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Contribution of invasive bivalves (*Dreissena* spp.) to element distribution: phase interaction, regional and seasonal comparison in a large shallow lake

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Abstract After introduction, the invasive bivalve dreissenids became key species in the biota of Lake Balaton, the largest shallow lake in Central Europe. The contribution of dreissenid soft tissue and shell, as biotic phases, in element distribution and its interaction with the water and upper sediment phases were examined in two basins with different trophic conditions in spring and autumn. Six metals (Ba, Cu, Fe, Mn, Pb, Zn) were detected in all investigated phases. In general, metals were abundant in the water and soft tissue in the eastern basin in spring, and in the sediment and shells in the western basin in autumn. This might be associated with the more urbanized surroundings in the eastern, and the enhanced organic matter production in the western basin. High relative shares of Ba, Cu, Mn, and Pb were associated with the water and shell samples, whereas high shares of Fe and Zn were noted in the soft mussel tissue and sediments. Results suggest that dynamics of metal uptake by dreissenids depend on the seasonal change in metabolic activity. Shell metal content is less changeable; shells might absorb metals from both the soft tissue and water phases. Metallothionein peptides, the scavengers of intracellular metals, were determined to be biomarkers of the bulk contaminants rather than only metals. The present study shows that invasive bivalves, with high abundance, filtering activity, and storing capacity can significantly contribute to element distribution in the shoreline of a shallow lake ecosystem.

Keywords Dreissenids · Metallothioneins · Metal partition · Sediment · Shallow lake · Shells

Introduction

The fate of inorganic elements and particularly metals in the aquatic environment is of great concern to

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understand interactions between abiotic and biotic phases. The bioavailability of metals, i.e. the metal content which can be assimilated by an organism, depends on the metal speciation form (Pempkowiak et al. 1999; Magalhães et al. 2015) and intrinsic factors [e.g. metabolic activity and biological usefulness of the given metal (Marsden and Rainbow 2004)]. As such, it is apparent that metal transfer in the aquatic environment depends on complex mechanisms and is very difficult to predict. Online monitoring programs (Elzwayie et al. 2017), and models for the assessment of metal speciation, bioaccumulation and/or toxicity (Adams et al. 2020; Brix et al. 2020) have been developed and conducted, which are promising to generalize the prediction of metal body burden in an organism from environmental data. However, as the behaviour of organisms is pretty complex, model applicability is site and metal specific, and needs validation with frequent measurements (Bourgeault et al. 2011; Le et al. 2011). Recently, a validation process has been recommended in setting protective values for aquatic life (PVALs, Garman et al. 2020). In doing so, uncovering rules of metal transportation between abiotic and biotic contributor of ecosystems is needed to become familiar with the activity-forced demand on essential metals, as well as to determine their actual toxic value and impact on the absorption/ingestion of other (non-essential) metals in the environment.

Freshwater bivalves have long been used in metal monitoring studies due to their filter-feeder mode of nutrition, sessile life style, long lifespan, size and abundance suitable for sampling throughout multiple seasons (Elder and Collins 1991). They also exhibit physiological responses to acute metal exposure and display accumulation capacity in situations of chronic exposure (Kraak et al. 1991; Gupta and Singh 2011). They have long been used in large scale biomonitoring programs, so called "Mussel watch" surveys, which aimed at estimating the contamination levels of target surface water bodies, and in completing risk assessments for biota (Goldberg 1975; Cantillo 1998; Maruya et al. 2014). So far, numerous field and laboratory studies have investigated particular heavy metal accumulation, toxicity, and protection mechanisms in bivalves (for review, see: Naimo 1995; Wang and Lu 2017). However, much less is known about the distribution and interaction of metals between bivalves and their environment (water and sediment phases), especially in seasons when environmental conditions and hence activity of bivalves change (Cummings and Graf 2010). In addition, many of the trace metals are essential for defined biological activities, which contributes to the complexity of this issue. Studies wherein metals essential for bivalves are counted and used in model assessments, are nowadays still in their initial phase (Le et al. 2022). In bivalves, besides the visceral organs (the soft tissue), the shell also accumulates and pools metals that can substitute Ca^{2+} in the inorganic matrix (Zuykov et al. 2013). The proteinaceous external layer of the shell is also capable of adsorbing and chelating metals from the environment (see in: Geeza et al. 2019). Dreissenids are invasive, r-strategist bivalves that, due to global increase in transport along interconnected waterways, nowadays are common in European and North American freshwaters (Karatayev et al. 2015). Dreissenids extensively use environmental resources, hence displace concurrent species and re-organize food webs (Ricciardi and Macisaac 2010). Due to their negative impact on biodiversity and biofouling of hydrotechnical appliances, they are considered as nuisance/pests (Pimentel et al. 2005). On the other hand, their applicability in biomonitoring studies and particularly in metal accumulation have long been a focus of research (Kraak et al. 1991, 1993, 1994a, b; Johns and Timmerman 1998; Gundacker 1999; Camusso et al. 2001; Johns 2001; Królak and Zdanowski 2001; Borcherding 2006; Marie et al. 2006; Matthews et al. 2015). Dreissenids are abundant in Lake Balaton, the largest shallow lake (600 km^2) in Central Europe. The population is estimated as 220,000 individuals per m^2 of the solid surface along the shoreline (Balogh et al. 2008). Dreissenids play a key role in the benthic food web of Lake Balaton by filtering planktonic algae and bacteria and serving as an important food source for fish (Specziár et al. 1997) and birds (Ponyi 1994). Together with the reduced loading of nutrients to the lake, dreissenids have contributed to the enhanced water transparency by controlling algal levels in the past few years. On the basis of laboratory filtering experiments, it was estimated that the entire dreissenid population in Lake Balaton can filter the total water volume in three years (Balogh 2008).

The principal goals of this study were to reveal interactions among elements and correlations of their distribution among four phases: the water, sediment, and biota (soft tissue and shell of dreissenid bivalves) in a shallow lake ecosystem. To achieve this goal, 17 element species were measured during two seasons (spring, autumn) in two basins differing in trophic status. To assess whether the environmental conditions, including metal content, are harmful to dreissenids, the metal-chelate metallothionein expression was also examined. Metal concentrations in Lake Balaton are discussed in a historical perspective by comparison of our data with those published in the past 50 years.

Materials and methods

Structure of the dreissenid assemblage in Lake Balaton and the history of studies on element contents in the lake

Two dreissenid species live in the lake, *Dreissena polymorpha* (Pallas, 1771) (from ~ 1932, Sebestyén 1938), and *Dreissena rostriformis bugensis*, Andrusov, 1897 (from ~ 2008, Balogh et al. 2018). The later invader rapidly colonized the lake, totally outcompeting *D. polymorpha* in the oligotrophic eastern basin, whereas both species co-exist in the meso-eutrophic western basin (Balogh et al. 2018).

Measurements of heavy metal concentration in dissolved and particulate phases of Lake Balaton, as well as in its biota, date back to the early 1980s (Müller 1981; Salánki et al. 1982), and continued before the turn of the millennium (Elbaz-Poulichet et al. 1996) until the 2000s (Hlavay and Polyák 2002; Nguyen et al. 2005a, b, c). The studies aimed at finding regional and seasonal differences in the metal contents in the lake (Hlavay and Polyák 2002; Nguyen et al. 2005a), determining the metal origins (geochemical background or pollution) (Elbaz-Poulichet et al. 1996; Weisz et al. 2000), tackling the pollution sources (Nguyen et al. 2005a), investigating the role of the wetland system connected with the main inlet river in retaining metals (Elbaz-Poulichet et al. 1996; Nguyen et al. 2005b), assessing the bioavailability of metals (Hlavay and Polyák 1998; Weisz et al. 2000), and comparing metal distribution among water, sediment, and biota (Nguyen et al. 2005c). Although these study periods are nearly decades apart, during which time the lake surroundings underwent economic changes and water management regulations, all the results showed that the heavy metal content of Lake Balaton did not exceed environmentally risky values. Metals mainly originated from local minerals, slightly influenced by seasonal contamination, e.g. from increased ship traffic in summer (direct pollution), and the activity of a coal combustion heating plant located near the lake (atmospheric impact). Bioconcentration and biomagnification of heavy metals were uncommon. Therefore, the metal burden of Lake Balaton seems to be stable and metal distribution is influenced by intrinsic factors. However, monitoring data on metals in the lake in the past fifteen years are missing.

