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Seasonal and size related variation of subcellular biomarkers in quagga mussels (Dreissena bugensis) inhabiting sites affected by moderate contamination with complex mixtures of pollutants

Ács, A.[a], Vehovszky, Á.[a], Győri, J.[a], Farkas, A[a].*

[a] MTA ÖK Centre for Ecological Research, Balaton Limnological Institute, Klebelsberg K. u. 3., PO Box 35, H-8237 Tihany, Hungary
* Corresponding author. Tel.: +36 87 448 244. E-mail address: farkas.anna@okologia.mta.hu, (A. Farkas).

Environmental Monitoring and Assessment

Abstract

Size-related differences in subcellular biomarker responses were assessed in *Dreissena bugensis* mussels inhabiting harbours moderately affected by pollution with complex mixtures of heavy metals and polycyclic aromatic hydrocarbons (PAHs). Adult *D. bugensis* samples were collected from three harbours of Lake Balaton (Hungary) characterized by moderate shipping activity, and as reference site, from a highly protected remote area of the Lake. Biomarkers of exposure (metallothioneins (MT), ethoxyresorufin-o-deethylase (EROD)), oxidative stress (lipid peroxidation (LPO), DNA strand breaks (DNAsb)) and possible endocrine disruption (vitellogenin-like proteins (VTG)) were analyzed in whole tissue homogenates of different size groups of mussels in relation to environmental parameters and priority pollutants (heavy metals and polycyclic aromatic hydrocarbons). Integrated Biomarker Response (IBR) indices were calculated for biomarker responses gained through *in situ* measurements to signalize critical sites, and to better distinguish natural tendencies from biological effects of contaminants. Biomarker responses showed close positive correlation in case of MT, EROD, LPO, DNAsb and negative correlation with VTG levels with mussel shell length in autumn, when higher levels of biomarkers appeared, possibly due to natural lifecycle changes of animals.

Keywords: Dreissena bugensis, integrated biomarker response, Biochemical markers, metallothionein-like proteins, ethoxyresorufine-O- deethylase (EROD), DNA damage, lipid peroxidation (LPO)

1. Introduction

Over the last decades the application of biomarker based assessment schemes has gained increasing interest in evaluating the environmental implications of anthropogenic pollution in aquatic ecosystems. These investigations rely on the assessment of a range of biomarkers of exposure and effects in selected bioindicator organisms, and proved to be efficient tools in identifying the pattern and level of contamination and its implications to biota (Astley et al. 1999; Galloway et al. 2002; van der Oost et al. 2003; O'Neill et al. 2004; Contardo-Jara and Wiegand 2008).

A major challenge of the biomarker investigative approach however, is to properly link harmful effects induced by often complex contaminant mixtures to ecological consequences at population- and finally to community level (Cajaraville et al. 2000; Narbonne et al. 2005; Voets et al. 2006; Hagger et al. 2010). The biomarker techniques are further complicated by a range of natural environmental and biological factors and processes (e.g. seasonality, reproductive cycle, body mass, quality of available food etc.) potentially interfering with the effects of contaminants on the biological responses of monitored organisms (Viarengo et al. 1991; Astley et al. 1999; Shaw et al. 2004; Lesser 2006; Faria et al. 2014).

Size related differences in bioaccumulation, uptake, elimination and/or leaching of chemical stressors both organic and inorganic have been extensively reported (Mills et al. 1993; Bruner et al. 1994; Gossiaux et al. 1996; Rutzke et al. 2000; Richman and Sommers 2005; Matthews et al. 2015). These data also suggesting, that size-related variability of biochemical markers may also be expected in samples form polluted waterbodies, including Lake Balaton.

For *in situ* pollution assessment of freshwater habitats bivalves, including the zebra mussel (*Dreissena polymorpha*) proved to be suitable bioindicator organisms due to their widespread distribution, sedentary and filter-feeding nature and their fairly good tolerance to physico-chemical stresses of both natural and anthropogenic origin (de Lafontaine et al. 2000; Klobucar et al. 2003; Binelli et al. 2006; Châtel et al. 2015). The suitability of *D. polymorpha* for integrated biomarker assessment studies has also been well demonstrated (de Lafontaine et al. 2000; Minier et al. 2006; Contardo-Jara et al. 2009; Faria et al. 2010). The widespread invasion of quagga mussel (*Dreissena bugensis*) in the last decades which shifted their dominance over the formerly established zebra mussel populations (Mills et al. 1996; Bij de Vaate et al. 2014), therefore, the already established biomarker assays should be performed on this new species as well.

