BASELINE

Changes in the tissue concentrations of trace elements during the reproductive cycle of Noah's Ark shells (*Arca noae* Linnaeus, 1758)

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Abstract

Concentrations of 23 trace elements (TEs; essential: Co, Cu, Fe, Mn, Mo, Se, V, Zn; non-essential: Ag, Al, As, Ba, Cd, Cr, Cs, Li, Ni, Pb, Rb, Sr, Ti, Tl, U) in whole soft tissues of Noah's Ark shell (*Arca noae*) were determined monthly during one year (March 2013 - February 2014) at two sampling sites in the central part of the Eastern Adriatic Sea. Our aim was to detect the influence of reproductive cycle and changes in the environmental factors on the variabilities of TEs' contents. Higher concentrations of Pb, Ba, V, Mo, Mn and Fe were found at potentially contaminated site in Pašman channel, whereas higher concentrations of Tl, Ni, Li, Cr, Cd, Ti and Se were found at reference site in Nature Park Telašćica. Since several bioaccumulated TEs were associated to mean gonadal index, in TEs monitoring in *A. noae*, animal gonadal status has to be considered.

Keywords: Noah's Ark shell, *Arca noae*; the Adriatic Sea; trace metals/metalloids; mean gonadal index; seasonal variability

Due to sedentary lifestyle, bivalves are suitable indicator species of coastal pollution because their body contaminant burdens can be attributed to local pollution sources or locally polluted water and sediment. Bivalves give time-integrated information on the bioavailability of chemicals in the water column and sediments (Regoli, 1998), and are known to accumulate high levels of metals in their tissues and yet survive in contaminated environments (Goldberg et al., 1978; Cantillo, 1998). A number of factors ranging from physico-chemical, environmental to biological, have been shown to affect the uptake and accumulation of trace elements by marine organisms. The accumulation of metals by marine organisms can vary through the year. Such seasonal changes can be caused by a combination of parameters including growth, reproduction and moulting cycles, food supply and environmental conditions (e.g. temperature, irradiance, nitrogen and phosphorus concentrations) acting directly on uptake or indirectly on growth rates (e.g. Beyer et al., 2017; Brown and Depledge, 1998; Phillips and Rainbow, 1992). Fattorini et al. (2008) found that seasonal fluctuations in metal content of *Mytilus galloprovincialis* appeared mostly related to phytoplanktonic blooms and especially to reproductive cycle which exhibited a certain inter-annual shift of the gametogenesis period during their 5-year study. In another multi-annual study on *M. galloprovincialis* an inverse relationship of mussel condition index (CI) and temperature with trace metals levels and metallothionein was found, which indicated the influence of food abundance and mussel annual reproductive cycle (Ivanković et al., 2005). Temperature has been recognised as a key factor that affects physiology of mussels (e.g. *Mytilus* sp.), influencing specifically the kinetics of metal uptake and excretion in marine biota (Brown and Depledge, 1998).

Noah's Ark shell (*Arca noae* Linnaeus, 1758) lives attached by solid byssus on rocks on all types of bottom that contain hard substrate and occurs either as solitary individual or in clumps. It filters the seawater in order to perform respiration and feeding and consequently it

efficiently accumulates nutrients, but also various toxins from the environment. As a slow growing organism *A. noae* can obtain length between 70 and 90 mm and live up to 16 years (Peharda et al., 2002 and 2003), but in unexploited populations the largest specimens with 120.3 mm in length and 24 years old were observed as well (Puljas et al., 2015). Therefore, *A. noae* could be a very efficient accumulator of various contaminants including trace metals/metalloids and it could be potentially a good bioindicator of a long term metal contamination in the coastal marine ecosystems. Unlike other bivalve species, for which there are a lot of data on concentrations of metals (Eisler, 2010), studies on metal concentrations in soft tissue of *A. noae* are generally lacking. According to our knowledge only four short reports on concentrations of several trace metals in tissues (Ozretić et al., 1990; Cuculić et al., 2010; Ghribi et al., 2016; Papadopoulou, 1973) and one report on elemental concentrations in the shells of this bivalve species (Kobelja et al., 2016) have been published so far.

The current study area includes one of the most important natural habitats of *A. noae* in central part of the Eastern Adriatic Sea, the area of the Pašman channel. This region is under considerable anthropogenic pressure of municipal and industrial wastewaters and native organisms could be exposed to contaminants originating from different sources. The presence of metals in the environment is a result of their natural origin on one hand and of the human activities on the other, and such anthropogenic pollution in particular affects coastal ecosystems. Main sources of anthropogenic impact in coastal areas are the residence in the coastal zone, fisheries and aquaculture, shipping, tourism and land-use practices (agriculture, industrial development).

Total of 23 elements (TEs) were studied: essential – Co, Cu, Fe, Mn, Mo, Se, V, Zn and non-essential – Ag, Al, As, Ba, Cd, Cr, Cs, Li, Ni, Pb, Rb, Sr, Ti, Tl, U. These specific elements were chosen due to their physiological importance for marine organisms (e.g. Cu, Co, Fe, Mn, Mo, Se, V and Zn), their possible toxicity (e.g. Ag, Al, Cd, Cr, Ni, Pb), as well as due to their

frequent occurrence in marine environment as a consequence of leaching from vessel parts and antifouling paints, leaching from agricultural lands, occurrence in communal wastewater discharge, in the industrial wastes and in the medical wastes.

The intention of this work was to extend our knowledge on the natural variability of TEs in tissues of commercially important bivalve species *A. noae*, which is currently lacking, focusing on seasonal fluctuations. This study presented for the first time the observations on concentrations of TEs in *A. noae* during one year. Accordingly, the aim of this study was to detect if: i) the physiological changes (reproductive cycle) and changes in food availability (phytoplankton bloom) influenced the variability of TEs' contents in the whole soft tissues of *A. noae* during one year; ii) the higher accumulation of TEs could be found at the sampling site more exposed to anthropogenic influence.

The study area is located in the central part of the Eastern Adriatic Sea in the Republic of Croatia, where two sampling sites were selected, as shown in Fig. 1. First sampling site was selected within the Pašman channel (PC), that is spreading in the northwest–southeast direction between the coastline and the island Pašman and is the harvesting area of *A. noae*. The expected anthropogenic impact on PC sampling site (at the depth of 5 – 7 m) originated from several sources: 1) municipal wastewaters of the town Biograd (hospital and marina) in the southeast, which are released into the marine water without prior treatment; 2) municipal and industrial wastewaters of the city of Zadar (international harbour - passengers and transport of chemicals) in the northwest, which are released into the marine water after the treatment in the wastewater treatment plant by submarine outlet placed at the depth of 40 m and distance of approximately 400 m from the shore; 3) the sewerage of Pašman island settlements, which is released directly into the marine water, without any pre-treatment; 4) the vicinity of major marine transportation routes and nautical tourism (marina in Sukošan with

1400 berths as the largest Croatian marina). Second sampling site was selected in the Nature Park Telašćica (NP; at the depth of 10-14 m) located on the island Dugi otok as the site of expectedly lower anthropogenic impact.

The *A. noae* from natural populations were sampled once per month during 12 months (from March 2013 to February 2014) by SCUBA diving and transported in dark and cool containers to the laboratory within 6 hours. Samples were immediately washed with seawater and the whole soft tissue dissected using stainless steel equipment. Nine individuals per station per sampling were randomly selected for metal analysis and their whole soft tissues were combined, without prior depuration, in three composite samples composed of whole soft tissues of three individuals. The length (mean±SD at NP site: 8.4±0.6 cm, n= 108 and at PC site: 8.1±0.6 cm, n=108) and the total weight (mean±SD at NP site: 58.8±12.5 g, n=108 and at PC site: 60.7±14.6 g, n=108) of 108 individuals selected at each sampling site for metal analysis were recorded during the study. The bivalve sampling was complemented by seawater temperature records measured near the sea bottom (at the same depth where bivalves were sampled).

