0.06 to 1.6  $\mu\text{g/g}$  d.w. at sites S1 and S2, respectively (Fig. 3). At site S1, Pb levels  
219 displayed similar trends, but significantly higher concentrations were recorded predominantly

220 in male lobsters and in spring. Opposite seasonal patterns with higher autumn values were  
221 observed at site S2 for both body size categories of females only. Concentration of Pb was  
222 significantly higher in small lobsters of both genders at site S1 in spring, and at site S2 in  
223 females in autumn (Table 2). Differences of Pb accumulation between sites were season-  
224 specific, that is, significantly higher at S1 and S2 in spring and autumn, respectively (Table 3).  
225 Concentration of Cr ranged from 1.1 to 8  $\mu\text{g/g}$  d.w. and from 1.1 to 5.45  $\mu\text{g/g}$  d.w. at sites S1  
226 and S2, respectively (Fig. 3). The season related pattern was more consistent at site S2, where  
227 significantly higher Cr concentrations were recorded in autumn. There were no differences  
228 between genders. Significant differences were detected between body size groups only for  
229 males at site S1, displaying higher Cr concentrations in large and small lobsters in autumn and  
230 spring, respectively (Table 2). Concentration of Cr was significantly higher at S1 only in spring  
231 (Table 3). The range of Mn concentrations for lobsters from sites S1 and S2 was between 7 to  
232 33.7  $\mu\text{g/g}$  d.w. and from 7.6 to 42.2  $\mu\text{g/g}$  d.w., respectively (Fig. 3). Seasonal differences were  
233 recorded only for females of both body size groups, displaying significantly higher levels in  
234 spring at site S1, and in autumn at site S2 (Table 2). Values for Mg concentration were  
235 significantly higher in small lobsters, but almost exclusively at site S1 (Table 2). Accumulation  
236 of Mn was significantly higher at site S2 for females only (Table 3).

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

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

249 A significant negative correlation was detected between MTs and metals ( $r_s$ = Mn, -0.4; Cd,  
250 -0.46; Cu, -0.46; Zn, -0.41,  $p<0.05$ ) for small lobsters (Table 5). Large organisms displayed  
251 significant ( $p<0.05$ ) positive correlation with Cd ( $r_s=0.25$ ), Hg ( $r_s=0.31$ ) and As ( $r_s=0.37$ ).  
252 Content of MTs was negatively correlated to ROS ( $r_s=-0.45$ ,  $p<0.05$ ) in digestive gland of small  
253 lobsters only. Large, but not small lobsters, displayed significant negative correlation with  
254 lipids ( $r_s=-0.31$ ,  $p<0.05$ ). Significant correlations ( $p<0.05$ ) were recorded between metal  
255 concentrations and lipids, namely for Hg, Pb, Mn and Cr ( $r_s=-0.38$  to  $-0.67$ ) and for Cd, As, Pb  
256 Hg, Mn and Cu ( $r_s= -0.3$  to  $-0.56$ ) in digestive gland of small and large lobsters, respectively.

257 A principal component analysis (PCA) was performed using data on MTs content, ROS  
258 level, metals accumulation and energy reserves, obtained from all individuals sampled of both  
259 body size groups, seasons, genders and sites. The first two principal components PC1 and PC2  
260 accounted for 59.2% and 51.6% for small and large lobsters, respectively (Fig. 5, upper and  
261 lower panel). Generally large variation was observed for samples spread on both PCA  
262 ordination plots. The contribution of the variables in each of the first two PCs varied between  
263 two body size groups. With exception of site S1 in autumn, small lobsters were mostly grouped

264 to the left part of the PC1, with contribution of Cd, As, Cu, Zn concentrations and ROS level  
265 that were negatively correlated to PC1, while MTs content was positively correlated to PC1.  
266 Small males and females from site S1 in autumn were separated along PC2 as a result of the  
267 differences in the accumulation of Mn, Cr, Pb and lipids content (negatively correlated to PC2).  
268 Large males and females from site S1 sampled in autumn were distributed at the left side of  
269 PC1 and had generally lower levels of metals and MTs content and higher lipids content. Large  
270 lobsters from site S2 were roughly separated along PC2, with spring samples being located in  
271 its positive side due to high accumulation of Hg and As, and autumn sample in the negative  
272 side due to association with higher ROS level and Cr and Zn concentrations.

