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Detoxification and cellular stress responses of unionid mussels *Unio tumidus* from two cooling ponds to combined nano-ZnO and temperature stress

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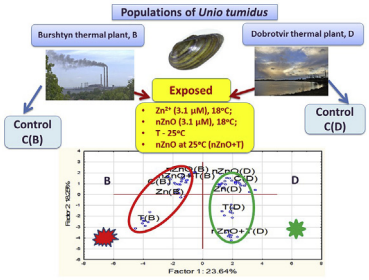
HIGHLIGHTS

- Mussels from two cooling ponds were exposed to Zn^{2+}, 25 °C, and nano-ZnO at 18 °C and 25 °C.
- A common stress response was the up-regulation of metallothioneins and gonad alkali-labile phosphates.
- Responses of HSPs and caspase-3 were distinct in the mussels from the two populations.
- Nano-ZnO modulated multixenobiotic-resistance protein activities.

GRAPHICAL ABSTRACT



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ABSTRACT

Bivalve mollusks from the cooling reservoirs of fuel power plants (PP) are acclimated to the chronic heating and chemical pollution. We investigated stress responses of the mussels from these ponds to determine their tolerance to novel environmental pollutant, zinc oxide nanoparticles (nZnO). Male *Unio tumidus* from the reservoirs of Dobrotvir and Burschtyn PPs (DPP and BPP), Ukraine were exposed for 14 days to nZnO (3.1 μM), Zn^{2+} (3.1 μM) at 18 °C, elevated temperature (T, 25 °C), or nZnO at 25 °C (nZnO + T). Control groups were held at 18 °C. Zn-containing exposures resulted in the elevated concentrations of total and Zn-bound metallothionein (MT and Zn-MT) in the digestive gland, an increase in the levels of non-metalated MT (up to 5 times) and alkali-labile phosphates and lysosomal membrane destabilization in hemocytes. A common signature of nZnO exposures was modulation of the multixenobiotic-resistance protein activity (a decrease in the digestive gland and increase in the gills). The origin of population strongly affected the cellular stress responses of mussels. DPP-mussels showed depletion of caspase-3 in the digestive gland and up-regulation of HSP70, HSP72 and HSP60 levels in the gill during most exposures, whereas in the BPP-mussels caspase-3 was up-regulated and HSPs either downregulated or maintained stable. BPP-mussels were less adapted to heating shown by a glutathione depletion at elevated temperature (25 °C). Comparison with the earlier studies on mussels from pristine

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habitats show that an integrative 'eco-exposome'-based approach is useful for the forecast of the biological responses to novel adverse effects on aquatic organisms.

1. Introduction

Freshwater ecosystems are exposed to multiple anthropogenic stressors leading to a rapid decrease in freshwater biodiversity (Dudgeon et al., 2006). Among the growing concerns about the integrity and sustainability of aquatic ecosystems, a key consideration is understanding and prediction of the effects of the multiple stressors and their interactions (Holmstrup et al., 2010; Griffen et al., 2016). Assessment of the combined effects of multiple stressors on aquatic organisms and their consequences for populations and ecosystems is impeded by the lack of mechanistic understanding of the multiple stressor effects which often quantitatively and/or qualitatively differ from the effects of single stressors (Amin et al., 2014; Noyes and Lema, 2015; Hrubik et al., 2016). Recent studies focused on human health emphasized the importance of understanding of the integrated cellular response (the so-called exposome) as a mechanistic basis for linking the stressor exposures to health outcomes (Escher et al., 2016); yet this approach has rarely been used in the wild populations of freshwater organisms. Studies of the integrated cellular responses to multiple stressors (i.e. the eco-exposome; Escher et al., 2016) can provide important clues for understanding of the impact of multiple stressors (such as pollution and climate change) on freshwater organisms' health and uncover critical mechanisms involved in stress tolerance of freshwater organisms to anthropogenically modified environments.

Warming and pollution are among major threats to freshwater ecosystems, yet their combined effects on the ecosystem health are not fully understood (Ficke et al., 2007; Woodward et al., 2010; Greaver et al., 2016). Temperature directly affects the rates of physiological and biochemical reactions in aquatic ectotherms and may thus strongly modulate their responses to pollution (Sokolova and Lannig, 2008; Sokolova et al., 2012; Nikinmaa, 2013). Laboratory studies show that elevated temperature may increase susceptibility of aquatic ectotherms to pollutants such as trace metals, metal-containing nanoparticles or organic pollutants (Sokolova and Lannig, 2008; Falfushynska et al., 2015a; Sulmon et al., 2015; Hallman and Brooks, 2016), although short-term acute warming may transiently enhance detoxification and biotransformation (Kennedy and Walsh, 1994; Bains and Kennedy, 2004). The effects of the chronic, multigenerational exposure to warming and pollution on stress tolerance of aquatic organisms and their ability to cope with additional challenges (e.g. introduction of novel chemicals or continuing warming) are not yet well understood. The cooling ponds of the electrical power plants represent excellent model systems to study the effects of long-term acclimatization to anthropogenically modified environments on the ability of freshwater organisms to cope with multiple stressors and to determine the physiological and molecular mechanisms setting limits to the multistressor tolerance (Falfushynska et al., 2010, 2013a, 2013b, 2014, 2016a, 2016b, Falfyshynska et al.2016). The fuel thermal power plants (TPPs) commonly release a mixture of pollutants (such as metals, radionuclides, aromatic and alkyl hydrocarbons and other hazardous compounds) in the discharge water and chronically elevate the temperature of the water in the vicinity of the discharge by as much as 5–8 °C (Baršienė and Rybakovas, 2008; Ruhl et al., 2012). Earlier studies showed that long-term exposures to thermal and chemical stress may improve the ability of organisms to cope with additional stressors due to the adaptation and/or phenotypic plasticity that enhance stress protection mechanisms (Hardivillier et al., 2006; Company et al., 2010; Falfushynska et al., 2014 Marigómez et al., 2017). However, stress overload may also occur during multiple stressor exposures leading to health deterioration and/or population declines (Romero et al., 2009; Sokolova, 2013).

Toxicity of the metal-based nanoparticles is a growing concern for freshwater ecosystems (Corsi et al., 2014). The manufactured nanoparticles such as nano-zinc oxide (nZnO) are widely utilized in electronics and personal care products and have been proposed as a major component of the semiconductor films for the effluent treatment (Kamat et al., 2002). As a result of the wide industrial and consumer use, nZnO particles are prevalent in the environment reaching high concentrations (in the microgram per liter range) in the surface waters (Dumont et al., 2015). nZnO may be damaging to the health of the filter feeders such as freshwater bivalves that accumulate metal-based engineering nanoparticles from the water and sediments (Gagnon et al., 2014; Trevisan et al., 2014; Gagné et al., 2015, 2016; Canesi and Corsi, 2016). The metal-containing nanoparticles can affect the health of freshwater bivalves through nanoparticle-specific mechanisms that differ from those elicited by the corresponding dissolved metals (Canesi et al., 2010; Canesi and Corsi, 2016; Falfushynska et al., 2015a). Understanding of the potential modulation of the stress tolerance by long-term exposure to pollution is thus important for understanding and predicting the potential effects of emerging pollutants (such as metal-containing nanoparticles) on keystone freshwater species including bivalves.

In our present study, we determined whether long-term acclimatization in the cooling ponds of TPPs modulates the integrated cellular stress response of the mussels (*Unio tumidus*) to nZnO (3.1 μM) and elevated temperature (25 °C). We hypothesized that chronic multigenerational acclimatization to the thermal and chemical pollution in the cooling ponds may enhance tolerance of freshwater mussels to additional stressors such as the metal-containing nanoparticles and warming. We anticipated that this enhanced tolerance will be reflected in the elevated background expression of stress protection mechanisms and blunted cellular response to additional stressors. To test this hypothesis, we measured the levels of cellular thiols (including metallothioneins (MTs) and glutathione) that play key roles in metal binding, redox balance and antioxidant defense (Valko et al., 2005; Kang, 2006), as well as the activity of the multixenobiotic resistance proteins (MXR) involved in the efflux of xenobiotics (including glutathione-bound metals) from the cells (Tommasini et al., 1996; Ivanina and Sokolova, 2008). The potential induction of oxidative stress and impact of nanoparticles of the membrane integrity and lysosomal function (Canesi et al., 2010; Della Torre et al., 2015) were also assessed. Reproductive dysfunction was evaluated by the level of gonad protein-related alkali-labile phosphates (ALP) commonly used as an indirect measure of vitellogenin (Vtg) in males (Gagné et al., 2016; Sánchez-Marín et al., 2017). The unfolded protein response (UPR) was determined by the expression of molecular chaperones, the heat stress proteins (HSPs). Immunity status was attested by the activity of phenoloxidase which is functionally associated with phagocytosis, self-nonself discrimination,

cytotoxicity and melanisation response (Cerenius and Söderhäll, 2004). Cellular injury was assessed by DNA damage and activity of apoptotic pathways (assessed by the activity of a key executor caspase-3). This comprehensive assessment of cellular protection mechanisms and stress-induced injury provides insights into the integrated cellular response (i.e. the eco-exposome) and the mechanisms contributing to the tolerance to multistressors in a sentinel freshwater species *U. tumidus* from anthropogenically modified habitats.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma Aldrich (St. Louis, USA) or Merck (Synbias, Kyiv, Ukraine), and were of the analytical grade or higher (Supplement 1).

2.2. Sampling and experimental exposures

Adult *Unio tumidus* (Unionidae) (~6 years old, 8 ± 1 cm length, and 42 ± 5 g weight, N = 200) were collected in the cooling ponds of two thermal power plants, Dobrotvir TPP (DPP, 50°12′ N 24°23′ E, the surface area of the cooling pond ~720 ha) and Burshtyn TPP (BPP, 49°15′ N, 24°35′ E, the surface area of the cooling pond ~1260 ha), located in the west part of Ukraine. BPP is included in the list of the ten worst environmental polluters in Ukraine (List of Top-100 companies in Ukraine responsible for pollution, 2017).

Bivalves (~150 mollusks per site) were collected within one week in early autumn of 2014 on the southern banks of the BPP and DPP ponds. Mollusks were transported to the laboratory in 10 L cages with aerated native water. Surface water samples were collected at the study sites and transported to the laboratory on ice for determination of the chemical parameters (Supplementary Table 1).

Bivalves were acclimated in the laboratory for up to seven days in 80 L tanks with aerated, dechlorinated, conditioned tap water prior to the experimental exposures. After the preliminary acclimation, mollusks from each site were randomly divided into five groups. One group from the each TPP site was maintained under the same conditions as during the preliminary acclimation and was considered control (C-DPP and C-BPP, respectively). Other groups were exposed to one of the following conditions: 1) 3.1 μM nZnO at 18 °C (nZnO); 2) 3.1 μM Zn^{2+} at 18 °C as a positive control for Zn exposure (Zn); 3) elevated temperature of 25 °C (T); and 4) a combination of 3.1 μM nZnO and elevated temperature of 25 °C (nZnO + T). The exposure conditions were the same as used earlier for the unionid mussels from a pristine site (Falfushynska et al., 2015a). The highest predicted exposure concentration of n-ZnO for aquatic organisms in the EU was ~500 ng/L (~8 nM) (NanoFATE, 2010–2014). In a pilot study, we determined the 96 h LC50 of n-ZnO for a unionid mussel *Unio tumidus* as 49 μM Zn, and EC_{50} calculated from the data of neutral red retention test (indicative of lysosomal stability) and micronuclei test (indicative of the genotoxicity), was 15 μM Zn (Stoliar, Falfushynska, unpublished data). Control and experimental exposures were conducted for 14 days. A static renewal design was used, with water changed and chemicals replenished every two days. Mollusks were fed with a commercial food ("Aquarius", Ukraine) prior to the water change. The Zn concentration in the tanks remained stable throughout experimental exposures (measured Zn levels in Zn^{2+} and nZnO exposures were 3.08 ± 0.05 μM and 3.08 ± 0.11 μM, respectively, N = 3). No mortality of the mussels occurred during the experimental exposures.

After 14 days of exposures, mollusks were dissected on ice. Sex was examined microscopically, and males were selected for further analyses. The mussels were examined under a light microscope for the presence of parasites in the digestive gland. Only parasite-free male mollusks were used for the investigation. Lysosomal membrane stability and nuclear lesions were determined in hemocytes, ALP in the gonads, all other traits in the digestive gland and gills. Hemocytes were studied immediately after sampling, while other tissues were shock-frozen and stored at −40 °C until further analyses.