Sampling

Ten sampling sites were selected along the shoreline of Lake Balaton at two different locations (in the eastern and western basins of the lake). The location of each site was situated near the mouth of a different inlet (Fig. 1, see also in Sebestyén et al. 2017). Characteristics of the catchment areas of each inlet are summarized in Online Resource T1. Sampling was conducted at the end of March and October 2019 after one week of windless weather. At each sampling site, 5 l of water was extracted with a column water sampler in three replicates, mixed and filtered through a 0.45 µm diameter plankton mesh, and stored and transported in pre-cleaned brand new glass bottles in the dark. Sediments were collected with a conventional mud column sampler (tube diameter = 7 cm) in three replicates. The upper 10 cm of the sediment was removed and dried in a heating oven at 120 °C overnight. Dreissenids were collected, depending on the surface availabilities, from stones or reed stems. The seasonal collection was adjusted to the seasonal activity of the species. The water temperature is $<$ 10 °C between October and March in Lake Balaton, and dreissenids rest in the dormant stage during this period; their metabolic activity and larval production is reduced (Claxton and Mackie 1998). Therefore, animals were collected to study the metal content at the beginning and at the end of the active period. In an earlier study, it was established that a minimum 25 individuals are statistically representative for the average metal content in a population (Gordon et al. 1980). Therefore, for measurements of metal concentration, around 300 animals (size range: 1–2 cm) were separated at each sampling site, cleaned gently with a tooth brush, and washed several times. Animals were transported to the laboratory where they were kept in

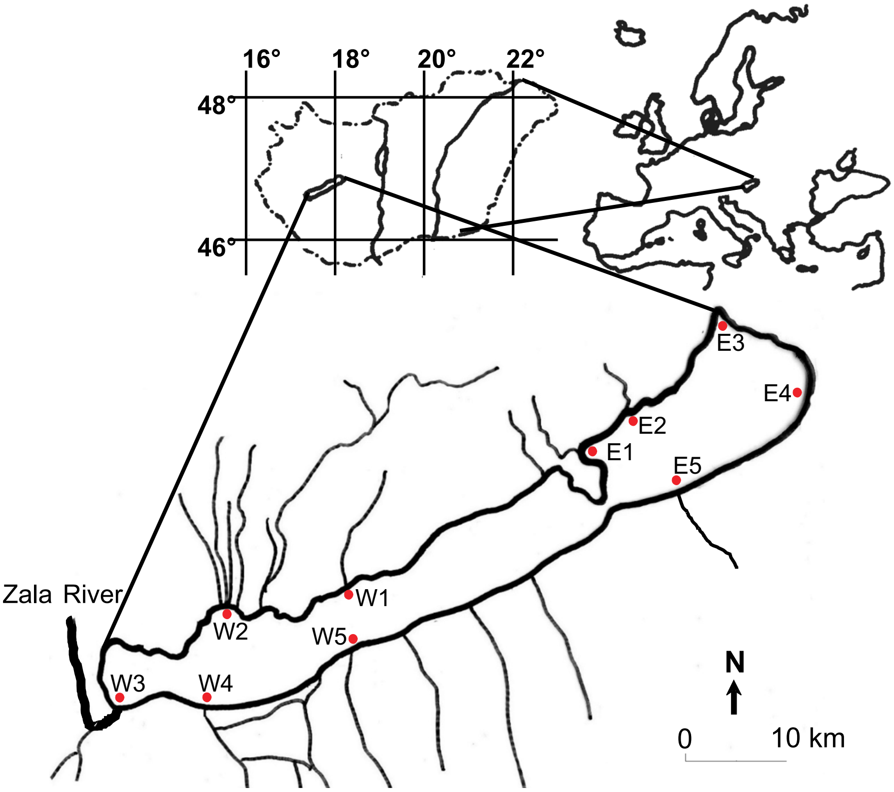

Fig. 1 Sampling sites in Lake Balaton. Lake Balaton is the largest shallow lake in Central-East Europe, situated in Hungary. Originally it used to be an endorheic lake, now the water level is regulated by an artificial outlet, the Sió canal. The main inlet is the Zala River together with other canals and smaller streams in the western basin. The lake has a trophic gradient along the longitudinal axis, with a decreasing order from west to east. Five sampling sites in the western as well as in the eastern basin were selected near the mouths of inlets. At each site, water, sediment and dreissenid samples were collected in early spring (March) and autumn (October). Environmental parameters were recorded on each occasion

barrels supplied with aerated Balaton water for 2 days. Then they were frozen and stored under -20 °C. Due to the high number of samples and the small size of the animals, instead of the custom manual dissection of each animal, mussels were cracked and milled in a mortar, further pulverized by adding some water in a motorized potter homogenizer, and the soft tissue and shells were separated by gentle rotation in a centrifuge in 2000×g for 5 min. The purity of the supernatant containing the soft tissue and the pellet shell debris was improved by discarding the border fraction, followed by decanting heavy particles from the supernatant, and floating the shell fraction under water in a beaker, respectively. The purity of the fractions were checked by the comparison of the applied method with the standard one, when 20 individuals from the stock of the spring samples from the sites E3 and W4 were dissected manually and analyzed. These preliminary trials confirmed that the results obtained with our method did not differ from those acquired in the standard way (Online Resource T2). Soft tissue homogenate was refrozen and freeze dried. For measurements of metallothionein gene expression at each sampling site (spring samples only), soft tissue of five midsize (1–2 cm) animals were removed from the valves, cut into small (< 1 mm^3) pieces, put in an RNA-later preservative (r0901, Sigma-Aldrich, Budapest, Hungary), and stored at 4 °C until use. Physicochemical properties of Lake Balaton water were recorded on each sampling occasion by a WTW Profiline Multi 3320 parameter device, Xylem Analytics, Germany).

Sample preparation and metal measurements

Sample preparation and metal measurements were carried out according to the standardized protocol of the related Hungarian regulation (sample preparation: National Standard [MSZ] 21470-50: 2006 3.1.2., International Classification for Standards [ICS] 13.080.05; metal measurement from ground water: MSZ 1484-3: 2006, ICS 13.060.45, ICS 13.060.01; from mud and dry tissues: MSZ 21470-50: 2006, ICS 13.080.05). Three independent subsamples were taken from each sample and measured subsequently. Briefly, 50 mg cc of nitrous acid was added to 10 ml taken from each water sample. Each sample of dry mud, freeze-dried soft tissue, and shell debris (0.1 mg) was added to 5 ml cc of nitrous acid. Starting with the first temperature program, the samples were destructed in a digester (Velp Scientifica, Usmate, Italy), then, after adding 2 ml cc of hydrogen peroxide, digestion was terminated using the second temperature program. The digested material was diluted 50×, and 10 ml of this dilution was used for measurement. Samples were analyzed for the following 17 metals: Ag, As, B, Ba, Cd, Co, Cr, Cu, Fe, Hg, Mn, Mo, Ni, Pb, Se, Sn, Zn by Inductively Coupled Plasma—Optical Emission Spectrometry (ICP-OES, IRIS Intrepid II XSP, ThermoFisher Scientific, Weltham, MA).

