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Metallothioneins, Caspase-3 and Oxidative Stress Responses in the Multi-Marker Study of Freshwater Mussel Inhabiting Sites of Various Human Impact

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Abstract

The aim of the present study was to ascertain whether the metallothioneins (MTs) of freshwater mussels can serve as a biomarker of environmental quality in the spontaneously polluted area. *Anodonta anatina* mussels from an agricultural site (A), the cooling pond of a nuclear power plant (N) and a forestry as a reference site (F) in Western Ukraine were studied during spring, summer and autumn. Concentrations of copper (Cu), zinc (Zn) and cadmium (Cd) in MTs and in the tissue, MTs protein (MT-SH) level, oxidative stress indices and apoptotic activities were determined in the digestive gland. The three sites were clear distinguished by biomarkers. In the A-group, the specimens, despite higher concentrations of Cu and Zn in the tissue, had low level of MT-SH level (in two seasons) making the metal-keeping function of MTs invalid. MTs-bound metals dropped to about 2.3% of Cu, 0.4% of Zn and 9.1% of Cd in the tissue in autumn in this polluted area. Lower level of glutathione redox-index reflected the oxidative stress in this group. The N-group displayed high levels of MT-bound Cu (corresponding to up to 57.7% of the total tissue metal) and caspase-3 activity (up to 7 times higher than in the reference F-group), indicating specific for cooling pond pollution by Cu and thermal effects. In group F, the set of markers showed the characteristic of the reference site. An extremely low redox–index of glutathione (RI GSH: 0.45) was observed in the A-group in spring, and high levels of protein carbonyls and TBA-reactive substances in autumn in group N.Hence, multi-marker approach needed to provetoxicityassesments in mussel sexposed byspontaneous and mixed pollution.

Keywords: *Anodonta anatine*, metallothionein, oxidative stress, apoptosis, spontaneous pollution.

Introduction

Metallothionein (MT) is one of the most approved markers of exposure to toxic metals in bivalve molluscs (Amiard *et al.*, 2006; Hagger *et al.*, 2006; Viarengo *et al.*, 2007) due to its unique metal-binding properties and inducibility by toxic metals. High variability of MTs in invertebrate animals was already recognized (Dallinger *et al.*, 2000; Gruber *et al.*, 2000; Ciocan and Rotchell, 2004; Jenny *et al.*, 2006). Cadmium (Cd) in particular can replace the essential metals zinc (Zn) and copper (Cu) in the composition of MTs and induce MT expression. Owing to their extremely high thiols content (about 30% of amino acids in their composition are represented by cystein), MTs could also be scavengers of reactive oxygen species (Cavaletto *et al.*, 2002; Dondero *et al.*, 2005). For bioindication purposes, the protein level of MTs is usually measured. The most approved method is based on SH groups assay (Viarengo *et al.*, 1997). Alternatively, MTs metal concentration or saturation (Hamza-Chaffai *et al.*, 2000; Amiard *et al.*, 2006) could be determined. To the best of our knowledge, comparison of MT levels by different approaches has seldom been combined in one study. However in the few studies, non-conformity of results obtained by different methods was shown (Van Campenhout *et al.*, 2004; Zorita *et al.*, 2006; Atli and Canli, 2007). Therefore we speculated that the juxtaposition of metal-binding activity and total concentration of MTs within the cells needs to be compared to assess the involvement of MTs in metal keeping and antioxidant defence depending on the environmental impact.

Our interest was connected to the typical for the areas that are not contaminated by certain metals but spontaneously polluted from different sources, mainly connected to agricultural and municipal activities. Run-off and sewage discharges and industrial processes could be important sources of phosphates, phenol, nitrites, ammonium, toxic metals and wide spectrum of pharmaceutical and personal care products are typical pollutants (Falfushynska *et al.*, 2010, 2014; Rzymski *et al.*, 2014).The kinds of

pollution in these areas are well described by Zeybek *et al.* (2012). The studied area in Western Ukraine corresponded to this criterion, since it is characterised by an unsteady level and composition of background pollution, punctuated by the occurrence of emergency situations. The use of bivalve molluscs to reflect the peculiarities of this type of pollution by a multi-marker approach was demonstrated in our previous reports (Falfushynska *et al.*, 2009, 2010, 2012).

To improve the understanding of MTs functionality in feral mussel populations, the aim of this work was to compare different MTs characteristics and their possible relation to other stress-related indices. Since the variability of responses can be controlled by seasonal changes in temperature and food availability more than by different levels of pollution (Robillard *et al.*, 2003; Geffard *et al.*, 2005), the mussels were compared over three seasons. To evaluate the stress syndrome and toxicity, the biomarkers of oxidative stress and apoptosis level were determined. To distinguish temporal and local regularities in field studies, simultaneous study of three groups of molluscs from different sites was conducted (Da Ros *et al.*, 2002).

Materials and Methods

Experimental Groups

The experiments were carried out during the middle of May, July and September of 2009 y. Adult *Anodonta anatina* Linnaeus, 1758 (8±1 cm length, 42±5 g, ~ 6 years old)were hand-collected from 0.5 to 1 m depth in three sites located in Western Ukraine (Figure 1). The forestry (F) site is located in the upstream portion of Seret River (near the village Ivachiv, 49°49′ N, 25°23′ E), where no sources of industrial contamination existsand close to the municipal water inlet; therefore, this was considered as a reference site. The agricultural (A) site is situated in the lower portion of the Nichlava River near the boroughs of the city of Borshchiv (48°48′ N, 26°00′ E) and received effluent from the region with intense agricultural activity and also from the city, in which a creamery is the most prominent source of pollution and wastewater treatment plants are absent. The site with stable higher water temperature (about 6-8°C higher compared with other sites) (N) is located on the southern bank of the cooling pond of Khmelnytskyi Nuclear Power Plant (NPP) in Netishyn in a forested area on the tributary of Goryn River, 50°21′ N, 26°38′ E). Additionally, at this location the highest concentrations of Cu and Cd in the water were consistently measured. The determining of other chemical indices of water in the pointed sites had confirmed their characteristics in general (Falfushynska *et al.*, 2010).