Hemolymph was withdrawn from the adductor muscle sinus. Hemocytes were pelleted at 250 × *g*, resuspended in phosphate-buffered saline/EDTA (v/v 1:1), diluted to 4 × 10^6 cells/mL, then aliquoted for analyses. For enzymatic analyses, the digestive gland and gill samples were homogenized (1/10 w/v) in 0.1 M phosphate buffer, pH 7.4, containing 100 mM KCl and 1 mM EDTA, and 0.1 mM PMSF to inhibit proteolysis. Homogenates were centrifuged for 10 min at 6000×*g*, Protein concentration in the supernatant was measured by the method of Lowry et al. (1951) using bovine serum albumin as a protein standard. All assays described in the following Sections 2.3–2.10 have been optimized to *U. tumidus* in our earlier studies (Falfushynska et al., 2015a).

2.3. Isolation and quantification of metallothioneins (MTs)

MTs from the digestive gland and gills were isolated as the thermostable proteins by size-exclusion chromatography on Sephadex G-50 (Roesijadi and Fowler, 1991). The fractions with high absorbance at 254 nm were identified as putative MTs-containing peaks and pooled for determination of the Zn content (used to assess Zn-MTs levels). The total concentration of MTs in the fraction was assessed as the concentrations of thiols using DTNB reduction method after the ethanol/chloroform extraction (Viarengo et al., 1997). The levels of MT-SH were expressed as μg of MT-SH per g of wet tissue mass (FW).

2.4. Zinc determination

The concentration of Zn was measured in the digestive gland and gills tissues and in the pooled eluate of MTs-containing fractions (see Section 2.3) after samples digestion in HNO_3 (conc). Zn content was evaluated by the atomic absorption spectrophotometry. Zn measurements were validated using *ERM-CE 278* certified reference material; the recovery of Zn was between 90% and 110%. Quality control was assessed by method of Standard Addition (Beukelman and Lord, 1960).

2.5. Activity of the multixenobiotic resistance proteins (MXR)

MXR activity was determined in freshly isolated digestive gland and gills tissues using the rhodamine B (RB) efflux assay as described elsewhere (Ivanina and Sokolova, 2008). The total efflux of RB expressed as pmol RB min^{-1} g^{-1} FW.

2.6. Oxidative stress markers

Superoxide dismutase (SOD, EC 1.15.1.1) activity was measured in digestive gland and gills based on the aerobic reduction of NBT at 535 nm by superoxide radicals (Beauchamp and Fridovich, 1971) and expressed as units·mg^{-1} soluble protein. Activity of Mn-SOD activity was determined using the supernatant preincubated with 5 mM KCN.

Glutathione-S-transferase (GST, EC 2.5.1.18) activity was measured using CDNB as the substrate (Habig et al., 1974). The GST activity was expressed as nmol min^{-1}·mg^{-1} soluble protein.

Total glutathione concentration in the digestive gland and gill tissues was quantified by glutathione reductase recycling assay

(Anderson, 1985). To estimate the oxidized glutathione (GSSG) level, the samples were treated with 2% 2-vinylpyridine (Griffith, 1980). The redox–index of glutathione (RI GSH) was calculated as the ratio of concentrations of reduced to total glutathione.

Lipofuscin concentration was determined in the chloroform:methanol extracts of the digestive gland tissues. A solution of quinine sulphate was used as a standard (Manibabu and Patnaik, 1997).

2.7. Immunoblotting of HSPs

Digestive gland and gills tissues were homogenized in ice-cold homogenization buffer (100 mM Tris, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton-X 100, 10% glycerol, 0.1% sodium dodecylsulfate, 0.5% deoxycholate, 0.5 μg mL^{-1} leupeptin, 0.7 μg mL^{-1} pepstatin, 40 μg mL^{-1} PMSF and 0.5 μg mL^{-1} aprotinin), sonicated three times for 10 s each), and centrifuged for 10 min at 14,000xg and 4 °C. Protein content was measured using Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA), and HSPs expression was determined using standard immunoblotting techniques as described elsewhere (Ivanina et al., 2008, 2016). The primary monoclonal antibodies against HSP70 (MA3-007, Affinity Bioreagents, Golden, CO, USA) or HSP60 (SPA-805, Stressgen Bioreagents, Ann Arbor, MI, USA) were used. The dilution of the primary antibody was 1:1000 v/v and the secondary antibiody - 1:2000 v/v. Densitometric analysis was performed by Gel-Doc 2000™ System with Quantity One 1D Analysis Software (Bio-Rad Laboratories Inc., Hercules, CA, USA) (Supplementary Fig. 1).

2.8. Toxicity markers

Possible genotoxic effects were assessed by the level of DNA strand breaks, frequency of the nuclear abnormalities, and activation of apoptotic pathways. Lysosomal membrane stability of hemocytes was measured as an index of lysosome injury.

For the determining of nuclear lesions, the smears of hemocytes fixed in methanol/acetic acid, stained with 5% Giemsa and mount in Canada balsam. The stained slides were analyzed under the light microscope. Frequency of MN and all other lesions (L + S + K) were and expressed per 1000 cells (Baršienė and Rybakovas, 2008).

Levels of protein-free DNA strand breaks in the digestive gland and gills was evaluated by the alkaline DNA precipitation assay (Olive, 1988) using Hoechst 33342 dye. To reduce the possible interference with traces of sodium dodecyl sulphate (SDS), the assay was conducted in the presence of 0.4 M NaCl, 4 mM sodium cholate, and 0.1 M Tris (pH 9) (Bester et al., 1994).

Activity of caspase-3 was assayed colorimetrically based on the hydrolysis of peptide acetyl-Asp-Glu-Val-Asp p-nitroanilide (Bonomini et al., 2004).

Lysosomal membrane stability was determined in hemocytes by the Neutral Red Retention (NRR) assay (Marchi et al., 2004).

2.9. Vitellogenin-like proteins

Concentration of the gonad alkali-labile phosphates (ALP) was measured as an indirect index of abundance of vitellogenin-like proteins (Vtg-LP) in male gonads (Blaise et al., 1999). Lipophosphoproteins were extracted using the *tert*-butyl methyl ether and subjected to an alkali treatment which releases the labile phosphates. The labile phosphates were quantified by the phosphomolybdenum assay (Blaise et al., 1999).

2.10. Phenoloxidase-like activity

The phenoloxidase-like activity of tyrosinase (EC 1.14.18.1) was determined by recording the formation of o-quinones (Luna-Acosta et al., 2011). p-Phenylenediamine were used as substrate. Phenoloxidase-like activity was monitored during 2 h at 420 nm. The amount of o-quinones was determined by using a molar extinction coefficient of 43 160 $M^{-1}cm^{-1}$. Data were expressed as nmol min^{-1} mg^{-1} protein.

2.11. Statistical analysis

Data were tested for normality and homogeneity of variance by Kolmogorov-Smirnoff and Levene's tests, respectively. As needed, the data were normalized by Box-Cox common transforming method. For the data that were not normally distributed after the transformation, non-parametric tests (Kruskall–Wallis ANOVA and Mann–Whitney *U* test) were performed. Pearson's correlation test was used to assess correlations between the studied traits. Normalized, Box-Cox transformed data were subjected to the principal component analysis (PCA) to reduce the dimensionality of the data set and identify the potential biomarker signatures of different experimental exposures.

The classification tree based on all studied trait was built using Classification and Regression Tree (CART) software using raw (non-transformed) data. All statistical calculations were performed with Statistica v. 12.0 and Excel for Windows-2013. Differences were considered significant if the probability of Type I error was less than 0.05. For MT analysis, sample size was 3 per group, each biological replicate consisting of pooled tissues from five mussels. For all other traits, sample size was 8. The data are presented as means ± standard deviation (SD) unless indicated otherwise.

3. Results

3.1. Zinc accumulation and binding

Zn levels in the digestive gland decreased in response to all experimental exposures in DPP mussels. The largest decrease observed in the mussels co-exposed to elevated temperature and nZnO (Fig. 1A and B). In the gill of the DPP mussels, Zn accumulated in response to Zn^{2+} and nZnO exposure at 18 °C (Fig. 1A and B). In BPP mussels, Zn content in the digestive gland increased during exposures to nZnO at 18 and 25 °C (Fig. 1A and B). In the gills of the BPP mussels, Zn content remained near the control levels in all exposures except the elevated temperature (25 °C). Notably, elevated temperature (25 °C) alone led to a decrease in Zn content in both studied tissues of DPP and BPP mussels indicating enhanced depuration under the experimental warming (Fig. 2A and B).

Tissue levels of Zn-MT in the digestive gland of the mussels increased in response to all Zn-containing exposures as well as during the warming alone (except the T group from Burshtyn) (Fig. 1C and D). Zn-MT content of the gills did not change in the DPP mussels except for a small but significant decline in the warming-exposed group (T). In the gills of the BPP mussels, Zn-MT was induced by exposure to Zn^{2+}. In all other exposures it remained at or below the control levels (Fig. 1C and D).

The level of MT-SH in the tissues was strongly elevated by all experimental exposures in the digestive gland and the gill tissues of *U. tumidus* (up to ~ 9 times in the digestive gland in the nZnO + T-exposed DPP mussels) (Fig. 1E and F). The only exception was nZnO-exposed mussels from the BPP site (Fig. 1E and F). The ratio of MT-SH to Zn-MT levels increased in all studied tissues and experimental exposures except for the digestive gland of nZnO exposed BPP mussels and the gills of Zn^{2+} exposed BPP mussels (Fig. 1J, H).

Levels of ALP in the gonad of the male *U. tumidus* increased in response to all experimental exposures (except Zn^{2+} exposed DPP mussels), more than doubling in nZnO-containing exposures (Fig. 3E).

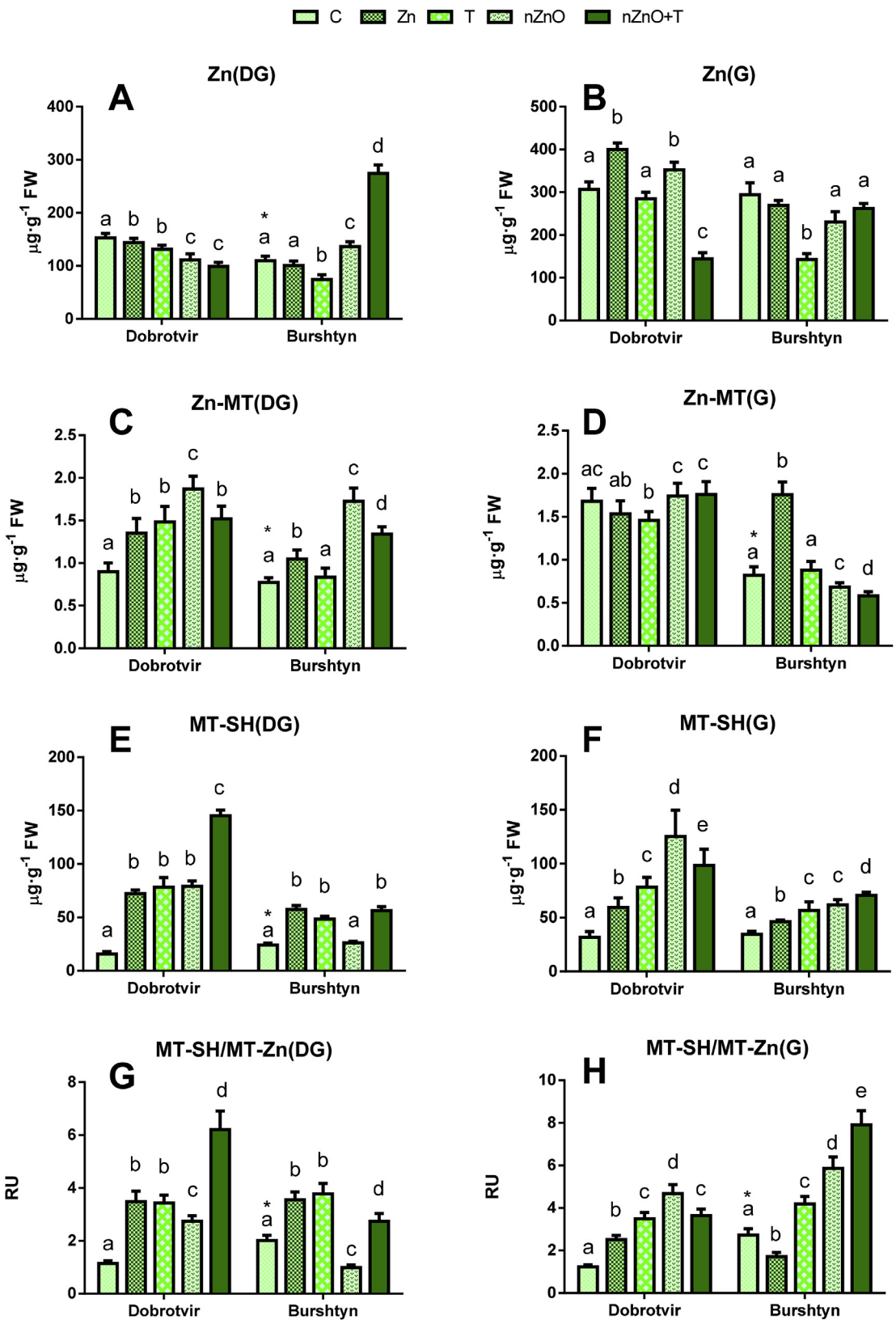

Fig. 1. Effects of experimental exposures to nanosized ZnO (nZnO), ionic zinc (Zn), elevated temperature (T) and the combination of nZnO + T on Zn content and metal binding proteins in the digestive gland (DG) and gills (G) of *Unio tumidus*: A, B - the total tissue Zn burden, C, D - levels of Zn-bound metallothioneins (MT-SH), E,F - total tissue content of metallothioneins (MT-SH), G, H - the ratio of the concentrations of MT-SH/Zn-MT. A, C, E, G - digestive gland, B, D, F, H - gills. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 8 except for the metallothionein-bound Zn, where N = 3.

