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Interspecies comparison of selected pollution biomarkers in dreissenid spp. inhabiting pristine and moderately polluted sites

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Abstract

Stress biomarkers, which can outline impacts of contaminants in aquatic biota at the biochemical level, are increasingly used as early warning tools in environmental monitoring. Reliable biomarker based assessment schemes, however, request appropriate knowledge of baseline levels of selected endpoints, and the potential influence of a range of natural influencing factors (both abiotic and biotic) as well. In this study, we examined the interspecies variability of various biomarkers (metallothioneins (MT), ethoxyresorufin-O-deethylase activity (EROD), lipid peroxidation (LPO), DNA strand breaks (DNA_sb), vitellogenin-like proteins (Vtg)) in *Dreissena polymorpha* and *Dreissena bugensis* inhabiting either pristine- or moderately impacted sites of Lake Balaton (Hungary). Levels of all biomarkers considered revealed low interspecies variability in the two dreissenid species at all sampling sites, with consistently higher (but statistically insignificant) values in *Dreissena polymorpha*. Levels of all biomarkers varied within the two investigated seasons, with significant influence of the reproduction cycle particularly on the levels of metallothioneins and vitellogenin-like proteins. Each biomarker considered was elevated by October, with significantly higher values in the mussels inhabiting harbours. Insignificant spatial and temporal variability in the general health indicators (condition index, total protein content) of dreissenids was observed, which, in parallel with evident rise in biomarker levels, apparently suggest that the anthropogenic impacts in harbours affect mussel fitness yet at sub organismal level. Our data might serve useful basis for future environmental monitoring surveys, especially in habitats where the progressive replacement of *Dreissena polymorpha* by

Dreissena bugensis is taking place, as the interspecies variability in susceptibility to chemical stress of the two species is well comparable.

Keywords: stress biomarkers, *D. polymorpha*, *D. bugensis*, interspecies variability

1. Introduction

Alterations of biochemical markers in bivalves have been efficiently used for decades to evaluate the ecological relevance of anthropogenic pollution affecting aquatic ecosystems (Lemaire et al., 1993; Regoli and Principato, 1995; Solé et al., 1996; Faria et al., 2010; Schmidt et al., 2013; Okay et al., 2014). This evaluation approach is based on the ability of these sessile organisms to filter large quantities of water, leading to high accumulation rates of anthropogenic contaminants in their tissues. The range of contaminants accumulated in bivalves inform about the contamination status of the habitat where they live in an integrated way, while the cellular and molecular alterations eventually evolved in these bioindicator organisms inform about the relevance of that contamination. (Goldberg, 1975; de Lafontaine et al., 2000; Galloway et al., 2004; Smolders et al., 2004; Minier et al., 2006). It has been recognized, however, that a range of biomarkers routinely screened in cells, body fluids, tissues and organs of bioindicator organisms to interpret contamination impacts, primarily participate in the normal homeostasis of the organism, therefore, they are significantly influenced also by several environmental and biological factors. The complexity of contaminant accumulation kinetics and metabolism, the synergistic/antagonistic effects of xenobiotics and a range of natural environmental parameters (temperature, salinity, nutrient availability etc.) proved to be additional significant influencing factors of biomarkers' modulation in environmental monitoring studies (Sheehan and Power, 1999; Hamer et al., 2004; Gauthier et al., 2014). Biological variables as age, sex, reproductive status, and the general health of indicator organisms (highly dependent on food availability and parasitism) proved to further complicate the linkage between biomarker alterations recorded in sentinel organisms and contamination status of the habitat subjected to anthropogenic pressure (Viarengo et al., 2007; Minguez et al., 2009; Pain-Devin et al., 2014). Interpretations of biomarker responses in environmental monitoring were therefore refined by focused research on bivalves' biological cycles and the seasonal variation of biomarkers' baseline levels (Sheehan and Power, 1999; Minier et al., 2000; Shaw et al., 2004; Depledge, 2009; Faria et al., 2014). While seasonality, reproduction status and food availability as confounding factors were addressed in several studies (Viarengo et al., 2007; Sheehan and Power, 1999; Shaw et al., 2004; Kopecka and Pempkowiak, 2008; Nahrgang et al., 2010; Faria et al., 2014), the relevance of interspecies variability of biomarker alterations to pollution were far less investigated (Habig and Di Giulio, 1991; Corsi et al., 2007; Lysenko et al., 2014). Evaluation of the interspecies variability of biomarker baseline levels in sentinel organisms is particularly justified when, due to bioinvasion, the gradual displacement of established populations by new invaders occurs, and the quality status of the habitats of concern is regularly assessed by biomonitoring techniques built on established sentinel species. Currently, investigation of the species specific variability in biomarkers baseline levels and their modulation due to natural environmental factors and anthropogenic impact appears to have actuality for dreissenid mussels: the zebra mussel (*Dreissena polymorpha*) and the quagga mussel (*Dreissena rostriformis bugensis*). The rapid range expansion of *D. bugensis* in freshwaters was noted both in North America and Europe, resulting in a relevant dominance shift from the established *D. polymorpha* populations to *D. bugensis* (Mills et al., 1996; Nalepa et al., 2010; Bij de Vaate et al., 2014). Several differences in the physiological attributes of the two species were suggested to support the gradual displacement of *D. polymorpha* by *D. bugensis*. *D.*

bugensis was shown to have higher assimilation efficiency (Baldwin et al., 2002), higher filtration rate (Diggins, 2001) and lower respiration rate (Stoeckmann, 2003), which imply higher growth rate. These features are particularly important when food resources are limiting. It was also suggested that *D. bugensis* is able to spawn at colder temperatures, enabling earlier breeding in the season, which results in a competitive advantage for larval settlement (Claxton and Mackie, 1998).

In the pollution assessment of freshwater habitats the zebra mussel *D. polymorpha* proved to be an efficient sentinel organism because its ecophysiological features (abundance, wide distribution, high filtration rate, and ability to accumulate large amounts of anthropogenic contaminants) enabled reliable *in situ* habitat contamination evaluations (Kraak et al., 1991; de Kock and Bowmer, 1993; Roper et al., 1996). More recently, zebra mussels have been successfully used even in integrated biomarker assessment studies when bioaccumulated contaminant data revealed good correlation with alterations of several cellular- molecular biomarker endpoints in these mussels (de Lafontaine et al., 2000; Minier et al., 2006; Contardo-Jara et al., 2009; Faria et al., 2010). Focused research provided relevant information about the natural variability of biomarker responses in zebra mussels, particularly related to seasonality and reproduction status, that helped establish validated biomonitoring approaches for the assessment of the environmental significance of chemical contamination in freshwater habitats (Stoeckmann and Garton, 2002; Binelli et al., 2005; Guerlet et al., 2007; Faria et al., 2014). Thus, the application of zebra mussels in monitoring the quality status of freshwaters appears a reliable approach. However, the widespread invasion of the quagga mussel (*Dreissena rostriformis bugensis*), resulting in an overgrowth of the quagga mussels within dreissenid populations (Mills et al., 1996; Bij de Vaate et al., 2014) calls for knowledge on the modulation of the routinely used biomarkers also in quagga mussels in response to anthropogenic pollutants. Interspecies differences in bioaccumulation of pollutants for the two dreissenid species have already been reported (Richman and Somers, 2005; Mathews et al., 2015). This variability was thought to be related to differences in both habitat preference characteristics and in physiological attributes of the two species such as energy consumption, filtration- and growth rate (Veltman et al., 2008; Le et al., 2011). Accordingly, interspecies differences in susceptibility to chemical stress between the two dreissenid species could also be expected. The goals of this study were therefore: a. to identify potential interspecies differences in baseline levels of selected biochemical parameters commonly used in pollution monitoring, between zebra mussels and quagga mussels; b. to assess the variability of these endpoints in two outlining seasons.