Based on the wealth of knowledge of previous researches of the field, the main goal of our study was to provide data by the *in situ* assessment on: i. the seasonal variability of selected biomarkers, known to be influenced by reproductive cycle, temperature, food availability and quality. ii. provide data about the natural differences in biomarker levels/responses related to mussel size, revealing effects of the life stage of mussels, which may influence the responses to environmental impacts, including chemical stress. iii. the relevance of impacts exerted by moderate to low level contamination on established *D. bugensis* populations in the littoral zone of Lake Balaton based on the measurement of a set of biomarkers of defence and damage, and the calculation of IBR indexes. By the application of the Integrated Biomarker Response (IBR) approach, seasonal- and site specific biomarker responses are expected to become more highlighted.

Seasonality and size dependent variation of biomarkers of defence (metallothionein (MT), ethoxyresorufine-O-deethylase (EROD)), biomarkers of damage (lipid peroxidation (LPO) and DNA damage (DNAsb)) and reproduction (vitellin-like proteins (Vtg)) were examined in *D. bugensis* from three harbours historically known as moderately affected by pollution due to ship traffic, and compared them with data measured in mussels collected from a highly protected remote area. EROD is considered as a specific bioindicator responding to organic contaminants like PAHs and PCBs in fish, nonetheless in case of bivalves it was considered as an ambiguous method to assess exposure to organic compounds (Viarengo et al. 2007). However, EROD activity assessment was included in this study 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) and laboratory experiments (Faria et al. 2009; Martin-Diaz et al. 2009; Sapone et al. 2016).

Studies applying the Integrated Biomarker Response (IBR) approach with mussels (Damiens et al. 2007, Zorita et al. 2008; Raftopoulou and Dimitriadis 2010; Dabrowska et al. 2013) and fish *L. aurata* and *Cyprinus carpio* (Oliveira et al. 2009; Kim et al. 2010), emphasize the

potential use of this index as an integrated view on biological effects of contaminants and signal critical areas. IBR can also be used as an indicator of environmental stress, and as a simple method for the qualitative evaluation of stress degree along contaminated sites (Kim et al. 2010; Raftopoulou and Dimitriadis 2010). In order to better distinguish natural tendencies from anthropogenically sourced stress related effects, integrated biomarker response (IBR) indexes were calculated for biomarker responses gained through *in situ* measurements.

2. Materials and Methods

2.1.Site selection and sampling

Three harbours were selected as sampling sites for the biomarkers assessment of established *Dreissena bugensis* populations, and as negative reference a highly protected littoral zone of the Lake. These sites were chosen on the basis of previously published contamination data of the bottom sediments (Hlavay and Polyák 2002; Bodnár et al. 2005; Nguyen et al. 2005; Ács et al. 2015). The above investigations have revealed a moderate pollution within- and in the close vicinity of harbor areas by heavy metals and polycyclic aromatic hydrocarbons predominantly resulting from the local shipping activities (Ács et al. 2015, Table 1, Figure 1). By sediment quality criteria (McDonald et al. 2000) none of the investigated contaminants exceeded the threshold effect concentration (TEC), but a distinct enrichment of contaminants was still observed in the harbours sediments compared to the sediments from remote (protected from any vehicle transport) or open areas.

The harbours around Lake Balaton are characterized by relatively shallow water with depths varying between 1.0 – 3.5 m. The harbours have wide openings enabling an intense water exchange, and the water level fluctuations are relatively small, no significant differences can be recorded in temperature, dissolved oxygen, salinity, pH and total dissolved solids compared to the values found in other, open areas (Tátrai et al. 2008; Szabó et al. 2011). Consequently, our selected sampling sites were also characterized by relatively low variations in basic environmental parameters as median summer temperatures (19 – 22 ^{o}C), pH (8.5 – 8.6), salinity (280 – 450 mg L^{-1}), dissolved oxygen (around 10 mg L^{-1}), conductivity (600 – 700 µS cm^{-1}), redox potential (400 – 600 mV).