Previous analyses of chemical data in the Pašman channel have shown that there were neither abnormally high concentrations of the nutrients nor low concentrations of the dissolved oxygen, meaning that eutrophication was not in the progress in this area (Vilibić et al., 1999). On the basis of the primary production and benthos investigations, the ventilation of the region seemed to be optimal (Vilibić et al., 1999).

Condition index was defined as the ratio between tissue dry weight and shell length: CI = (dry flesh weight, g / shell lenght, cm) × 100 (Lundebye et al., 1997). The whole soft tissues were dried using lyphilizer FreeZone® 2.5 L (Labconco, USA) for 72h (T = -45 – -49 °C; p = 12 – 16 Pa).

Total of 285 individuals collected from April 2013 to February 2014 at two sampling sites were processed for histological analysis. Gonad samples were fixed in 4% formaldehyde and stored for histological analysis. Each sample was dehydrated in ethanol, embedded in paraffin (Histowax, LeicaR), sectioned at 7 μm and stained using hematoxylin and counterstained with eosin. Histological sections were examined at 100 and 400× magnification, sexed and assigned to one of six qualitative categories of reproductive stages according to Walker and Power (2004) and adopted by Peharda et al. (2006) for *A. noae*. Since 95.3% of collected individuals were females and according to fact that accumulations of trace elements can vary between male and female individuals during gametogenesis (Fitzpatrick et al., 2008; Meistertzheim et al., 2009; Richir and Gobert, 2014, 2016), only female individuals were used in the analysis. In addition, a mean gonadal index (MGI) was calculated for each sampling month and site. This value was obtained by multiplying the number of individuals from each development stage by the numerical ranking of that stage, and dividing the result by the total number of individuals in each sampling month (Gosling, 2003). Qualitative categories of reproductive stages and corresponding numerical values used for calculation of mean gonadal index were following: inactive (0), early active (3), late active (4), mature (5), partially spawned (2) and spent (1).

Wet digestions of *A. noae* soft tissues were performed in duplicate by weighing approximately 100 mg homogenized freeze-dried (the average water content in soft tissue was ~80%) whole soft tissues of *A. noae* with a mixture of 5 mL HNO_3 (65% Suprapur, Merck) and 1 mL H_2O_2 (30% Suprapur, Merck) in a tightly capped Teflon vials by heating at 80°C for 3h (mild digestion using single-step simulation of hot-plate method). Digested samples were quantitatively transferred into the volumetric flasks of 50 mL, filled to the mark with

deionised water (Milli-Q, 18.2 MΩcm) and stored in polyethylene bottles until analyses. Procedural blank samples were also prepared and analysed.

Analyses of metals in digested soft tissues of *A. noae* were performed using high resolution inductively coupled plasma mass spectrometer (HR ICP-MS, Element 2, Thermo Finnigan) using an autosampler (ASX 510, Cetac Technologies) and sample introduction kit consisting of SeaSpray nebulizer and cyclonic spray chamber Twister. Prior to measurements indium (Fluka) was added to all samples as internal standard (1 μg L^{-1}). Measurements of ^{7}Li, ^{82}Se, ^{85}Rb, ^{98}Mo, ^{109}Ag, ^{111}Cd, ^{133}Cs, ^{205}Tl, ^{208}Pb and ^{238}U were operated in low-resolution mode, whereas ^{27}Al, ^{47}Ti, ^{51}V, ^{52}Cr, ^{55}Mn, ^{56}Fe, ^{59}Co, ^{60}Ni, ^{63}Cu, ^{66}Zn, ^{86}Sr and ^{138}Ba were measured in medium-resolution mode and ^{75}As in high-resolution mode. External calibration was performed using standards prepared in 2% HNO_3 (Suprapur, Merck) by appropriate dilutions of 100 mg L^{-1} multielement stock standard solution (Analytika).

Standard reference materials IAEA-452 (scallop tissue, International Atomic Energy Agency Reference Materials), SRM 1566a (oyster tissue, standard reference material, distributed by U.S. Department of Commerce, National Bureau of Standards, Gaithersburg) and CRM 278R (mussel tissue reference material, Environment Institute, Ispra, Italy and Institute for Reference Materials and Measurements, Geel, Belgium) for trace elements were analysed for quality control check for the determination of metals in the *A. noae* tissues (Table 1). In this study the range of good agreement with the reference material was set at ±30% for the soft tissue of *A. noae*. All measured metal concentrations were within this range except Zn concentrations. Therefore, Zn concentrations were recalculated using the respective correction factor.

Data analysis was carried out using the Statistica 8 (StatSoft Inc.) software package. The one-way ANOVA was applied for testing the effect of sampling month on TEs concentrations and

CI of *A. noae*. Differences in concentrations of TEs between two sampling sites were tested using Mann-Whitney U test. Differences in CI between two sites were tested using t-test. Pearson's correlations analysis was applied to the data to determine the degree of association between TEs concentrations and MGI, as well as associations between all measured TEs. The significance level for all applied tests and correlations was set at $p < 0.05$.

Values of MGI, CI and seawater temperatures are presented in Fig. 2 and the results of histological analysis of gonads presented as relative frequencies of different development phases are shown in Fig. 3. It was evident that *A. noae* in this area of the Adriatic Sea had only one annual spawning peak, with minima in MGI occurring in September at NP site and in August-September at PC site, respectively (Fig. 2). There was almost no gonad inactivity period, and new female gametogenesis cycle began as early as September (Fig. 3). From February to April (spring) female gametes were predominantly in the stage of late development and maturity was reached already in May (late spring) and lasted till August (Fig. 3). Spawning started in July and occurred during August and September at PC site, while it started later in August and occurred mainly in September at NP site. Gametogenic development started immediately after spawning. There was no correlation between MGI and temperature at both sampling sites ($r = -0.0195$, $p = 0.914$ at NP and $r = -0.0248$, $p = 0.891$ at PC).

Condition index and MGI were significantly correlated at both sampling sites ($r = 0.648$, $p = 0.00005$ at NP site and $r = 0.575$, $p = 0.00047$ at PC site). There was a significant effect of temporal variability on CI in *A. noae* over the sampling year at both sampling sites (NP site: $F = 2.366$, $p = 0.0377$; PC site: $F = 2.308$, $p = 0.042$). In the first six months of the study high similarity in CI and MGI patterns was observed. Later on, a small peak in CI that occurred in October coincided with slight increase in seawater temperature (Fig. 2). Even though the CI

values at two sampling sites showed similar patterns throughout the year (Fig. 2) the difference in CI between NP and PC sites was significant with higher CI values noted at NP site ($p = 0.002$).

To get an overview of levels of 23 TEs studied in the soft tissue of *A. noae*, ranges of measured values and the median values of all measured samples from reference (NP) and contaminated (PC) site during one year are presented in Table 2. Concentrations measured in soft tissues increased in the following order: Tl < Cs < U < Co < Pb < Ni < Li < Cr < Ba < V < Cd < Ti < Mo < Ag < Cu < Rb < Se < Mn < Sr < As < Al < Zn < Fe covering the range from several $ng/g_{(dry\ weight)}$ to several hundred $\mu g/g_{(dry\ weight)}$ (i.e. range of five orders of magnitude). Significant differences in yearly medians of TE concentrations between two sampling sites were observed for Tl, Pb, Ni, Li, Cr, Ba, V, Cd, Ti, Mo, Se, Mn, Fe ($p < 0.05$, Mann-Whitney U test; Table 2), with higher concentrations of Pb, Ba, V, Mo, Mn and Fe at potentially contaminated site in Pašman channel (PC) and with higher concentrations of Tl, Ni, Li, Cr, Cd, Ti and Se at reference site in Nature Park Telašćica (NP).