For this purpose various biomarkers of defence (metallothionein (MT), ethoxyresorufin-O-deethylase (EROD)), biomarkers of damage (lipid peroxidation (LPO) and DNA damage (DNA_sb)) and reproduction (vitellogenin-like proteins (Vtg)) were recorded in established populations of *D. polymorpha* and *D. bugensis* within four harbours of Lake Balaton known to be affected by moderate pollution due to ship traffic, and compared with biomarker levels of mussels collected from a highly protected pristine habitat. In addition, as indicators of nutritional state, the condition index and total protein levels were used to assess the overall mussel fitness also at individual level. Samplings were scheduled after the main spawning period of mussels in June and in the resting stage in October, when gametes are almost completely lacking.

2. Materials and Methods

2.1. Site descriptions

Four harbours (H1, H2, H3, H4) and a highly protected remote area, as reference site (R) (Fig. 1), were selected for the biomarkers assessment of *Dreissena* spp. populations. Harbours, as moderately impacted sites, were selected based on historical contamination data published (Hlavay and Polyák, 2002; Nguyen et al., 2005; Bodnár et al., 2005) as well as according to contaminant- and toxicity data recorded for bottom sediments of these locations in our recent investigations performed in 2013 (Ács et al., 2015).

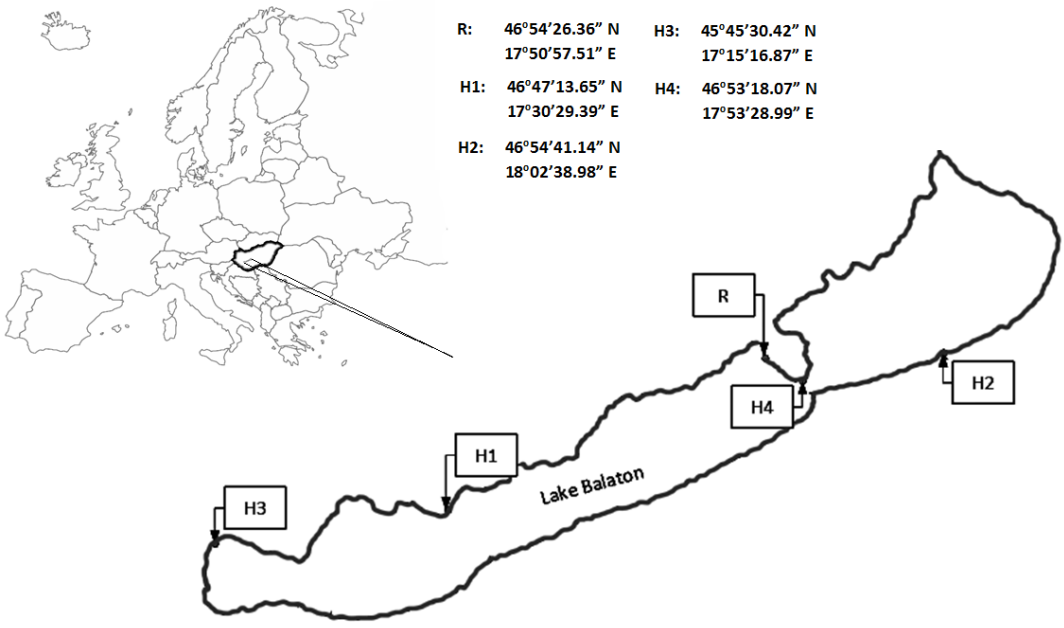

Fig. 1. Location and geographical coordinates of sampling sites along Lake Balaton (R = pristine site; H1, H2, H3, H4 = harbour sites)

Selected harbours are shallow (2.0 – 3.5 m depth) with wide openings, enabling intense water exchange therefore, the hydrodynamic features and the physico-chemical characteristics of the water do not significantly differ from the conditions in open areas of the lake (Tátrai et al, 2008; Szabó et al., 2011). During samplings, at all locations water temperatures varied between 18 - 21 ^{o}C; the pH was between 8.5 – 8.6; salinity spanned 310 – 460 mg L^{-1}; dissolved oxygen was around 10 mg L^{-1}; conductivity was 600 – 700 µS cm^{-1} and the redox potential varied between 400 – 600 mV. Data on metals and polycyclic aromatic hydrocarbons determined in harbour sediments in midsummer 2013, were considered as descriptors of harbour contamination (Table 4., data retrieved from Ács et al., 2015).

2.2. Dreissenid spp. sampling

Mussel sampling and biomarker measurements were performed in 2014 in June after the main spawning period of mussels and in October. The littoral zone of the lake is populated by mixed populations of *D. polymorpha* and *D. bugensis*, with the later species prevailing in general. Mussels were sampled randomly at mid shore level from each mussel bed area. Overall, three groups of ca. 50 mussels tied on rocks (distance between replicates was approx. 10 – 15 m) belonging to the 10 – 20 mm length range were detached from the substrates by byssus excision and used for biomarker analysis (approx. 150 - 200 individuals per site).

The mussels were transported to laboratory in containers filled with lake water of the site. Mussels were cleaned of shell debris and allowed to flush sediment and gut contents overnight in aerated filtered lake water in 200 L flow-through system aquaria.
Separation of mussel samples in groups of *D. polymorpha* and *D. bugensis* was performed based on the differences in ventral surface morphology, which is distinctly flattened for zebra mussels and slightly rounded for quagga mussels (Dermott and Munawar, 1993; Claxton et al., 1998). Except for a single harbour (H3), where the mussel population was dominated by *D. polymorpha* (approx. 90% in June and 65% in October), at each sampling location significantly higher preponderance (70 – 95%) was found for *D. bugensis*.
For each individual mussel the length (maximum anterior-posterior axis) and wet weight were determined to the nearest 0.1 mm and 0.1 g respectively. Following morphometric measurements the soft tissues were excised, immediately weighed then, pooled in batches of 10 – 20 individuals (depending on size), frozen and stored at -80^{o}C until biomarker measurements were taken.