Specimens of *Dreissena* sp. were collected and the biomarker measurements performed in June and October 2014, considered as the beginning and the end of the main spawning period of mussels. Recently, the littoral zone of the lake is predominantly populated by *Dreissena bugensis*, with significantly lower incidence (10 – 30%) of *Dreissena polymorpha*.

Mussels were sampled randomly at mid shore level from each area. Overall, three groups of mussels tied on rocks with an approximate plane surface area of 20 – 50 cm^2 were photographed per site (distance between replicates was ca. 10 – 15 m) in the presence of a ruler, of which one randomly selected group was separated from the substrate by byssus excision and used for biomarker analysis (approx. 400 - 600 individuals).The mussels transported to laboratory in containers filled with lake water from the same site were cleaned

of shell debris, then kept overnight in aerated filtered lake water in 200 L flow-through system aquaria allowing to flush their sediment and gut contents.

Specimens of *D. bugensis* were identified by the morphological characteristics described by May and Marsden (1992) and Claudi and Mackie (1994). Within 24 hours after collection, live mussels were separated into four size groups based on their relative shell length (11 – 13 mm, 14 – 16 mm; 17 – 19 mm; 20 – 22 mm; referred later as 12, 15, 18, 21 mm size category respectively). Ten to twenty individuals in each size category were blotted dry and then weighed to obtain whole wet weight. For each individual the length (maximum anterior-posterior axis) to the nearest 0.1 mm was measured using Vernier callipers. Then mussels were immediately frozen and stored at -80 ^{o}C until biomarker analyses were performed on whole tissue homogenates of 10 – 20 pooled individuals.

2.2. Tissue preparation

Whole soft tissues for biochemical measurements were homogenized on ice in a general buffer (25 mM Hepes-NaOH, 130 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 7.4) at a weight to volume ratio of 1:5. Subsamples of homogenate were frozen at -80 ^{o}C for analysis of DNA damage (DNA), lipid peroxidation (LPO) and total protein content. The remaining homogenate was centrifuged at 12,000 g for 10 min at 4 ^{o}C, and aliquots of the supernatant (S12) were frozen at -80 ^{o}C for evaluation of metallothioneins (MT), ethoxyresorufine-O-deethylase (EROD), vitellin like proteins (Vn) and total protein content. The values of each biomarker were normalized against the protein content of either the homogenate or supernatant (S12) determined according to Bradford (1976).

2.3.Metallothionein-like proteins

Metallothionein like proteins (MT) were quantified by partial purification of MTs according to Viarengo et al. (1997), followed by the reaction of the MT-containing fraction with the Ellman's reagent and spectrophotometrical quantification using reduced glutathione as standard. Blanks of re-suspension buffer and standards of glutathione (GSH, Sigma-Aldrich) were included in each run. The results were expressed as nMol metallothionein mg^{-1} of protein.

2.4. EROD activity

EROD activity was determined by means of the method of Burke and Mayer (1974). The method is based on determining the efficiency of a given biological sample to hydrolyze the ethoxyresorufin substrate to its fluorescent product resorufin (Grzebyk and Galgani, 1991). Calibration was performed with serial dilutions of 7-hydroxyresorufin (Sigma-Aldrich). Results were expressed as pmol min^{-1} mg^{1} protein.

2.5. Lipid peroxidation

Lipid peroxidation was determined by quantifying the levels of malondialdehyde (MDA) in tissue homogenates by the thiobarbituric acid method (Wills, 1987). Since malondialdehyde is a degradation product of peroxidised lipids, levels of MDA serves as an index for determining the extent of lipid peroxidation from the breakdown of polyunsaturated fatty acids. Calibration was performed with serial dilutions of tetramethoxypropane (Sigma-Aldrich), and results were expressed as μmol TBARS mg^{-1} protein.