Sampling was carried out at all sites simultaneously. About 30 individuals from each site were transported to the laboratory in cages with native water and treated within a day after sampling. For each biochemical parameters, eight digestive gland

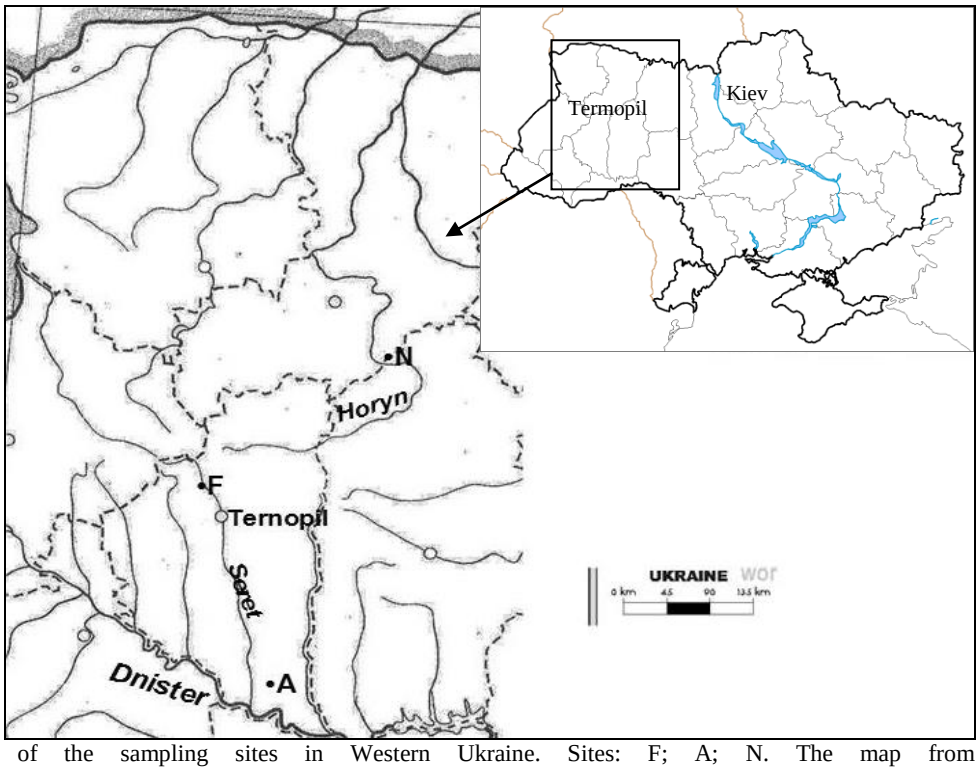


Figure 1. Location of the sampling sites in Western Ukraine. Sites: F; A; N. The map from http://www.lib.berkeley.edu/EART/x-ussr/M35W.gif was utilized.

samples were prepared individually. Tissue preparation for the analyses was carried out at a temperature of around 4°C.

Materials

Acetyl-Asp-Glu-Val-Asp p-nitroanilide, chymotrypsinogen, cytohrome*c*, EDTA, Glutathione Reductase from baker's yeast (*S. cerevisiae*), 2,4-dinitrophenylhydrazine (DNPH), 5,5'-dithio-bis(2-nitrobenzoic acid) (DTNB), insulin chain B oxidized, β-mercaptoethanol, metallothionein MTII from rabbit liver, myoglobin, β-NADPH, nitrobluetetrazolium (NBT), reduced glutathione (GSH), phenazinemethosulfate, phenylmethylsulfonyl fluoride (PMSF), Sephadex G-50, serum albumin, thiobarbituric acid (TBA), and ubiquitin were purchased from Sigma. All other chemicals were of analytical grade.

Metallothionein Isolation

MTs from the digestive glands were obtained as the thermostable proteins (Roesijadi and Fowler**,** 1991). Tissue samples from five individuals of each group were pooled in aliquot quality (total mass 350 mg) and homogenized in an ice-cold 10 mM Tris-HCl buffer, pH 8.0, containing 10 mM 2-mercaptoethanol and 0.1 mM PMSF, and centrifuged at 16,000 × g for 45 minutes at 4°C. The supernatant was incubated at 85°C for 5 minutes and subsequently centrifuged at 16,000 × g for 45 minutes at 4°C. The thermostable supernatant obtained was applied to the size-exclusion chromatography on Sephadex G-50 superfine column (1.5 × 50 cm) in the same buffer, at a flow rate of 0.33 ml·minute^{-1}. Fractions (5 ml) were collected and analyzed for absorbance at 280 and 254 nm. Column calibration was achieved by applying a mixture of the following standards: chymotrypsinogen (25.8 kDa), myoglobin (17.0 kDa), cytohrome*c* (12.3 kDa), ubiquitin (8.4 kDa), insulin chain B oxidized (3.5 kDa). The fractions of peaks with the high absorbance at 254 nm were pooled (total 10 ml) for the ultraviolet (UV) absorption spectra and the analysis of metals. The MTs-containing fraction was identified based upon peculiar spectral features (comparatively high density ratio D_{254}/D_{280}), thermostability and low molecular mass.

Quantification of Metallothioneins from Thiols Measure

MTs were determined according to the method of Viarengo *et al.* (1997). The digestive gland samples were homogenised in three volumes of 20 mM Tris-sucrose buffer with 0.1 % ß-mercapto ethanol and 0.5 mM PMSF, followed by Ethanol/ chloroform extraction. After incubation with DTNB, the samples' absorbance was read at 412 nm. The levels of MT (MT-SH) were calculated assuming the relationship: 1 mol MT-SH = 20 mol GSH and expressed as μg of MTs per g of fresh weight (FW).