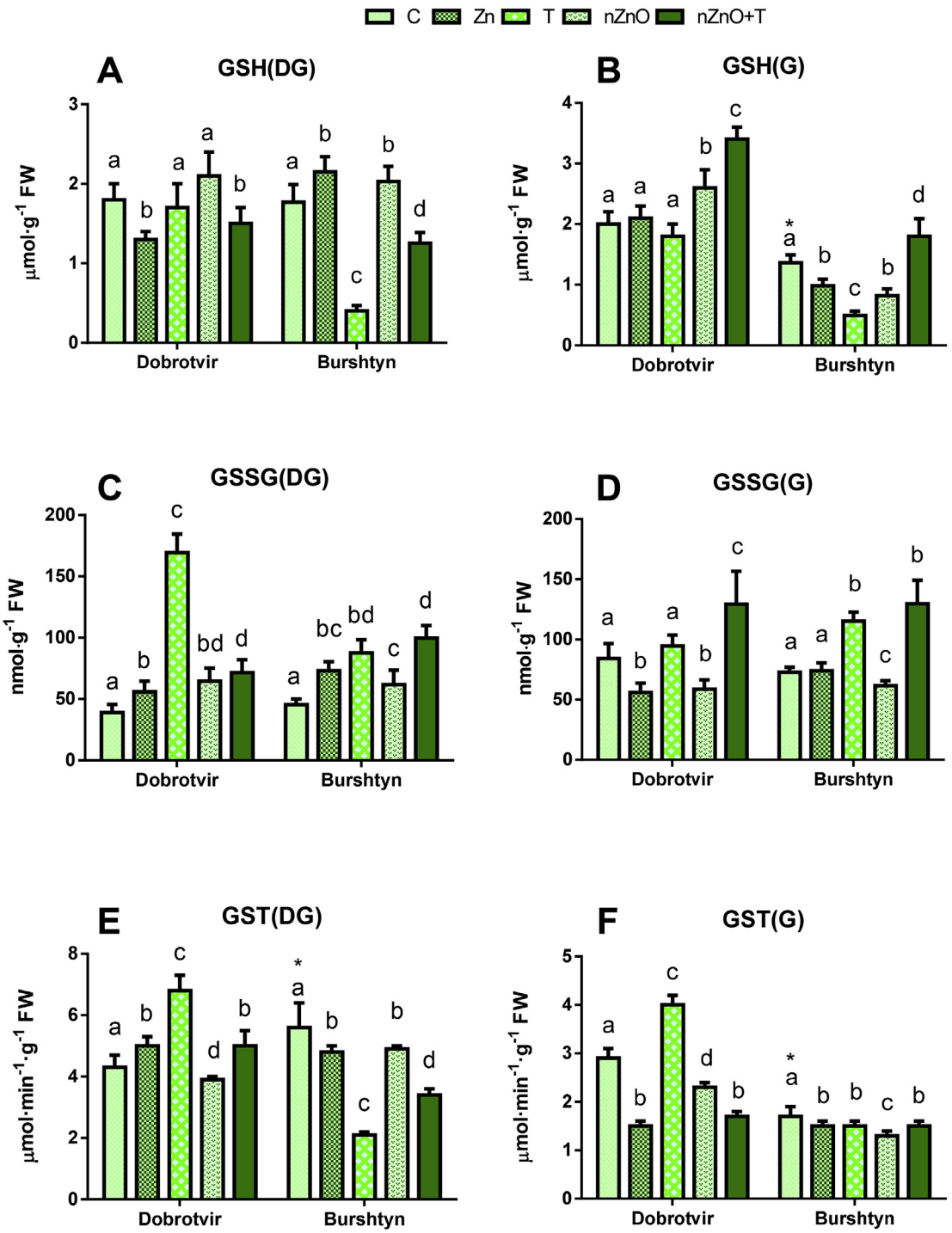

Fig. 2. Effects of experimental exposures to nanosized ZnO (nZnO), ionic zinc (Zn), elevated temperature (T) and the combination of nZnO + T on the glutathione pool of the mussel tissues: A, B - concentration of the reduced glutathione, C, D - concentration of the oxidized glutathione, E, F - activity of glutathione-S-transferase. A, C, E - digestive gland, B, D, F - gills. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 8.

3.2. Glutathione system response

Elevated temperature had a strong effect on the tissue levels of GSH and GSSG in mussels (Fig. 2A–D). In the BPP mussels exposed to 25 °C, GSH levels in the digestive gland and the gills decreased by ~4 and 3 fold, respectively. Combined exposure to nZnO and elevated temperature had a similar albeit less pronounced effect on the GSH levels in digestive gland (Fig. 2A and B). This was accompanied by an increase (up to ~4 fold) in the tissue GSSG levels of the BPP mussels exposed to elevated temperatures alone or in combination with nZnO (Fig. 2C and D). In the DPP mussels, GSH levels remained relatively stable in the digestive gland except slight but significant decrease during exposures to Zn^{2+} and nZnO + T (Fig. 2A). In the gills of the DPP mussels, GSH levels increased in

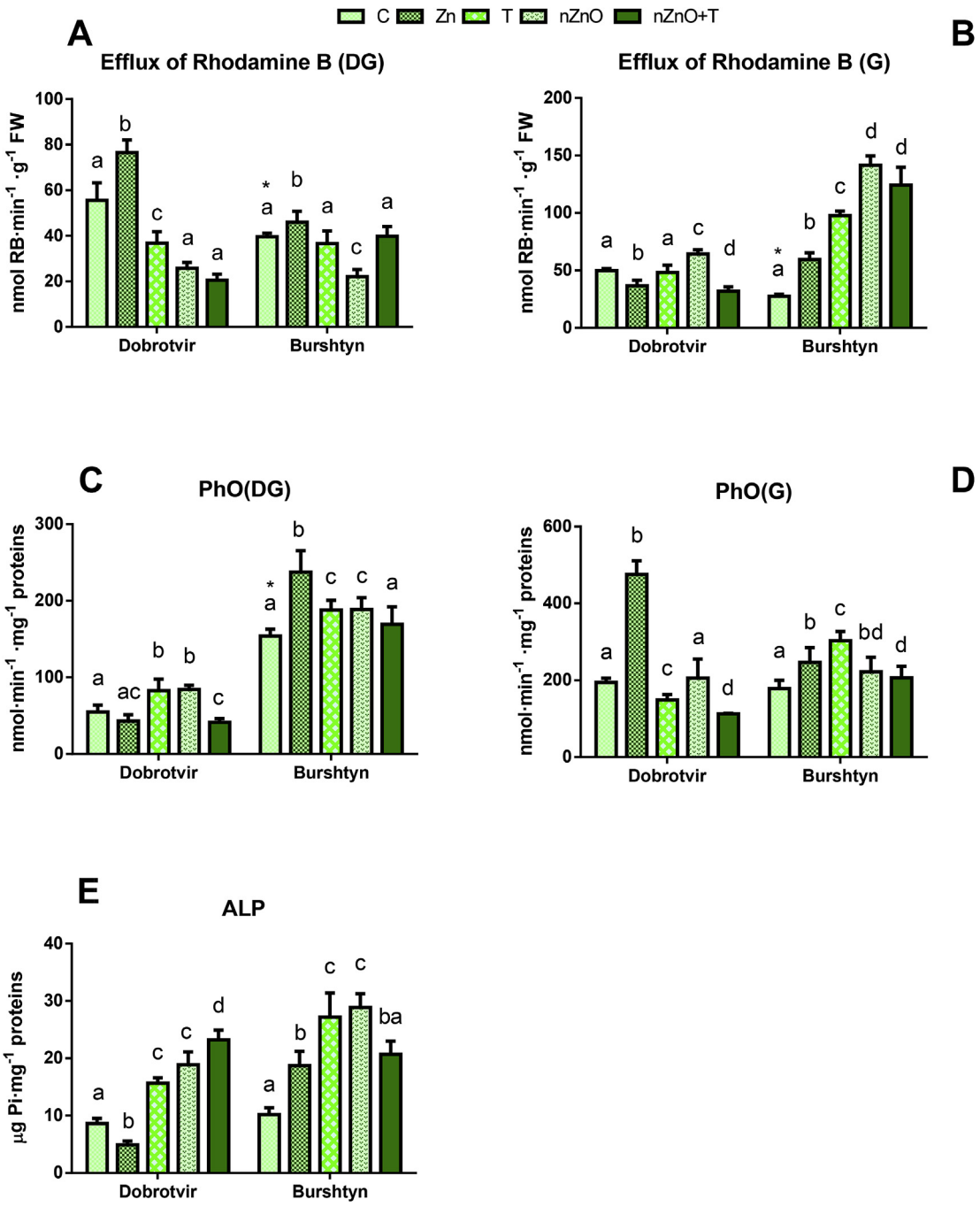

Fig. 3. Efflux of Rhodamine B (A, B), phenol-oxidase activity (C,D) and alkali-labile phosphates (ALP) levels (E) in *Unio tumidus* exposed to nanosized ZnO (nZnO), ionic zinc (Zn), elevated temperature (T) and the combination of nZnO + T during 14 days: A, C - digestive gland, B,D - gills, E - gonads. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 8.

response to nZnO and nZnO + T. GSSG levels in the digestive gland of the DPP mussels were elevated in all experimental exposures (Fig. 2C). In the gills of the DPP mussels, the GSSG response was more variable with an increase in nZnO + T exposed group and decreases in nZnO and Zn^{2+} exposures (Fig. 2D). Elevated temperatures led to significant decreases of the RI ratio from 0.98 ± 0.01 in control to 0.91 ± 0,02 in the digestive gland of the T-exposed DPP mussels and from 0.95 ± 0,01 to 0.81 ± 0,03 in both studied tissues of the T-exposed DPP mussels ($p < 0.05$).

The activity of GST in the digestive gland of DPP mussels increased in response to all experimental exposures except nZnO (Fig. 2E and F). In the gills of DPP mussels, the GST activity was stimulated by the elevated temperatures and suppressed in response to all other exposures (Fig. 3 E, F). In BPP mussels, there was a general suppression of GST activity in the digestive gland and the gills by all experimental exposures (Fig. 2E and F).

3.3. MXR and phenoloxidase activity

The rates of efflux of an MXR substrate, rhodamine B (indicative of the MXR activity) was lower in the tissues of mussels from BPP compared to DPP under the control conditions (Fig. 3A and B). In

the gill tissues, MXR activity was stimulated by nZnO (in DPP mussles) and by all experimental exposures (in BPP mussels). In the digestive gland, MXR activity was stimulated by Zn^{2+} and either suppressed or remained unchanged in all other exposures, depending on the population. Notably, exposure to nZnO caused a decrease of this activity in the digestive gland and an increase in the gills in the mussels from both ponds.

Phenoloxidase activity in the digestive gland of BPP mussels increased in response to all experimental treatments, whereas in the DPP groups the response was variable with most prominent increase (by 2.4 times) in the gills of Zn^{2+} exposed mussels (Fig. 3C, D).

3.4. Oxidative stress parameters

The baseline levels of Mn-SOD in the digestive gland and Cu,Zn-SOD in the both tissues of mussels were higher in the DPP mussels compared to their counterparts from the BPP site (Fig. 4A and B). Experimental exposures affected the SOD activity differentially in the DPP and BPP mussels. In the digestive glands of the DPP mussels, Cu, Zn-SOD activity increased while Mn-SOD activity declined or remained unchanged in response to experimental treatments (Fig. 4A). An opposite pattern was seen in the gills (Fig. 4B). In the digestive gland of the BPP mussels, Cu, Zn-SOD activity declined and Mn-SOD activity increased in experimental exposures, and while in the gills Cu, Zn-SOD activity increased and Mn-SOD activity decreased or remained unchanged (Fig. 4A and B).