Metallothionein RT-PCR

RNA isolation from the whole soft tissue was carried out according to the manufacturer's instructions using the phenolic reagent TRIZOL (Invitrogen, Karlsruhe, Germany). Following centrifugation (20,000×*g*) the aqueous phase was extracted from the lipoid phase with chloroform. From the aqueous phase, the RNA was recovered by precipitation with isopropyl alcohol. The pellet obtained by centrifugation (20,000×*g*) was re-dissolved in 50 µl RNase-free diethyl pyrocarbonate-treated water (DEPC water). RNA purification using RNeasy Mini Kit (Qiagen, Hilden, Germany) was performed according to the supplier's protocol with an additional step of on-column DNA digestion using RNase-Free DNase Set (Qiagen, Hilden, Germany). The quality and quantity of the isolated RNA were measured with NanoDrop1000 Version 3.8.1. (Thermo Fisher Scientific). Reverse transcription from 1000 ng of total RNA was performed with the High Capacity cDNA Archive Kit (Applied Biosystems, Foster, CA, USA) in a total volume of 50 µl according to the manufacturer's protocol.

PCR Primers for metallothionein gene expression (both sense and mutated) were designed according to the sequences published in the National Center of Biotechnology Information (NCBI, Sense sequence: GCGTTGAAACCGGTGATTGC; mutated sequence: GCCACAACAGTTGGGTTTGC Acc. No.: U67347, efficiency: 1.96). These primers were also successfully used elsewhere (Contardo-Jara et al. 2010).

Real-time PCR assays were run in a LightCycler® 96 System (Roche, Basel, Switzerland), using the gene-specific primers with SYBR Green protocol. For cycling, each 10 µl PCR reaction contained 1 µl cDNA (20 ng), 250 nM primers, and 5 µl qPCRBIO SyGreen Mix Lo-ROX (2X, PCR Byosystems, London, UK). The PCR protocol was as follows: Enzyme activation at 95 °C for 2 min, 45 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C, and extension at 60 °C for 10 s. All the qPCRs were performed with three replicates. After amplification, the melting curve was checked to verify the specificity of the PCR reactions. The Ct values were normalized to ß-actin gene for each time point.

Statistical analysis

Principal Component Analysis (PCA) (based on a correlation matrix, with varimax rotation of axes) was conducted to: (i) find groups of correlated metals; (ii) reduce the number of variables to be analysed (comparisons between phases, seasons and basins). Only metals found above the detection limits in more than 75% of all samples were included in the PCA (Ba, Cu, Pb, Zn, Fe, Mn). Below detection limit measurements were replaced with X/2 values, where X is the detection limit for a particular metal (for reference, see EPA, 1991). This approach was necessary as for some data groups (phase × season × basin combinations), most of the data points were below the detection limits. To compare metal content in water with those in the other phases (expressed as µg/l and µg g/DW, respectively), we ran the PCA using percentage shares of metals in particular samples. Furthermore, we ran another PCA using metal concentrations in the three solid phases (excluding water, which was not directly comparable due to different measurement units).

General Linear Models (GLM) were run on the three established principal components (PCs), with basin (western and eastern), season (spring and autumn) and phase (mussel soft tissue, shell, water and sediment) as categorical factors. The models also included all interactions among the factors. Phase was included in the models as a within-subject factor, as all phases were measured simultaneously at each site. Additionally, to test the effect of basin (eastern and western) and season (spring and autumn) on the concentrations of the most common elements in particular phases (water, sediments, soft tissue and shells), Kruskal–Wallis tests (with 4 groups: basin*season combinations) followed by pairwise Mann–Whitney U tests as a post-hoc procedure were carried out.

Furthermore, to check relationships among different phases, multiple regressions were conducted, with PC1-3 scores (from the PCA based on percentage shares of metals) for a given phase as a dependent variable and corresponding PC scores for the remaining phases as independent variables. It was assumed that the tissue metal content is likely to be related to those in all other phases (shell, water and sediments), shell content: to those in water and sediments, and sediment content to that in water. This approach allowed us to determine whether metal contents in different phases were related to one another across basins and seasons.

Metallothionein gene expression levels (means per sampling sites) were correlated with corresponding PC1-3 scores (from the PCA based on metal concentrations) for mussel soft tissue and shell using Pearson linear correlations (6 analyses). Moreover, metallothionein levels were compared between the basins using a t-test for independent samples.

Pairwise comparisons for significant effects in GLMs were carried out using sequential Bonferroni corrected Fisher LSD tests.

Results

Based on metal concentrations measured in four phases (dissolved and SPM in water, particulate matter in the upper sediments, dreissenid soft tissue and shell, separately) at ten sampling sites in two seasons, metals can be grouped into three categories: (1) metals below the detection limit in all phases: Ag, As, Cd, Hg, Se, Sn; (2) metals detected sporadically in certain phases, sites, or seasons: B, Co, Cr, Mo, Ni; and (3) metals present above the detection limit in more than 75% of the samples: Ba, Cu, Fe, Mn, Pb, Zn.

Background parameters—basin and seasonal characteristics (Online Resource T1, F1)

In both basins, the water temperature was between 11 and 15 °C, with an average of 13.3 °C in spring, and 15–17 °C (average: 16.6 °C) in autumn. The Secchi transparency and conductivity means were always higher in the eastern basin than in the western basin, with higher scores in the former, and lower in the latter in autumn. Water pH was in the alkaline range, with lower values in the western basin and in autumn. Chlorophyll concentration was generally higher in the western basin than in the eastern basin. Among the sampling sites, E5 and W3 showed marked differences compared to the average of the given basin. At E5, the pH and transparency were the lowest and the chlorophyll content was the highest in the eastern basin. In contrast, at W3 the relatively high water transparency coincided with the low conductivity and chlorophyll content.

Sporadic occurrence of metals (group 2: B, Co, Cr, Mo, Ni, Fig. 2)

Boron was detected only in dreissenids, reaching considerable concentrations in the western basin in autumn. Boron concentrations were approximately three times higher in the shells than in the soft tissue. *Chromium* accumulated in the sediments, with a higher concentration in spring in both basins. In the eastern basin, Cr was detected in the mussel soft tissue where its spring concentration in the sediment was the highest in the entire study. *Cobalt* was detected in the water samples in autumn, with the highest concentration in the western basin. It was much less concentrated in the sediments and in shells collected in spring, and below the detection limit in the soft tissue. *Molybdenum* appeared in the autumn water samples, and was at the border of the detection limit in dreissenids. In the abiotic phases, *Ni* could only be detected in the eastern basin sediment in autumn. Moreover, it was found in the soft tissue in both basins in spring.