The concentrations of majority of analysed TEs showed significant temporal variability at NP site, whereas the same was observed for only half of analysed TEs at PC site. The variability of TEs concentrations measured once per month during one year was significant (ANOVA, $p < 0.05$) at NP sampling site for all measured elements except Cu, Pb, Sr, Zn, As and Rb (Table 3). Similarly to NP, at PC site variability was also not significant for Cu, Pb, Zn and As, and additional seven TEs (Mn, Se, Ag, Cr, Ni, Li, Al) showed no significant variability as well (Table 3).

Mean values and standard deviations of 23 analysed TEs during the reproductive cycle of *A. noae* at two sampling sites are presented in Figs. 4 – 6. Studied TEs could be classified in

three groups according to their correlation to MGI: i) negatively correlated TEs (Ba, Cd, Co, Fe, Li, Mn, Ni, Se and V; Fig. 4); ii) positively correlated TEs (As, Rb, Tl and Zn; Fig. 5); iii) non-correlated TEs (Ag, Al, Cr, Cu, Cs, Mo, Pb, Sr, Ti and U; Fig. 6). The results from our study showed that first group of 9 out of 23 TEs analysed in *A. noae* (Ba, Cd, Co, Fe, Li, Mn, Ni, Se and V; Fig. 4) followed the pattern with lower TEs levels in the late spring and early summer, and higher levels in the period after bivalve spawning. Second group of TEs in *A. noae* (As, Rb, Tl and Zn; Fig. 5) showed the opposite sinusoidal pattern, i.e. a higher level of metal during the period of intensive gonadal and somatic growth in late spring and early summer (the pre-spawning period) and lower in the late summer and autumn after bivalve spawning when soft tissue weight minimum occurred. Third group of TEs (Ag, Al, Cr, Cu, Cs, Mo, Pb, Sr, Ti and U; Fig. 6) either at one or at both sampling locations, partially followed or generally did not follow the aforementioned sinusoidal patterns of seasonal variations in metal levels. Another feature of TEs' patterns in this group was presence of sharp peaks noticed for Al, Cs and Ti in September 2103, November 2013 and January 2014 (Fig. 6) and for Ag and Cu in November 2013 and January 2014 at NP site, but not at PC site (Fig. 6).

In general, reproductive and condition indices observed in this study were in accordance with previous studies reported for *A. noae* in the Adriatic Sea. The pattern in development stages of *A. noae* recorded in this study (Fig. 3) was similar to the pattern already observed in the populations inhabiting Mali Ston Bay in the south Adriatic Sea which indicated that only one spawning peak occurred each year of that study (Peharda et al., 2006). Although MGI appeared to increase with rising temperature and decrease with falling temperature (Fig. 2) no correlation between MGI and temperature was found, which was in accordance with previous observations of Peharda et al. (2006) who found no correlation between MGI and environmental conditions in Mali Ston Bay. In this study a significant temporal variability in

CI was observed. Any increase or decrease in the condition index (Fig. 2) depends on the balance between the rates of food assimilation and of catabolism (metabolic breakdown of complex organic molecules with liberation of energy). The CI therefore responds to anthropogenic stress, but also to the use of metabolic reserves when accumulating gametes (Phillips and Rainbow, 1992). The pattern of CI observed in this study was in accordance with CIs of *A. noae* from Pašman channel recorded by Župan et al. (2014). In that study CI values were greatest from April to June 2008, which corresponded to the period preceding spawning, while minimal values of CI were recorded in November 2008 and December 2008, when low concentrations of chlorophyll *a* were also recorded (Župan et al., 2014).

With regard to the differences in the yearly medians of TEs concentrations in *A. noae* between two sampling sites (Table 2), we can relate higher Ni concentration at NP site to a slightly increased Ni concentrations in sediments that were previously found at that site (Ivanković et al., 2016). It was probably related to the boating activities and leaching from the chromate coated parts of boats and yachts, as it was already observed by Mihelčić et al. (2010) at the anchorage points in NP aquatorium, which is visited by organised tourist boats as well as by individual nautical vessels. In the previous study significantly higher concentration of Ni was found in shells of *A. noae* sampled in Nature park Telašćica, while Mn concentration was higher in samples from Pašman (Kobelja et al., 2016), which is in agreement with our findings of Ni and Mn concentrations in soft tissues of *A. noae* (Table 2). Concentrations of TEs in the whole soft tissues of *A. noae* obtained in this work were generally in the same concentration ranges as those reported for the soft tissue of *A. noae* from relatively unpolluted areas of the Island Mljet in the southern Adriatic Sea (Cuculić et al., 2010) and the Island Susak in the northern Adriatic Sea (Ozretić et al., 1990) (Table 2). Metal concentrations reported for *A. noae* from Bizerta Lagoon (Tunisia) (Ghribi et al., 2016) were higher for Pb, Zn, Fe, similar for Cu and lower for Cd compared to *A. noae* from the Adriatic Sea (Table 2).

Exceptionally high concentration of Mo was reported by Papadopoulou (1973) for soft tissue of *A. noae* bivalves collected from coastal water of Greece which was 10 times higher than maximal value of Mo measured in this study (Table 2).

Concerning the obtained values of tissue concentrations variable standard deviations observed in Figs. 4 – 6 were expected although the individuals analysed in this study were of comparable sizes/ages, because bivalve growth depends on food availability and energy used by an organism to survive. Differences in growth would lead to differences in weight/size and it would be expected that even animals of similar size/age would have a range of different weights and some variation in trace metal concentrations. The reason for using adult mature bivalves in this study was based on the findings of Robinson et al. (2005) who showed that younger animals generally have more variable trace metal concentrations, which is often attributed to highly variable metabolic rates in younger, non-mature animals. These results indicated that in order to reduce intrinsic variability in trace metal concentrations, older mature animals should be selected. However, with mature bivalves there are complications because of the effects of spawning, i.e. sudden loss of trace metals or body weight, and thus the reproductive cycle does contribute to the variation in trace metal concentrations. In this study we observed that Ba, Cd, Co, Fe, Li, Mn, Ni, Se and V (Fig. 4) were negatively correlated to MGI and they followed the sinusoidal curve pattern suggested by Borchardt et al. (1988), i.e. a lower level of metal in the late spring and early summer due to the rapid increases in biomass caused by growth of gonadal and somatic tissues (an effect of tissue dilution) and a higher level in the period after bivalve spawning. This pattern would imply that the incorporation of the aforementioned TEs in gametes was lower than in non-gonadal tissues. However, a small deviation from the sinusoidal pattern in TE concentration was observed for Cd, Co and Fe at PC site compared to NP site (Fig. 4). Although for both sampling sites the correlation was negative, several sharp peaks were noticed at PC site (e.g.

September and December 2013 for Cd, December 2103 for Co, Fe and Mn; Fig. 4) indicating that some environmental or possibly anthropogenic factors were superimposed over the physiological variability.

Second group of TEs positively correlated with MGI (As, Rb, Tl and Zn; Fig. 5) and they showed the opposite sinusoidal pattern. This pattern would imply that concentrations of these TEs were higher in gametes than in non-gonadal tissues, because after releasing gametes their concentrations in the whole soft tissue decreased.