Metals Determination

The Zn, Cu and Cd concentrations were measured in weighed samples of digestive gland tissue (250 mg) of each specimen, and in the pooled eluate of each fraction of low weight thermostable proteins after chromatography (10 ml) in each group of mussels (in triplicate). The samples were dried for 24 hours at 105°C, and then digested under pressure with 5 ml HNO_3 for 3 hours at 105°C, using an acid-cleaned Teflon bomb. The Cu and Zn concentrations were analyzed by atomic absorption spectrophotometry against certified standards using spectrometer C-115, ("Lomo", Russia) and Cd concentrations, using graphite furnace atomic absorption spectrometer S-600 ("Selmi", Ukraine). The reliability of the measurements of several selected elements was assessed by analysing *Ermce278 Fluka (Sigma-Aldrich)* certified reference material; recoveries of metals were between 90% and 110%.

Metal concentration in tissues and MTs forms was expressed as μg·g^{-1} fresh weight (FW), and in MTs also as nmol·g^{-1} FW. MTs metal-bindingcharacteristic (MT-Me) was calculated from the concentrations of metals in the low thermostable proteins peaks (MT-(Cu+Zn+Cd)), considering that one molecule of Mtsbinds seven Zn^{2+}or Cd^{2+}ions or twelve Cu^{+} ions (Nielson and Winge, 1984). These values were expres sedin μg g^{-1} FW.

Enzyme Assays

The digestive gland tissue was homogenized (1/10 w/v) in 0.1 M pH 7.4 phosphate buffer containing 100 mM KCl and 1 mM EDTA, as well as 0.1 mM PMSF for the inhibition of proteolysis. The homogenates were centrifuged at 6,000×g for 10 minutes and the resulting supernatant was used immediately for measurement.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured according to the method of Beauchamp and Fridovich (1971) based on aerobic reduction of NBT at 535 nm by superoxide radicals and was expressed as units·mg^{-1} soluble protein; 1 unit of SOD activity being defined as the amount of protein causing 50% inhibition of the rate of NBT reduction. In order to assess Mn-SOD activity, the supernatant was preincubated for 60 minutes at 0°C in the presence of 5 mM KCN, which produced total inhibition of Cu, Zn-SOD. The latter activity was calculated as the difference between the activities in the absence and in the presence of KCN.

Caspase-3 colorimetric assay was based on the hydrolysis of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) by caspase-3, resulting in the release of the p-nitroaniline (pNA) moiety. p-

Nitroaniline was detected at 405 nm (ε_{mM}=10.5). The concentration of the pNA released from the substrate was calculated from the absorbance values at 405 nm (Bonomini *et al.*, 2004). This value was express edinpmole × mg^{-1} proteins.

Oxidative Damage and Glutathione level Determination

Lipid peroxidation (LPO) was determined in the supernatant of 1/10 w/v homogenate by the production of TBA-reactive substances (TBARS) (Ohkawa *et al.*, 1979). A molar extinction coefficient of $1.56 \cdot 10^5$ $M^{-1} \cdot cm^{-1}$ was used.

Protein carbonyl (PC) content, as an index of protein oxidation, was measured in the resulting supernatants through the reaction with DNPH (Reznick and Packer, 1994). The differences in absorbance between the DNPH- and the HCl-treated samples were determined by spectrophotometry at 375 nm, and the amount of carbonyl was determined by using a molar extinction coefficient of $2.2 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$. Data were expressed as nmol carbonyl·mg^{-1} of soluble extracted protein.

Total glutathione (GSH) concentration was quantified by the glutathione reductase recycling assay (Anderson, 1985). To estimate the oxidized glutathione (GSSG) level, the protein free sample was treated with 2-vinylpyridine prior to the assay (60 minutes) at final concentration 2% (Griffith, 1980). The rate of 5-thionitrobenzoic acid formation was monitored spectrophotometrically at 412 nm. The redox–index of glutathione (RI GSH) as the ratio of concentrations ([GSH]–[GSSG])/[GSH] was calculated. Standards were prepared from reduced glutathione, and concentrations were expressed as μmol per g wet weight.

The protein concentration in the supernatant (soluble protein) of the digestive gland and of the gonad tissue was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard.

Statistical Analysis

Analysis of the thermostable solution was carried out in triplicate, and all the other measurements on 8 specimens. The results were expressed as means ± standard deviation (S.D.). Data were tested for normality and homogeneity of variance using Kolmogorov-Smirnoff and Levene's tests, respectively. Since data were not normally distributed (Lilliefors' test), non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney U-test) were performed (significant at $P<0.05$). When it was possible, data were normalized by common transforming methods.For detection of correlation, the Pearson's correlation test was also performed at a 0.05 level of significance (Mead *et al.*, 2002).

Discriminant functional analysis was used to determine the statistical significance of the individual biochemical variables among sites and seasons (significant at $P<0.05$). A classification tree was built using Classification and Regression Tree (CART) software on the basis of all determined biological characteristics both jointly in three seasons and for each season separately. All statistical calculations were performed with Statistica v 8.0 and Excel for Windows-2000.

Results

Metallothioneins Quantification and Their Participation in Metal Binding

Gel-filtration of the thermostable fractions obtained from the digestive gland revealed a low molecular weight fraction with an apparent molecular mass of 8 kDa (Figure 2). This was present in all samples except in the group N in spring. In this case, peaks with an apparent molecular mass of about 16 kDa and 4 kDa resulted. In summer, 4 kDa peaks were found in samples from the sites F and A. UV absorbance patterns of the material of these peaks corresponded to the MTs with the maximum at about 254 nm, indicating the presence of characteristic metal-thiolate clusters, typical for MTs (Kagi and Schaffer, 1988). Some exceptions for mussels from site A were revealed as a shift of this peak to the near-*UV* spectral region (260-270 nm) (Figure 2).

Based on these characteristics, low molecular weight peaks (II, III, and IV on Figure 2A,B,C) were considered as containing MTs or products of their partial hydrolysis or olygomerization, and were combined for the metal analyses. Corresponding metal concentrations were signed as MT-Me. The total and MT-related concentrations of Cu, Zn and Cd in the digestive glands are shown in Figure 3. Prominent seasonal differences were found for metal concentrations in the tissue and in MTs. In the summer, in the F- and A-groups, the highest levels of metals in MTs were detected, and in group N, the lowest values of the summer season were revealed. When the three groups were compared, the concentration of Cu in MTs was always highest in group N. In spring and autumn, MTs in this group also contained the highest concentrations of Zn.