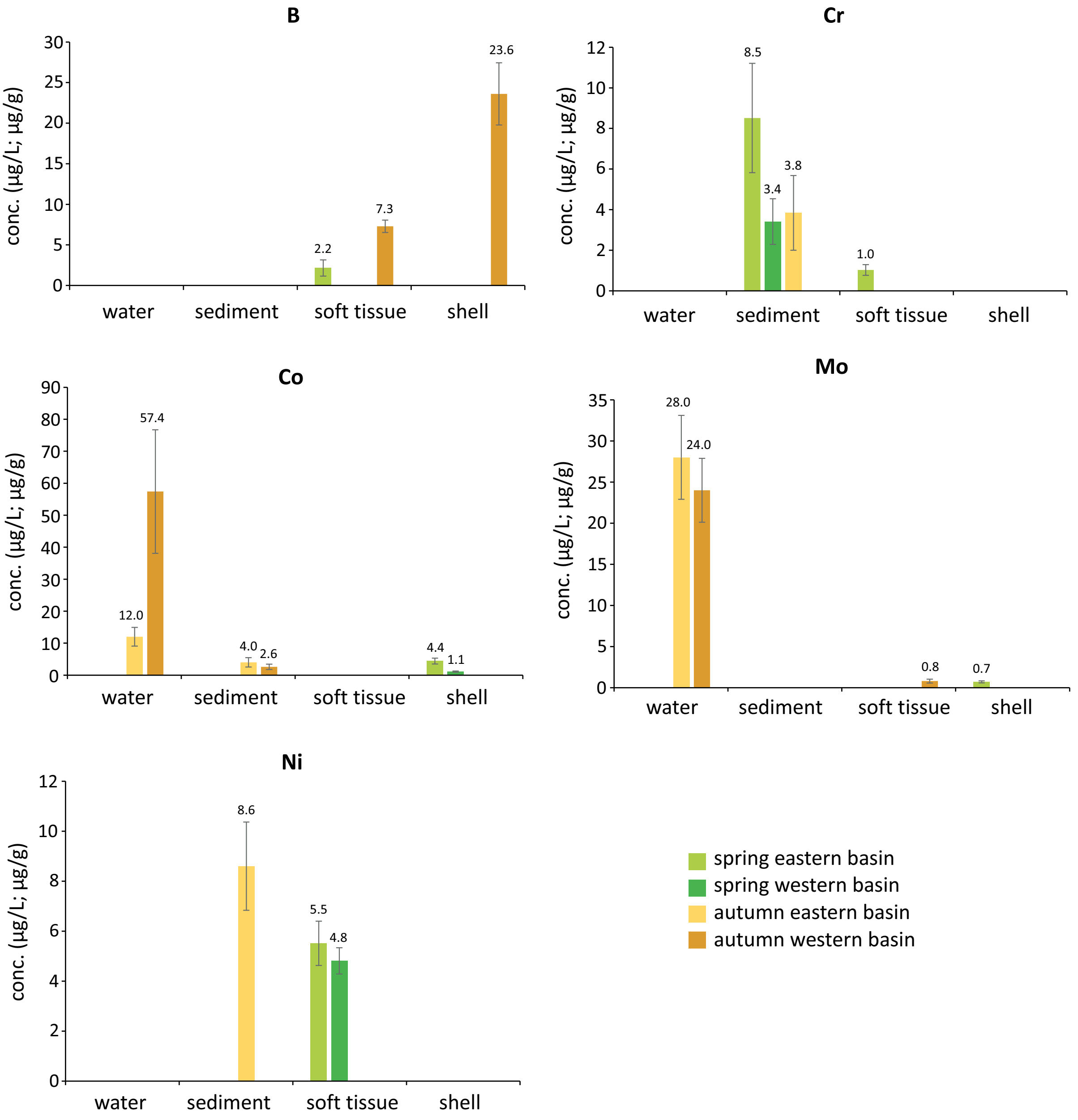

Fig. 2 Non-essential trace metals detected in particular phases (water, sediments, dreissenid soft tissue and shells) of Lake Balaton in two basins and two seasons. Metal quantity and distribution showed seasonal alterations, suggesting dynamic inter-phasic movement of metals

Distribution of abundant metals (group 3: Ba, Cu, Fe, Mn, Pb, Zn) among abiotic and biotic (dreissenid tissues) phases (Fig. 3, Online Resource T3)

Barium content in sediments was higher in the eastern basin than in the western part of the lake, as well as in spring than in autumn. In water and shell matrices, Ba was uniformly distributed with low to moderate concentration levels in both basins and seasons. Nevertheless, in the eastern basin, its concentration in spring was significantly higher than in autumn. In the soft tissue, Ba was detected in both basins only in autumn. *Copper* concentration was higher in the

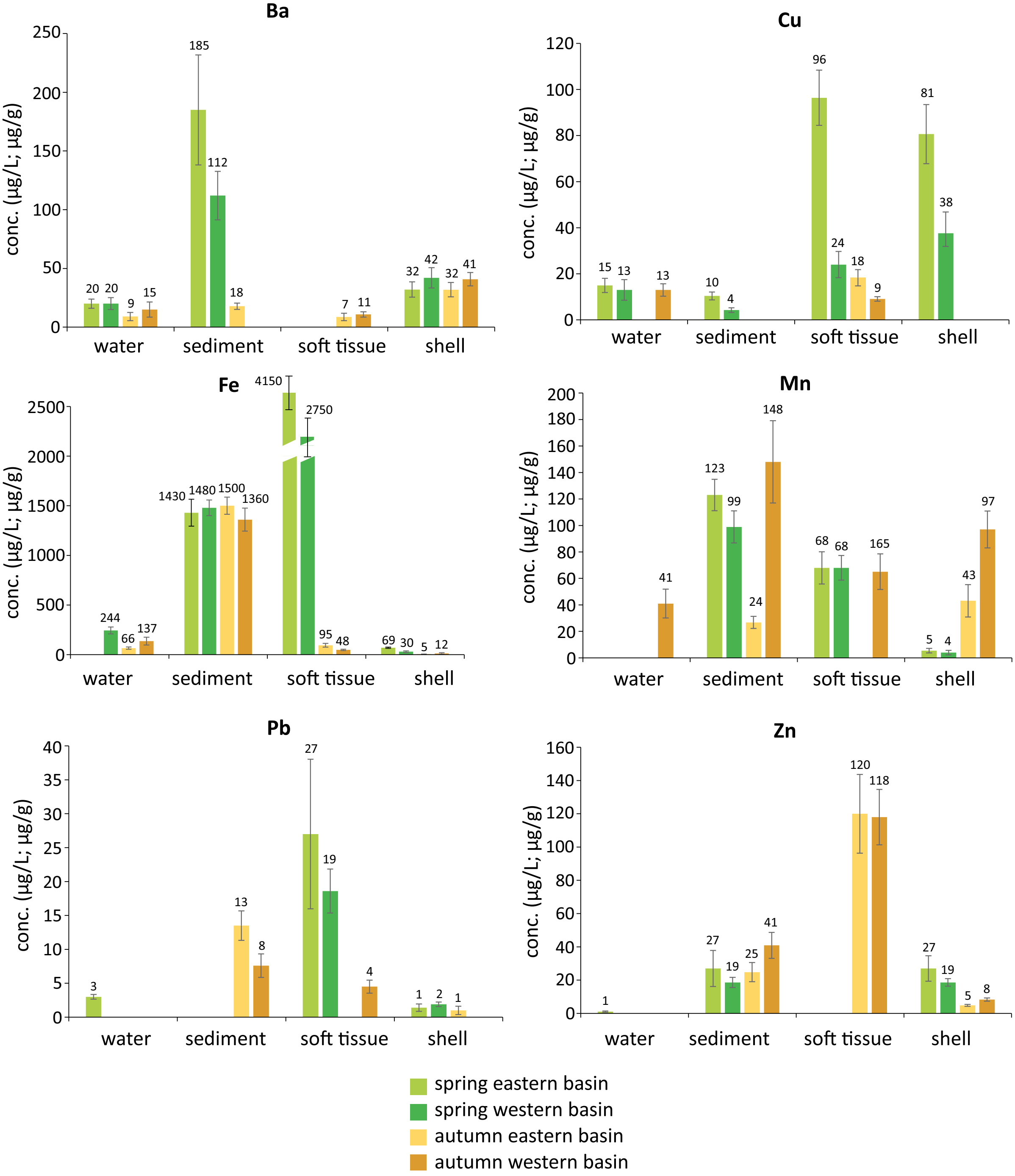

Fig. 3 Quantity and distribution of major metals in particular phases (water, sediments, dreissenid soft tissue and shells) of Lake Balaton in two basins and two seasons. Note that, except lead, major metals are essential in bivalves and other freshwater living organisms. The upper layer of the sediments (equivalent to the suspended particulate matter in the water phase), and the soft tissue are the main metal sources in the samples. In general, the seasonal distribution map shows more metals in spring, and more bioavailability in the eastern basin