Third group of TEs (Ag, Al, Cr, Cu, Cs, Mo, Pb, Sr, Ti and U; Fig. 6) was not correlated to MGI and they expressed deviation from the sinusoidal pattern suggested by Borchardt et al. (1988). This deviation from the sinusoidal patterns may be due to different anthropogenic input in the water at certain location as well as changes in environmental parameters such as salinity, temperature, oxygen concentrations or increased dissolved organic carbon during the sampling period. At PC sampling site variations in salinity were reported, due to the presence of submarine freshwater springs that can be active in winter season with increased atmospheric precipitation typical for the Mediterranean climatological area (Župan et al., 2014). Additionally, since the settlements at the island of Pašman are still not connected to the sewerage system, at PC site there was a possibility of outflow of sewage waters from the cesspits through the porous karst terrain to the nearby sea. Thus, for example, levels of Pb at the NP site almost completely followed the seasonal pattern (i.e. sinusoidal curve) suggested by Borchardt et al. (1988), while at the PC site its levels were similar in both reproductive periods. Thus, the absence of typical seasonal variations for Pb on PC location may be attributed to the higher anthropogenic availability of Pb on this location (Ivanković et al. 2016). Additionally, the presence of sharp peaks noticed for some TEs (Al, Cs and Ti in September 2103, November 2013 and January 2014, Fig. 6; Ag and Cu in November 2013

and January 2014 at NP site, Fig. 6) imply that some environmental or anthropogenic factors could have influenced the bioaccumulation in that specific time and location.

Since no reports on variability of TEs related to reproductive cycle in *A. noae* were available in the literature, we compared our results with some other bivalve species commonly investigated (e.g. mussels and oysters). Similar TEs behaviour was observed in the study of rope-grown *M. galloprovincialis* from the Diane pond (east Corsica), where TEs were analysed before and after the spawning and it was found that Al, Fe, Cr, Mn, Ni, Sn, Mo, Be and Bi were less concentrated prior to spawning (effect of a tissue dilution during the gametogenesis); V and Ag were more concentrated prior to spawning (they were sufficiently abundant to mask the dilution effect due to the gametogenesis); Se, Cd, Sb, As and Pb showed similar tissue concentrations during both reproductive stages but with contents a little higher in one occasion prior to spawning (they were more accumulated prior to spawning and displayed similar concentrations with mussels having spawned, although they were also diluted during gametogenesis); Co, Cu and Zn displayed lower concentrations prior to spawning and similar contents during both physiological stages (Richir and Gobert, 2014). In oyster *Saccostrea glomerata* Cd and Se were significantly higher in the gonadal tissues, but Zn was found in significantly higher concentrations in the non-gonadal tissues, while Pb and Cu concentrations were not different between tissue types (Robinson et al., 2005). Studies of Cu, Zn, Cd and Ni concentrations in *Mytilus edulis* found higher concentrations in the non-gonadal tissues than gonadal tissues (LaTouche and Mix, 1982; Lobel and Wright, 1982). It was concluded that seasonal dynamics of Zn in *M. edulis* can be explained in terms of somatic growth rather than growth of germinal tissue (Lobel and Wright, 1982). Contrary, Martinčić et al. (1984) found higher trace metal concentrations of Zn in gonadal tissues compared to total and soft edible part of Mediterranean mussel (*M. galloprovincialis*) and oysters (*Ostrea*

edulis) from the same location with the same physicochemical conditions. Increased concentrations of Cu and Cd in gonads of oysters and Pb in gonads of mussels were found as well (Martinčić et al., 1984).

Since uptake and loss of TEs in marine bivalves are greatly affected by changing body weights related to reproduction cycle and condition, they have to be closely considered when reporting on levels of trace elements in bivalves, especially in terms of concentration.

Pearson's correlation analysis between each and every measured TEs concentration was performed in order to detect possible coaccumulation pattern of TEs in *A. noae*. Similar study was made by Robinson et al. (2005) who found a positive correlation between Cu, Zn and Se in the whole animal body, which indicated that the coaccumulation of some trace elements may be a natural accumulation strategy of oysters *S. glomerata*. In the current study we found significant (all $p < 0.05$) positive correlations between Co, Fe, Mn, Ni and Pb at NP sampling site ($r_{Co:Fe} = 0.755$, $r_{Co:Mn} = 0.691$, $r_{Co:Ni} = 0.679$, $r_{Co:Pb} = 0.641$, $r_{Fe:Mn} = 0.616$, $r_{Fe:Ni} = 0.672$, $r_{Fe:Pb} = 0.799$, $r_{Mn:Ni} = 0.887$, $r_{Mn:Pb} = 0.656$, $r_{Ni:Pb} = 0.837$). According to their chemical properties Fe, Mn, Co and Ni are transition metal elements. They could be found jointly in the marine sediments where Co^{2+}, Ni^{2+} and Pb^{2+} cations are adsorbed on Fe- and Mn-oxides and oxyhydroxides (Ansari et al., 2004). These elements can become biologically available under certain environmental conditions (e.g. intensified reductive conditions in the sediments) when dissolution of oxides commences causing subsequent release of adsorbed metals and results in concentration increase of their dissolved forms (remobilization). Thus, in such occasions increased bioavailablity of TEs (Co, Fe, Mn, Ni and Pb) could lead to their increased bioaccumulation in *A. noae*. Since such association was observed only at NP site and not at PC site, we could assume that sediment characteristics, geochemical fractions and/or conditions in the sediments differed between two sampling sites. However, we cannot be

certain of this because bioavailability is influenced by both physico-chemical properties of metals and sediments and by biological strategies of the organisms involved (Ansari et al., 2004). Contrary, a rather strong association between TEs was found at both sampling sites between Al, Fe and Ti (NP: $r_{Al:Fe}$ = 0.793, $r_{Al:Ti}$ = 0.967, $r_{Fe:Ti}$ = 0.848; PC: $r_{Al:Fe}$ = 0.685, $r_{Al:Ti}$ = 0.780, $r_{Fe:Ti}$ = 0.715). These associations were interesting because the coaccumulation of Fe, Al and Ti in *A. noae* reflected the fact that Al, Ti and Fe often occur together as the major lithogeneous contributors to the marine sediment (Dolenec et al., 1998). Such lithogeneous components of the sediments at NP and PC sites were apparently present in relatively shallow coastal habitat where bivalves were sampled. Also, cross-correlation coefficients computed for all metal pairs in sediment cores from bottom sediments in Southern Baltic showed highly significant association of Ni, Ti, Co, Th, K and partly Mg with Al and Fe (Szefer, 1990), which corresponded to strong significant associations between Ti, Al and Fe in tissues of *A. noae* found in our study. Lead and Cs also showed strong association at both sites (NP: $r_{Pb:Cs}$ = 0.750; PC: $r_{Pb:Cs}$ = 0.747) and in this case coaccumulation reflected their similar entry pathway to the marine environment from atmospheric deposition (aeolian source). The strongest association was observed between Ba and V concentrations, also at both sites (NP: $r_{Ba:V}$ = 0.929, PC: $r_{Ba:V}$ = 0.936, both $p < 0.001$), and based on their concentrations patterns (Fig. 4) we can assume that similar physiological and/or environmental factors affected bioaccumulation of both elements. Observed high increase in their concentrations in September after the spawning could be the consequence of extremely low concentrations of V and Ba in gonadal tissues. This is in agreement with previous findings in scallops (*Pecten maxiumus*) exposed to V as a consequence of the "Erika" oil spill, when this element was stored in new growth bands of the bivalve shell, i.e. non-metabolically active tissue (Chiffoleau et al., 2004). Barium is also deposited in shells and it has been verified in both the field and laboratory, that background Ba/Ca ratios in *M. edulis* shells were directly related to