Levels of lipofuscin in the digestive glands of the DPP and BPP mussels was not affected by nZnO alone and changed by less than 12% compared to the control values in other experimental exposures, although these slight changes were significant in some groups (Fig. 4C).

3.5. Lysosomal membrane destabilization

The lysosomal membrane stability was 22–25 min in the control groups of the DPP and BPP mussels and declined to ~15 min in all exposures except in the groups exposed to the elevated temperature alone (Fig. 4D).

3.6. Molecular chaperones

The expression of molecular chaperones showed tissue- and site-specific response in *U. tumidus* (Fig. 5). Levels of the inducible HSP69 isoform remained unchanged or decreased in the digestive gland and the gills of the DPP mussels in all exposures except the combined nZnO + T exposure which induced elevated HSP69 expression (Fig. 5A and B). The levels of the constitutive HSP72 were not affected by most experimental exposures in the DPP mussels. Only in the digestive gland of the Zn^{2+} and nZnO exposed mussels the HSP72 levels were suppressed (Fig. 5C and D). In the BPP mussels, HSP69 levels were suppressed in all experimental exposures in the digestive gland but elevated during exposures to Zn^{2+}, nZnO or warming in the gills (Fig. 5A and B). HSP72 levels were suppressed by Zn^{2+} exposure or warming in the digestive gland and increased during exposures

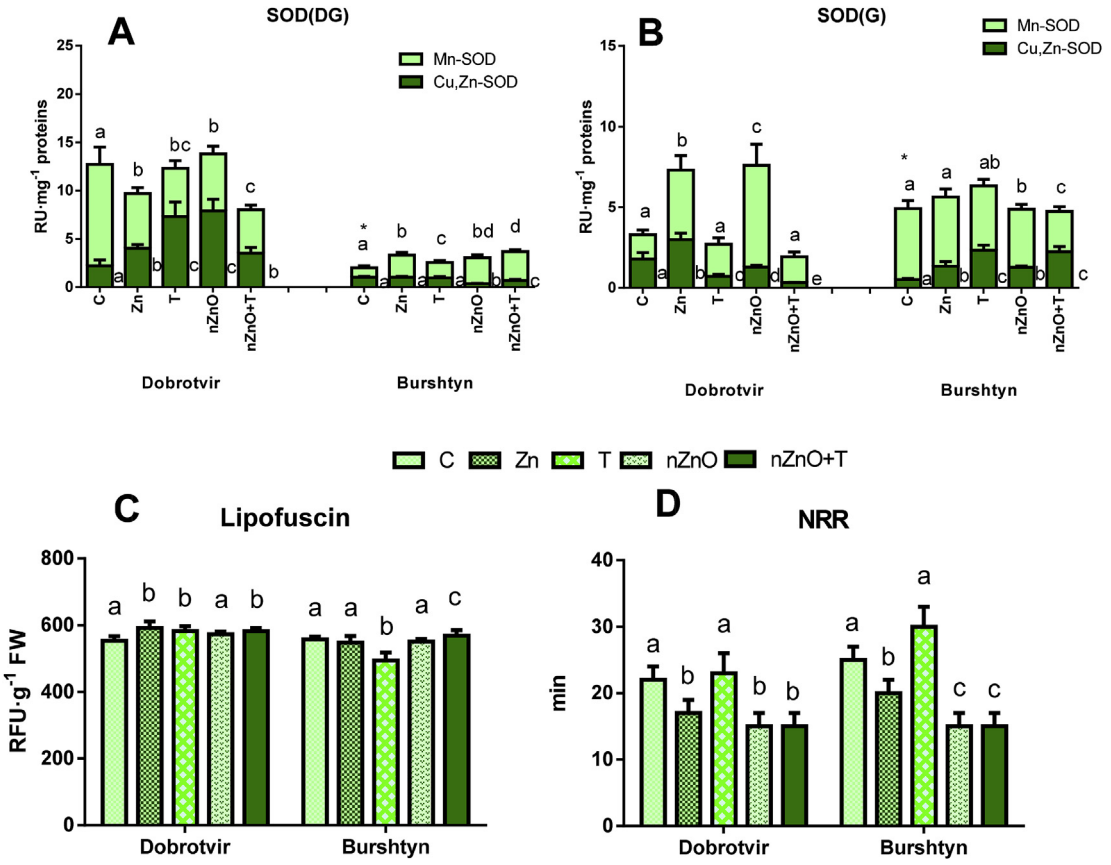

Fig. 4. Effects of experimental exposures to nanosized ZnO (nZnO), ionic zinc (Zn), elevated temperature (T) and the combination of nZnO + T on oxidative stress parameters in *Unio tumidus* from cooling ponds of Dobrotvir and Burshtyn power plants: A, B - superoxide dismutase (SOD) activity; C - lipofuscin concentration, D - lysosomal membrane stability assessed by neutral red retention. A, C - digestive gland, B - gills, D - hemocytes. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 8.

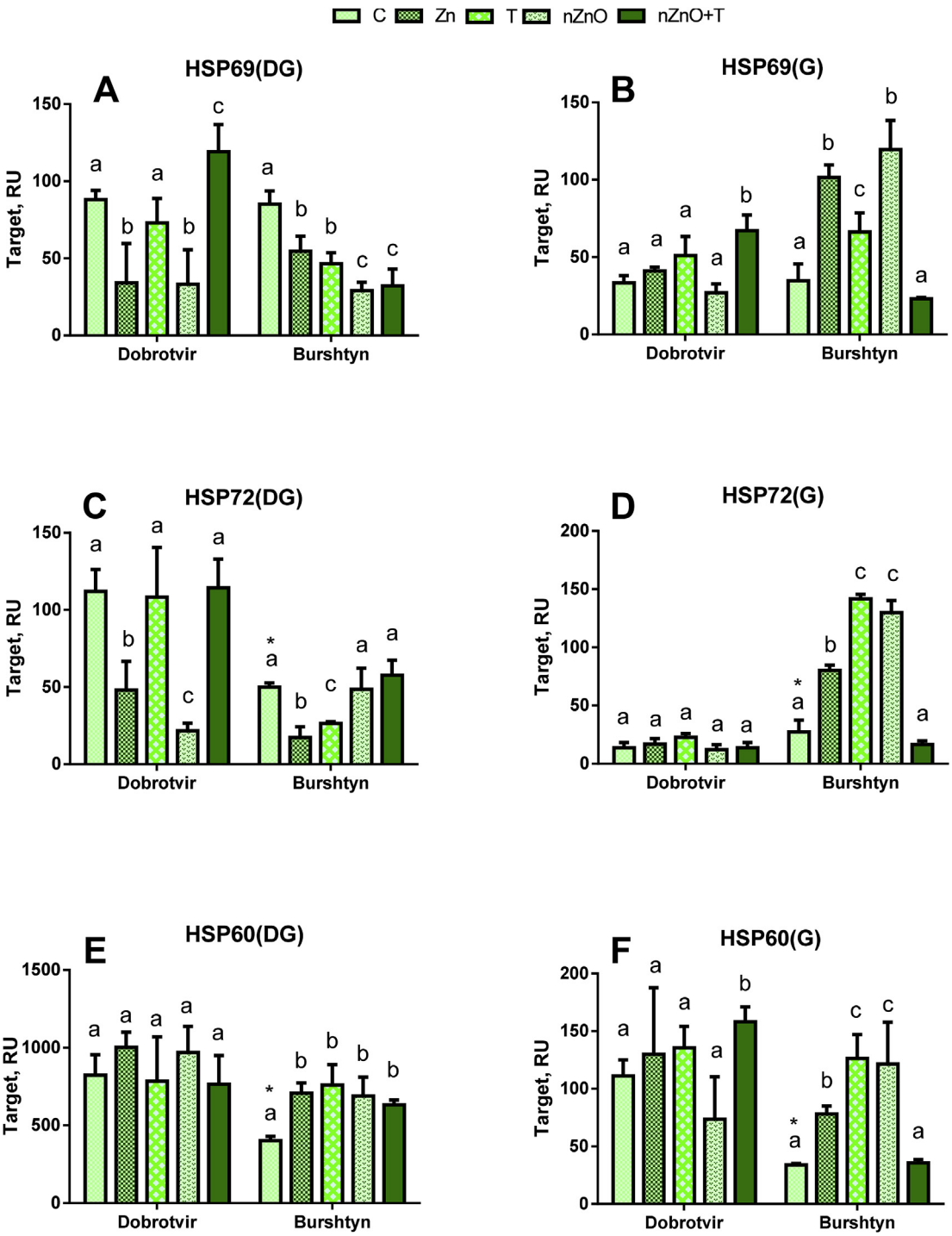

Fig. 5. Molecular chaperons in Unio tumidus exposed to nanosized ZnO (nZnO), ionic zinc (Zn), elevated temperature (T) and the combination of nZnO + T during 14 days: A, B - HSP69; C,D - HSP72; E,F - HSP60. A,C,E - digestive gland, B,D,F - gills. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 5.

to Zn, warming or nZnO in the gills of the BPP mussels (Fig. 5C and D).

In DPP mussels, there was no significant effect of experimental exposures on the levels of the mitochondrial HSP60 except a slight but significant increase in the gills of nZnO + T exposed mussels (Fig. 5E and F). In the BPP mussels, all experimental exposures (except the nZnO + T exposure in the gill) strongly increased the HSP60 expression (Fig. 5E and F).

3.7. Genotoxicity and apoptosis markers

In DPP mussels, elevated DNA fragmentation was induced by Zn^{2+} and nZnO + T exposures in the digestive gland and by Zn^{2+} exposure and warming in the gills (Fig. 6A and B). In BPP mussels, all experimental exposures (except nZnO in the digestive gland) led to elevated DNA fragmentation (Fig. 6A and B). All experimental exposures resulted in the increased frequencies of micronuclei

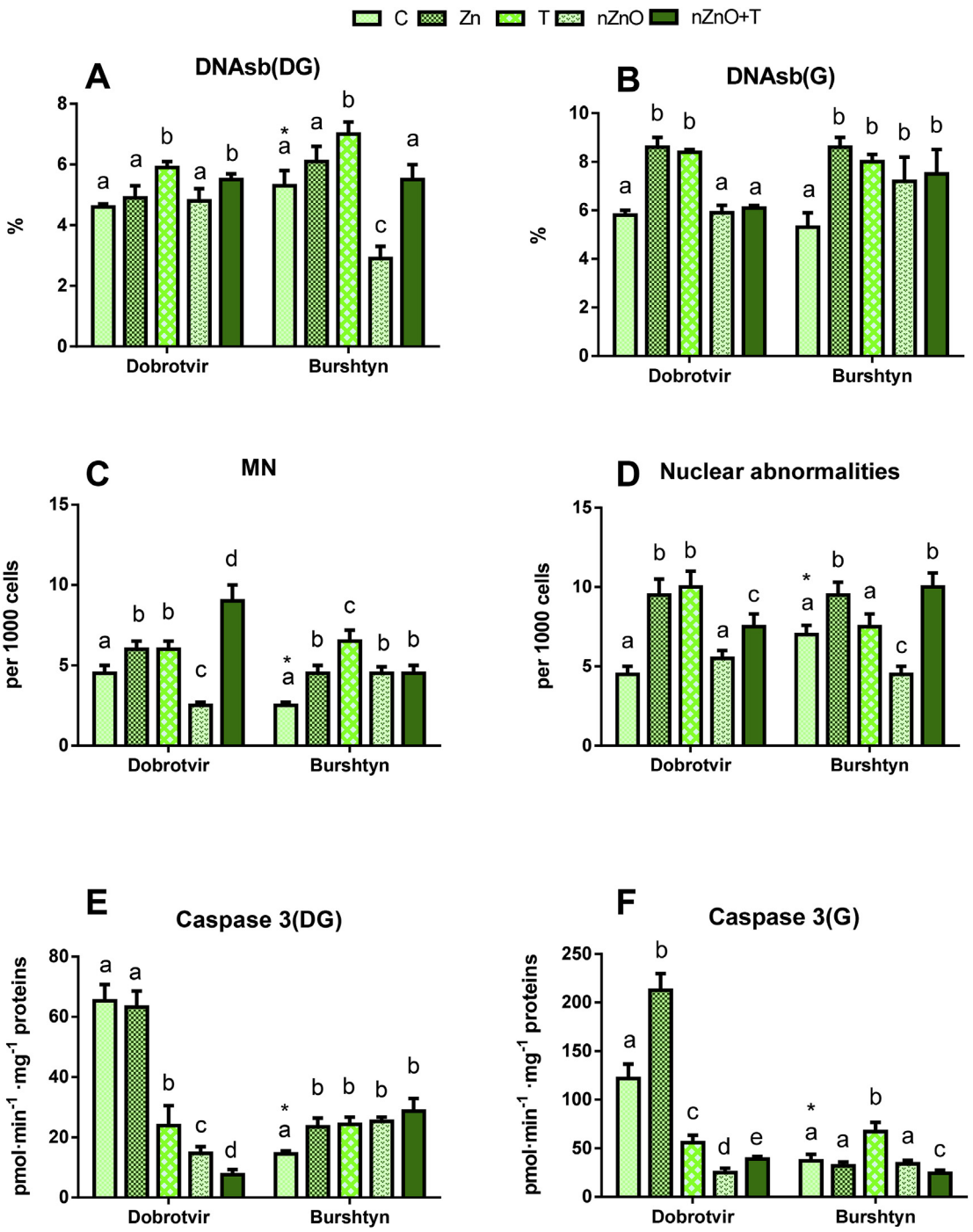

Fig. 6. Cytotoxicity markers in the tissues of *Unio tumidus* exposed to nanosized ZnO (nZnO), ionic zinc (Zn), elevated temperature (T) and the combination of nZnO + T: A,B - frequencies of the DNA strand breaks; C - micronuclei frequency in hemocytes; D, - nuclear abnormalities frequency in hemocytes; E,F - caspase-3 activity. A, E - digestive gland, B-F - gills, C,D - hemocytes. The columns that share the same letters indicate the values that are not significantly different ($P > 0.05$). Asterisks indicate the control values of the traits that significantly differ between the DPP and BPP mussels ($P < 0.05$). N = 8.