2.3. Biomarker measurements

2.3.1. Condition index and total protein content

Based on the weight data recorded after field sampling and soft tissue collection, the condition index of mussels was determined according to formula: CI = soft body weight/total wet weight. As second indicator of mussel fitness we considered the total protein content of tissue homogenates, which was determined according to Bradford (1976) using bovine serum albumin (Fluka Chemie GmBH, Buchs, Germany) as standard.

2.3.2. Biochemical biomarkers

Biomarker measurements were run in pooled whole soft tissues of several mussels (10 – 20 individuals, n = 3 – 4 samples per site) homogenized in ice-cold Hepes-NaOH buffer (25 mM, pH 7.4) containing 130 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol, at a weight to volume ratio of 1:5 w/v. Subsamples of homogenates were stored at -80^{O}C until analysis.

Metallothionein-like proteins were quantified according to the method developed by Viarengo et al. (1997). Subsamples of tissue homogenates were centrifuged at 12,000g for 30 min at 4 ^{o}C. Then, 500 µL of supernatant was mixed with 500 µL of ethanol (95%) containing 8% chloroform and centrifuged at 6000g for 10 min at 2 ^{o}C. Next, 700 µL of resultant supernatant was further mixed with 50 µL RNA (1 mg mL^{-1}), 10 µL HCl 6 M and 1.2 mL of ethanol (4 ^{o}C), then held at -80 ^{o}C for 25 min. The mixture was further centrifuged at 6000g for 10 min at 2 ^{o}C. The retained pellet was re-suspended with 87% ethanol:1% chloroform (300 µL) and centrifuged again at 6000g for 1 min at 2^{o}C. The retained pellet was re-suspended this time with 150 µL of 0.25 M NaCl and 150 µL HCl 0.2 M containing 4 mM EDTA. Three hundred microliter of Ellman's reagent (dithionitrobenzoate 0.4 mM, 2 M NaCl and 0.2 M Tris base, pH 8) was added to the reaction mixture, and the absorbance was measured at 412 nm after 10 min incubation at room temperature. Replicates of re-suspension buffer as blanks and standards of glutathione (GSH) were included in each run. Results were expressed as nmol of MT-equivalents per milligram protein.
EROD activity was determined in the 12,000g microsomal fraction according to Burke and Mayer (1974). Subsamples of tissue homogenates were centrifuged at 12,000g for 30 min at 4 ^{o}C (S12, fraction). Two hundred microliters of S12 supernatant were incubated at 30 ^{o}C for 60 min in a final volume of 1,8 mL containing 100 mM phosphate buffer, pH 7.4, 100 µM reduced NADPH and 10 µM 7-ethoxyresorufin. The reaction was started by the addition of NADPH, and stopped

by the addition of 100 µL of 0.5 M NaOH. The resultant 7-hydroxyresorufin was determined by fluorometry at 520 nm excitation and 590 nm emission wavelengths. Calibration was performed with serial dilutions of 7-hydroxyresorufin. Results were expressed as pmol min^{-1} mg^{-1} total protein.

Lipid peroxidation was evaluated based on the formation of malonaldehyde in tissue homogenates by the thiobarbituric acid method elaborated by Wills (1987). A 450 µL homogenate was mixed with 900 µL of 10% TCA containing 1 mM $FeSO_4$ and 450 µL of 0.67% thiobarbituric acid. The mixture was heated to 80 ^oC for 10 min then, precipitates were removed by centrifugation (10,000g for 10 s). The supernatant was subjected to fluorescence measurement at 516 excitation/600 nm emission. Blanks and standards of tetramethoxypropane were prepared in Hepes homogenization buffer. Results were expressed as µmoles of thiobarbituric acid reactants per milligram of homogenate protein.

DNA strand breaks were quantified by an adaptation of the alkaline precipitation assay of Olive (1988). A 100 µL tissue homogenate was mixed with 800 µL of 2% SDS containing 10 mM EDTA, 10 mM Tris-base and 40 mM NaOH and shaken for 1 min. Eight hundred microliter of 0.12 M KCl was added, the mixture was further heated at 60 ^oC for 10 min, mixed by inversion and cooled at 4 ^oC for 30 min then, centrifuged at 8000g for 5 min at 4 ^oC. Five hundred microliter of supernatant was added to 1500 µL of Hoechst dye (1 µg mL^{-1}, in buffer containing 0.4 M NaCl, 4 mM sodium cholate and 0.1 M Tris-acetate, pH 8.5-9 and mixed for 5 min on a plane shaker. Fluorescence was measured at 360 excitation/450 nm emission wavelengths. Blanks contained identical constituents, with 100 µL Hepes buffer replacing the tissue homogenate. Salmon sperm DNA standard (Sigma) was used for DNA calibration and the results were expressed as DNA_sb µg mg^{-1} protein.

Vitellogenin-like proteins (Vtg) were determined in the 12,000g microsomal fraction following the alkali-labile phosphate (ALP) method developed by Blaise et al. (1999). Four hundred microliter of sample homogenate was mixed with 220 µL acetone (35% final concentration) for 10 min and centrifuged at 10,000g for 5 min. The retained pellet was then dissolved in 200 µL 1 M NaOH and mixed for 30 min at 60 ^oC. The total phosphate was then determined by the colorimetric phosphomolybdenum method developed by Stanton (1968). To a 200 µL sample 1250 µL H_2O, 50 µL 100% TCA, 250 µL of molybdate reactive and 250 µL ascorbate 1% were added, mixed for 10 min and the absorbance was read at 815 nm and 444 nm. Rainbow trout vitellogenin was used for calibration and aliquots of NaOH (1M) were used as blanks. Vtg levels were expressed as µmoles of ALP per milligram of protein.

The values of each biomarker were normalized against the protein content of either the whole homogenate or supernatant (S12) (Bradford, 1976).

Proteins and enzyme activities were determined in triplicate using a microplate reader (VICTOR$^3{}_{TM}$ 1420 Multilabel Counter, Perkin Elmer, USA) at 25 °C.

2.4. Statistical analysis

Analysis of variance (ANOVA) or Kruskal Wallis One Way Analysis of Variance on Ranks was applied to evaluate the significance of inter-species differences in biomarker responses in relation to site- and sampling ocasion. If significant, pairwise multiple comparisons were performed using the Tukey or Dunn's tests. Statistical analyses and graphical plotting were performed in OriginPro.