The metal compositions of MTs differed between sites and seasons (Table 1). The calculation of metal concentrations in MTs forms showed that the concentration of Zn in MTs was up to ten times higher than that of Cu, and only in the N-group in summer was the concentration of Cu in MTs 1.5 times higher than that of Zn. Cd was rather negligible in the composition of MTs, no more than 1 atom Cd per 9 atoms of Cu and Zn.

The relative metal concentrations in MTs and in the tissue decreased in the following order: Cd>Cu>Zn. Less metal-binding ability of MTs was frequently found in the A-group. It corresponded to

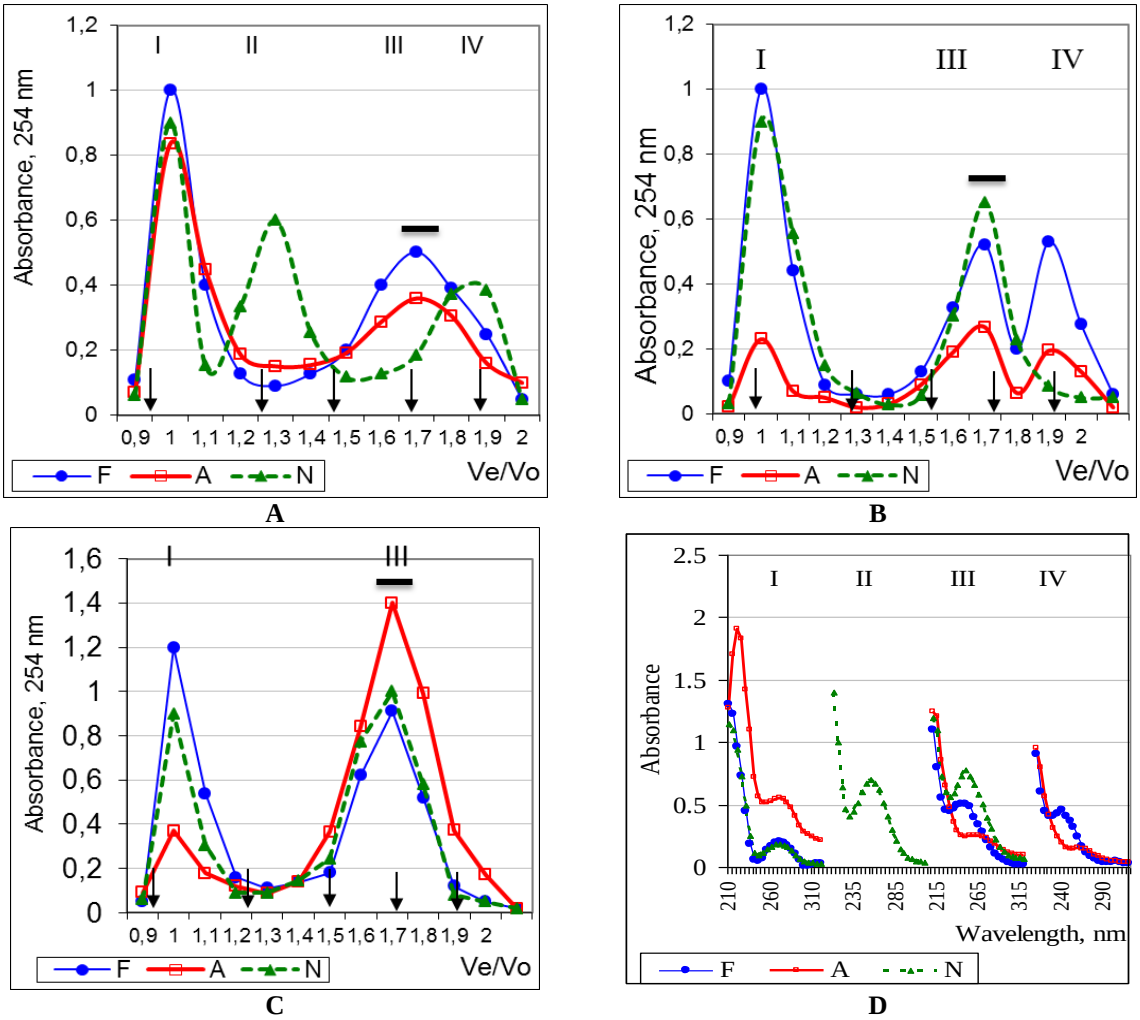


Figure 2. The elution profiles on Sephadex G-50 in spring (A), summer (B) and autumn (C) of the thermostable proteins from the digestive gland of mollusks and typical UV-spectra of obtained fractions (D). I, II, III and IV indicates fraction of thermostable proteins; in Figure 2A,B,C, arrows highlight the elution volume of markers: 25.8 kDa, 17.0 kDa, 12.3 kDa, 8.0 kDa, 3.4 kDa appropriate to 1.06; 1.31; 1.51; 1.74; 1.91 Ve/Vo correspondingly; Ve, elution volume; Vo, void volume of the column; the bar indicates the elution volume of standard metallothionein from rabbit liver.

only 2.3% of metal concentration in the tissue for Cu, 0.4% for Zn and 9.1% for Cd in autumn. Consequently, the most prominent rates of unbound Cu, Zn and Cd were detected in A-group.

The concentration of MTs protein (MT-SH) increased significantly from spring to summer–autumn, especially in the N-group (Figure 4). In summer and autumn, inter-site differences of MT concentration were detected with the highest value for the N-group and lowest for the A-group. The values of MT-Me and MT-SH were not correlated (r=0.03, P>0.05).

Markers of Oxidative Stress and Apoptosis

Characteristics of antioxidant defence and markers of apoptosis in mussels from the three sites showed clear seasonal differences (Figure 5). Cu,Zn-SOD activity varied seasonally over an order of magnitude. The levels of Mn-SOD and TBARS changed dramatically between the seasons, but frequently these changes were contrary in different sites. The same seasonal dynamic for all three groups was shown only for GSH concentration (r = 0.79, P<0.01; r = 0.83, P<0.001, r = 0.82, P<0.001 for F-/A-, F-/N- and A-/N- groups correspondingly).