(Fig. 6C and D) and other nuclear abnormalities (Fig. 6E and F) in the hemocytes of the DPP and BPP mussels.

Basal levels of caspase-3 activity were considerably higher (by 3–4 fold) in the BPP mussels compared to their DPP counterparts ($p < 0.05$) (Fig. 6G and H). Caspase-3 activity was strongly suppressed (by up to 9 fold) in the digestive gland and gills of the DPP-mussels exposed to warming, nZnO and nZnO + T co-exposure but increased in the gills of the Zn^{2+} exposed mussels (Fig. 6G and H). In the BPP mussels, caspase-3 was up-regulated in the digestive gland in all exposures and in the gills by warming (Fig. 6G and H).

3.8. Data integration

The PCA analyses of the studied traits reflect species- and population-specific differences in the biomarker response profiles to Zn^{2+}, nZnO and temperature exposures (Fig. 7, Supplementary Tables 2 and 3). In the DPP population, the PCA analysis revealed the first two principal components that explain 33% and 23% of the

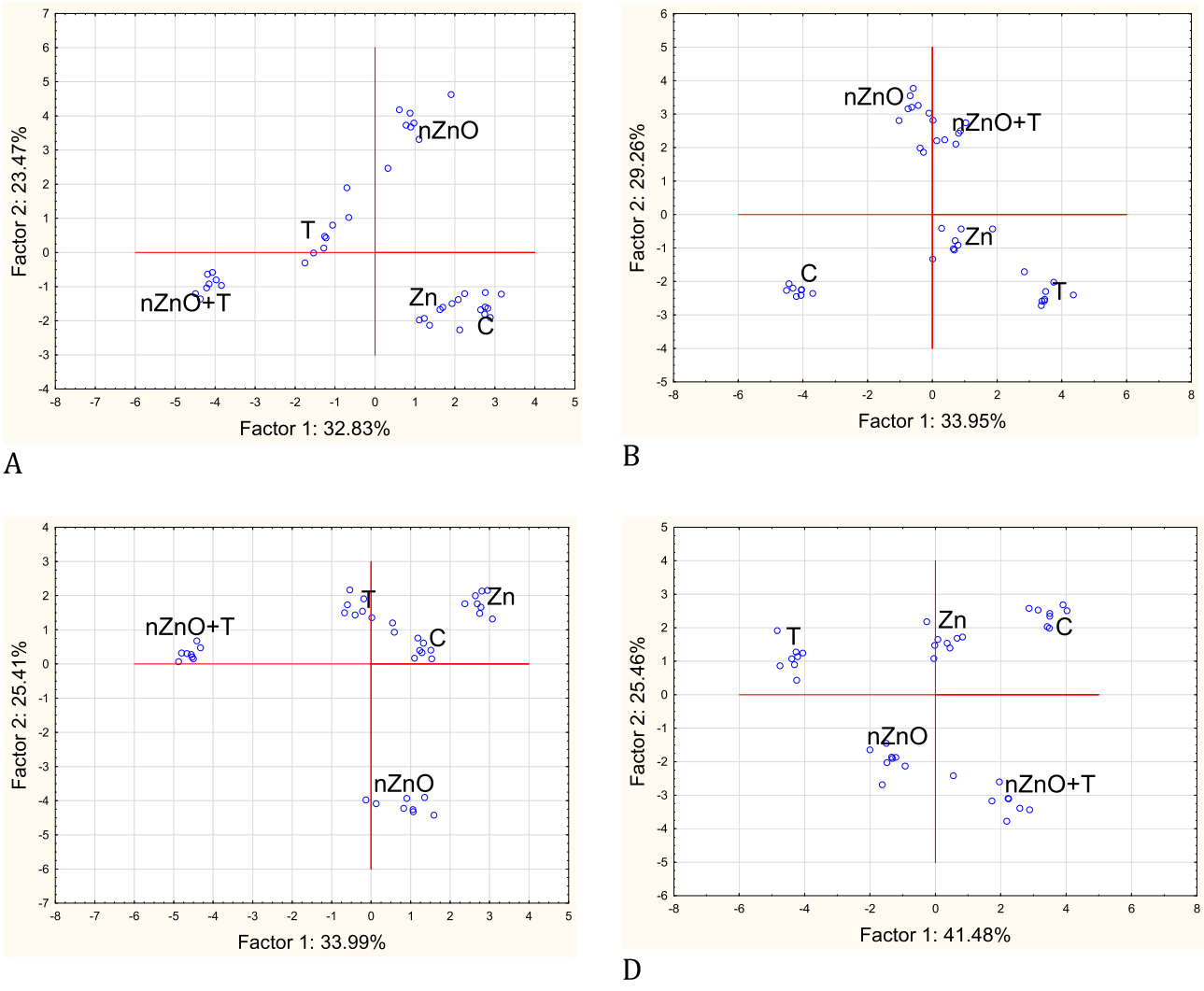

Fig. 7. Results of the principal component analysis of the studied biological traits in digestive gland (A, B) and gills (C, D) of *Unio tumidus* from cooling ponds of Dobrotvir (A, C) and Burshtyn (B, D) power plants

total variation of the biomarkers in the digestive gland, and 34% and 25% of the total variation in the gill, respectively (Fig. 7A, C). In the digestive gland, the nZnO + T and T groups were separated from the control, Zn^{2+} and nZnO groups along the PC1 axis. The PC1 has high positive loadings (>0.6) of caspase-3 activity, tissue Zn content and MXR-like activity, and high negative loadings (<-0.6) of ALP, DNA damage, frequency of micronuclei and the ratio of MT-SH to Zn-MT (Supplementary Table 2). The PC2 which separated the control and Zn^{2+} exposed mussels from the nZnO-exposed group based on the digestive gland biomarker responses, had high positive loadings of the GSH content, phenoloxidase activity and Zn-MT and high negative loadings of the micronuclei frequency.

The pattern of the separation of different exposure groups of DPP mussels based on the biomarker responses in the gills was similar to those of BPP mussels. Thus, the nZnO + T group was separated from the rest along the PC1 axis (high positive loadings of SOD, caspase-3 and phenoloxidase activities and tissue Zn content, and high negative loadings of GSH, GSSG and ALP concentrations, as well as the micronuclei frequency and HSP69 content). nZnO group was separated from the other groups along the PC2 axis (high positive loadings of the DNA and nuclear damage, as well as the HSP60 content, and high negative loadings of MT-SH, MXR -like activity and the ratio of MT-SH to Zn-MT) (Supplementary Table 2).

In the digestive gland of BPP mussels, the PC1 (33% of the total variation) separated the control mussels from those exposed to Zn^{2+} or temperature stress alone, whereas the nZnO-exposed groups (including nZnO and nZnO + T) were separated from the others along the PC2 axis (Fig. 7B). The PC1 had high positive loadings of GSSG, ALP, micronuclei frequency, caspase-3 activity, HSP60 content and the ratio of MT-SH to Zn-MT, and high negative loadings of the GST activity and lipofuscin content. The 2nd principal component was positively associated with SOD and caspase-3 activity, total Zn and Zn-MT content, and negatively associated with the lysosomal stability and HSP69 content (Supplementary Table 2).

Separation of different exposure groups based on the biomarker responses in the gills of the BPP mussels was similar to that found in the digestive gland. The control mussels were separated from those exposed to Zn^{2+} or temperature stress alone along PC1 axis, and the nZnO-exposed groups (including nZnO and nZnO + T) separated from the others along the PC2 axis (Fig. 7D). The PC1 in the gills had high positive loadings of GSH, lipofuscin, tissue Zn

content, and high negative loadings of the SOD, caspase-3 and phenoloxidase activities, ALP, micronuclei frequency, DNA damage and HSP72 and HSP60 content. The 2nd principal component was positively associated with the lysosomal stability and negatively associated with the MT-SH content, MT-SH to Zn-MT ratio and MXR-like activity (Supplementary Table 3, Supplementary Fig. 2).

4. Discussion

Our study demonstrates the variability of the molecular responses to common environmental stressors (including an emerging environmental pollutant nZnO, ionic Zn^{2+}, elevated temperature and their combination) in unionid mussels from two cooling ponds of electrical power plants. The comparison of two control groups suggest lower baseline activity of detoxification and stress protection systems in the BPP mussels. Notably, the two study sites have comparable levels of chemical parameters (Supplementary Table 1). However, the differences in the molecular markers baselines between the two studied populations are persistent for 21 days of acclimation under the common garden conditions in the laboratory. It is presently unknown what causes the differences in the baseline levels of the studied biomarkers between the mussels from the two study sites. Potential explanations may include the differences in the genetic make-up or acclimation history of the two studied populations, and/or reflect the influence of some environmental variables that were not measured in the present study. Regardless of the sources of variation, differences in the biomarker baselines presents challenges for environmental risk assessments in the native populations and might require establishing the marker baselines for each studied population used as a sentinel for species or ecosystem health.

4.1. Uptake and binding of Zn in mussels

Generally, DPP and BPP mussels did not accumulate high Zn in Zn^{2+} or nZnO exposures indicating that this metal is either not bioavailable or strongly regulated in the cooling pond populations commonly exposed to elevated levels of trace metals in their habitats. Exposure to the environmental sources of Zn in the form of Zn^{2+} or nZnO led to accumulation of Zn only in the gills of the mussels from the DPP pond. Similar results were reported by Trevisan et al. (2014) in the acute exposure of oysters *Crassostrea gigas* to 4 mg L^{-1} (62 μM Zn) of nZnO. In other cases (including the gill tissues of the BPP mussels and the digestive glands of the mussels from both study sites (except nZnO + T group of BPP mussels)) no Zn accumulation was detected. Furthermore, Zn burden in the digestive gland of the DPP mussels decreased during exposures to Zn^{2+} or nZnO. A similar decrease in Zn burden when exposed to Zn^{2+} or nZnO was found in *U. tumidus* from a pristine site (Falfushynska et al., 2015a). This may indicate stimulation of the Zn efflux mechanisms by Zn exposure to prevent over-accumulation of this metal (Eide, 2006) leading to a slight but significant net loss of Zn from the mussel tissues. Exposure to Zn^{2+} or nZnO led to a strong upregulation of the total metallothionein levels and increased binding of Zn to metallothioneins in *U. tumidus*. This response may mitigate the potential Zn toxicity while preventing excessive loss of this essential metal from the tissues (Eide, 2006; Kimura and Kambe, 2016).

An ability to upregulate the levels of Zn-bound MTs during Zn^{2+} or nZnO exposures was more pronounced in the mussels from the polluted ponds of the power plant (this study) than in the population of *U. tumidus* from a pristine site (Falfushynska et al., 2015a). Thus, the levels of MTs and MT-SH/Zn-MT ratio increased by up to ~9 times in response to Zn-containing exposures whereas in the mussels from a pristine site this elevation was ~30% above the control level (Falfushynska et al., 2015a; this study). Furthermore, unlike in *U. tumidus* from a pristine site (Falfushynska et al., 2015a), the heating did not abolish the response of MTs during co-exposure to Zn or nZnO and elevated temperatures in the mussels from the cooling ponds. This indicates that a long term acclimatization to a warm metal-polluted environments (such as the cooling ponds of the power stations) may enhance metal-detoxification capacities of the mussels over a broad range of environmental temperatures and compensate for the negative effects of acute warming on metal homeostasis (Sokolova and Lannig, 2008; Falfushynska et al., 2015a).