Since we could not collect both dreissenid species from each sampling site at both sampling occasions, the resultant data matrix did not fulfil ANOVA-like design. Therefore, we performed partial direct gradient analysis supplemented with variance partitioning (Cushman and McGarigal, 2002) to rate the influence of inter-specific differences, size of individuals, season and sampling site (explanatory variables) on stress indicators (response variables) in molluscs. For analyses, stress indicator values were standardized by their standard deviation and lnx transformed to improve their normality. Of the potential explanatory variables,

species (*D. bugensis* and *D. polymorpha*), season (June and October) and sampling site (S, H1, H2 and H3) were recoded into binary dummy variables prior to analysis. According to a preliminary detrended correspondence analysis (DCA), our data represented only a relatively short gradient length (0.624 in standard deviation units), and therefore redundancy analysis (RDA) was applied for data evaluation (Lepš and Šmilauer, 2003). A forward stepwise selection procedure (at $P<0.05$) based on Monte Carlo randomization test (full model and 9,999 unrestricted permutations) was performed to exclude explanatory variables with insignificant explanatory power. This selection revealed that all non-redundant dummy variables (i.e. species: *D. bugensis*; sampling occasion: June; and site: R, H1, H2 and H3) and also size of individuals contributed significantly to the overall RDA model. To show their position in the ordination plane, we also included the redundant dummy variables (i.e. *D. polymorpha*, October and H4) as inactive supplementary variables to the analysis. Then, a series of RDA and partial RDAs were performed to rate relative effects of significant explanatory variables on the stress level of molluscs (Cushman and McGarigal, 2002). Significances of the overall model and each partial model were evaluated again with the Monte Carlo randomization test (full model and 9,999 unrestricted permutations). DCA and RDA analyses were run with CANOCO version 4.5 software (ter Braak and Šmilauer, 2002).

3. Results

3.1. Comparisons of size and the nutritional state of investigated mussels

Dreissenid spp. collected in June 2014 at the reference site were slightly larger in mean size (*D. bugensis* length: 17 ± 3; *D. polymorpha* length: 16 ± 3 mm) comparative to the samples collected from harbour areas (Table 2.), and mussels collected from the H2 harbour location in both sampling campaigns (June, October) were consistently smaller than the exemplars collected from other sites, but even in these cases differences in the size of mussel samples were statistically not significant.

Table 1. Mean length, condition index and protein content of dreissenid spp. subjected to biomarker investigations. Data are presented as mean ± SD. For each set of data normality and homogeneity of variances were met (Levene's test, $p>0.05$). For none of the parameters considered were detected any statistically significant differences at $p<0.05$ (ANOVA, Bonferoni test).

			Reference	Harbor areas			
				H1	H2	H3	H4
June	*D. bugensis*	Number of mussels	50	60	70	0	60
		Length [mm]	16.6 ± 2.8	16.1 ± 1.6	13.0 ± 1.0	-	13.7 ± 1.2
		CI [ww ww^{-1}]	0.30 ± 0.05	0.26 ± 0.02	0.26 ± 0.03	-	0.23 ± 0.05
		protein [mg ml^{-1} supernatant]	2.08 ± 0.26	2.07 ± 0.25	1.82 ± 0.14	-	1.88 ± 0.20
	D. polymorpha	Number of mussels	40	50	0	60	0
		Length	16.3 ± 2.5	14.5 ± 2.1	-	13.6 ± 2.6	-
		CI [ww ww^{-1}]	0.31 ± 0.05	0.22 ± 0.03	-	0.25 ± 0.05	-
		protein [mg ml^{-1} supernatant]	1.8 ± 0.22	1.92 ± 0.15	-	1.86 ± 0.32	-
October	*D. bugensis*	Number of mussels	50	60	70	50	50
		Length	15.8 ± 2.9	17.6 ± 1.5	13.7 ± 1.9	16.5 ± 3.4	15.5 ± 2.3
		CI [ww ww^{-1}]	0.33 ± 0.05	0.25 ± 0.05	0.22 ± 0.04	0.30 ± 0.09	0.21 ± 0.05
		protein [mg ml^{-1} supernatant]	2.33 ± 0.19	1.87 ± 0.17	2.12 ± 0.17	2.22 ± 0.33	2.09 ± 0.31
	D. polymorpha	Number of mussels	50	0	0	40	35
		Length	1.6.6 ± 2.9	-	-	17.2 ± 2.2	15.1 ± 1.0
		CI [ww ww^{-1}]	0.29 ± 0.07	-	-	0.32 ± 0.06	0.18 ± 0.02
		protein [mg ml^{-1} supernatant]	2.25 ± 0.35	-	-	2.04 ± 0.42	1.88 ± 0.33

Insignificant seasonal- and site specific differences in the general condition of mussels, reflected by condition index and total protein content, were detected. Additionally, insignificant interspecies differences related to size and general condition of mussel samples subjected to biomarker investigations were evidenced.

3.2. Biomarkers baseline levels in dreissenid spp.

The overall comparison of biomarker baseline levels in the two dreissenids collected in the pristine area revealed low interspecies variability in both sampling campaigns (Table 2.). Relevant seasonal variation was recorded for the metallothionein- and vitellogenin like proteins (three- and four fold increase in October, respectively), and closely similar increase in DNA alteration (by 70%) was recorded for both dreissenid spp. by October.

Table 2. Baseline levels of biomarkers in dreissenid spp. populating pristine area (average ± S.D.; n = 4 cumulative samples per site of 10 – 20 individuals).

Biomarker	Unit	*D. bugensis*		*D. polymorpha*	
		June	October	June	October
MT	nmol mg protein^{-1}	1.59 ± 0.54	4.64 ± 0.86***	1.48 ± 0.28	4.44 ± 0.54***
EROD	pmol min^{-1} mg protein^{-1}	14.8 ± 4.4	19.5 ± 3.2	11.8 ± 3.5	16.0 ± 2.2
LPO	µg TBARS mg protein^{-1}	2.01 ± 0.43	2.55 ± 0.58	1.9 ± 0.29	2.69 ± 0.19**
DNA_sb	µg mg protein^{-1}	46.7 ± 15.3	79.9 ± 17.9*	53.3 ± 17.7	92.3 ± 21.3*
Vtg	µg ALP mg protein^{-1}	0.46 ± 0.15	1.86 ± 0.49**	0.57 ± 0.11	2.10 ± 0.69***

Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between measured data recorded in the two sampling campaigns (ANOVA, Tukey test). For each set of data normality and homogeneity of variances were met (Shapiro-Wilk, Levene's test, $p < 0.05$). In both sampling campaigns, interspecies comparison of biomarkers in mussels inhabiting the pristine site revealed statistically non-significant differences.

3.3. Biomarker alterations in dreissenid spp. inhabiting harbour areas

In June, relatively low spatial variability of antioxidant defence status in mussels was observed. Compared to the biomarker baseline levels recorded in the mussels inhabiting the pristine area, distinctly higher levels of metallothionein like proteins (by 50- and 80% respectively, $p < 0.05$) were recorded in *D. polymorpha* at two harbours (H1, H3) (Fig. 2. A), and significantly higher EROD activity (2.7 fold higher level, $p < 0.01$) was detected in *D. bugensis* populating site H4, (Fig. 2. C).