When the three groups were compared, the A-group differed from the other groups with lower Cu, Zn- and Mn-SOD activities in spring and summer and the highest of these in autumn. In this group, the levels of GSH and RI GSH were always (with one exception) lower than in the other groups. The extremely low RI GSH (0.45) was observed in the A-group in spring. The levels of protein carbonyls and TBARS had not shown consequent differences between groups and seasons, but were correlated (r = -0.64, P<0.01; r = -0.81, P<0.001, r = 0.90, P<0.001 for F-/A-, F-/N- and A-/N- groups correspondingly). High levels of both these indices were detected in autumn in group N.

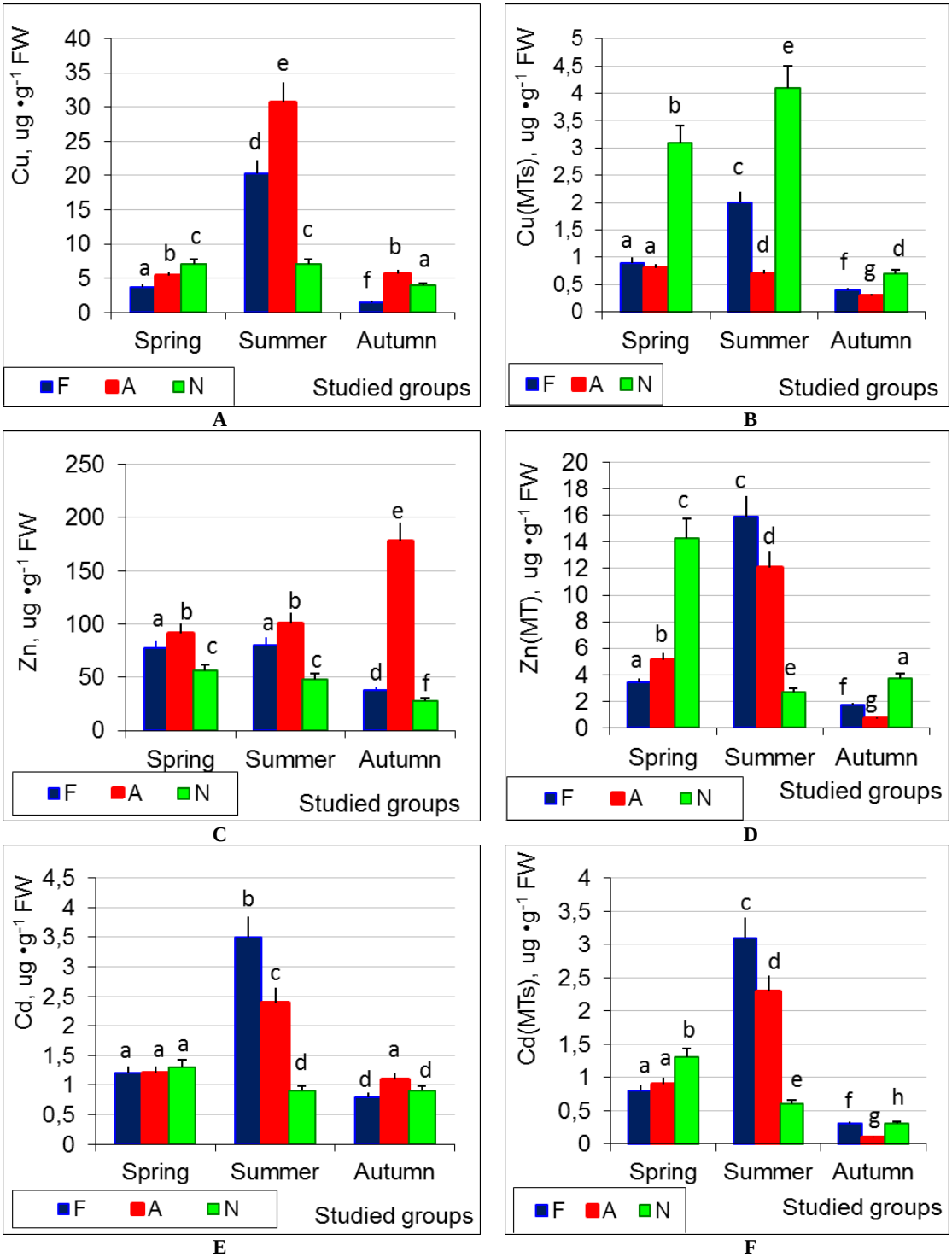
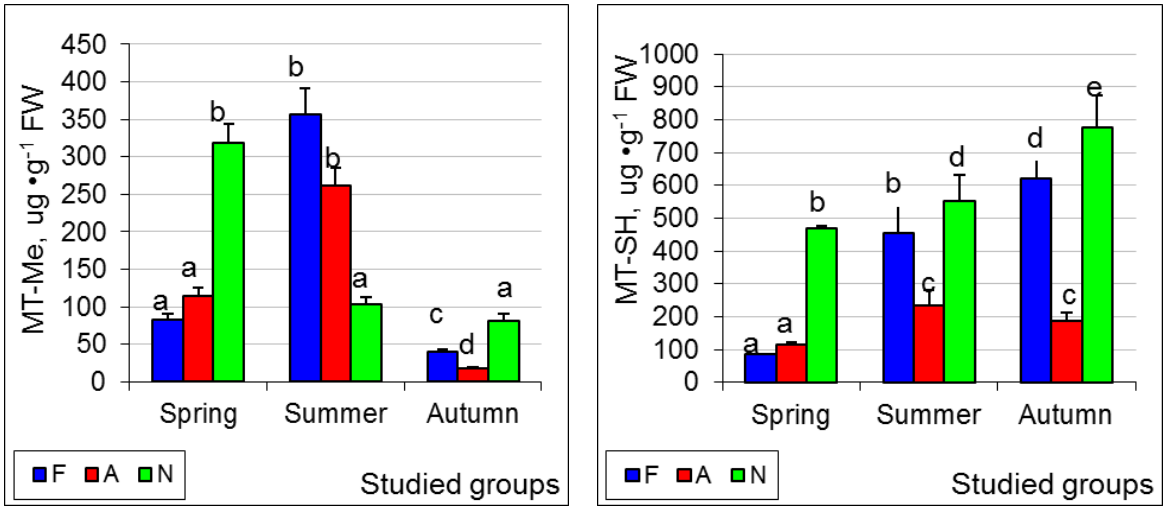


Figure 3. Metal copper (A, B), zinc (C, D) and cadmium (E, F) concentrations in the digestive gland tissue (A, C, E) and in its low weight thermostable peaks (MTs) (B, D, F) obtained by size-exclusive chromatography, µg g^{-1} FW, Mean±S.D. For each separate index, the values do not display a significant difference (P>0.05) are marked by the same letter.