2.6. DNA damage (strand breaks)

DNA damage was determined by the alkaline precipitation assay developed by Olive (1988). The assay is based on the alkaline precipitation of protein-linked genomic DNA leaving protein-free DNA strand breaks in the supernatant (Bester et al., 1994). The number of DNA strand breaks results from the DNA repair of DNA adducts and alkali-labile sites. The results were expressed as μg of DNA mg^{-1} protein. Calibration was performed with salmon sperm DNA (Sigma-Aldrich).

2.7. Vitellogenin-like proteins

Vitellogenin-like proteins (Vn) were determined by the indirect alkali-labile phosphate (ALP) technique developed by Blaise et al. (1999). This assay is based on the determination of labile phosphates released by vitellin-like proteins after hydrolysis with alkali. Rainbow trout vitellogenin was used as positive control and samples substituted with NaOH were used as blanks. Vn levels were expressed as μmoles of ALP mg^{-1} protein.

2.8. Integrated biomarker response (IBR) index calculation

The integrated biomarker response (IBR) was computed for each mussel size group from each sampling site according to the method of Beliaeff and Burgeot (2002) with modification by Broeg and Lehtonen (2006) to evaluate the overall mussel status. Briefly, calculation of the mean and standard deviation for each biomarker and each group, was followed by standardization of data for each sampling site so that the variance = 1 and the mean = 0. This was achieved by calculating a standardized value of biomarker using a formula of $x_i' = (x_i - x)/s$, where x_i= standardized value of the biomarker; x_i' = mean value of a biomarker from each group, x = mean value of the biomarker for all groups, s = standard deviation for the station-specific values of each biomarker. Biomarker scores (B_i) were then calculated by summing the standardized value obtained for each group and the absolute minimum value in the data set ($B_i = x_i' + |x'_{min}|$). The calculation of the star plot areas was performed then by multiplying the scores of each biomarker (B_i) with the score of the next biomarker ($B_i + 1$) and dividing each calculation by 2. Finally, the IBR index was calculated by summing of all star plot areas $\{[(B_1 \times B_2)/2] + [(B_2 \times B_3)/2] + \ldots [(B_{n-1} \times B_n)/2]\}$. This sum was divided by the number of biomarkers measured to yield a normalized IBR (Broeg and Lehtonen 2006).

2.9. Statistical analysis

Four groups of 10 – 20 mussels each (depending on size) were anlysed at each site and subjected to biomarker analysis ($n = 4$). Data are expressed as site means per size class with standard deviations (mean ± SD). Data were tested for normality and variance using Kolmogorov-Smirnov and Levene's tests, respectively. Data that passed these tests were analysed via parametric analysis. Data that failed normality and/or variance assumptions were analysed using non-parametric statistics. A two-way ANOVA with mussel size (shell length) and season as the two factors were performed to assess their individual and interactive influence on the biomarker datasets. The results of this analysis are summarized in the Supplementary Material. The significance of differences in biomarkers was assessed by pairwise multiple comparisons performed using the Tukey or Dunn's tests. Relationships between endpoints and mussel shell length were examined using regression analysis. The level of significance was set at $p \leq 0.05$. The effect of site contamination on biomarker datasetes was investigated for the two seasons individually by two-way ANOVA with mussel size and site contamination (expressed by either ΣMe or ΣPAHs concentrations in the sediments) as the two factors. The significance of site related differences in parametric biomarkers was assessed by pairwise multiple comparisons performed using the Tukey or Dunn's tests.For biomarker data where no significant correlations related to size were found, site specific differences were assessed by the Mann-Whitney *U*-test, at a significance level of $p \leq 0.01$. Analyses and graphical plotting were conducted using Origin Pro 9.0 software.

3. Results

In the whole soft tissue homogenates of mussels collected in June 2014, insignificant spatial variability in biomarker levels was evidenced moreover, insignificant size related differences were detectable (Table 2). By October a relevant increase for each biomarker endpoint was observed, with significantly higher elevation in the mussels inhabiting the harbours. Regarding the two biomarkers of defence (MT and EROD), significant positive correlation with the size of mussels was evident at each sampling location ($p<0.001$; Table 2). Overall, the level of metallothionein like proteins and EROD activity were 2.5 – 3-, and 1.5 higher respectively, than the means recorded in mussels from the pristine site (Figure 2). In the mussels populating the reference site, significant rise was recorded only for metallothioneins by October (Table 2).