eastern basin than in the western basin, as well as in spring than in autumn. It was prominent in the soft tissue, especially in spring, but was not detected at all in autumn sediment and shell samples. *Iron* was the most abundant metal investigated. It was uniformly present in the sediments regardless of the basin and season. Conversely, it was less often detected in the water phase, where it was more abundant in the western vs. eastern basin, especially in spring. A huge amount of Fe accumulated in the soft tissue of dreissenids in spring (significantly more in the eastern than the western basin), but the concentrations drastically decreased in autumn. Much less Fe accumulated in shells, with higher concentration in spring, especially in the eastern basin. *Manganese* distribution was heterogeneous among phases. The concentration was relatively high in the western basin in autumn. Comparing the two basins, Mn concentrations were similar in spring, whereas they were different from each other in autumn. The shells accumulated Mn in autumn, whereas its concentration in soft tissue was higher in spring, though only in the eastern basin. *Lead* accumulated in the sediments in autumn and in the mussel soft tissue in spring. In the eastern basin, sediment concentrations in autumn seemed slightly higher than in the western basin. The shell contained only small amounts of Pb, except the autumn samples from the western basin, from which it was totally absent from. In the water phase, Pb was only present in the spring samples from the eastern basin. *Zinc* more accumulated in the autumn sediments from the western basin than elsewhere. It was undetectable in water except very small amounts found in the eastern basin in spring. In dreissenid soft tissue, Zn was found at high concentrations in autumn in both basins, whereas it was totally absent in spring. The shells also accumulated Zn, especially in spring.

Differences in percentage metal shares among phases, basins and seasons (Fig. 4, Online Resource T4, T5)

The PCA run on percentage shares of metals in samples established three principal components (PC1-3) explaining 47, 23 and 19% of the total variability, respectively. All the components discriminated among different phases, seasons and basins, resulting in significant 3-way interactions in General Linear Models (Online Resource T4).

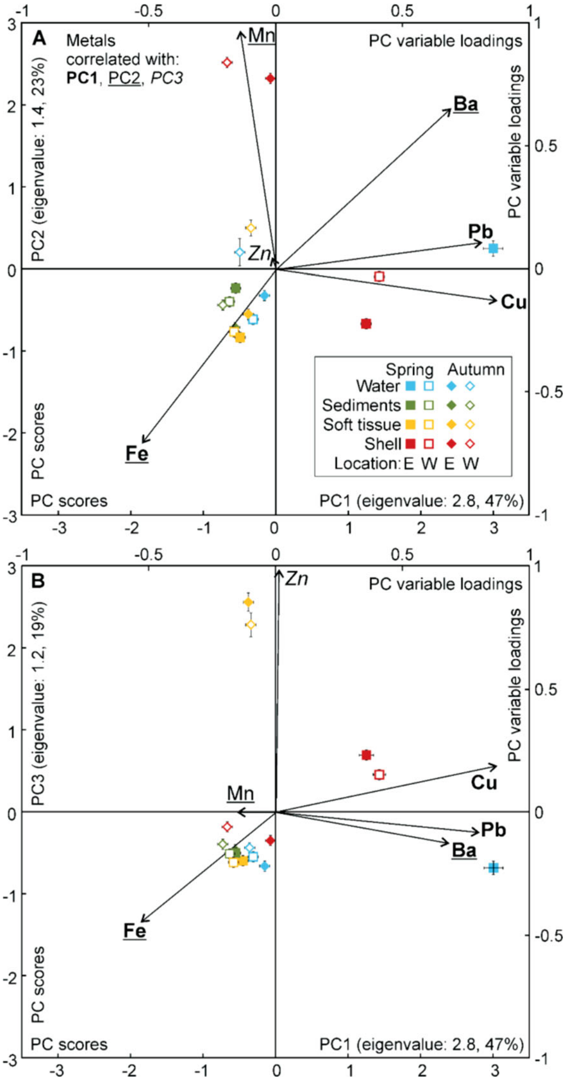

Fig. 4 The Principal Component Analysis based on percentage shares of the major metals in the four analysed phases (water, sediments, mussel soft tissue and shells). Three Principal Components (PC1-3) were established. The points show mean principal component scores (± SE) for each phase, season and basin. Solid symbols—eastern basin, open symbols—western basin; squares—spring, diamonds—autumn. Color meanings: blue—water; green—sediments; yellow—soft tissue; red—shell

PC1 was highly positively correlated with relative shares of Cu, Pb and Ba, and negatively associated with Fe (Fig. 4A). The highest PC1 scores were assigned to spring water samples from the eastern basin, and to a lower extent, to shells collected in both basins in spring. On the other hand, sediment and soft tissue samples had the lowest PC1 scores. Moreover, in autumn shells and sediments from the eastern basin had higher PC1 scores than those from the western basin, and soft tissues from the western basin had higher scores in autumn than in spring (Fig. 4A, Online Resource T5A).

PC2 was positively correlated with relative shares of Mn and Ba, and negatively related to Fe (Fig. 4A). The highest PC2 scores were assigned to shells sampled in autumn. The lowest scores were usually exhibited by soft tissue (except the western basin in autumn) and water (except the eastern basin in spring) samples. PC2 scores were higher in autumn than in spring in mussels (soft tissue and shells), as well as in water samples from the western basin. Conversely, PC2 scores for water and sediment samples from the eastern basin were higher in spring than in autumn. PC2 scores for the western basin samples were generally higher than those from the eastern basin in autumn and in shells collected in spring. In contrast, the eastern basin had higher PC2 scores than the western basin abiotic phases (water and sediments) in spring (Fig. 4A, Online Resource T5B).

PC3 was positively related to the percentage share of Zn (Fig. 4B) and had the highest scores for soft tissue samples from autumn and shells from spring. Moreover, PC3 scores of abiotic samples (water and sediments) from the western basin were higher than those from the eastern basin (Fig. 4B, Online Resource T5C).

Differences in metal concentrations among solid phases, basins and seasons (Fig. 5, Online Resource T6, T7)

The PCA run on metal concentrations in solid phases (sediments and mussels) established three principal components (PC1-3) explaining 40, 28 and 15% of the total variability, respectively. All the components discriminated among different phases, seasons and basins, resulting in significant 3-way interactions in General Linear Models for PC1 and PC2, and all significant 2-way interactions for PC3 (Online Resource T6).

PC1 was positively related to the concentrations of Fe and Pb (Fig. 5A), and had the highest scores assigned to spring soft tissue samples and autumn sediment samples. PC1 scores in mussel samples (soft tissue and shells) collected in spring were higher at the eastern basin compared to the western basin, whereas the opposite was true for shells collected in autumn (Online Resource T7A).