Table.1. Metal (Cu:Zn:Cd) ratio (nmol·g^{-1} FW) in the thermostable low weight fractions

Season	Groups		
	F	A	N
Spring	2:7:1	3:20:2	4:20:1
Summer	1:11:1	1:19:2	12:8:1
Autumn	2:10:1	5:12:1	4:21:1



A **B**

Figure 4. Concentration of metallothioneins measured by metal concentrations in low weight thermostable proteins (A) and by thiols measurement method (B) in the digestive gland of mussels, μg·g^{-1} FW, Mean±S.D., N=8.For each separate index, the values do not display a significant difference (P>0.05) are marked by the same letter.

Stable high caspase-3 level was demonstrated in the group N (Figure 5). In the F-group, it was lower during all seasons.

Statistical Interpretation of the Received Results

Using multivariative statistics, we have determined the influence of environmental and biological factors on the response of several molecular biomarkers in the mussels collected from three sites. Discriminant functional analysis showed the significance of both seasonal (F (24.80)=132.65 P<0.0001) and spatial (F (24.80)=69.3 P<0.0001) differences between groups. Within tested indices, GSH and TBARS concentrations, and Mn-SOD and Cu,Zn-SOD activities were only significant seasonally. On the other hand, total (MT-Me) metal-binding capacity of MTs, and caspase-3 activity were only significant across sites.

To determine the main markers that distinguish the groups, a classification tree was built (Figure 6). When all biomarkers and all three groups were included, only caspase-3 activity and total Zn concentration in the digestive gland were partitioning criteria. The tree had one pure leave, containing all mollusks from site F (and only one from site N) with low level of caspase-3 activity. The other terminal nodes contained molluscs from the sites A and N that were separated by Zn concentration. The resulting confusion matrix shows an overall classification accuracy of 85%. The best classification was predicted for site F, followed by sites N and A.

Discussion

In the case of spontaneous pollution, it is difficult to select both sites with certain types and constant levels of toxic substances or reference clean sites due to the numerous pollution sources, variable in time, for example, the co-occurrence of small farming and municipal effluents. The variability of several biochemical and morphological indices between the groups and seasons without distinct regularity could complicate the assessment of the health status in the feral animals from these areas (Falfushynska *et al.*, 2009, 2010, 2012). The high variability of the oxidative stress indices values, and their dependence more on the season than on the site (for GSH and TBARS, Mn-SOD and Cu,Zn-SOD), indicates this in the present study.Therefore, the assessment of several biomarkers is the most relevant way to evaluate the toxicity in the environment (Hagger *et al.*, 2006; Cravo *et al.*, 2013).

In the present study, the assessment of the metal-binding function of MTs and apoptotic activity allowed us to distinguish the nature of the environmental impact of the three sites most legibly. At site A, higher levels of Cu and Zn in the digestive gland tissue indicated its spontaneous pollution by agricultural and municipal wastes (Falfushynska *et al.*, 2010, 2012). Despite this, the typical MT response to metal toxicity was absent: the level of metals in MTs and MT concentration was lower in these mussels throughout the three seasons. This phenomenon could be explained by the suppression of these stress-related proteins in mussels in chronically polluted sites (Doyen *et al.*, 2008; Lilja *et al.*, 2008; Bigot *et al.*, 2010) or by their post-translation injury. The latter is in accordance with distorted spectral features of low weight thermostable proteins. The study of clams (*Ruditapes decussatus*) from three coastal ecosystems in Southern Portugal subjected to different anthropogenic stressors demonstrated that metallothionein-like protein concentration did not correspond to high levels of metals in the area (Carreira *et al.*, 2013).

The variability of forms with the spectral characteristics of MTs could be the result of partial