It is worth noting that upregulation of MTs (and the corresponding increase in the MT-bound pool of Zn) was also induced by exposure to the elevated temperature in the absence of Zn^{2+} or nZnO exposures. This may reflect an important involvement of MTs in the general stress response and antioxidant protection in mollusks (Hardivillier et al., 2006; Serafim and Bebianno, 2007; Gagné et al., 2008; Falfushynska et al., 2013a, 2013b). The same was trough for ALP in gonads in this study and for the mussels from cooling plant from Nuclear PP, studied recently (Falfyshynska et al., 2016). Temperature-induced oxidative stress is common in aquatic ectotherms including mollusks (Lushchak, 2011; Paital et al., 2016), and upregulation of MTs at elevated temperatures contributes to maintaining of the redox homeostasis. The hypothesis of temperature-induced oxidative stress in *U. tumidus* is supported by an increase of the oxidized glutathione levels in the digestive gland and the gills of the mussels, as well as by an increase in the GST activity in the gills. Notably, there was no consistent upregulation of the SOD activity in response to the elevated temperature (25 °C) across the studied tissues and populations of *U. tumidus* indicating that the thiol-based antioxidants (such as MTs and GSH) play the predominant role in the redox control under these conditions. Notably, the basal levels of lipofuscin in the mussels from the TPPs' reservoirs (this study) is over 10-fold higher than in the mussels from a pristine site (Falfushynska et al., 2015a). Earlier studies conducted in the mussels from a pristine site reported prominent accumulation of lipofuscin in the lysosomes of the mussels during stress, such as nZnO and acute heating (Falfushynska et al., 2015a). Similarly, exposure of naïve bivalves (*Mytilus* spp.) to metal-containing nanoparticles led to accumulation of lipofuscin in the absence of warming or other stressors (Canesi et al., 2012). In *U. tumidus* from TPPs' reservoirs, experimental exposures did not lead to accumulation of lipofuscin above the high baseline levels. This may indicate that upregulation of antioxidants during stress exposures in these organisms affords sufficient protection against lipid peroxidation. Alternatively, it is possible that stress-induced accumulation of lipofuscin in experimental exposures are too small to show against the high background levels of lipofuscin in *U. tumidus* from TPP reservoirs.

An increase (of up to three times) of the levels of ALP in the gonad of the male *U. tumidus* was another common response to Zn-containing experimental exposures (except Zn^{2+} exposed DPP mussels). Elevated ALP levels in the male mussels are commonly regarded as a sign of the endocrine disruption reflecting stimulation of Vtg production in males caused by estrogenic contaminants (xenoestrogens). However, this interpretation has been currently subject of controversy (Porte et al., 2006; Matozzo et al., 2008; Cubero-Leon et al., 2010; Morthorst et al., 2014; Sánchez-Marín et al., 2017) because the standard ALP method may release phosphate from other proteins in addition to vitellogenin (Sánchez-Marín et al., 2017). In our present study, a strong increase in ALP in Zn-containing exposures may reflect an increase in the Zn-binding phosphoproteins that commonly serve as Zn carrier for the cell proliferation in gonads (Macdonald, 2000; Thompson et al., 2012) rather than endocrine disruption and upregulation of Vtg.

Notably, a Zn-binding protein termed the major yolk protein playing a role as a carrier to transport Zn to the gonads of both female and male sea urchins during the nonreproductive season (Unuma et al., 2011). A similar increase in ALP levels during exposures to Zn^{2+} and nZnO was shown for the unionid mussels from a pristine habitat (Data published in a local periodic (Falfushynska et al., 2015b)). Furthermore, ALP levels increased in the mussels from the cooling pond of a nuclear PP in response to warming (Falfyshynska et al., 2016).

4.2. The molecular signatures of nZnO effects

The biomarker responses to nZnO particles were population- and tissue-dependent in the studied populations of *U. tumidus*. Due to the large interpopulational variation in the baseline values of the studied biomarkers, it was impossible to identify a threshold value for each biomarker that would unequivocally indicate stress or identify nZnO exposure. However, analysis of the integrated battery of biomarkers by CART and PCA analyses shows that the biomarkers that consistently distinguish nZnO-exposed groups from their control and Zn^{2+}–exposed counterparts involve nuclear abnormalities (the frequency of micronuclei), apoptotic markers (caspase-3 activity), and tissue and/or metallothioneins Zn load (Supplementary Figs. 2 and 3). However, the degree and direction of change in these biomarkers differed between the studied populations. Combination of nZnO exposure and elevated temperature modulated the responses leading to an increase in the genotoxic damage. Notably, while the total Zn content of the tissue was highly associated with the principal components separating the nZnO-exposed experimental groups, the changes in the Zn content showed no consistent pattern with both increases and decreases found depending on the tissue and population. This, along with the observed lack of strong Zn accumulation, indicates that tissue Zn burdens are of limited value in assessing the exposures to or physiological consequences of the environmental nZnO.

Lysosomal membrane stability was a general stress biomarker that distinguished all Zn-exposed mussels (including those exposed to nZnO and Zn^{2+}) from the control groups. The sequestration and efflux of metal ions and engineered metal-containing nanoparticles is mediated by lysosomes (Kukic et al., 2014; Jimeno-Romero et al., 2017). Nanosized particles could cause mechanical damage of lysosome membranes, whereas dissolved metal can affect the lysosomal membrane indirectly, through multiple pathways including the metal-induced oxidative damage to the membrane (Canesi et al., 2012). In our current study, the intracellular Zn imbalance, caused by Zn depletion and/or trace metal release from oxidized MTs, might contribute to the decrease in lysosomal membrane stability (Lee and Koh, 2010; Matias et al., 2016). Similar to our findings, an earlier study in marine mussels (*Mytilus galloprovincialis*) to nanosilver and silver ions showed that both forms of metal exposure result in accumulation of silver in the lysosomes and lysosomal membrane destabilisation (Jimeno-Romero et al., 2017). Overall, these data indicate that lysosomal membrane destabilization may serve as a useful index for general metal toxicity in the mussels but cannot be used to distinguish between the exposures to dissolved *vs*. nanosized metals.

A consistent pattern of biomarker change that distinguishes mussels exposed to Zn or nZnO was the MXR-like activity in the digestive gland which was activated by Zn^{2+} and inhibited by nZnO and nZnO + T. In contrast, in the gills a non-specific activation of the MXR-like activity by different stressors was observed, notably in the BPP mussels. MXR transporters (such as P-glycoprotein) are located in the plasma membrane (Paterson and Gottesman, 2007) and can transport hundreds of structurally unrelated hydrophobic amphipathic compounds in an ATP-dependent fashion. MXR transporters can also contribute to the efflux of trace metals such as Cd^{2+}, likely in the complex with glutathione (Broeks et al., 1996; Ivanina and Sokolova, 2008). This may explain upregulation of the MXR protein expression and activity in the organisms and cells exposed to trace metals such as Cd^{2+} (Ivanina and Sokolova, 2008) or Zn^{2+} (present study). The mechanisms of nZnO-induced inhibition of the MXR-like activity in the digestive gland of *U. tumidus* are presently not known. An inhibiting effect of nZnO on drug efflux was earlier reported in a multidrug-resistant cancer cell line (Liu et al., 2016). Possibly, suppression of the MXR functions by exposure to the nanoparticles may reflect mechanical damage to the membrane and the consequent dysregulation of membrane transporters activity. Further studies are needed to determine the mechanisms of nZnO-dependent modulation of MXR activity. However, regardless of the specific molecular mechanisms, nZnO-induced inhibition of MXR activity may have negative effect on mussels in polluted habitats acting as a chemosensitizer and increasing vulnerability to other toxins transported by the MXR pumps (Kurelec et al., 2000).

Activation of the cellular antioxidant defense in the digestive gland by most (but not all) experimental exposures indicates non-specific stimulation of oxidative stress pathways by the metal and temperature stress. In the digestive gland, markers of oxidative stress (SOD and GST activities and concentrations of GSH and lipofuscin) were significantly and positively correlated. In the BPP mussels this oxidative stress was also associated with the unfolded protein response and overexpression of molecular chaperones (including both mitochondrial and cytosolic HSPs) indicating that the cellular protection systems are overwhelmed resulting in the damage to cellular protein. In contrast, the more resistant DPP mussels did not show the unfolded protein response. Notably, the activity of GST, a key enzyme of Phase II of detoxification and a crucial executor in the GSH functions, decreased in response to nZnO exposure in the digestive gland of DPP-mussels, whereas in the gills of DPP-mussels and in the both tissues of BPP-mussels GST activity decrease was detected in almost all exposures. The reduction in the activity of GST was also detected under the acute exposure to 32 μg mL^{-1} (0.78 mM) nZnO in freshwater snail *Lymnaea luteola* (Ali et al., 2012) and in the clam *Ruditapes philippinarum* exposed to nZnO (1 and 10 μg L^{-1}) and $ZnCl_2$ (10 μg L^{-1}) for 7 days (Marisa et al., 2016). Tissue levels of ALP and MT-Me levels negatively correlated with the levels of oxidative lesions traits in BPP and DPP mussels (Fig. 4B; Supplementary Tables 4 and 5) reflecting potential involvement of these proteins in the antioxidant activity in addition to their role in metal binding. Antioxidant roles of MTs are well described (REFS), and phosphoproteins such as vitellogenin can also act as radical scavengers in invertebrates (Corona et al., 2007) supporting this interpretation. Overall, our study indicate that warming and/or pollutant exposures shift the redox balance and result in oxidative stress response in *U. tumidus*, but these changes are not diagnostic for Zn^{2+} or nZnO exposures and reflect the general cellular stress.

4.3. Limits of the thermal protection mechanisms in the chronically stressed mussels

Long-term acclimatization and/or adaptation of mollusks from the human-modified native habitats could contribute to their tolerance to further impacts of the stressors (such as chemical pollution and/or warming) common in these habitats. Earlier studies showed that the mussels from a cooling pond of a nuclear power plant had more efficient stress protection mechanisms to warming, and mussels from an agricultural site were more tolerant to the stress effects of a fungicide, whereas the effect of unusual stressor, irradiation, was similar in the mussels from pristine and

human-modified habitats (Falfushynska et al., 2013a, 2013b, 2014, 2016a). The response to additional impacts may be non-specific when the ecosystem is close to degradation and the adaptive ability is exhausted (Hook et al., 2014). Hence the degree of the specificity of stress responses in a population may serve as an indicator of the cumulative stress load in the ecosystem. Our current results indicate that responses to stressors such as warming or nZnO are population-specific rather than stress specific in the mussels from the cooling ponds, and general cellular stress responses are induced by a variety of different stressors and their combinations. This suggests that the stressor-specific protection mechanisms may be overwhelmed in the mussels from the cooling pond habitats so that they rely on the general stress protection mechanisms (such as metallothioneins and antioxidants) for survival.

Chronic stress in the mussels from the thermal PP cooling ponds was indicated by the considerably low basal level of the membrane stability in comparison with that of the mussels from the pristine population (Falfushynska et al., 2015a). Experimental warming (25 °C) did not cause the lysosomal membrane destabilization in the DPP and BPP mussels indicating resistance of the cellular membranes to heat. In contrast, several cellular traits of mussels from the DPP and BPP cooling ponds were vulnerable to the heating in comparison with other stress exposures. Notably, warming resulted in a stronger shift in the GSH/GSSG ratio than any other studied stressors indicating that warming disrupts cellular redox status. High interpopulational difference in responses of the GST activity to warming (elevated in the temperature-stressed DPP mussels and stable or suppressed in their BPP counterparts) is consistent with the high sensitivity of GSH metabolism of the mussels to temperature. Elevated temperature also resulted in the strongest depletion of the tissue Zn pool compared to other exposures. This may negatively affect Zn-dependent cellular functions such as antioxidant defense, metabolism and DNA repair (Vallee and Falchuk, 1993; Hogstrand and Wood, 1996). Negative shifts in the redox balance, GSH profile and disturbance of Zn homeostasis might result in elevated levels of cellular damage as shown by high levels of DNA fragmentation in heat-stressed mussels. Overall, our data show that chronically stressed mussels from the thermal plant cooling pond do not have elevated thermal tolerance despite acclimatization in a permanently warmed habitat.

Heat shock proteins play a key role in thermal tolerance of aquatic ectotherms protecting against stress-induced protein damage. Temperature-induced increase in the expression levels of cytosolic HSP70 family as well as the mitochondrial HSP60 were higher in the gills compared to the digestive gland of *U. tumidus*. This agrees with the earlier findings of the higher inducibility of HSPs in the gills and the greater dependence of gills on HSPs for protection against environmental stressors such as toxic metals compared to the digestive gland (Ivanina et al., 2008, 2009). Furthermore, inducibility of HSPs differed between the two studied populations. In the BPP mussels, HSP69, HSP72 and HSP60 increased in response to warming in the gills as well as the digestive glands, whereas in the BPP mussels the HSP levels remained stable or decrease. Similar differences were found between two species of freshwater mussels, a thermally tolerant *Villosa lienosa*, and thermally sensitive *Villosa nebulosa* with higher upregulation of HSP90 and HSP70 in *V. lienosa* during acute heat shock and greater induction of *HSP60* during the chronic warming in *V. nebulosa* (Payton et al., 2016).