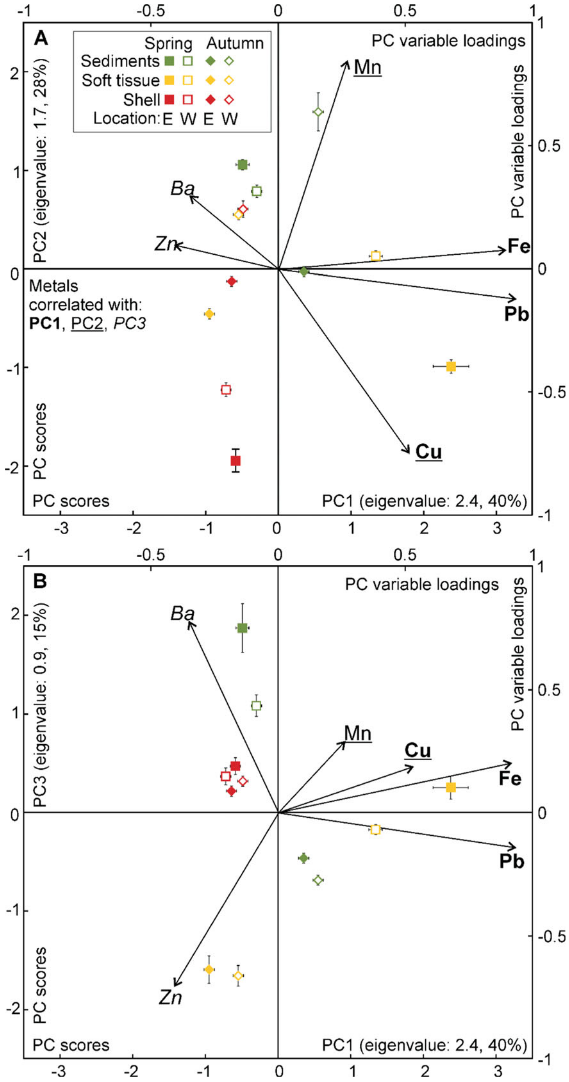

Fig. 5 The Principal Component Analysis based on concentrations of major metals in the three solid phases (sediments, mussel soft tissue and shells). Three Principal Components (PC1-3) were established. The points show mean principal component scores (± SE) for each phase, season and location. Solid symbols—eastern basin, open symbols—western basin; squares—spring, diamonds—autumn. Color meanings: blue—water; green—sediments; yellow—soft tissue; red—shell

PC2 was positively correlated with Mn concentration and negatively associated with Cu (Fig. 5A). The highest scores along this PC were evident for sediment samples, whereas the lowest PC2 scores were assigned to shells collected in spring. PC2 scores for autumn samples were generally higher than in spring except sediments from the eastern basin. Moreover, scores assigned to the western basin were higher than those from the eastern basin except spring sediments (Online Resource T7B).

PC3 was positively associated with concentration of Ba and negatively correlated with Zn (Fig. 5C). Soft tissue samples, particularly from autumn, were assigned the lowest PC3 scores in both basins and seasons. The highest scores were exhibited by sediments collected in spring and shells sampled in autumn. Spring sediments and soft tissue samples had higher PC3 scores than in autumn. Moreover, scores for sediments in spring were higher in the eastern basin than in the western basin (Online Resource T7C).

Relationships in percentage metal shares among phases (Online Resource T8)

Percentage shares of metals in soft tissue samples were positively related to those in sediments and shells with regard to PC2 scores, and negatively correlated with percentage metal contents in shells with regard to PC1 and PC3. Metal shares in shells were positively associated with those in water samples along PC1 and PC2 and negatively related to metal shares in sediments along PC2. Finally, metal shares in sediments and water were positively associated with each other with regard to PC3.

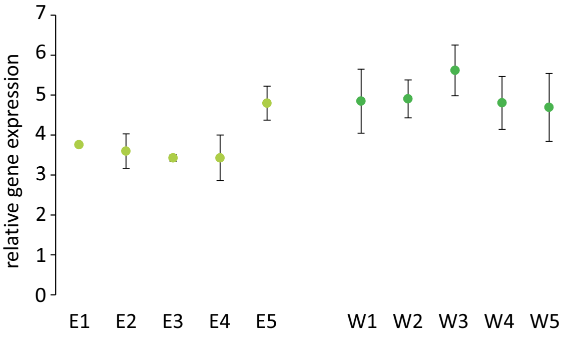

Fig. 6 Metallothionein RT-PCR in dreissenids obtained from the sampling sites (E1-5; W1-5). In the western basin, dreissenids contain more metallothioneins. E5 and W3 sampling sites showed values higher than averages for their basins, which refers to special environmental conditions. Metallothionein levels seem to coincide more feasibly with cumulative stress than with the metal content alone

Relationships between metallothionein expression and metal concentrations in the mussel body (Fig. 6, Online Resource T9)

Metallothionein expression was generally higher in the western than in the eastern basin (t-test: $t_8 = 3.76$, $P = 0.006$) by ca. 30%. At site E5, metallothionein expression was higher than the average for the eastern basin, and more similar to the values detected in the western basin. The highest level of metallothionein expression was found in animals collected from W3, at the mouth of the main inlet, the Zala River.

Metallothionein expression was negatively correlated with concentrations of metals associated with PC1 in shells, whereas it was positively correlated with PC2 scores for mussel shells and soft tissues.

Discussion

Metal concentrations in historical perspective (Online Resource T10)

Earlier studies on the lake element contents provide the possibility to make comparisons between these and the present datasets. However, because of the lack of data coherency with regard to the detection method, matrix, site, and season, these comparisons can address only trends rather than exact changes. Six out of the seventeen metals we examined (Ag, As, Cd, Hg, Se, Sn) were present below the detection limit in all samples. Among them, As, Cd and Hg were found earlier in the water and/or sediments of Lake Balaton (Müller 1981; Elbaz-Poulichet et al. 1996; Polyák and Hlavay 1999; Nguyen et al. 2005a, c), as well as in the biota, including mussel species other than dreissenids (Salánki et al. 1982; Nguyen et al. 2005a, c). As the SPM amount in shallow waterbodies is sensitive even to minor disturbances in the environment, data on the water phase are not appropriate for long time comparisons, and hence for monitoring purposes, as it can also be seen in the case of Lake Balaton (Nguyen et al. 2005c). In contrast, sediments are considered as the best phase for such comparisons because of their relative stability and potential of storing of inorganic components. Indeed, most of the data on sediment metal concentrations in Lake Balaton show a decreasing time-trend for all the metals detected. This means that, on one hand, sources of the metal load to the lake have been reduced, and, on the other hand, mineralization and chelation of metals regularly remove metals from the dissolved phase and bury them into the deeper layers of the sediment in the recent decades. The lake sediment metal concentrations remain below the average levels measured in Hungarian soils (Kádár 1995), which has already been noted in the current millennium (Hlavay and Polyák 2002; Nguyen et al. 2005a, c). Mussel soft tissues show a more heterogeneous picture of metal accumulation. Concentration of Cu and Pb is higher, those of Fe, Zn, Cr, Ni similar, and that of Mn lower in dreissenids compared to *Unio* and *Anodonta* spp. studied 20 and 40 years ago (Salánki et al. 1982; Nguyen et al. 2005c). In the view of the general attenuation of sediment metal concentration in time, differences in soft tissue datasets are due to different accumulation potential and capacity of the species rather than due to the changes in the metal load. Lukashev (2008) determined the background level of metal content in soft tissues of *Dreissena rostriformis bugensis* in the Dnieper River and reservoir, the place of origin of this subspecies. Comparing our data with that of Lukashev's, only Pb concentration is higher than the native range of dreissenid living in the Dnieper. Bivalve shells have not been investigated for their metal content in Lake Balaton yet. A comparison of the molar ranking from the current study with the data obtained for *D. polymorpha* in a French river (Immel et al. 2016), and for marine mussels (Szefer and Szefer 1990; Huanxin et al. 2000; Protasowicki et al. 2008), confirms that metal content in shells is highly influenced by environmental factors and species.