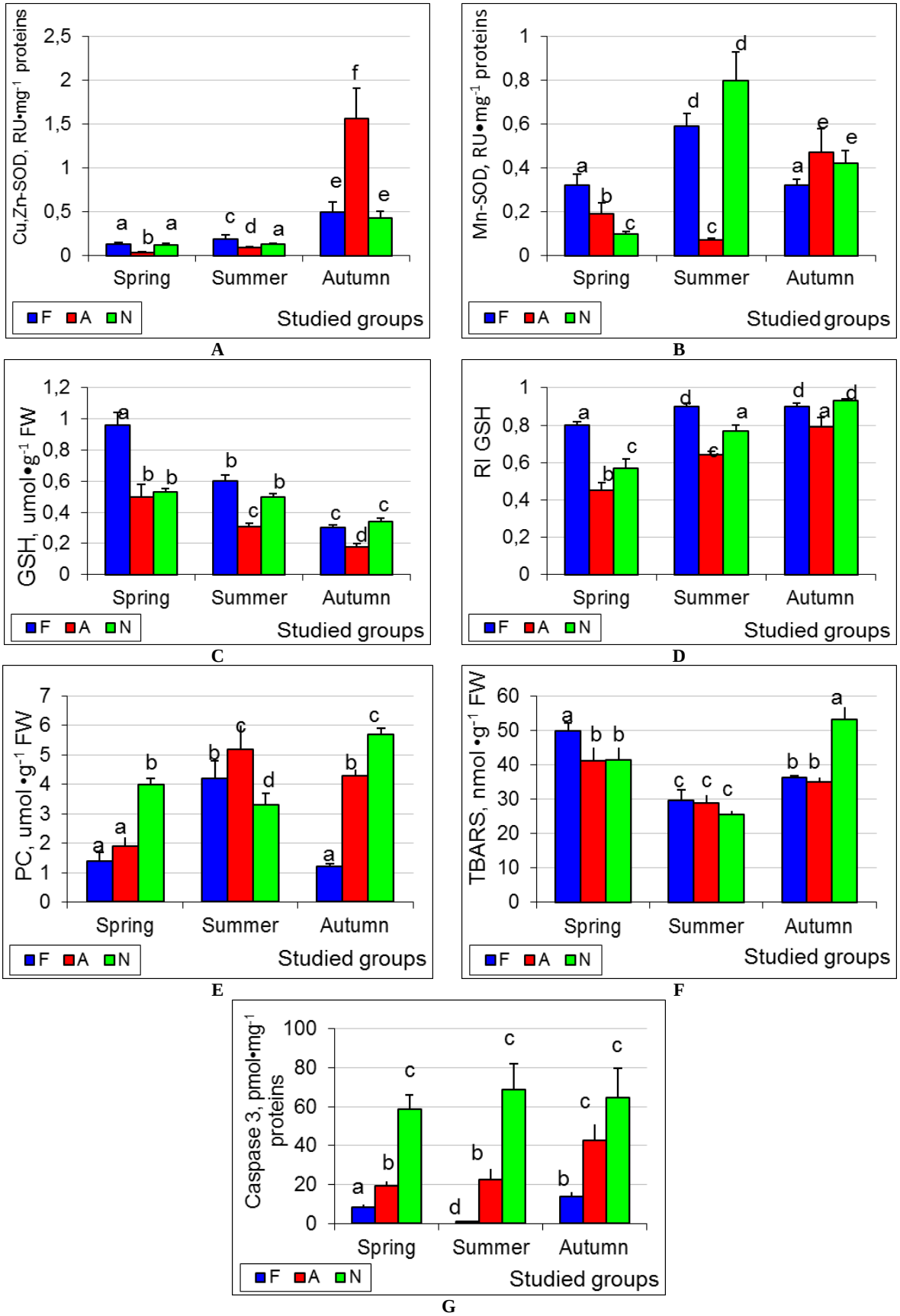


Figure 5. Biomarkers of health status in mussels from three sites. A, Cu,Zn-superoxide dismutase activity; B, Mn-superoxide dismutase activity; C, Total glutathione concentration, D, redox index of glutathione; E, protein carbonyls concentration; F, TBARS concentration; G, Caspase-3 activity (all in digestive gland),Mean ± S.D., N=8.For each separate index, the values do not display a significant difference (P>0.05) are marked by the same letter.

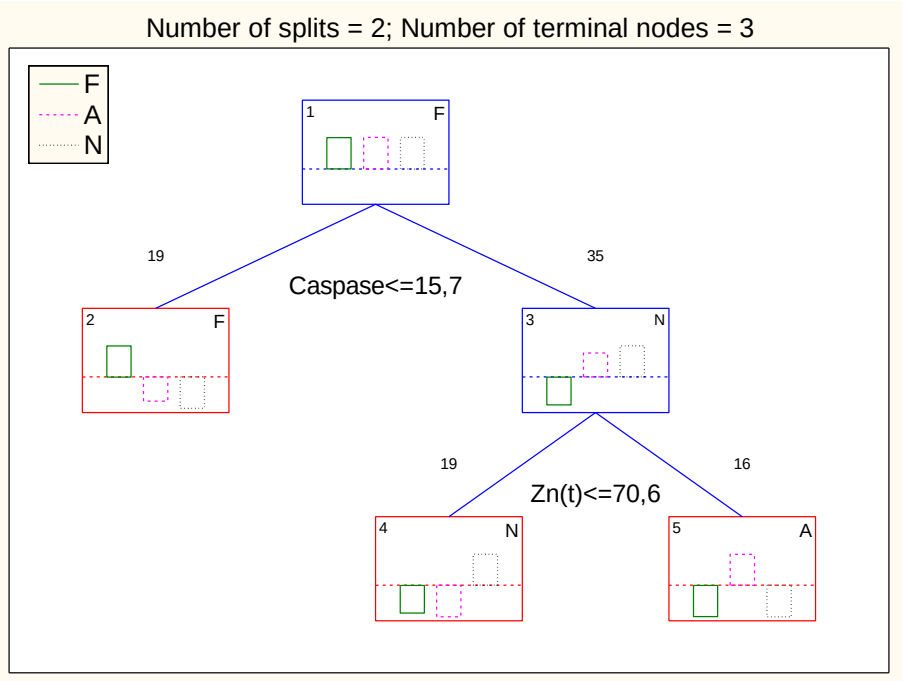


Figure 6. Classification tree model.Terminal nodes of the tree identify the dominant site and the number of mussels represented in total for three sites. "Caspase" refers to caspase activity; "Zn(t)" to the total concentration of Zn in digestive gland.

hydrolysis and/or olygomerization of MTs that is frequently caused by its oxidation *in vitro* and *in vivo*, even in the presence of β-mercaptoethanol (Wilhelmsen *et al.*, 2002). Similar results are known for other invertebrates. For example, the elution of MT from white shrimp *Litopenaeusvannamei* exposed to Cd and Zn was accompanied by Zn-containing low-weight fraction (Wu al., 2005). The presence of cytosolic heat-stablethiolicccompoundswith apparent molecular mass of 4 to 15 kDa and binding Ag, Cu and Zn has been reported in sponges and *Hedistediversicolor* (Polychaete) from the French Mediterranean coastal sites (Berthet *et al.*, 2003, 2005). Two proteins with molecular weight 10,100 and 4,100 respectively (as estimated by Sephadex G-50 chromatography) with the typical for MTs shoulder at 254 nm occur in the hepatopancreas of *Carcinusmaenas*, binding variable amounts of Cu, Zn and Cd (Wong and Rainbow, 1986). In the present study, the distortion of typical MTs UV-spectra of these proteins was typical for the mussels from polluted site A.