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

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

290 Seasonal variations and gender dependent differences of Cd and other metals that exert  
291 high MTs binding affinity, were already reported for *N. norvegicus* digestive gland (Canli and  
292 Furness, 1993a). Nevertheless, MTs content for larger body size lobsters displayed weak  
293 positive correlation ( $r_s=0.25$ ) to Cd that, according to previous reports, predominantly  
294 accumulates in digestive gland tissue of *N. norvegicus* (Canli and Furness, 1993b). The notion  
295 of elevated MTs content in response to Cd could be supported by laboratory studies  
296 demonstrating Cd as an effective inducer of MTs synthesis in *N. norvegicus* (Canli et al., 1997)  
297 and other crustaceans (Moltó et al., 2005; Pan and Zhang, 2006). However, considering the  
298 fairly weak correlation, it is difficult to speculate to what extent the level of Cd in *N. norvegicus*  
299 digestive gland might be related to MTs content fluctuations in this particular case. According  
300 to recent data on sediment metal concentrations (Cukrov et al., 2011, 2014; Ilijanić et al., 2014),  
301 there are no indications of particular sediment Cd enrichment at sampling sites in comparison  
302 to other offshore areas of unpolluted Adriatic and Mediterranean regions. Besides,  
303 concentrations of Cd for digestive gland were in line with data previously reported for *N.*  
304 *norvegicus* (Canli and Furness, 1993a) and lobsters *H. gammarus* and *H. americanus* (Barrento  
305 et al., 2009; Leblanc and Prince, 2012) from unpolluted sites of Atlantic coast. Similarly, low  
306 enrichment of off-shore sediments and generally decreasing contamination trend for Cu and Zn  
307 in Kvarner Bay (Cukrov et al., 2011, 2014) could explain the lack of correlation between these



308 metals and MTs content in *N. norvegicus*. The essential metals like Cu and Zn also possess high  
309 MTs binding ability and MTs induction potential when accumulated in excess (Amiard et al.,  
310 2006), but it seems that a pool of MTs that bind these metals could be considered as storage  
311 and donor for metalloproteins, such as for apohemocyanin and carboanhydrase (Henry et al.,  
312 2012).

313 The experimental evidences on MTs-inductive potential of other toxic metals such as Hg  
314 and As are generally scarce for crustaceans (Barka et al., 2001; Barka, 2007; Vellinger et al.,  
315 2013), but when these organisms were used for studying the adverse effect of anthropogenic  
316 contaminants in the field revealed correlations of both metals with MTs content (Faria et al.,  
317 2010, Martín-Díaz et al., 2009). A mild positive correlation of MTs was detected in the present  
318 study for Hg ( $r_s=0.31$ ) and As ( $r_s=0.37$ ) but again only for larger lobsters, indicating that for  
319 this body size class the level of Hg and As accumulated in digestive gland tissue could be  
320 sufficient to surpass the necessary threshold for MTs induction. This is consistent with the  
321 essential physiological role of MTs in detoxification and storage of metals in the form of  
322 insoluble MTs complex. Noteworthy, values for Hg concentrations exceeded those previously  
323 reported for digestive gland of *N. norvegicus* (Canli and Furness, 1993a) and other crustaceans  
324 from the Atlantic (Barrento et al., 2009) and the Pacific (Frías-Espericueta et al., 2016).  
325 Moreover, data reported herein are in agreement with consistently high Hg concentrations in  
326 the soft tissues of *N. norvegicus* and other organisms from Mediterranean waters that, as widely  
327 emphasised before, could be related to the large cinnabar deposits in the Mediterranean basin  
328 (up to 55% of total world reserves) and slow turnover of Mediterranean waters through the  
329 Gibraltar strait (Perugini et al., 2009; Renzoni et al., 1998). Besides, Hg enrichment of  
330 sediments and biota in the Northern Adriatic is predominantly linked to continuous discharge  
331 from nearby industrialized zones and large Hg mining sites (Kotnik et al., 2015). Levels of As  
332 were higher than in digestive gland of crustaceans from the Atlantic (Barrento et al., 2009;  
333 Leblanc and Prince, 2012) and the Pacific (Lewtas et al., 2014; Metian et al., 2010). A relatively  
334 high amount of total As was already reported for *N. norvegicus* digestive gland from the same  
335 Adriatic area (Sekulić et al., 1993). The findings of high As levels in comparison to crustaceans  
336 from other geographical areas are consistent with previous reports for temperate Mediterranean  
337 and tropical Caribbean seas (Fattorini et al., 2006) and could be linked to the influence of  
338 environmental conditions, particularly temperature and salinity fluctuations (Valentino-Álvarez  
339 et al., 2013; Vellinger et al., 2012).