Behaviour of elements non-essential for dreissenids

Most of the detected elements are not compatible with the life of bivalves and their source is mostly anthropogenic, like in the case of heavy metals: Co, Cr, Ni, Pb, and the metalloid B. On the other hand, the alkaline earth metal, Ba, has natural geological origin (Cserny and Nagy-Bodor 2000). Although for some elements the source of pollution could be organic rich effluents in the western basin (B), or atmospheric precipitation of ashes from the nearby heating power plant (Ni, Nguyen et al. 2005a), the majority of the anthropogenic pollution comes from yacht harbours and urban run-offs due to human population overexploiting the eastern basin in the holiday season (Cr, Ni, Pb, Weisz et al. 2000). This accounts for the elevated concentration of Ni and Pb in the autumn sediment samples, particularly those taken from the eastern basin. Chromates and other chromium derivatives are applied for hull impregnation in yacht harbours, which explains the level of Cr in spring sediments after ship repairing in winter (Nguyen et al. 2005a). In spring soft tissue samples, the accumulation of Cr, Ni, and Pb suggests the increased bioavailability and uptake of these metals by dreissenids in this season. Hlavay and Polyák (2002) also noted the increased bioavailability of metals in spring. They found that low oxygen level due to the lack of phytoplankton activity in winter might lead to acidification by microorganisms at least at the sediment–water interphase, which could liberate metals from stable compounds. Metal uptake by dreissenids increases parallel with metabolic and filtration activities (Secor et al. 1993) and metals are passively absorbed in the soft tissue directly from the water (Kraak et al. 1994b). This could explain why Cr, Ni and Pb concentrations increased in the soft tissue in the beginning of the active season of dreissenids. B and Ba distributions among phases and seasons showed marked differences from distributions of Cr, Ni, and Pb. They both have physiological roles in phytoplankton (Brown et al. 2002; Gillikin et al. 2006), which influences their appearance seasonally in the biota, including the algal consuming dreissenids. It is possible that the spring bloom of benthic diatoms living on the sediment surface and accumulating Ba from the water column, or collapsed phytoplankton sinking to the bottom in winter contribute to high Ba concentrations in the spring sediment. Detection of Ba even in low concentrations in the spring soft tissue samples might be the consequence of the co-uptake with Ca during the active season (Fritz et al. 1990), However, the high Ca content in the lake does not favour Ba substitution and incorporation to the shell (Markich and Jeffree 1994), as it is indicated by the constant level of Ba in the shell regardless of the season. Boron is an essential metalloid in diatoms and cyanobacteria (Brown et al. 2002), hence its high abundance in dreissenids after summer in the western basin is expected due to anoxic conditions at the sediment–water interface, driving phosphorus-dependent cyanobacterial blooms in long warm periods (Istvánovics 2010). The accumulation of Ni and Pb in the sediment and soft tissue phases across seasons showed an opposite tendency than that of Ba, which can be explained by their different origin (Ni, Pb are anthropogenic, whereas Ba is geological, Cserny and Nagy-Bodor 2000) and carriers (Ni, and Pb as free ions, whereas Ba in phytoplankton) through which dreissenids uptake these elements.

Behaviour of metals essential for dreissenids

Major metals: Cu, Fe, Mn, and Zn, as well as some minors (Co [Watanabe and Bito 2018], Mo [Mendel 2005]) integrate into organic complexes and build in enzymes in living organisms. Although they are essential only in small amounts in a single organism, their accumulation by the entire population on the ecosystem scale can be measureable, and influence element distribution among the examined phases. Those found in all phases and with amounts exceeding 100 µg/g in particular phases are ground materials, associated with the aluminiosilicate (Fe, Mn) or carbonate fraction (Mn, Zn) of the sediment transported by the inlets (Elbaz-Poulichet et al. 1996; Hlavay and Polyák 1998). Others, such as Cu, Co, and Mo, having anthropogenic origin, leach from the watershed areas either through inlets (Co, Nguyen et al. 2005a), or directly from the runoff rainwater (Cu, Hlavay and Polyák 2002). Co and Mo could be more frequent in autumn water samples, indicating their association with enhanced tourist activity, and/or phytoplankton productivity in summer. Both possible explanations have been confirmed in earlier studies (Elbaz-Poulichet et al. 1997; Tabouret et al. 2012). However, in our samples, the concentrations of these elements in dreissenids were low, possibly due to small saturation rate in and intense depuration from the soft tissue (Belivermis et al. 2016). The main source of Cu in the lake are the vineyard fields extending around the lake where Cu-based fungicides are brought to the soil from decomposing leaves and washed out between autumn and spring (Hlavay and Polyák 2002), increasing the Cu concentration in all examined phases in spring samples. Notably, dissolved Cu was the only element in the study exceeding the limit of the Environmental Quality Standard from the European Water Framework Directive (1.4 µg/L for waters with $CaCO_3 > 200$ mg/L).

The accumulation of major metals (all belonging to the ground minerals except Cu) in dreissenids depends on their bioavailability and uptake frequency. Iron and Zn concentrations seem constant in the water column and sediments, meaning that their annual net amount did not change during the survey. Their low concentrations in water could be explained by the fact that they can be hardly mobilized from minerals (Hlavay and Polyák 1998). In contrast, Mn lability, changing sensitively with temperature (Ullmann et al. 2013), redox conditions (Zhao et al. 2017), and phytoplankton activity (Langlet et al. 2007), could lead to its diverse appearance in water and sediments. High Mn concentrations in all phases in the western basin in autumn confirm this, as periods of low oxygen concentration followed by acidification at the sediment–water interface often happen in this area (Istvánovics 2010), mobilizing Mn and making it bioavailable.

Similar to Cr, Ni, and Pb, concentrations of Fe, Cu and Mn were significantly increased in the soft tissue in spring. This could be partly due to the mussel need for essential elements at the beginning of the active season [Fe-ferritin for shell secretion (Zhang et al. 2003), Cu-hemocyanin as oxygen carrier, Mn as a part of anti-oxidative systems]. However, only a small portion of metals are actually used for physiological purposes (Markich and Jeffree 1994), most of them are stored in extracellular granules (Markisch et al. 2001). In spring, more drivers of elemental uptake should coincide simultaneously, including the bioavailability, shell secretion (Ca influx), feeding rate, phytoplankton productivity (Ravera et al. 2007), organic matter content in the SPM (Magalhães et al. 2015). Organic matter forms chelates with metals, reducing bioavailability, which might contribute to the lower levels of Cu and Fe in dreissenids living in the organic-rich western basin. Zn was the only metal which significantly accumulated in the soft tissue exclusively in autumn samples. Dreissenids accumulate zinc in the soft tissues regardless of the variation in the ambient environmental concentration (Karouna-Renier and Sparling 2001; Immel et al. 2016), and this was also described for other bivalve species (Huanxin et al. 2000), and for zooplankton in Lake Balaton (Nguyen et al. 2005a). Therefore, Zn concentration in the soft tissue seems to be associated with some physiological/metabolic changes in dormancy.

While the composition of bivalve soft tissue reflects recent condition changes, shell is considered as a lifespan integrator of metals (Ravera et al. 2007), even it is as low as 2–3 years for dreissenids in Lake

Balaton (Balogh 2008). Hence, the shell metal concentration is expected to be season independent. In contrast, in the present study, most of the metals showed significant seasonal differences in their shell concentration, suggesting that the incorporation and depuration dynamic in shells is frequent even though it did not reach the amount observed in the soft tissue. If shell secretion, which brings Ca substituting metals from the soft tissue to the shell matrix, would be the dominant form of metal sequestration by the shell, then the concentration of metals in the shell would have been higher in autumn than in spring. However, this is only the case for B and Mn in our study. In addition, we found that Ba, which content changes parallel with Ca in the shell (Fritz et al. 1990; Vander Putten et al. 2000; Geeza et al. 2019) did not change with season. Therefore, it is suggested that in general, the seasonal fluctuation of the shell metal concentration is rather due to the direct interaction of the shell with the water phase than to the different secretion activity. This is also supported by the phase interaction analysis showing the positive correlation between the water and shell phases for PC1 and PC2 metals. Periostracum, the outermost proteinaceous layer of the shell, is an active metal absorbent where both indigenous metal excretion and deposition from the environment occur (Dermott and Lum 1986; Bolotov et al. 2015). Intensive cleaning of the animals before processing and the lack of correlation between the sediment and shell, particularly for the ground constituents Fe and Mn (similar to Al as the background shell contaminant reference, Bolotov et al. 2015), suggest that metals associated with the shell do not belong to the SPM that may cover the surface. Although the mechanism of reversible shell metal binding is uncertain, but according to our results and those of other studies (Gundacker 1999; Wilson et al. 2018), particular metal concentrations in shells may be indicative for actual environmental happenings, like Cu and Mn liberation, as it is suggested from the present results.