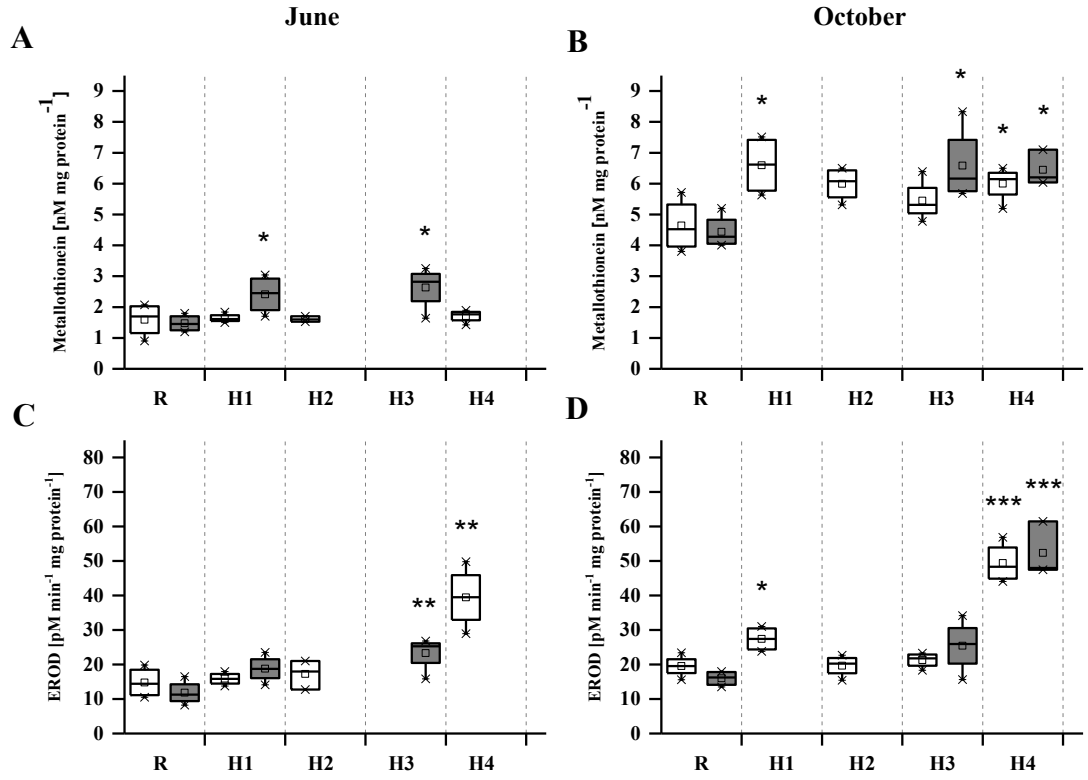

Fig.2. Metallothionein like protein concentrations (A,B) and EROD activity (C,D) recorded in whole tissue homogenates of dreissenid mussels inhabiting sites of Lake Balaton (R = pristine area, H_{1-4} = harbours). Plots represent the median (square), 25 – 75% percentiles (box) and 10^{th} – 90^{th} percentiles range (whiskers) values (n = 4, except for *D. bugensis* at site H2 in June and for *D.* polymorpha at site H4 in October where n = 3). Empty box corresponds to *D. bugensis*, grey box corresponds to *D. polymorpha*. Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between data measured in mussels inhabiting harbours versus pristine area within same season (ANOVA, Tukey test). For each set of data normality and homogeneity of variances were met (Shapiro-Wilk, Levene's test, $p < 0.05$).

By October significant elevation in metallothionein like proteins was detected in both dreissenid species and at all sampling locations. While in dreissenids inhabiting the pristine area a threefold increase in metallothioneins levels was observed in both species, in the mussels species collected in harbour areas a significantly higher fourfold increase was characteristic ($p < 0.001$). Significant increase in EROD activity was observed for both dreissenids at the H4 sampling point (~ 2.5 and 3.3 fold increase respectively, $p < 0.001$). Overall, low interspecies variability in the antioxidant defence markers was noted in both sampling campaigns (statistically insignificant, data not shown).

Of the three biomarkers of damage assessed (Fig. 3.), in June distinct site-specific alterations were recorded for DNA damage only (Fig. 3. C.). In both dreissenids significantly higher DNA strand breaks (an overall 2 fold increase, $p < 0.01$, ANOVA, Tukey test) were observed in mussels populating the H_{1-3} sites.

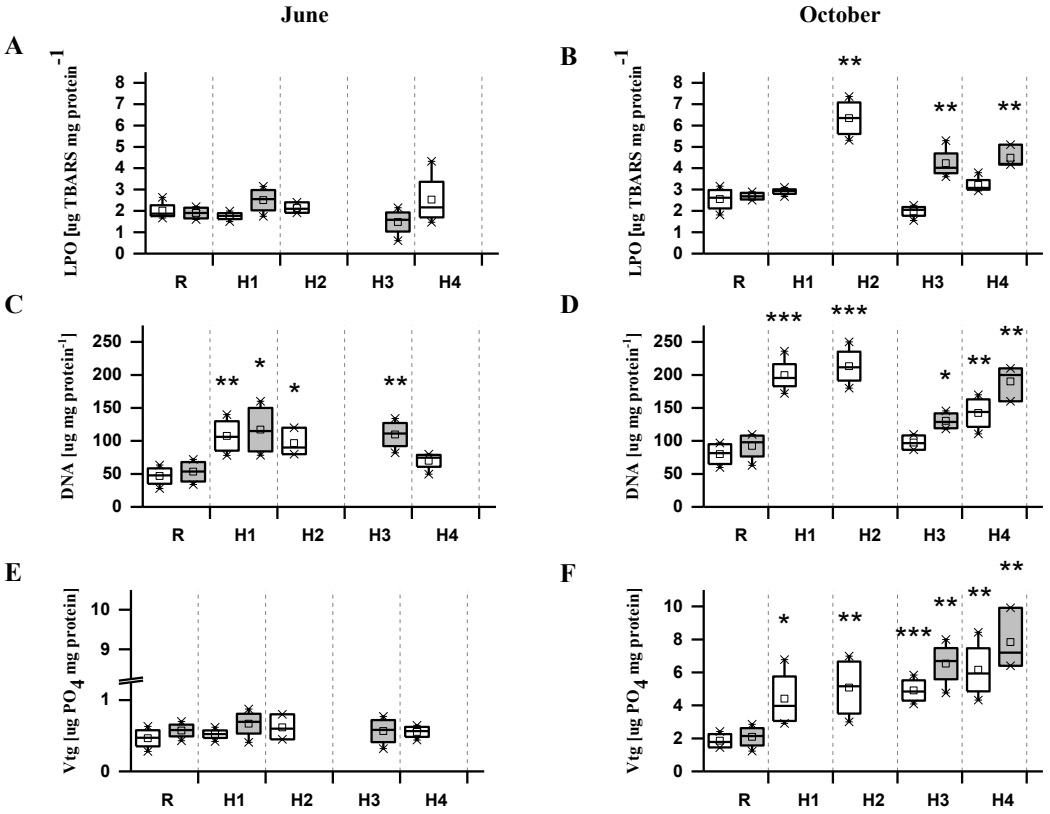

Fig.3. Lipid peroxidation (A, B), DNA strand breaks (C, D) vitellogenin- like proteins concentration (E, F) recorded in whole tissue homogenates of dreissenid mussels inhabiting the five study sites (R = pristine area, H_{1-4} = harbours). Plots represent the median (square), 25 – 75% percentiles (box) and 10^{th} – 90^{th} percentiles range (whiskers) values (n = 4, except for *D. bugensis* at site H2 in June and for *D.* polymorpha at site H4 in October where n = 3). Empty boxes correspond to *D. bugensis*, grey boxes correspond to *D. polymorpha*. Asterisks indicate significant differences (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) between data measured in mussels inhabiting harbours versus pristine area within same season (ANOVA, Tukey test). For each set of data normality and homogeneity of variances were met (Levene's test, $p < 0.05$).