Notably, upregulation of the HSPs in the heat-stressed BPP mussels went hand-in-hand with the suppressed levels of the apoptotic marker (caspase-3) indicating that molecular chaperones mitigated the cellular damage and alleviated injury-induced apoptosis. Interestingly, co-exposure to nZnO + T induced elevation of HSP 70 and HSP60 in the gills in the DPP but not the BPP mussels. This may indicate that co-exposure to nZnO and heat stress overwhelmed the chaperone-dependent protection mechanisms in the more sensitive BPP mussels, as was earlier shown during co-exposures to elevated temperatures and metal stress in oysters *Crassostrea virginica* (Ivanina et al., 2009). Taken together, the predominant dependence on the non-specific stress protection mechanisms and high inducibility of the unfolded protein response suggests that the BPP mussels have exceeded the limits of their molecular stress protection mechanisms making them vulnerable to additional stressors.

4.4. Conclusions and significance

Our study shows that the typical stress responses detected in the mussels from a pristine site (Falfushynska et al., 2015a) were diminished in the specimens from the cooling reservoirs of the thermal power plants. However, common features of responses to nZnO stress can be discerned including the modulation of the MXR-like activity and activation of the antioxidant and metal-binding proteins, metallothioneins. nZnO and Zn^{2+} exposures led to changes in the levels of metallothioneins, MT-SH/Zn-MT ratios and lysosomal membrane destabilization indicating that these traits can serve as biomarkers for contamination with Zn in both dissolve and nanoparticulate forms. Long-term acclimatization of the mussels to the combined thermal and chemical pollution in their habitats made their cellular responses to additional stressors, both novel and common for their habitats, unpredictable, likely reflecting the site-specific variations of the chronic load on the stress protective systems in mussels from the human-modified environments.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.chemosphere.2017.11.079.

References

Ali, D., Alarifi, S., Kumar, S., Ahamed, M., Siddiqui, M.A., 2012. Oxidative stress and genotoxic effect of zinc oxide nanoparticles in freshwater snail *Lymnaea luteola L.* Aquat. Toxicol. 124–125, 83–90.

Amin, M.T., Alazba, A.A., Manzoor, U., 2014. A review of removal of pollutants from water/wastewater using different types of nanomaterials. Adv. Mater. Sci. Eng., 825910, 2014.

Anderson, M.E., 1985. Determination of glutathione and glutathione disulfide in biological samples. Meth. Enzymol. 113, 548–555.

Bains, O.S., Kennedy, C.J., 2004. Energetic costs of pyrene metabolism in isolated hepatocytes of rainbow trout, *Oncorhynchus mykiss*. Aquat. Toxicol. 67, 217–226.

Baršienė, J., Rybakovas, A., 2008. Cytogenetic damage in gill and gonad cells of bivalve mollusks. Ekologija 54, 245–250.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. Anal. Biochem. 44, 276–287.

Bester, M.J., Potgieter, H.C., Vermaak, W.J.H., 1994. Cholate and pH reduce interference by sodium dodecyl sulfate in the determination of DNA with Hoechst. Anal. Biochem. 223, 299–305.
Beukelman, T.E., Lord, S.S., 1960. The standard addition technique in flame spectrometry. Appl. Spectrosc. 14, 12–17.
Blaise, C., Gagne, F., Pellerin, J., Hansen, P.D., 1999. Determination of vitellogenin-like properties in *Mya arenaria* hemolymph (Saguenay Fjord, Canada): a potential biomarker for endocrine disruption. Environ. Toxicol. 14, 455–465.
Bonomini, M., Dottori, S., Amoroso, A., Arduini, A., Sirolli, V., 2004. Increased platelet phosphatidylserine exposure and caspase activation in chronic uremia. J. Thromb. Haemost. 2, 1275–1281.
Broeks, A., Gerrard, B., Allikmets, R., Dean, M., Plasterk, R.H.A., 1996. Homologues of the human multidrug resistance genes MRP and MDR contribute to heavy metal resistance in the soil nematode *Caenorhabditis elegans*. EMBO J. 15, 6132–6143.
Canesi, L., Corsi, I., 2016. Effects of nanomaterials on marine invertebrates. Sci. Total Environ. 565, 933–940.
Canesi, L., Ciacci, C., Vallotto, D., Gallo, G., Marcomini, A., Pojana, G., 2010. *In vitro* effects of suspensions of selected nanoparticles (C60 fullerene, TiO_2, SiO_2) on *Mytilus hemocytes*. Aquat. Toxicol. 96, 151–158.
Canesi, L., Ciacci, C., Fabbri, R., Marcomini, A., Pojana, G., Gallo, G., 2012. Bivalve molluscs as a unique target group for nanoparticle toxicity. Mar. Environ. Res. 76, 16–21.
Cerenius, L., Söderhäll, K., 2004. The prophenoloxidase-activating system in invertebrates. Immunol. Rev. 198, 116–126.
Company, R., Serafim, A., Cosson, R.P., Fiala-Médioni, A., Camus, L., Serrão-Santos, R., Bebianno, M.J., 2010. Sub-lethal effects of cadmium on the antioxidant defence system of the hydrothermal vent mussel *Bathymodiolus azoricus*. Ecotoxicol. Environ. Saf. 73, 788–795.
Corona, M., Velarde, R.A., Remolina, S., Moran-Lauter, A., Wang, Y., Hughes, K.A., Robinson, G.E., 2007. Vitellogenin, juvenile hormone, insulin signaling, and queen honey bee longevity. Proc. Natl. Acad. Sci. U. S. A. 104, 7128–7133.
Corsi, I., Cherr, G.N., Lenihan, H.S., Labille, J., Hassellov, M., Canesi, L., Dondero, F., Frenzilli, G., Hristozov, D., Puntes, V., Della Torre, C., 2014. Common strategies and technologies for the ecosafety assessment and design of nanomaterials entering the marine environment. ACS Nano 8, 9694–9709.
Cubero-Leon, E., Ciocan, C.M., Hill, E.M., Osada, M., Kishida, M., Itoh, N., Kondo, R., Minier, C., Rotchell, J.M., 2010. Estrogens disrupt serotonin receptor and cyclooxygenase mRNA expression in the gonads of mussels (*Mytilus edulis*). Aquat. Toxicol. 98, 178–187.
Della Torre, C., Balbi, T., Grassi, G., Frenzilli, G., Bernardeschi, M., Smerilli, A., Guidi, P., Canesi, L., Nigro, M., Monaci, F., Scarcelli, V., 2015. Titanium dioxide nanoparticles modulate the toxicological response to cadmium in the gills of *Mytilus galloprovincialis*. J. Hazard. Mater. 297, 92–100.
Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.-I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.-H., Soto, D., Stiassny, M.L.J., Sullivan, C.A., 2006. Freshwater biodiversity: importance, threats, status and conservation challenges. Biol. Rev. 81, 163–182.
Dumont, E., Johnson, A.C., Keller, V.D., Williams, R.J., 2015. Nano silver and nano zinc-oxide in surface waters–Exposure estimation for Europe at high spatial and temporal resolution. Environ. Pollut. 196, 341–349.
Eide, D.J., 2006. Zinc transporters and the cellular trafficking of zinc. Biochim. Biophys. Acta 1763, 711–722.
Escher, B.I., Hackermüller, J., Polte, T., Scholz, S., Aigner, A., Altenburger, R., Böhme, A., Bopp, S.K., Brack, W., Busch, W., Chadeau-Hyam, M., et al., 2016. From the exposome to mechanistic understanding of chemical-induced adverse effects. Environ. Int. 99, 97–106.
Falfushynska, H.I., Gnatyshyna, L.L., Farkas, A., Vehovszky, Á., Gyori, J., Stoliar, O.B., 2010. Vulnerability of biomarkers in the indigenous mollusk *Anodonta cygnea* to spontaneous pollution in a transition country. Chemosphere 81, 1342–1351.
Falfushynska, H., Gnatyshyna, L., Stoliar, O., 2013a. Effect of in situ exposure history on the molecular responses of bivalve mollusks to trace metals. Ecotoxicol. Environ. Saf. 89, 73–83.
Falfushynska, H., Gnatyshyna, L., Stoliar, O., 2013b. In situ exposure history modulates the molecular responses to carbamate fungicide Tattoo in bivalve mollusk. Ecotoxicology 22, 433–445.
Falfushynska, H., Gnatyshyna, L., Yurchak, I., Ivanina, A., Stoliar, O., Sokolova, I., 2014. Habitat pollution and thermal regime modify molecular stress responses to elevated temperature in freshwater mussels (*Anodonta anatina*: unionidae). Sci. Total. Environ. 500–501, 339–350.
Falfushynska, H., Gnatyshyna, L., Yurchak, I., Sokolova, I., Stoliar, O., 2015a. The effects of zinc nanooxide on cellular stress responses of the freshwater mussels *Unio tumidus* are modulated by elevated temperature and organic pollutants. Aquat. Toxicol. 162, 82–93.
Falfushynska, H.I., Gnatyshyna, L.L., Yurchak, I.V., Sokolova, I.M., Stoliar, O.B., 2015b. Endocrine disruption and cytotoxicity in bivalve mollusk under the complex exposure to zinc nanooxide. Наук. запис. терноп. нац. пед. ун-ту. Серія Біологія 64 (3–4), 787–790.
Falfushynska, H., Gnatyshyna, L., Yurchak, I., Stoliar, O., Sokolova, I.M., 2016a. Interpopulational variability of molecular responses to ionizing radiation in freshwater bivalves *Anodonta anatina* (Unionidae). Sci. Total Environ. 568, 444–456.
Falfushynska, H.I., Phan, T., Sokolova, I.M., 2016b. Long-term acclimation to different thermal regimes affects molecular responses to heat stress in a freshwater clam *Corbicula fluminea*. Sci. Rep. 6, 39476.
Falfyshynska, G.I., Gnatyshyna, L.L., Yurchak, I.V., Mudra, A.Y., Ivanina, A., Sokolova, I., Stoliar, O.B., 2016. Responses of the clam *Anodonta anatina* to thermal impact depending on peculiarities of occurrence in natural habitat. Hydrobiol. J. 52, 71–82.
Ficke, A., Myrick, C., Hansen, L., 2007. Potential impacts of global climate change on freshwater fisheries. Rev. Fish. Biol. Fish. 17, 581–613.
Gagné, F., Auclair, J., Turcotte, P., Fournier, M., Gagnon, C., Sauve, S., Blaise, C., 2008. Ecotoxicity of CdTe quantum dots to freshwater mussels: impacts on immune system, oxidative stress and genotoxicity. Aquat. Toxicol. 86, 333–340.
Gagné, F., Auclair, J., Peyrot, C., Wilkinson, K.J., 2015. The influence of zinc chloride and zinc oxide nanoparticles on air-time survival in freshwater mussels. Comp. Biochem. Physiol. 172–173C, 36–44.
Gagné, F., Auclair, J., Trépanier, S., Turcotte, P., Pilote, M., Gagnon, C., 2016. The impact of zinc oxide nanoparticles in freshwater mussels exposed to municipal effluents. Invertebr. J. Surviv 13, 281–290.
Gagnon, C., Turcotte, P., Trépanier, S., Gagné, F., Cejka, P.J., 2014. Impacts of municipal wastewater oxidative treatments: changes in metal physical speciation and bioavailability. Chemosphere 97, 86–91.
Greaver, T.L., Clark, C.M., Compton, J.E., Vallano, D., Talhelm, A.F., Weaver, C.P., Band, L.E., Baron, J.S., Davidson, E.A., Tague, C.L., Felker-Quinn, E., Lynch, J.A., Herrick, J.D., Liu, L., Goodale, C.L., Novak, K.J., Haeuber, R.A., 2016. Key ecological responses to nitrogen are altered by climate change. Nat. Clim. Change 6, 836–843.
Griffen, B.D., Belgrad, B.A., Cannizzo, Z.J., Knotts, E.R., Hancock, E.R., 2016. Rethinking our approach to multiple stressor studies in marine environments. Mar. Ecol. Prog. Ser. 543, 273–281.
Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione reductase an 2-vinylpyridine. Anal. Biochem. 106, 207–212.
Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases. The first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249, 7130–7139.
Hallman, T.A., Brooks, M.L., 2016. Metals-mediated climate susceptibility in a warming world: larval and latent effects on a model amphibian. Environ. Toxicol. Chem. 35, 1872–1882.
Hardivillier, Y., Denis, F., Demattei, M.V., Bustamante, P., Laulier, M., Cosson, R., 2006. Metal influence on metallothionein synthesis in the hydrothermal vent mussel *Bathymodiolus thermophiles*. Comp. Biochem. Physiol. 143C, 321–332.
Hogstrand, C., Wood, C.M., 1996. The physiology and toxicology of zinc in fish. In: Taylor, E.W. (Ed.), Toxicology of Aquatic Pollution: Physiological, Cellular and Molecular Approaches. Cambridge University Press, Cambridge, pp. 61–84.
Holmstrup, M., Bindesbol, A.-M., Oostingh, G.J., Duschl, A., Scheil, V., Koehler, H.-R., Loureiro, S., Soares, A.M.V.M., Ferreira, A.L.G., Kienle, C., Gerhardt, A., Laskowski, R., Kramarz, P.E., Bayley, M., Svendsen, C., Spurgeon, D.J., 2010. Interactions between effects of environmental chemicals and natural stressors: a review. Sci. Total Environ. 408, 3746–3762.
Hook, S.E., Gallagher, E.P., Batley, G.E., 2014. The role of biomarkers in the assessment of aquatic ecosystem health. Integr. Environ. Assess. Manag. 10 (3), 327–341.
Hrubik, J., Glisic, B., Tubic, A., Ivancev-Tumbas, I., Kovacevic, R., Samardzija, D., Andric, N., Kaisarevic, S., 2016. Toxicological and chemical investigation of untreated municipal wastewater: fraction-and species-specific toxicity. Ecotoxicol. Environ. Saf. 127, 153–162.
Ivanina, A.V., Sokolova, I.M., 2008. Effects of cadmium exposure on expression and activity of P-glycoprotein in eastern oysters, *Crassostrea virginica* Gmelin. Aquat. Toxicol. 88, 19–28.
Ivanina, A.V., Cherkasov, A.S., Sokolova, I.M., 2008. Effects of cadmium on cellular protein and glutathione synthesis and expression of stress proteins in eastern oysters, *Crassostrea virginica* Gmelin. J. Exp. Biol. 211, 577–586.
Ivanina, A.I., Taylor, C., Sokolova, I.M., 2009. Effects of elevated temperature and cadmium exposure on stress protein response in eastern oysters *Crassostrea virginica* (Gmelin). Aquat. Toxicol. 91, 245–254.
Ivanina, A.V., Nesmelova, I., Leamy, L., Sokolov, E.P., Sokolova, I.M., 2016. Intermittent hypoxia leads to functional reorganization of mitochondria and affects cellular bioenergetics in marine molluscs. J. Exp. Biol. 219, 1659–1674.
Jimeno-Romero, A., Bilbao, E., Izagirre, U., Cajaraville, M.P., Marigómez, I., Soto, M., 2017. Digestive cell lysosomes as main targets for Ag accumulation and toxicity in marine mussels, *Mytilus galloprovincialis*, exposed to maltose-stabilised Ag nanoparticles of different sizes. Nanotoxicology 11, 168–183.
Kamat, P.V., Huehn, R., Nicolaescu, R., 2002. A "sense and shoot" approach for photocatalytic degradation of organic contaminants in water. J. Phys. Chem. B 106, 788–794.
Kang, Y.J., 2006. Metallothionein redox cycle and function. Exp. Biol. Med. 231, 1459–1467.
Kennedy, C.J., Walsh, P.J., 1994. The effects of temperature on the uptake and metabolism of benzo[a]pyrene in isolated gill cells of the gulf toadfish, Opsanus beta. Fish. Physiol. Biochem. 13, 93–103.
Kimura, T., Kambe, T., 2016. The functions of metallothionein and ZIP and ZnT transporters: an overview and perspective. Int. J. Mol. Sci. 17, 336.
Kukic, I., Kelleher, S.L., Kiselyov, K., 2014. Zn^{2+} efflux through lysosomal exocytosis prevents Zn^{2+}-induced toxicity. J. Cell Sci. 12, 3094–3103.
Kurelec, B., Smital, T., Pivèeviæ, B., Eufemia, N., Epel, D., 2000. Multixenobiotic resistance, P-Glycoprotein, and chemosensitizers. Ecotoxicology 9, 307–327.
Lee, S.J., Koh, J.Y., 2010. Roles of zinc and metallothionein-3 in oxidative stress-induced lysosomal dysfunction, cell death, and autophagy in neurons and astrocytes. Mol. Brain 3, 30.
List of Top-100 companies in Ukraine responsible for pollution. 2017. Available at [http://old.menr.gov.ua/images/news/04072017/TOP_100.pdf]. Accessed on