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

345 Previous laboratory and field studies demonstrated positive correlation of Pb, Cr and Mn  
346 to MTs content in some crustaceans (Lavadras et al., 2014; Martín-Díaz et al., 2009). On the  
347 other hand, at exposure concentrations of Pb relevant for contaminated marine environment,  
348 the accumulation of Pb in digestive gland tissue of shrimp *L. vannamei* was not observed and  
349 could not be linked to MTs content increase (Nunez-Nogueira et al., 2010). Hence, the lack of  
350 correlation to MTs could be explained by relatively low concentrations of Pb that were two to  
351 tenfold lower than values previously reported by Canli and Furness (1993a) for digestive gland

352 of *N. norvegicus*, and within values for other crustaceans from low to moderately contaminated  
353 marine areas (Canli et al., 2001; Leblanc and Prince, 2012; Lewtas et al., 2014; Yilmaz and  
354 Yilmaz, 2007). Similarly, concentrations of Cr reported herein were comparable to those  
355 reported for shrimps from unpolluted aquaculture site in the Pacific (Metian et al., 2010),  
356 although some studies reported lower levels for other decapode crustaceans collected from sites  
357 of varying contamination degree (Çiftçi et al 2011; Leblanc and Prince, 2012; Pereira et al  
358 2009). It is also important to consider that both metals (Pb in particular) accumulate more  
359 effectively in other tissues of *N. norvegicus*, such as the gills (Cenov, 2017) further limiting the  
360 possibility for linking the observed fluctuations of these metals to MTs content in digestive  
361 gland. Accumulation of Mn was higher than previously found for digestive gland of lobsters *N.*  
362 *norvegicus* (Baden et al., 1999) and *H. americanus* from low to moderately contaminated areas  
363 in the Atlantic (Leblanc and Prince, 2012) but generally lower than in blue shrimp *Litopenaeus*  
364 *stylirostris* from the Pacific (Metian et al., 2010). The lack of correlation between Mn and MTs  
365 content is consistent with the tendency of slow Mn accumulation in the digestive gland with  
366 respect to gills or exoskeleton in particular (Cenov, 2017), and the variable pattern of  
367 accumulation recorded for *N. norvegicus* might reflect dietary intake Mn rather than its level in  
368 the surrounding environment (Ericsson and Baden, 1998).

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

373 While Cd is mostly distributed in soluble cytosol (Pedersen et al., 2014) some metals are  
374 mainly detoxified as insoluble metal-rich granules, as reported for some crustaceans (Barka,  
375 2007; Legras et al., 2000; Mouneyrac et al., 2001; Nunez – Noguiera et al., 2010). Since the  
376 total concentration of metals was taken into account here, their potentially toxic effect reflected  
377 in elevated MTs content might have remained obscured. Clearly, information on partitioning of  
378 metals between soluble and insoluble fraction is needed to further explain the MTs content  
379 fluctuations trends in relation to metal accumulation in *N. norvegicus*.

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

394 A gender-related specificity of MTs content in digestive gland of lobsters was not detected  
395 in the current study. Our findings are opposite to recent evidences of gender dependent MTs

396 content variations reported for crabs *Neohelice granulata* (Buzzi and Marcovecchio, 2016) and  
397 *Callinectes* sp. (Lavradas et al., 2014). Moreover, the use of only one gender was suggested for  
398 investigations of metals accumulation and biochemical responses in the tissues of crustaceans  
399 (Giarratano et al., 2016; Martín-Díaz et al., 2009). The absence of gender dependent Cd  
400 concentrations clearly contrasted previous findings of significantly higher level of this metal in  
401 the digestive gland of *N. norvegicus* females (Canli and Furness, 1993a). Conversely, a notable  
402 and consistently higher accumulation in males was displayed for As and Hg for both body-size  
403 groups and in both seasons, in accordance to previous study on *N. norvegicus* (Canli and  
404 Furness, 1993a). The observed differences may be due to larger dimensions of males than  
405 females sampled within the frame of the present study. In fact, Barrento et al (2009) found  
406 higher As concentration in digestive gland of females that in that particular survey displayed  
407 faster growth rate.