By October, all three biomarkers of damage indicated significant alterations in the dreissenid populations inhabiting harbour areas compared to the pristine site. The pattern of spatial variability of the three biomarkers revealed in general a common trend with highest alteration rate observed at the H2 harbour for LPO and DNA damage, while the highest levels of Vtg like proteins for both dreissenid spp. were recorded at harbour H4 (~ 11 fold increase vs. reference site, $p < 0.001$, ANOVA, Tukey test).

3.4. Relationships between variables

The RDA analysis explained a remarkable 80.4% of the variance in stress indicators of mussels (Table 3).

Table 3. Percentage explained variance by explanatory variable groups in the redundancy analysis exploring stress levels of *D. bugensis* and *D. polymorpha* in pristine and altered harbor habitats of Lake Balaton, Hungary. Results revealed the predominant and highly independent influence of seasonality and sampling site on the set of biomarkers considered.

	Variance explained (%)				
	pure	**shared**	**total**	**F**	***p***
Season	40.3%	10.5%	50.8%	57.8	<0.001
Site	25.8%	4.7%	30.5%	5.82	<0.001
Length	2.3%	2.2%	4.5%	2.66	0.07
Species	2.3%	-2.1%	0.2%	0.14	0.97
Full model	-	-	80.4%	29.25	<0.001

Sampling season and site proved to be powerful explanatory factors accounting for the 40.3% and 25.8% of variance as pure effect and 50.8% and 30.5% in total, respectively. Results also showed that effects of season and site were highly independent with moderate proportions of shared effects in the explained variance. On the other hand, interspecies and size-dependent variability in the stress indicators of molluscs seemed to be inconsiderable. The first RDA axis captured most of the explained variance (62.3%) suggesting lower stress in June and for the pristine reference site (S) compared to October and human impacted harbour sites (H1-H4) (Fig. 4).

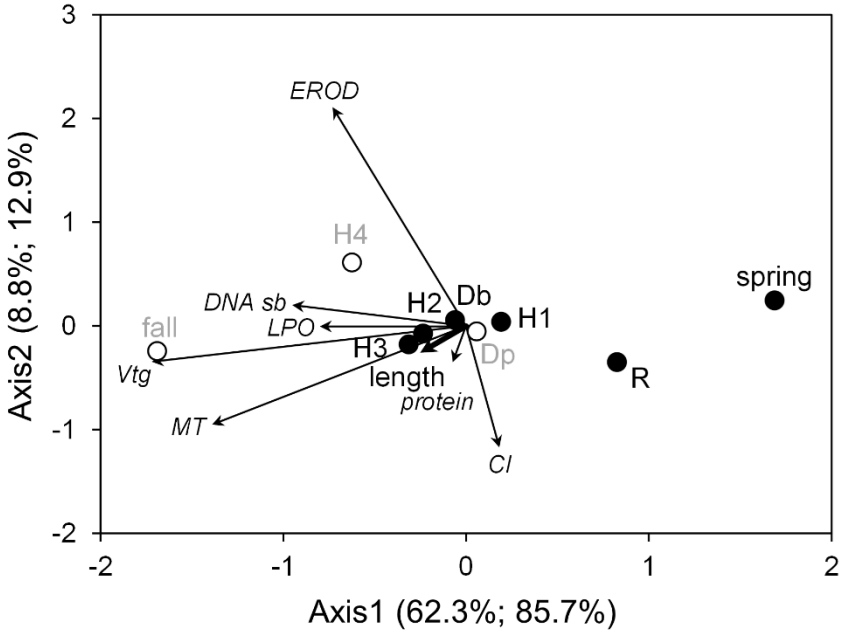

Fig. 4. Results of redundancy analysis depicting the relationships between the selected effective explanatory variables (filled circles represent dummy coded and bold arrow continuous variables, both written in normal letters) and stress markers (thin arrows and written in small italic letters) of *D. bugensis* (Db) and *D. polymorpha* (Dp) in pristine (R) and altered harbour habitats (H1-H4) of Lake Balaton, Hungary. Inactive dummy coded

variables (empty circles and written in normal grey letters) are also plotted. Percentage variances represented by axes are indicated in brackets (of species data; of species-vegetation relation) after the axis name.

4. Discussion

Differences in the basic physiological attributes of *D. bugensis* and *D. polymorpha* are reported in the literature, which might well imply also differential susceptibility/tolerance and adaptive abilities of the two species to anthropogenic pollution pressure. Consequently, a species-specific modulation of stress biomarkers in response to contaminants in *D. bugensis* and *D. polymorpha* could be expected as well. *D. bugensis* was proven to allocate more energy to soft tissue relative to shell length (Roe and MacIsaac, 1997), has higher assimilation efficiency (Baldwin et al., 2002), grows faster (Baldwin et al., 2002; Karatayev et al., 2011; Le et al., 2011), filters at a higher rate (Diggins, 2001; Veltman et al., 2008) and was reported to have a lower respiration rate (Stoeckmann, 2003). Interspecies differences were also found for the accumulation of metals and organic contaminants, in general with higher contaminant burdens in *D. polymorpha* (Rutzke et al., 2000; Richman and Somers, 2005; Schäfer et al., 2012; Matthews et al., 2015). In the meantime, while several studies have addressed the interspecies variability in biomarker responses to chemical stress in different aquatic invertebrates, in the case of dreissenids these issues have seldom been addressed. For example, species-specific susceptibility to xenobiotics as revealed by the differential expression of several biomarkers of effect or damage have been reported for amphipods (Timofeyev et al., 2008; Quintaneiro et al., 2015) or clams (Carregosa et al., 2014). The incidence of interspecies variability in biomarker baseline levels, and the potential for their differential modulation under pollution pressure in *D. bugensis* and *D. polymorpha* was assessed at sites of Lake Balaton owing its well-known environmental characteristics as well as its low-to-moderate pollution state. Pollution surveys performed over decades have outlined an overall low contamination of the lake, with low spatial variability and insignificant seasonal pattern (Kiss et al., 1997; Kiss et al., 2001; Hlavay and Polyák, 2002; Bodnár et al., 2005; Nguyen et al., 2005). The pollution of harbour areas was evidenced only by significantly higher contaminant burdens in bottom sediments compared to open areas (Hlavay and Polyák; 2002; Kiss et al., 2001; Nguyen et al., 2005; Bodnár et al., 2005; Ács et al., 2015). Within harbours, significant variability in the contamination of the sediment compartment was never recorded, which results from the fact that these harbours are characterized by very similar usage. Sediments in the selected study sites did not significantly differ in terms of grain-size composition and organic matter content (Ács et al., 2015). According to the most recent pollution survey (Ács et al., 2015), the sediment compartment was characterized by a relatively low level of anthropogenic contamination as according to the consensus-based sediment-quality criteria of MacDonald et al. (2000), none of the investigated contaminants exceeded the threshold effect concentration (TEC) below which no biological effects could be expected (Table 4.).