For the biomarkers of damage (LPO. DNAsb, VTG) at the beginning of the shipping season in June insignificant spatial- or size related variability could be observed in the mussel populations investigated. By autumn in turn, an overall high elevation was observed for the content in VTG-like proteins in mussels. This rise in VTG concentration was observed at each sampling location, and revealed a strong negative correlation with the size of mussels ($p<0.001$; Table 2, Figure 3). Rise in the VTG content of mussels was distinctly higher for the individuals collected form harbour areas (10 – 25 fold increase). Additionally by autumn, in the mussels inhabiting harbor sites significant rise in LPO (1.5 – 3 fold increase) and DNA damage was also observed. For both LPO and DNAsb in general significant positive correlation with size was evidenced ($p<0.001$; Table 2, Figure 3) except for DNAsb in the mussel population inhabiting the H_1 harbor, where this endpoint showed a strong negative correlation with the size of mussels ($p<0.001$; Table 2, Figure 3).

Biomarker scores and IBR indices computed for each mussel size class per site and season revealed low spatial variability in mussels in June (Table 3). Differences in the constitutive levels of biomarkers were apparent for the mussels inhabiting the H3 harbour area for the smallest size group (12 mm), characterized by increased EROD and LPO activity. By October, an elevation of biomarker levels are recognisable, with significantly higher intensity in mussels inhabiting harbour areas, also suggesting site specific effects. In case of R and H1 October values, the most powerful effects are apparent for the smallest size groups (15 and 12 mm respectively) characterized by increased levels of VTG and DNA. In case of H2 harbour site, pronounced elevation of biomarkers by October were evidenced for the largest size groups (18 and 15 mm), and biomarker scores for these size sets are very similar. The three different size groups of mussels show a very different biomarker pattern in case of H3, also size related differences and biomarker level changes are not so pronounced like in case of other harbour sites.

By October the IBR values tended to increase compared to values computed for June. IBR values showed no considerable seasonal difference in case of remote site. Given that the IBR is an indicator of environmental stress, elevated IBR values for harbour sites, and low values with no seasonal change in case of remote area confirm the higher level of pollutant load in harbour sites. Size related change of IBR values seemed also to be present: IBR values for H1 site suggest a negative correlation with mussel size. IBR values computed for H3 site suggest only very weak seasonality, since values are in closely similar magnitude for both seasons, in contrast to other harbour sites, were IBR values show two and three orders of magnitude increase.

4. **Discussion**

Seasonal variation in stress marker values of mussels have often been related to changes in food availability and quality, changes of ambient temperature (Leiniö and Lehtonen 2005; Bocchetti and Regoli 2006; Rank et al. 2007; Ochoa et al. 2012; Nahrgang et al. 2013). More recently, Faria et al. (2014) suggested reproductive cycle as the major factor affecting variation of biomarker values in *D. polymorpha*. Several studies are reporting peeking of antioxidant defence system activities in Dreissenid species in late winter, when gonads are in early spawning stage (Faria et al. 2010; Palais et al. 2012; Parolini et al. 2013), and reach their minimum levels in summer. Levels of lipid content, the putative substrate of lipid peroxidation, are reported to increase later from March and peak in June, in most cases in parallel to LPO values. Seasonal changes in DNA replications (and thus DNA damage/strand brakes), and VTG values can also be associated to lifecycle changes of mussels. Since the method of measuring DNA strand breaks measures the abundance of single DNA strands, it is also a measure of DNA replication and transcription (Faria et al. 2010). VTG-like proteins are precursors of vitellin synthesis in vertebrates and in some invertebrates also, including bivalve molluscs (Pipe 1987; Suzuki et al. 1992). Thus, besides indicating exposures to substances perturbing endocrine functions, or causing DNA damage, elevation in DNAsb is also influenced by the reproductive cycle of mussels. MTs are regarded as specific bioindicators responding to trace metals however, previous studies pointed out that MTs play a role also in

heavy metal cation homeostasis, ROS scavenging activity and are found to be induced also by organic aromatic compounds (Sato and Bremner 1993; Viarengo and Nott 1993; Viarengo et al. 1999). Moreover, during gametogenesis increased MT levels in molluscs have been detected, irrespective of temperature regime (usually elevated temperature in the warm season) or ambient metal bioavailability (Raspor et al. 2004; Geffard et al. 2005; Bochetti et al. 2008). Seasonal variability of EROD activity has been reported also, being high in autumn, and declining during gametogenesis (Kirchin et al. 1992; Sheehan and Power 1999).