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

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

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

452 Finally, the comparison of MTs content between *N. norvegicus* digestive gland from the  
453 Kvarner Bay (present study) and that of other crustacean species from worldwide coastal and  
454 off-shore areas is impaired due to the well-known discrepancies between MTs content data  
455 related to differences in the method for MTs quantification (Pedersen et al., 2008). Considering  
456 the results previously obtained by spectrophotometric sulphhydryl method (Viarengo et al.,  
457 1997), the values for MTs content presented herein are of the same order of magnitude as that  
458 of crab *Neohelice granulata* (Buzzi and Marcovecchio, 2016). Values higher by approximately  
459 one order of magnitude were reported for blue crab *Callinectes sapidus* from unpolluted sites  
460 (De Martinez Gaspar Martins and Bianchini, 2009).

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

472 This study presents the first report on the metallothioneins content in the digestive gland  
473 of *N. norvegicus* taking into account the effect of body size, gender and spatio - temporal  
474 variations. Body size had significant influence on MTs content variations that displayed  
475 generally inconsistent seasonal patterns, raising questions whether and to what extent the effect  
476 of metals exposure could be masked and remain undetected. By contrast, differences between  
477 males and females were negligible. Despite seasonal fluctuations of both MTs content and metal  
478 accumulation, the observed mild positive relationship with Cd, Hg and As and was in  
479 accordance to the well-known metal-scavenging function of these proteins. A negative  
480 relationship with ROS reinforced the notion of possible MTs involvement in antioxidative  
481 response. This study also stressed that variations of biochemical and chemical parameters  
482 measured in *N. norvegicus* digestive gland tissue could be linked to energy reserves, particularly

483 lipids. Thus, data presented here provide a solid starting point for future studies that should be  
484 aimed in particular to filling the knowledge gaps concerning MTs response to increased metal  
485 body burden.

486

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490

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## Figure captions

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

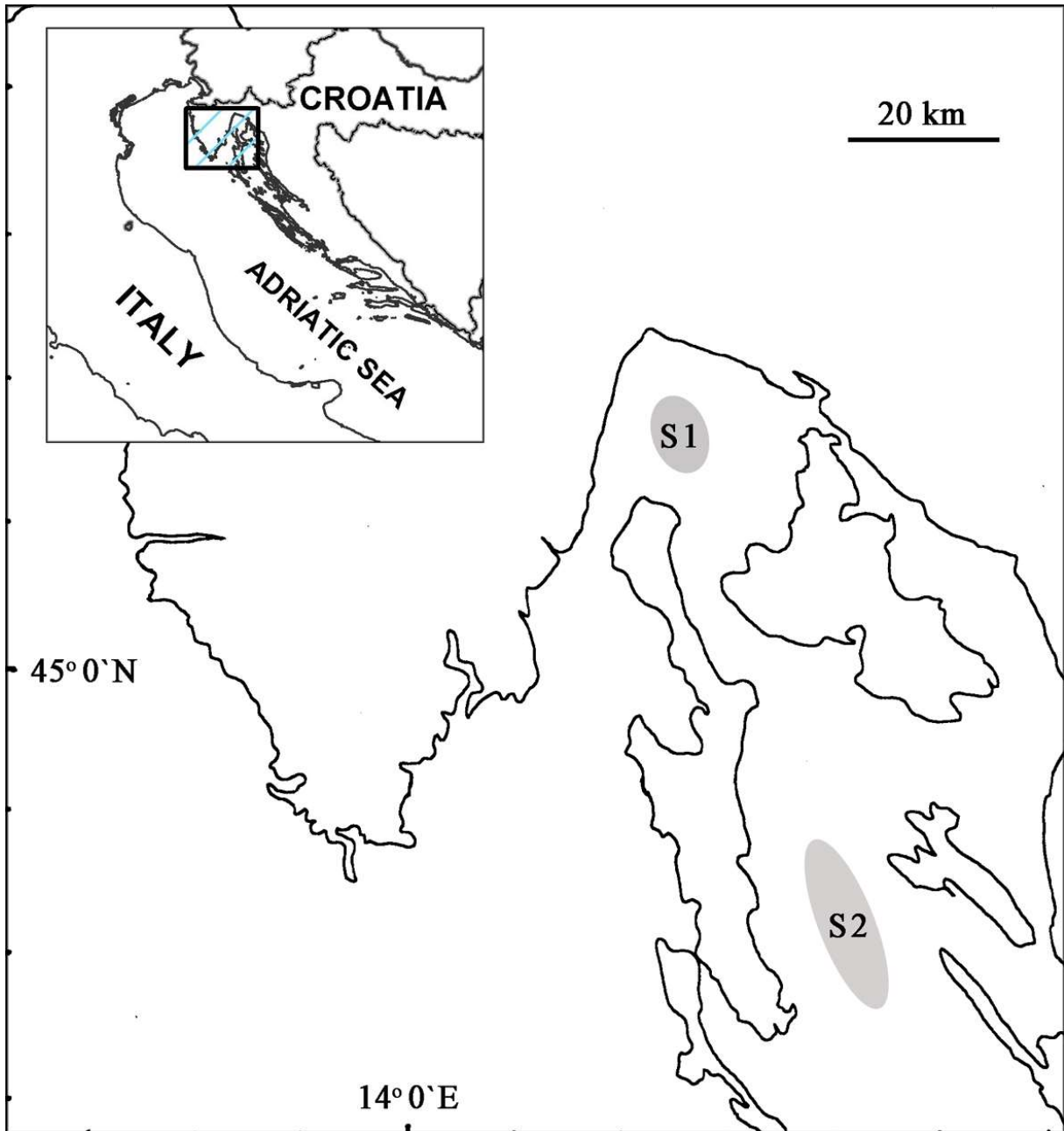
Fig 2. MTs content (nmol/g w.w.) in the digestive gland of small (<36mm) and large (>36 mm) *Nephrops norvegicus* from sites S1 and S2 in autumn (Aut) and spring (Spr). Square boxes indicate lower and upper quartile and whiskers represent minimum and maximum data values (1.5 interquartile range). Medians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