October 29, 2017.
Liu, J., Ma, X., Jin, S., Xue, X., Zhang, C., Wei, T., Guo, W., Liang, X.J., 2016. Zinc oxide nanoparticles as adjuvant to facilitate doxorubicin intracellular accumulation and visualize pH-responsive release for overcoming drug resistance. Mol. Pharm. 13, 1723–1730.
Lowry, O.H., Rosebrough, H.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
Luna-Acosta, A., Thomas-Guyon, H., Amari, M., Rosenfeld, E., Bustamante, P., Fruitier-Arnaudin, I., 2011. Differential tissue distribution and specificity of phenoloxidases from the Pacific oyster *Crassostrea gigas*. Comp. Biochem. Physiol. B. Biochem. Mol. Biol. 159, 220–226.
Lushchak, V.I., 2011. Environmentally induced oxidative stress in aquatic animals. Aquat. Toxicol. 101, 13–30.
Macdonald, R.S., 2000. The role of zinc in growth and cell proliferation. J. Nutr. 130, 1500S–1508S.
Manibabu, P.V., Patnaik, B.K., 1997. Lipofuscin concentration of the brain shows a reduction with age in male garden lizard. Comp. Biochem. Physiol. 117C, 229–232.
Marchi, B., Burlando, B., Moore, M.N., Viarengo, A., 2004. Mercury- and copper-induced lysosomal membrane destabilisation depends on $[Ca^{2+}]i$ dependent phospholipase A2 activation. Aquat. Toxicol. 66, 197–204.
Marigómez, I., Múgica, M., Izagirre, U., Sokolova, I.M., 2017. Chronic environmental stress enhances tolerance to seasonal gradual warming in marine mussels. PLos One 12, e0174359.
Marisa, I., Matozzo, V., Munari, M., Binelli, A., Parolini, M., Martucci, A., Franceschinis, E., Brianese, N., Marin, M.G., 2016. In vivo exposure of the marine clam *Ruditapes philippinarum* to zinc oxide nanoparticles: responses in gills, digestive gland and haemolymph. Environ. Sci. Pollut. Res. Int. 23, 15275–15293.
Matias, A.C., Manieri, T.M., Cerchiaro, G., 2016. Zinc chelation mediates the lysosomal disruption without intracellular ROS generation. Oxid. Med. Cell. Longev. 2016, 13. https://doi.org/10.1155/2016/6724585, 6724585.
Matozzo, V., Gagné, F., Marin, M.G., Ricciardi, F., Blaise, C., 2008. Vitellogenin as a biomarker of exposure to estrogenic compounds in aquatic invertebrates: a review. Environ. Int. 34, 531–545.
Morthorst, J.E., Holbech, H., Jeppesen, M., Kinnberg, K.L., Pedersen, K.L., Bjerregaard, P., 2014. Evaluation of yolk protein levels as estrogenic biomarker in bivalves; comparison of the alkali-labile phosphate method (ALP) and a species-specific immunoassay (ELISA). Comp. Biochem. Physiol. 166C, 88–95.
Nikinmaa, M., 2013. Climate change and ocean acidification—interactions with aquatic toxicology. Aquat. Toxicol. 126, 365–372.
Noyes, P.D., Lema, S.C., 2015. Forecasting the impacts of chemical pollution and climate change interactions on the health of wildlife. Curr. Zool. 61, 669–689.
Olive, P.L., 1988. DNA precipitation assay: a rapid and simple method for detecting DNA damage in mammalian cells. Environ. Molec. Mutagen. 11, 487–495.
Paital, B., Panda, S.K., Hati, A.K., Mohanty, B., Mohapatra, M.K., Kanungo, S., Chainy, G.B.N., 2016. Longevity of animals under reactive oxygen species stress and disease susceptibility due to global warming. World J. Biol. Chem. 7, 110–127.
Paterson, J.K., Gottesman, M.M., 2007. P-Glycoprotein is not present in mitochondrial membranes. Exp. Cell Res. 313, 3100–3105.
Payton, S.L., Johnson, P.D., Jenny, M.J., 2016. Comparative physiological, biochemical and molecular thermal stress response profiles for two unionid freshwater mussel species. J. Exp. Biol. 219, 3562–3574.
Porte, C., Janer, G., Lorusso, L.C., Ortiz-Zarragoitia, M., Cajaraville, M.P., Fossi, M.C., Canesi, L., 2006. Endocrine disruptors in marine organisms: approaches and perspectives. Comp. Biochem. Physiol. C Toxicol. Pharmacol. 143C, 303–315.
Roesijadi, G., Fowler, B., 1991. Purification of invertebrate metallothioneins. Meth. Enzymol. 205B, 263–273.
Romero, L.M., Dickens, M.J., Cyr, N.E., 2009. The reactive scope model — a new model integrating homeostasis, allostasis, and stress. Horm. Behav. 55, 375–389.
Ruhl, L., Vengosh, A., Dwyer, G.S., Hsu-Kim, H., Schwartz, G., Romanski, A., Smith, S.D., 2012. The impact of coal combustion residue effluent on water resources: a North Carolina example. Environ. Sci. Technol. 46, 12226–12233.
Sánchez-Marín, P., Fernández-González, L.E., Mantilla-Aldana, L., Diz, A.P., Beiras, R., 2017. Shotgun proteomics analysis discards alkali labile phosphate (ALP) as a reliable method to assess vitellogenin levels in *Mytilus galloprovincialis*. Environ. Sci. Technol. 51, 7572–7580.
Serafim, A., Bebianno, M.J., 2007. Kinetic model of cadmium accumulation and elimination and metallothionein response in *Ruditapes decussatus*. Environ. Toxicol. Chem. 26, 960–969.
Sokolova, I.M., 2013. Energy-limited tolerance to stress as a Conceptual framework to integrate the effects of multiple stressors. Integr. Comp. Biol. 53, 597–608.
Sokolova, I.M., Lannig, G., 2008. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: implications of global climate change. Clim. Res. 37, 181–201.
Sokolova, I.M., Frederich, M., Bagwe, R., Lannig, G., Sukhotin, A.A., 2012. Energy homeostasis as an integrative tool for assessing limits of environmental stress tolerance in aquatic invertebrates. Mar. Environ. Res. 79, 1–15.
Sulmon, C., van Baaren, J., Cabello-Hurtado, F., Gouesbet, G., Hennion, F., Mony, C., Renault, D., Bormans, M., El Amrani, A., Wiegand, C., Gérard, C., 2015. Abiotic stressors and stress responses: what commonalities appear between species across biological organization levels? Environ. Pollut. 202, 66–77.
Thompson, E.D., Mayer, G.D., Glover, C.N., Capo, T., Walsh, P.J., Hogstrand, C., 2012. Zinc hyperaccumulation in Squirrelfish (*Holocentrus adscenscionis*) and its role in embryo viability. PLoS One 7 e46127.
Tommasini, R., Evers, R., Vogt, E., Mornet, C., Zaman, J.R.G., Schinkel, A.H., Borst, P., Martinola, E., 1996. The human multidrug resistance-associated protein functionally complements the yeast cadmium resistance factor 1. PNAS 93, 6743–6748.
Trevisan, R., Delapedra, G., Mello, D.F., Arl, M., Schmidt, É.C., Meder, F., Monopoli, M., Cargnin-Ferreira, E., Bouzon, Z.L., Fisher, A.S., Sheehan, D., 2014. Gills are an initial target of zinc oxide nanoparticles in oysters *Crassostrea gigas*, leading to mitochondrial disruption and oxidative stress. Aquat. Toxicol. 153, 27–38.
Unuma, T., Sawaguchi, S., Yamano, K., Ohta, H., 2011. Accumulation of the major yolk protein and zinc in the agametogenic sea urchin gonad. Biol. Bull. 221, 227–237.
Valko, M., Morris, H., Cronin, M.T., 2005. Metals, toxicity and oxidative stress. Curr. Med. Chem. 12, 1161–1208.
Vallee, B.L., Falchuk, K.H., 1993. The biochemical basis of zinc physiology. Physiol. Rev. 73, 79–118.
Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic Molluscs. Mar. Environ. Res. 44, 69–84.
Woodward, G., Perkins, D.M., Brown, L.E., 2010. Climate change and freshwater ecosystems: impacts across multiple levels of organization. Philos. Trans. R. Soc. B Biol. Sci. 365, 2093–2106.