Extremely low RI GSH, found in group-A, indicates the toxic effect at this site (Regoli and Principato, 1995). At the same time, other indices of oxidative injury were not consistent throughout the three seasons, despite the rather high protein carbonyls level and frequently lower GSH level in this group. The characteristically high variability of SOD activities imbalanced the regularity of the oxidative stress response in this group. The particular role of Cu, Zn-SOD in the response of indigenous mussels from highly polluted sites has been demonstrated (Manduzio *et al.*, 2004). Significant induction of SOD was shown in marine mussels *Mytilusgalloprovincialis* in the contaminated sites in the lagoon of Venice (Nasci *et al.*, 2002). Other studies show the increase of Cu, Zn-SOD under excessive oxidative stress and no induction of Mn-SOD in invertebrate animals (Datkhile *et al.*, 2009). However, in the present study, the responses of SOD were highly seasonally dependent. Therefore, we can conclude that MT and GSH were the most valid characteristics of the toxic effect in this chronically polluted site.

Group N was distinguished throughout the three seasons by Cu-enriched MTs, and high effectiveness of MTs towards Cu and Zn binding within the tissue (up to 57.7% of Cu and up to 13.3% of Zn of total tissue metal). It corresponded to highly elevated MT concentration in two of the seasons. This group was also characterised by high levels of caspase-3 (constantly) and protein carbonyls (in two of the seasons). The cooling ponds are characterized by distinctive thermal regimes, with stably elevated temperatures, typically 3-5°C above similar natural reservoirs (Sylayeva *et al.*, 2012). Elevated levels of metals, radionuclides, aromatic and alkyl hydrocarbons and other hazardous compounds were also reported in the vicinities of a nuclear power plant (NPP) in Lithuania (Baršienė and Rybakovas, 2008). However, the peculiarities of the environment in the cooling ponds of NPPs are seldomly studied. In the study of Baršienė and Rybakovas (2008), the highest levels of chromosome abnormalities were found in bivalves from the cooling reservoir. Notably, earlier

studies of several NPPs in Ukraine, including Chernobyl NPP did not find significant effects of heated water discharge on benthic animals (Fetisov *et al.*, 1992; Silayeva and Protasov, 2005). However, later studies identified significant shifts in important cellular and biochemical characteristics indicating stress in the mussels from Khmelnytskyi NPP (Falfushynska *et al.*, 2010, 2012). High levels of Cu in the environment could be the reason behind it (Falfushynska *et al.*, 2010). To date, a two-year study in the vicinity of a NPP in north eastern France shown low sensitivity of indigenous *Dreissenapolymorpha* to the spatial effects of high levels of Cu in the area (Guerlet *et al.*, 2007). Elevated ambient temperature at this site can provoke specific effects as shown in this study. These results correspond well with results concerning the expression of heat shock proteins in goldfish inhabiting Gaobeidian Lake in Beijing, China. The water of this lake was reported to be moderately polluted and have an elevated temperature. The up-regulation of the heat shock proteins suggested that fish under these specific environmental conditions were experiencing a complex stress process (Wang *et al.*, 2007). Piano *et al.* (2004) reported that the levels of MT were significantly increased in the tissues of individuals of *Ostreaedulis* exposed to thermal stress (35°C, 1 hour) and allowed to recover at 18°C for 24 hours.

Despite the scant information concerning the activity of caspases in bivalve molluscs, caspase-3 activity seems to represent a valuable marker for the determination of toxic effects, as confirmed by CART analysis for the mussels from sites A and N (Figure 6). The important finding of Romero *et al.* (2011), that caspase genes in *Mytilus galloprovincialis* have extremely high expression levels within the gland and gills due to the clearance of damaged cells, contributes to the rationale for including this assay in the set of biomarkers of the effect of warming on molluscs.

At site F, the studied indices reflect the sustainable environmental quality. The level of GSH and RI GSH was rather high and caspase-3 activity lower compared between the three groups. Elevated MT-Cd concentration in summer did not contradict this conclusion, because the effective ability to accumulate Cd from the surroundings could attest to the adequacy of the MTs functioning. Besides that, the ratio of Cd in the composition of MTs was very low. The elevated level of Cd in the mussels and their MTs in this area was shown previously, despite other markers characterizing this area as a reference site (Falfushynska *et al.*, 2009). MTs are able to accumulate metals from the environment with particular sensitivity for Cd (Hamza-Chaffai *et al.*, 2000; Amiard *et al.*, 2006). MTs usually bind about 80-90% of Cd in the cells, for example in freshwater bivalves *Pyganodongrandis* living along a Cd concentration gradient (Giguère *et al.*, 2003). In comparison, at site A in autumn, mussels accumulated only about 9.1% of Cd in the tissue. Discriminant functional analysis indicated the significance of MTs metal-binding properties for the distinguishing of mussels from different sites, independently of season.

Participation of MTs in the antioxidant defence of mussels is expected from the high concentration of thiols and the particular metal binding/release characteristics of these proteins (Viarengo *et al.*, 2007). On the other hand, the concentration of MTs (MT-SH) was not correspond with their metal-binding characteristics (MT-Me).Available data demonstrate alsoa discrepancy between two characteristics of MTs in some cases, particularly in fish (Van Campenhout *et al.*, 2004), providing evidence for the up-regulation of this protein expression in the apoform. Hence, enhanced antioxidant potential of this thiol-reach protein can be expected due to the high concentration of unbound SH-groups and the stability of metal-free apo-MT (Kelly *et al.*, 2006). This advantage for antioxidant defence can be realised particularly in mussels from site N.

To summarize, the applied multi-marker approach allowed the differentiated assessment of toxicity in mussels inhabiting areas subjected to spontaneous and mixed pollution. Among characteristics, oxidative stress indices reflected best the seasonal peculiarities of the health status in each site, whereas elevated caspase-3 activity reflected the stable inappropriate effects in the area, and MTs characteristics indicated the exceeding of the adaptive response to pollution in the mixed polluted area and particular response to the pollution by Cu in the cooling pond.

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