Table 4. Range of basic physico-chemical characteristics and contaminant concentrations recorded in the sediments of study sites in 2014 (retrieved from Ács et al., 2015).

Contaminant	R	H_{1-4}	TEC - PEC[a]
	N = 2	N = 4	
% < 63 µm	15 - 29	16 - 34	
TOC (%)	5	5 - 10	
Metals [µg g^{-1}]			
Pb	6.8 – 8.1	**16.0 – 25.0**	47 – 220
Cd	0.08 – 0.18	**0.16 – 0.31**	1.2 – 9.6
Cr	2.50 – 3.25	**4.8 – 7.0**	81 – 370
Cu	5.5 – 6.3	**10.3 – 20.3**	34 – 270
Ni	5.7 – 7.5	3.1 – 6.7	20.9 – 51.6
Zn	3.0 – 8.9	**11.1 – 61.3**	150 - 410
PAHs [ng g^{-1}]			
Naphthalene	1.20 – 4.46	0.65 – 5.33	160 - 2100
Acenaphthene	1.57 – 1.71	1.26 – 1.91	16 – 500
Fluorene	0.47 – 2.20	1.28 – 3.80	19 – 540
Phenanthrene	4.26 – 6.90	**12.1 – 40.1**	240 – 1500
Anthracene	0.28 – 0.76	**2.13 – 2.74**	85.3 – 1100
Fluoranthene	4.6 – 12.4	**20.5 – 37.8**	600 – 5100
Pyrene	4.5 – 11.9	**25.3 – 40.6**	665 – 2600
Benz[a]anthracene	0.49 – 2.48	**8.0 – 10.4**	261 – 1600
Chrysene	1.03 – 3.66	**8.9 – 11.9**	384 – 2800
Benzo[b]fluoranthene	9.2 – 16.8	13.3 – 30.6	-
Benzo[k]fluoranthene	0.93 – 3.93	**6.6 – 10.7**	-
Benzo[a]pyrene	1.2 – 5.7	**12.2 – 16.6**	430 – 1600
Dibenz(a,h)anthracene	0.39 – 0.82	**1.12 – 2.27**	4022 – 44792
Benzo(ghi)perylene	1.8 – 8.9	**10.9 – 21.0**	63.4 - 260
Indenopyrene	1.3 – 7.0	**10.7 – 17.6**	-

TOC = total organic carbon

R = reference location; H_{1-4} = harbour areas; N = number of individual measurements per reference- i.e. harbor locations

Contaminant concentrations of harbour sediments outlined in bold were distinctly higher than the concentration ranges recorded in the pristine area.

[a]TEC = threshold effect concentration; PEC = probable effect concentration (McDonald et al., 2000)

However, distinctly higher loads for some metals and polycyclic aromatic hydrocarbons were evidenced in harbour areas compared to the values recorded in the sediments of the pristine area. This fact was evidenced by the 2- to 2.6-fold increase for summed metal concentrations and the 2- to 4-fold increase for summed PAHs concentrations in the bottom sediments of harbours compared to the sediments from the pristine site or open areas (Ács et al., 2015).

In the current study, investigated mussels showed insignificant temporal and spatial variability in the condition index and total protein content, suggesting their closely similar physiological states. The range of biomarker levels (MT, EROD, LPO, DNA_sb, Vtg) detected in dreissenids inhabiting Lake Balaton were comparable with previously reported data for zebra mussels populating various aquatic habitats, either pristine- or affected by certain anthropogenic impact, as the St Lawrence River in Canada (de Lafontaine et al., 2000), Lago Maggiore in Italy (Binelli et al., 2005) or Lough Ree in Ireland (Quinn et al., 2011). Moreover, the spatial and temporal alternation of these biomarkers fell within ranges and followed similar pattern with that previously reported for pure *D. bugensis* populations inhabiting Lake Balaton (Ács et al., 2016).

The statistical evaluation of data outlined insignificant interspecies differences for the studied biomarkers, although in general consistently higher expression of all endpoints considered was observed for *D. polymorpha*. Inconsistent interspecies differences in susceptibility to contaminants for the two dreissenids have been previously reported by Schäfer et al., (2012) who have recorded in a laboratory study significantly higher DNA damage in the gills of *D. bugensis*, and conversely significantly higher heat-shock protein content (hsp70) in *D.*

polymorpha following the simultaneous exposure to differentially polluted sediments from a tributary of the Elbe River (Germany). These apparently inconclusive results indicate some differential sensitivity to chemical stress of the two mussel species, which to fully clarify, needs further consideration.

Application of redundancy analysis to our biomarker datasets revealed the predominant and highly independent influence of season and sampling site (by 50%- and 30% total variance, respectively). While in June, relatively low spatial variability of biochemical markers were recorded, by October significant rise for each endpoint (MT, EROD, LPO, DNA_sb, Vtg) was observed in mussels at all sampling sites, with distinctly more pronounced elevation for the mussels inhabiting harbour areas. In the mussels populating the pristine site significant increase by October was observed for metallothionein- and vitellogenin like proteins (3- and 4 fold increase respectively), moderate increase in DNA strand breaks (by 70%), and insignificant rise in LPO and EROD activity.

In interpreting the time related changes in biomarker levels of dreissenids populating the pristine site, first we relied on the knowledge that this location is safe of anthropogenic impact. This fact was demonstrated by the trace metal datasets repeatedly recorded over time in this area, which always fell within the regional geochemical background (Hlavay and Polyák, 2002; Nguyen et al., 2005; Ács et al., 2015). Similarly for PAHs, their concentration in the sediment was always very low and did not significantly differ from the values characteristic for open areas of the lake (Kiss et al., 1997; Kiss et al., 2001; Bodnár et al., 2005; Ács et al., 2015). Secondly, we considered the basic knowledge that contamination by metals and polycyclic hydrocarbons in aquatic organisms above certain level may: a. exceed the antioxidant capacity of the defence system; b. decrease the function of key antioxidant proteins and enzymes. Both mechanisms imply an excessive ROS formation that leads finally to oxidative cell damage, including proteins, lipids and DNA (Livingstone, 2001). Induction of the antioxidant system was proven to usually imply both significant rise in DNA damage and lipid peroxidation (de Lafontaine et al., 2000; Barhoumi et al., 2012; Maranho et al., 2015). As in the dreissenids populating the pristine site just moderate increase in DNA_sb with unaltered LPO status were observed by October, we consider that rise in MT and Vtg levels were most probably related to the progression of gametogenesis in mussels.