the Ba/Ca ratios of the water in which they grew, and that the nearly ubiquitous Ba/Ca peaks found in bivalve shells were related to phytoplankton blooms in a complex manner (Gillikin et al., 2006). Besides Ba:Ca ratios also Mo:Ca ratios have been investigated in shells of the tropical scallop *Comptopallium radula* and the synchronism of Ba:Ca and Mo:Ca peaks discovered in their shells suggested that the process or processes responsible for their temporal variations might share, to some extent, a common origin. For the formation of Ba:Ca peaks the most plausible hypothesis was the ingestion of diatom cells enriched in Ba (adsorbed on iron oxyhydroxides associated with the frustules), while Mo:Ca peaks would probably come from the ingestion of phytoplankton cells containing nitrate reductase requiring high levels of Mo for the enzyme activity (Thébault et al., 2009). In our study the pattern of Mo concentrations (Fig. 6) determined in soft tissues of *A. noae* was closely related to the pattern of Ba and V concentrations (Fig. 4), especially in the period from July to December 2013, which corroborated the aforementioned behaviour of Ba, Mo and V in shells of bivalves.

Comparing the correlations between all TEs at both sampling sites, it became obvious that As was only associated with Rb (NP: $r_{As:Rb}$ = 0.694; PC: $r_{As:Rb}$ = 0.717). Fattorini et al. (2008) already observed in their multi-annual study that As was not correlated with gonadic development, neither with other elements. Arsenic had different seasonal cycle compared to other studied elements. The same authors found that arsenobetaine and arsenocholine (organic forms of As) were always the predominant forms (up to 85% of total As), while a significant increase of dimethylarsine and trimethylarsine oxide in spring (24% of total arsenic) might reflect the effect of phytoplanktonic bloom on both geochemistry and trophic transfer of this element (Fattorini et al., 2008).

These findings would imply that bioaccumulation in *A. noae* was influenced by biological factors, as well as by inherent chemical properties of TEs and their physico-chemical forms in

different environmental compartments (i.e. controlled by biogeochemical processes in marine systems).

This study showed that phases of reproduction cycle and condition of Noah's Ark shell *A. noae* have to be closely considered when reporting on the concentrations of TEs in this organism. Gametogenesis diluted concentrations of several TEs due to the important tissue production prior to spawning. However, in the same period the concentrations of several other TEs were increased. Based on these observations, in case of monitoring of TEs in *A. noae*, the gonadal status of animals should be taken into account.

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Declarations of interest: none

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Figure captions

Figure 1. Study areas in the central Adriatic Sea in Croatia with indicated sampling locations (PC sampling site in the Pašman channel and NP sampling site in the Telašćica Nature Park)

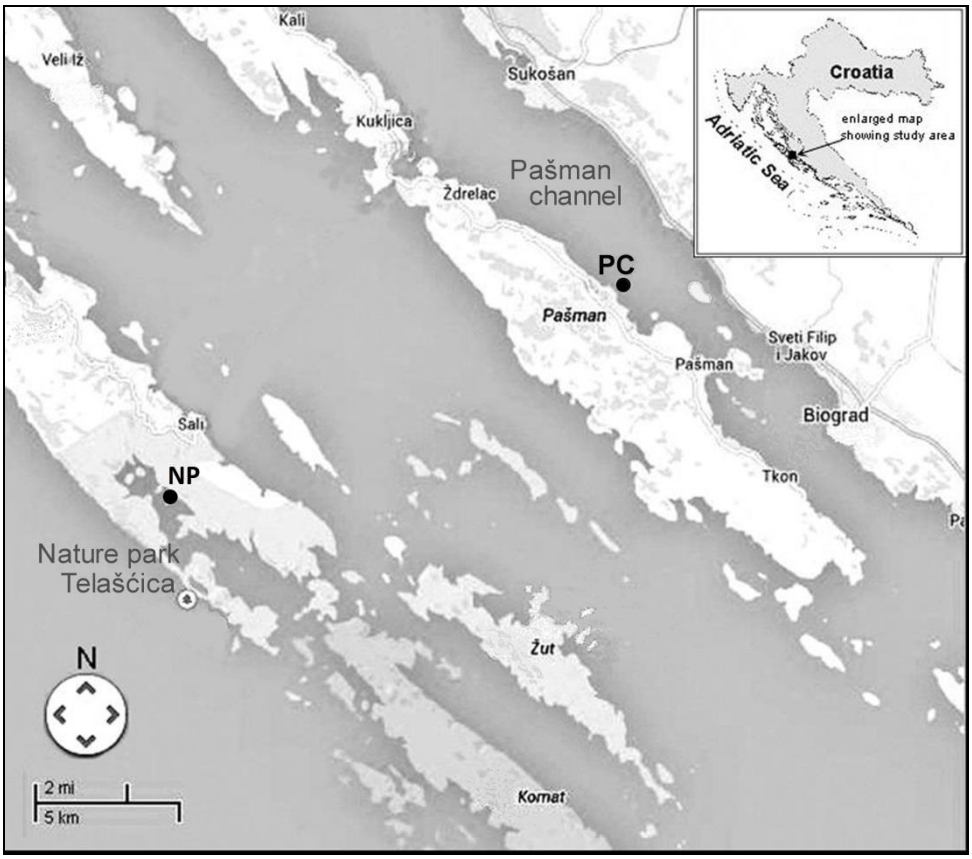
Figure 2. Values of mean gonadal index (MGI), condition index (CI; mean values and standard deviations are presented, n = 9) and seawater temperatures at two studied sampling sites NP – Nature Park Telašćica and PC – Pašman channel.

Figure 3. Relative frequency of each development phase of *A. noae* females recorded during 12 moths from April 2013 to February 2014 at two sampling sites NP – Nature Park Telašćica and PC – Pašman channel.

Figure 4. Mean values and standard deviations (n = 3) of concentrations of the TEs measured in the soft tissues of *A. noae* from March 2013 to February 2014, that are negatively correlated with MGI at both sampling sites (NP and PC) with negative Pearson's correlation coefficient higher than 0.3 (significant correlations are marked in bold with asterisk, $p < 0.05$). Sampling sites: NP – black circles and solid line, PC – grey circles and dashed line.

Figure 5. Mean values and standard deviations (n = 3) of concentrations of the TEs measured in the soft tissues of *A. noae* from March 2013 till February 2014, that are positively correlated with MGI (Pearson's correlation coefficients are indicated at each graph for both sampling sites; significant correlations are marked in bold with asterisk, $p < 0.05$). Sampling sites: NP – black circles and solid line, PC – grey circles and dashed line.

Figure 6. Mean values and standard deviations (n = 3) of concentrations of the TEs measured in the soft tissues of *A. noae* from March 2013 till February 2014, that are not correlated with MGI at both sampling sites. Sampling sites: NP – black circles and solid line, PC – grey circles and dashed line.