Our results demonstrated distinct alterations by October (versus conditions in June) in both the biomarkers of damage (LPO, DNAsb and VTG) and in the biomarkers of defence (MT and EROD) for *D. bugensis* inhabiting harbour areas. In mussels inhabiting the pristine site distinct rise was evident only for metallotionein- and vitellogenin like proteins. In interpreting the seasonal variations of biomarkers in the mussels from the pristine area we have based first on the fact that exposure to ubiquitous anthropogenic contaminants as metals and polycyclic aromatic hydrocarbons generally induce ROS production and may overwhelm the antioxidant capacity or decrease the function of the antioxidant defence system. Both mechanisms may lead to excessive ROS formation and oxidative damage to DNA, proteins and lipids (Livingstone 2001). This cascade of toxic effects was reported in both field surveys and laboratory exposures when moderate pollution pressure by metals and PAHs triggered the elevation of metallothioneins content and EROD activity in aquatic invertebrates and fish. Such induction of the antioxidant system was accompanied also by alterations in DNA damage and lipid peroxidation (La Fontaine et al. 2000; Gagné et al. 2012; Gagné et al. 2015). As in the mussels inhabiting the pristine area just moderate elevation in DNAsb with unaltered LPO status were observed by autumn. We therefore hypothesize that the rise in MT and VTG levels were most probably related to the higher metabolic rate during the summer season and also reflects the progression of gametogenesis. The incidence of anthropogenic pressure in the pristine area is very unlikely, as the site is located at reasonably high distance by any populated settlement, within a highly protected natural reserve area. The low anthropogenic influence of the pristine area was demonstrated by the low metal and PAH concentrations sequestered in the bottom sediments reported previously (Ács et al. 2015).

Biomarker scores and IBR values computed for sampling sites mirror the different feature of habitats: In June, the set of biomarker scores and IBR values computed for mussels from remote site differs only slightly from the characteristics recorded in October, suggesting virtually unaltered status in environmental stress. Biomarker scores draw a very different picture of harbour areas regarding their seasonal fluctuations and environmental impacts. The pattern of biomarker scores in June are very similar to that established for the remote site, only in mussels from harbour H3 slightly elevated LPO and EROD level were observed. By October, however, a remarkable increase of biomarker values is obvious for all mussels inhabiting harbour areas, although in site H3 this increase less remarkable compared to H1 and H2 harbours.

Size related differences in biomarker responses of Dreissenid mussels, to our best knowledge, were not investigated until now, although some marine and freshwater mussels such phenomenon were described before, but the size-dependent responses of mussel species are much less investigated, than for example in case of fish (Lau and Wong 2003). Size reflects

the life stage of an organism, which may influence the responses to environmental impacts, including chemical stress. While size effects can be easily minimized or eliminated in laboratory studies, this may not be the case in field studies employing organisms for monitoring purposes. Thus, size is always a factor to be addressed in case of field sampling. Most biomonitoring studies employing field-collected Dreissenid mussels are aware of the size factor reporting size ranges with 1 to 5 mm precision of the mean shell length of the animals applied (de Lafontaine et al. 2000; Binelli et al. 2006, 2010; Faria et al. 2010, 2014). The mean shell length range, however varied on a relatively wide scale from app. 10 mm to 30 mm, in different studies, mostly depending on the available animal sizes, and not on a pre-desired size range deliberately set out. As a consequence, it is hard to make a direct comparison of the results obtained in different studies.