Seasonal fluctuation in the constitutive levels of several biochemical markers (particularly MT and Vtg) in both aquatic invertebrates and fish have been commonly reported and were related to rise in ambient temperature, food availability and gonad development (Sheehan and Power, 1999; Leiniö and Lehtonen, 2005; Izagirre et al., 2008; Schmidt et al., 2013; Lekube et al., 2014; Faria et al., 2014). This phenomenon is attributed to the close functional relationship between the digestive system and gonad development as periods of food abundance and gonad development (both evolving in the warm season) are often coincident (Mackie, 1984; Sheehan and Power, 1999). Significant increase in MT levels during gametogenesis were reported for several aquatic invertebrates inhabiting unpolluted sites (Raspor et al., 2004; Geffard et al., 2005; Geffard et al., 2007; Bochetti et al., 2008). As for example, fourfold increase in metallothionein-like proteins was reported by Baudrimont et al. (1997) for the Asiatic clam (*Corbicula fluminea*) inhabiting a remote site in France, and this elevation was unrelated to metal exposure but seemed to be strongly correlated with the reproductive cycle of this bivalve. It was demonstrated that during the progress of gametogenesis, MTs act in the homeostatic regulation of essential metals (Cu, Zn) as an essential metal storage and aim at meeting enzymatic and other metabolic demands (Roesijadi, 1996). Meistertzheim et al. (2009) have reported for *Crassostrea gigas*, encountering large temperature fluctuations as stress factor, insignificant variation of metallothionein levels in gills, mantle and digestive glands of oysters, while endogenous metallothionein levels in gonad increased significantly during gametogenesis, reaching more

than 3-fold rise by full maturation of oocytes, then suddenly decreased after spawning. This rise of MTs in the gonads of mussels suggest a role for these proteins during meiosis (Anderson and Nusslein-Volhard, 1984; Meistertzheim et al., 2009).
Similarly for vitellogenins, a common biomarker of estrogenic effects (Neubert, 1997; Blaise et al., 2003; Pampanin et al., 2005) natural fluctuations related to the reproductive cycle were recorded (as reviewed by Matozzo et al., 2008). Vitellogenins (Vtg) are the main precursors of the egg-yolk proteins, vitellins, which serve as energy reserves during embryonic development in oviparous organisms. In mature females, Vtg are generally synthesized in response to endogenous estrogens, such as 17ß-estradiol (E2), released into the bloodstream and then stored in developing oocytes. Studies have outlined that levels of E2 in molluscs display a seasonal alternation associated with the reproductive cycle and are synchronized with variations of oocyte diameter and assumed Vitellogenin mRNA levels from early vitellogenic stage to spawning stage (Matsumoto et al., 1997, 2003; Osada et al., 2004; Ni et al., 2014). Ni et al. (2014) in their study recorded several magnitude higher vitellogenins expression in the ovary of *C. gigas* in the maturation and ripeness stages than in the partially spent stage.
This study outlined distinct alterations by October (versus conditions in June) in all the biomarkers investigated for both dreissenids populating harbours. Significantly higher elevation in metallothioneins compared to that observed in the pristine area was apparent for dreissenids from three harbours (H1, H3, H4), while moderate rise in EROD activity (by 70- and 35%, respectively) was recorded in two harbours (H1, H4). Although EROD activity assessment in bivalves was considered a disputable method in outlining exposure to organic chemicals (Viarengo et al., 2007), we have included this endpoint based on evidences of significant induction of CYP-like enzymes and associated mixed function oxidase components in mussels either in *in situ* studies (de Lafontaine et al., 2000; Binelli et al., 2005; Okay et al., 2014) or laboratory experiments (Faria et al., 2009; Martin-Diaz et al., 2009; Sapone et al., 2016). Our results outlined just moderate spatial and temporal variability in the EROD activity of dreissenids inhabiting the investigated sites therefore, we considered for the moment these results as inconclusive. In this interpretation we have based first on the evidence that the molluscan mixed function oxidase components seem to respond less efficiently to chemical stress than in vertebrates (Peters et al., 2002). Secondly, it was evidenced that the natural variability of EROD activity in mussels may span several fold range as reported by Jimenez et al., (1990), and considered within the 2 – 6 fold range also by de Lafontaine et al. (2000) as baseline level. Another important issue that has to be considered here is the fact that certain ubiquitous contaminants as pharmaceuticals (diclofenac, carbamazepine, clofibrate etc.), organochlorine residues and some metallic contaminants (organotins, mercury) were proven to inhibit the CYP-like enzymes and associated mixed function oxidase components (Thibaut et al., 2006; Lavado et al., 2006; Edwards et al., 2007; Faria et al., 2010) in several biological models. Thus, for sites as harbours, which are usually affected by complex mixtures of contaminants, the antagonism of chemicals on the EROD activity of aquatic biota might also be expected.
The biomarkers of damage (DNA strand breaks, LPO and Vtg-like proteins) measured in both dreissenid species populating harbours were characterized by even greater alterations. Although these biomarkers cannot be considered as specific to a particular group of contaminants, they represent an integrative response to the impact of multiple toxic and environmental stress factors (Mayer et al., 1992; de Lafontaine et al., 2000). By October, lower amplitude of variation within sampling sites was recorded for LPO in both dreissenid spp., while distinctly higher alterations in all harbours were apparent in DNA damage (twofold increase). Mean concentrations of Vtg-like proteins achieved the highest elevation in mussels inhabiting harbours that cannot be attributed to the natural variability of these

proteins related to the progress of gametogenesis. The alterations observed for the biomarkers of damage in the mussels inhabiting harbours suggest that various metabolic functions in dreissenids were negatively affected.

5. Conclusions

The focus of our study was to assess the relevance of interspecies variability and the seasonal alternation of ubiquitous biomarkers of defence (metallothioneins, ethoxyresorufin-O-deethylase activity), biomarkers of damage (lipid peroxidation, DNA damage) and reproduction (vitellogenin-like proteins) in *D. polymorpha* and *D. bughensis*, within an *in situ* monitoring survey.
Our results indicated statistically insignificant interspecies variability for the biomarkers tested in the two dreissenid species, and the seasonal variation of these endpoints was also comparable. The fact that season proved to be the most powerful explanatory factor for the biomarker variations observed in dreissenids, outlines its major importance that has to be considered in future environmental assessment programs. The significant influence of site location on the levels of investigated biomarkers, evidenced by consistently higher values in harbour areas, raises the question of pollution impact on mussel populations at these sites which, to fully clarify, would require further investigations.

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