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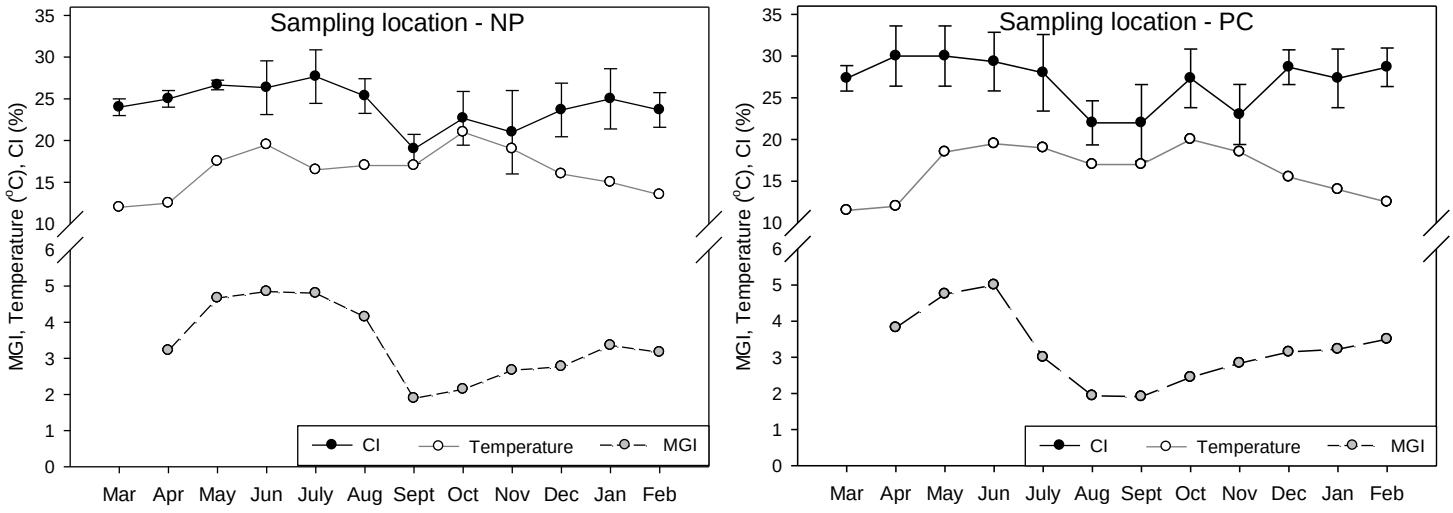

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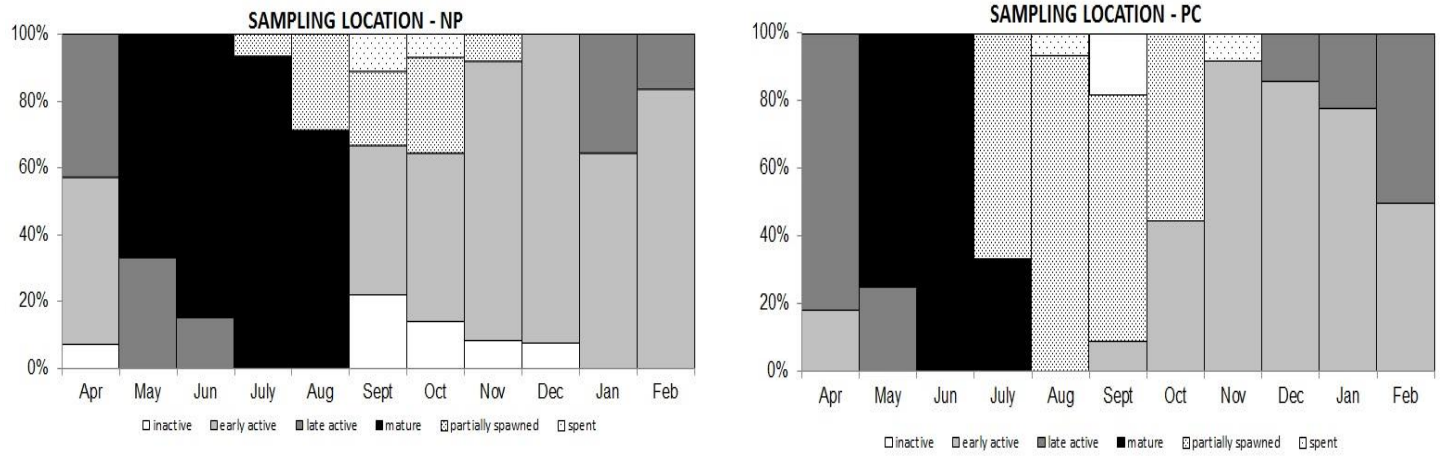