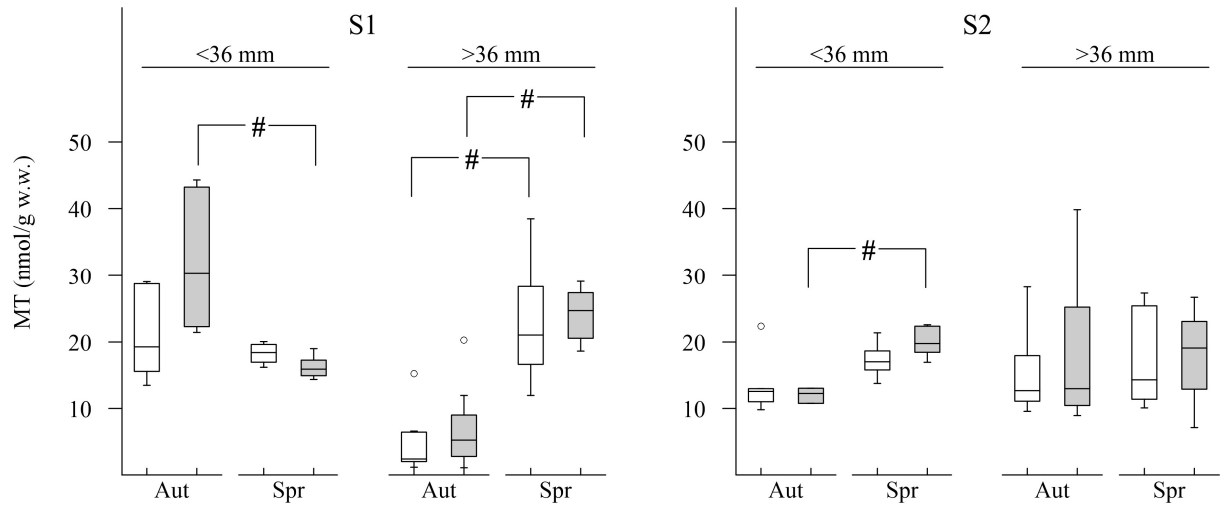
Fig 3. Concentrations of Cd, As, Hg, Pb, Cr, Mn ( $\mu\text{g/g}$  d.w.) in the digestive gland of small (<36mm) and large (>36 mm) *Nephrops norvegicus* from sites S1 and S2 in autumn (Aut) and spring (Spr). Square boxes indicate lower and upper quartile and whiskers represent minimum and maximum data 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; ■ – 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.





S1

S2