In the present study, the biomarker responses of different size ranges were normalized to protein concentrations of the sample, and graphical presentations suggested correlation with shell length. However, for spring samples statistics did not confirmed correlation of biomarker responses with animal size. In contrast, the samples collected in autumn showed a rather unified picture of size-dependent biomarker responses: all values strongly correlated with shell length of the mussels, with a correlation factor around or above 0.9 in absolute values. MT, EROD, LPO, DNA assays displayed positive correlation, and VTG values decreased with growing shell length. The only exception was the level of DNA strand breaks for mussels from site H1 in October, which showed a negative correlation to animal size. Sets of biomarker scores of different size groups of *D. bugensis* showed a very toned picture for the mussel samples collected in October: in case of H1 site environmental stress seemed to affect more the smaller mussel groups and the disturbing effects appeared to be reduced with growing shell length. The opposite of this apparent correlation is shown in mussels from harbour H2, where larger mussels seem to be more affected by environmental effects. In addition, size dependent variation also appears to relate to gametogenesis and spawning stage of mussels, as reported previously by Faria et al. (2014).

Taking into account local characteristics of the sampling sites, due to relatively low variations of basic environmental parameters (Tátrai et al. 2008; Szabó et al. 2011), the selected sites may differ only in the contamination status mainly deriving from the temporal ship traffic present from spring to late autumn. The sampling areas, according to previous sediment quality studies, are slightly polluted by metals and PAHs as none of the investigated contaminants exceeded the threshold effect concentration (TEC) below which no biological effect can be expected. Size dependent results could also be attributed to size related differences in metabolic-, uptake-, and loss rates of contaminants. Larger individuals may compensate for higher metabolic demands by increasing their respiration rates, thereby increasing their exposure to waterborne contaminants (Bruner et al. 1994). This may cause additional energetic stress to larger individuals resulting the higher impact of the chemical stress, compared to smaller exemplars. In literature, positive correlation with the size/age of mussels was recently reported by Izagirre et al. (2014) for several stress biomarkers in *Mytilus galloprovincialis*. In interpreting these results we have to count also with the specific depuration rates of contaminants that for ex. for metals as cadmium and mercury are particularly low (Merian ed., 1991). This implies that larger/older mussels even at relatively

low pollution pressure are more affected by chemical stress than smaller individuals. Additionally, at relatively low concentration of pollutants the higher growth dilution characteristic for younger individuals may partially reduce the accumulation rates of pollutants as reported by Richman and Somers (2005).

Biomarker responses of *D. bugensis* samples obtained from the slightly polluted habitats of Lake Balaton showed strong seasonality and size-dependent correlation in October, coinciding with the end of the shipping season. These site and season-related differences in biomarker values were properly demonstrated by the IBR indices, and facilitated the comparison of biomarker changes between different mussel colonies. The alteration patterns of biomarkers in mussels by October may also suggest different short-term environmental stress routes, and/or natural change of biomarker levels. Therefore, in order to properly reveal environmental stress related alteration in biomarker responses and to eliminate natural size-, and seasonal variations, our results also suggest further studies after the main spawning season and/or before the very start of gametogenesis.

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Figure legends

Fig. 1 Localization of sampling sites along Lake Balaton (R= reference site; H1, H2, H3= harbour sites)

Fig. 2 Responses of biomarker of defence (a= metallothionein-like proteins (MT); b= ethoxyresorufin-O-deethylase (EROD)) recorded in whole tissue homogenates of *Dreissena bugensis* inhabiting the four study sites (R = pristine area, H1-3 = harbours). Empty symbols represent the median and standard error of data recorded in June, solid filled symbols represent the median and standard error of data recorded in October. Dashed lines reveal significant correlation patterns within data sets. For each set of data normality and homogeneity of variances were met (Shapiro-Wilk, Levene's test, $p < 0.05$)

Fig. 3 Responses of biomarkers of damage (a= vitellogenin-like proteins (VTG); b= DNA strand breaks (DNAsb); c= lipid peroxidation (LPO)) recorded in whole tissue homogenates of *Dreissena bugensis* mussels inhabiting the four study sites (R = pristine area, H1-3 = harbours). Empty symbols represent the median and standard error of data recorded in June, solid filled symbols represent the median and standard error of data recorded in October. Dashed lines highlight significant correlations between variables. For each set of data normality and homogeneity of variances were met (Shapiro-Wilk, Levene's test, $p < 0.05$).