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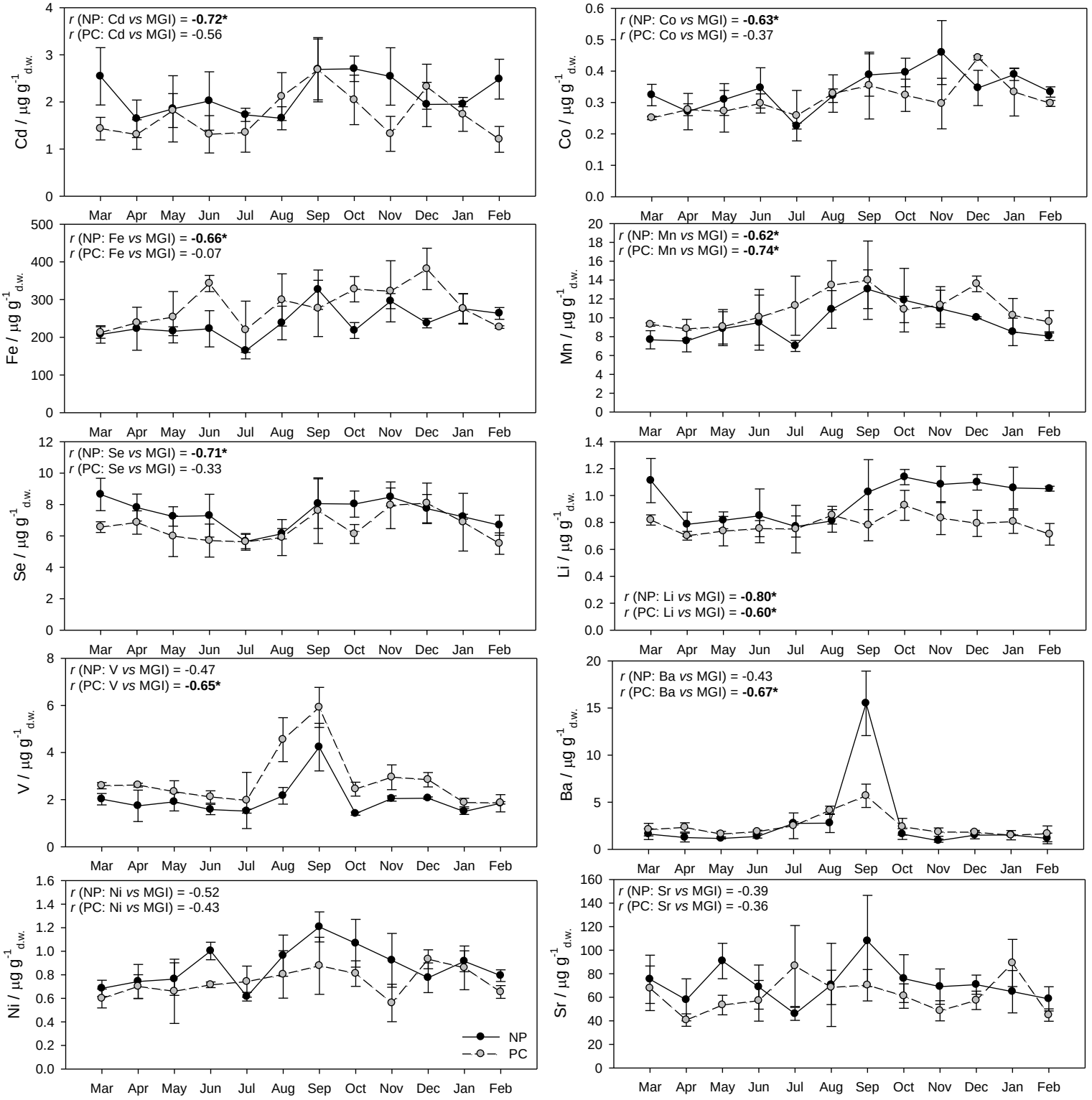
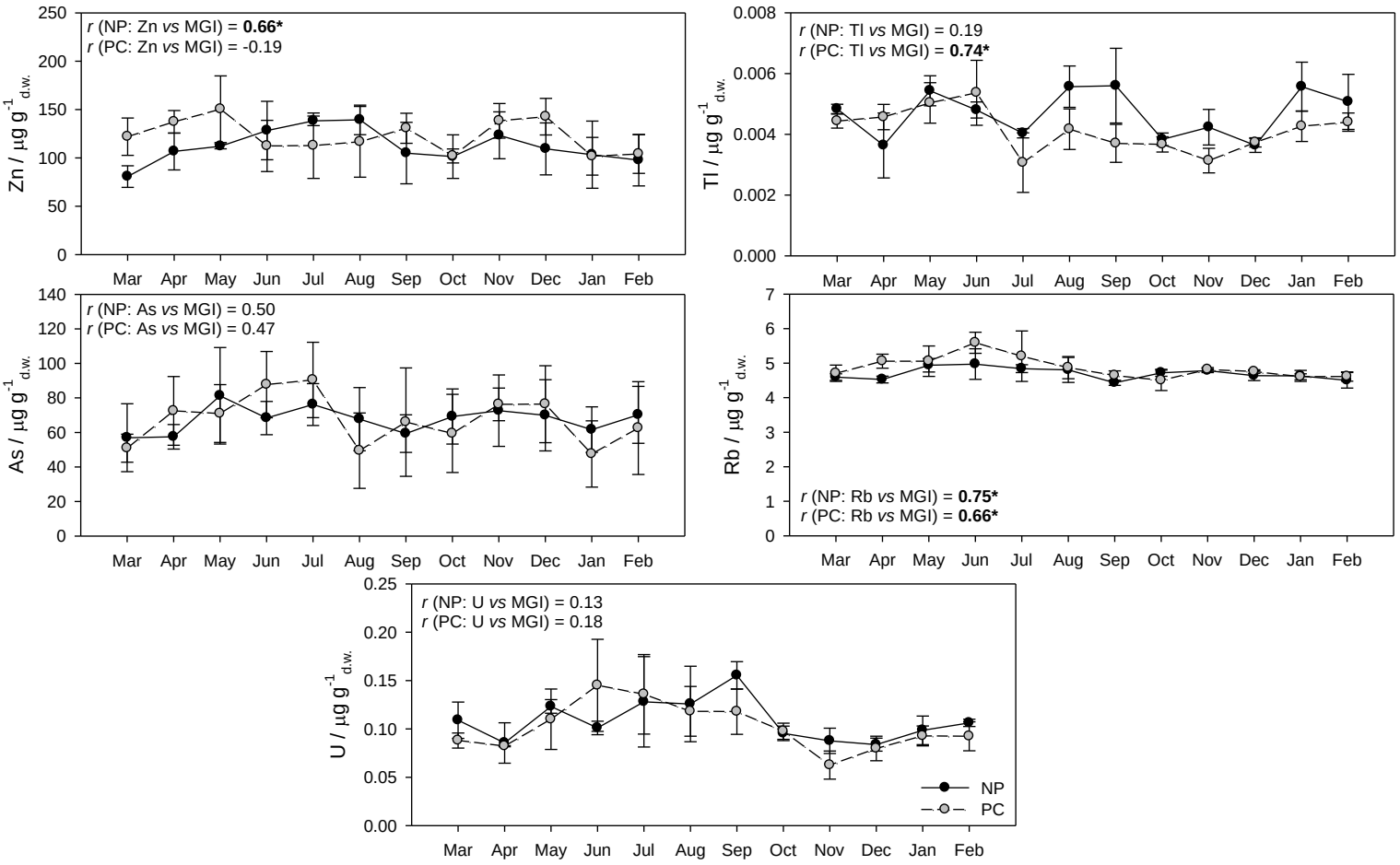


Figure 4. Mean values and standard deviations (N=3) of concentrations of the TEs measured in the soft tissues of *A. noae* from March 2013 to February 2014, that are negatively correlated with MGI at both sampling sites (NP and PC) with Pearson's correlation coefficient higher than -0.3 (significant correlations are marked in bold with asterisk, $p < 0.05$). Sampling sites: NP – black circles and solid line, PC – grey circles and dashed line.



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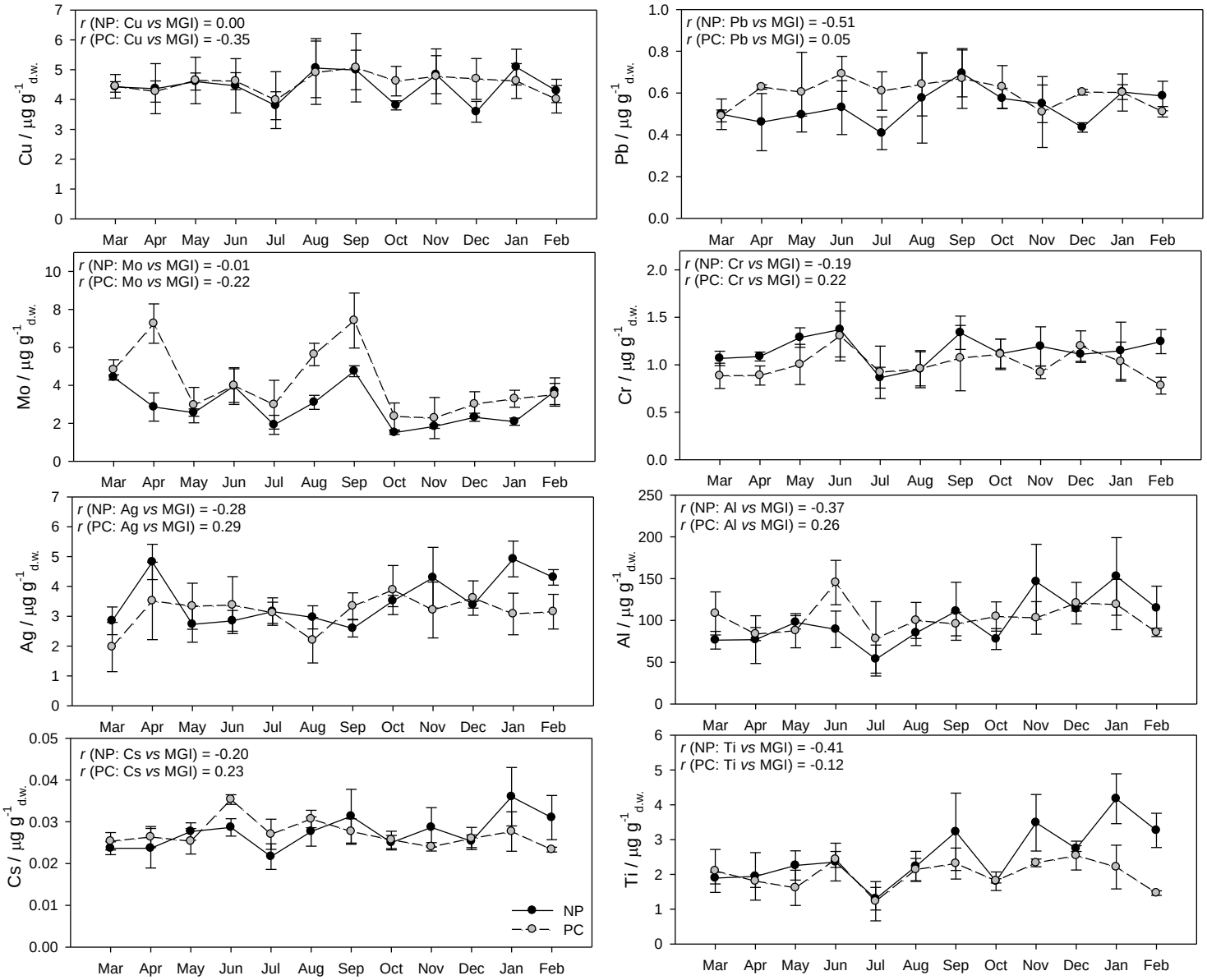

Figure 6. Mean values and standard deviations (N=3) of concentrations of the TEs measured in the soft tissues of *A. noae* from March 2013 till February 2014, that have weak positive or negative correlations with MGI (correlations are not significant at both sampling sites NP and PC). Sampling sites: NP – black circles and solid line, PC – grey circles and dashed line.

Table 1. Quality control for metal determination in shellfish tissue was based on following reference materials: IAEA-452 (trace elements in scallop tissue, standard reference material, distributed by International Atomic Energy Agency Reference Materials, certified values and the standard deviations (SD) are presented), SRM 1566a (oyster tissue, standard reference material, distributed by U.S. Department of Commerce, National Bureau of Standards, Gaithersburg; the uncertainty is obtained from 95% prediction interval) and CRM 278R (mussel tissue reference material, prepared by Environment Institute, Ispra, Italy and Institute for Reference Materials and Measurements, Geel, Belgium, certified values and half-width of the 95% confidence interval are presented). Measured values for IAEA-452 (n = 6), for SRM 1566a (n = 9) and for CRM 278R (n = 6) are presented as means and SD.

	Metal concentrations (mg/kg d.w.)	
	Reference values	Measured values
Ag[a]	1.68 ± 0.15	1.94 ± 0.23
As[b]	17.5 ± 2.2	14.5 ± 0.7
Cd[b]	29.6 ± 3.7	25.3 ± 0.8
Co[b]	1.62 ± 0.20	1.62 ± 0.06
Cr[c]	0.78 ± 0.06	0.63 ± 0.08
Cu[b]	10.8 ± 1.3	8.94 ± 0.34
Fe[b]	1020 ± 130	798 ± 21
Li[b]	2.01 ± 0.25	2.02 ± 0.12
Mn[b]	273 ± 34	268 ± 15
Ni[a]	2.25 ± 0.44	1.93 ± 1.35
Pb[a]	0.371 ± 0.014	0.340 ± 0.045
Rb[b]	7.85 ± 0.98	5.47 ± 0.15
Sb[b]	0.100 ± 0.013	0.093 ± 0.011
Se[a]	2.21 ± 0.24	2.64 ± 0.56
Sr[b]	82.9 ± 10.3	85.6 ± 4.0
V[b]	6.36 ± 0.79	6.75 ± 0.19
Zn[b]	166 ± 21	113 ± 5

Reference material: [a] SRM 1566a; [b] IAEA-452; [c] CRM 278R

Table 2**.** Median values and min-max ranges (in parentheses) of concentrations of 23 TEs (metals and metalloids) in the soft tissue of *A. noae* sampled over 12 months from reference (NP) and contaminated (PC) location. Significant differences between locations ($p < 0.05$, Mann-Whitney U test) are indicated with an asterisk next to the higher value. Literature values are presented as well.

TE concentrations (µg/g d.w.)	NP (n = 36)	PC (n = 36)	[a]Mljet, Croatia	[b]Susak, Croatia	[c]Bizerte lagoon, Tunisia
Tl	0.005 (0.003-0.007)*	0.004 (0.003-0.007)			
Cs	0.027 (0.019-0.041)	0.026 (0.022-0.036)			
U	0.103 (0.067-0.181)	0.093 (0.046-0.199)			
Co	0.343 (0.205-0.565)	0.293 (0.196-0.448)			
Pb	0.521 (0.318-0.821)	0.601 (0.380-0.822)*	0.59-0.69	0.55	0.65-2.00
Ni	0.856 (0.585-1.349)*	0.717 (0.447-1.018)			
Li	0.985 (0.674-1.276)*	0.793 (0.580-1.043)			
Cr	1.14 (0.79-1.70)*	0.97 (0.66-1.60)			
Ba	1.49 (0.62-19.44)	1.99 (0.98-6.79)*			
V	1.76 (1.10-5.36)	2.52 (1.20-6.85)*			
Cd	2.02 (1.12-3.32)*	1.67 (0.89-3.47)	1.52-2.84	3.35	0.70-1.55
Ti	2.49 (1.03-4.64)*	1.99 (0.86-2.91)			
[d]Mo	2.67 (1.38-5.06)	3.77 (1.28-8.89)*			
Ag	3.33 (2.04-5.50)	3.09 (1.27-4.83)			
Cu	4.47 (3.29-5.77)	4.41 (3.08-6.26)	2.85-13.23		4.85-7.80
Rb	4.66 (4.34-5.46)	4.76 (4.20-5.93)			
Se	7.38 (5.31-9.81)*	6.25 (4.50-9.79)			
Mn	8.84 (6.12-15.44)	10.15 (6.93-16.76)*			
Sr	64 (40-130)	58 (36-124)			
As	65 (43-111)	70 (33-111)		95.05	
Al	97 (39-196)	95 (47-179)			
Zn	113 (68-157)	124 (77-179)	89.7-167.6		187.2-375.1
Fe	235 (160-361)	261 (151-438)*			150.3-234.7

[a]Cuculić et al., 2010 (Mljet Island is located in the southern Adriatic Sea; for comparison purposes metal concentrations were multiplied by 5 to obtain dry weight values.)
[b]Ozretić et al. (1990) (Susak Island is located in the northern Adriatic Sea; for comparison purposes metal concentrations were multiplied by 5 to obtain dry weight values.)
[c]Ghribi et al., 2016 (For comparison purposes metal concentrations were multiplied by 5 to obtain dry weight values.)
[d]Papadopoulou, 1973 in Eisler, 2010. Concentration of Mo in soft parts of *A. noae*: 88.0 µg/g d.w.

Table 3. Probabilities of analysis of variance of TEs concentrations at two sampling sites during one year (significant variations are marked bold)

Variable (TE concentrations)	*p* (NP)	*p* (PC)
Tl	**0.0020**	**0.0016**
Cs	**0.0166**	**0.0015**
U	**0.0101**	**0.0117**
Co	**0.0016**	**0.0415**
Pb	0.0948	0.4559
Ni	**0.0006**	0.1445
Li	**0.0031**	0.3019
Cr	**0.0486**	0.1583
Ba	**0.0000**	**0.0000**
V	**0.0000**	**0.0000**
Cd	**0.0483**	**0.0031**
Ti	**0.0001**	**0.0091**
Mo	**0.0000**	**0.0000**
Ag	**0.0000**	0.2166
Cu	0.0573	0.8051
Rb	0.0642	**0.0119**
Se	**0.0077**	0.1292
Mn	**0.0083**	0.0666
Sr	0.1238	**0.0179**
As	0.8210	0.2424
Al	**0.0031**	0.0823
Zn	0.1375	0.1857
Fe	**0.0005**	**0.0101**