Metal interrelation, partitioning and redistribution among phases

Percentage-based all-phase analysis

PCA analysis and GLM statistics were performed on the percentage of shares of metals for comparison of metal distribution in fluid and solid phases. Variance in Fe shares had the strongest effect on differences among phases, basins and sampling dates due to much higher concentration of this metal than other metals. Thus, Fe presence might have masked fine differences related to the other metals. Nevertheless, Cu, Pb, and partly Ba distribution (PC1) were similar among phases. In spring, the more urbanized areas around the eastern basin could be responsible for the higher scores for these metals in water samples due to run-off from hard surfaces. The higher PC1 scores of the samples found in the sediment and shell phases from the eastern basin in autumn could be due to a similar reason and the delayed metal translocation to the solid phases. As shells do, but sediment and soft tissue do not, show high PC1 scores for spring samples in both basins, it is suggested that these metals could be directly and promptly adsorbed onto the surface of dreissenid shells in spring. It also seems that in the western basin, the spring peaks of Cu and Pb shares in the shell are followed by the corresponding peaks in the soft tissue in autumn after a seasonal delay. Mn and partly Ba formed a second group (related to PC2), which was coherent in their distribution, however to a lesser degree than PC1 metals. In autumn, these metals were strongly associated with the shell, and were relatively more common in the western than eastern basin in all the phases, which might be explained by the intensive phytoplankton production in the western basin in summer and autumn. Supporting this view, Mn is known to accumulate in intensively photosynthesising algae (Sunda and Huntsman 1985), and both metals can accumulate in the SPM and upper sediments due to precipitation by algae onto their extracellular surface, which helps to form assemblages in decaying and sinking cells (Bishop 1988; Richardson et al. 1988). The notable appearance of these metals in the water and sediment from the eastern basin in spring might be due to similar reasons and associated with the diatom bloom which characterizes the lake phytoplankton activity in early spring, as was suggested by others (see Vander Putten et al. 2000; Zhao et al. 2017). The relative share of Fe was negatively correlated with both PC1 and PC2. This means that, in contrast to Cu, Pb, and Ba, Fe neither increases in water in the eastern basin in spring, nor in the sediment and shell in the western basin in autumn (which Mn and Ba do), but rather is accumulated in the soft tissue in spring independently of environmental circumstances. These findings support the known stability and high abundance of Fe in the environment (Hlavay and Polyák 1998) and its physiological role in bivalve shell production (Zhang et al. 2003). In the percentage-based all-phase analysis, Zn was found to behave in an opposite manner compared to other metals (PC3): it concentrated in the soft tissue in autumn and in the shell in spring, and was found more commonly in the water and sediment in the western basin. This suggests that Zn exists in specific complexes in the SPM, transported by a unique mechanism, and has a definite role in dreissenids, which makes the regulation of Zn level independent of the environment, as was described earlier (Marie et al. 2006).

Solid phase analysis

The advantage of the solid phase comparison (excluding the water samples) over the percentage-based analysis is that metal concentrations are expressed as absolute values, rather than as a ratio relative to the total metal contents. Although in this case the water phase containing solubilized metals that can be assimilated by the organisms had to be removed from the analysis (due to different measurement units), the SPM, which is filtered out by dreissenids from the water column, remained, as the upper sediment contains large amounts of SPM in windless conditions. Thus, it is suspected that metals associated with the SPM are the integrative part of the analysis, whereas metals associated to planktonic algae and ionic forms dissolved in water are not. Therefore, the differences between the results obtained from the two analyses might also reflect the role of phytoplankton in metal distribution. Solid phase metal concentrations showed that the strongest inter-metal correlation occurred between Fe, Pb, and, to a lesser extent, Cu (PC1 in Fig. 5). Similar findings were recorded in studies on metal partitioning between mussels and marine environments (Koide et al. 1982; Szefer and Szefer 1990). In the current study, the highest scores for PC1 were found in spring soft tissue samples which indicates that these metals bioconcentrate in dreissenids at the beginning of the active season. Intensive absorption of essential metals, such as Fe and Cu, and substitution of ligands in cationic transports by non-essentials, such as Pb, and possibly also Cr and Ni (not verified statistically in the present study) by dreissenids might explain the observed distribution of these metals. The higher external load and/or bioavailability of metals could explain the higher scores for the soft tissue obtained from the eastern basin. By autumn, as the dreissenid dormanence period starts, Fe, Pb, and Cu were eliminated from the soft tissue and, concomitantly, the sediment and shell scores increased, suggesting a dynamic interchange of these metals between the living and non-living solid phases in the benthic zone. In the solid phase analysis, Mn distribution in space and time did not show substantial differences from that found in the all-phase analysis, indicating that free Mn dissolved in water had no substantial role in the Mn cycle, which is highly connected to phytoplankton production and sedimentation. The opposite behaviour of Cu can be due to other mechanisms: (i) increased flow of run-off water from herbicide-treated vineyards, and/or (ii) biochemical need for Cu exhibited by active dreissenids in spring. Among the examined solid phases, Ba represents the metal whose abundance in spring was prominent in the sediment, but negligible in the soft tissue. In contrast, Zn seemed to be negatively correlated with Ba. According to the discussion on the environmental behaviour of each metal (*see* Sect. 4.3), the opposite effects between Zn and Ba can be explained by different mechanisms in their cycling rather than antagonistic behaviour.

Multiple regression analysis on metal shares

Mn and Ba content in the dreissenid soft tissues correlated with that of the sediment and shell content, suggesting that the uptake of these elements occurs by filtering the solid SPM, and the soft tissue may excrete an excess amount of Mn and Ba to the shell. On the other hand, metal absorption on the shell surface is also a possible phenomenon, as contents of Mn and Ba in water were correlated with that of the shell. The inverse relationship between Mn and Ba content in the shell with that in the sediment suggests a mutual exchange of these elements between the two phases. Alternatively, the benthic diatom activity forming Ba precipitates and Mn-rich dead phytoplankton (Vander Putten et al. 2000; Geeza et al. 2019), which sinks down and increase sediment Ba/Mn concentration, might negatively affect the shell Ba/Mn absorption (possibly in the organic periostracum). Similar to Mn and Ba, the content of Cu, Pb and Zn in water coincided with that in the shell, however, unlike the positive relationship of Mn and Ba between soft tissues and shell, Cu, Pb and Zn behaved in an opposite manner. Among these metals, only changes in Zn concentration were parallel in abiotic phases, suggesting that Zn exhibits a dynamic balance between water and sediments. It is thought that metals such as Ba, Cu, Mn, and Pb adhere directly from the water to the shell external surface. Among these metals, Ba and Mn can also be extracted from the sediment and transported into the shell internal matrix along Ca through mantle secretion. In addition, a negative correlation between the shell and soft tissue phases in the case of Cu, Pb and Zn, indicates a possible interchange of metals between the non-living shell matrix and the living parts of an organism. In the case of Cu and Zn, their usefulness in biochemical processes may explain their rescue, whereas for Pb, a possible substitution of some bivalent cations between these two biotic phases is postulated.

Dreissenid metal content and metallothionein synthesis

At first glance, a slightly but definitively higher metallothionein expression in the western compared to the eastern basin would indicate a higher metal load and amount in dreissenid soft tissues in the western basin. However, this was not confirmed by the results, which showed that in general, metal concentrations were higher in the eastern basin. Metallothioneins play an important role in the homeostasis of essential metals and detoxification of excess amounts of essential and non-essential metals in aquatic invertebrates, but their use as a toxicity biomarker is limited (Le et al. 2016) because of their competition with other metal sequestration cellular systems (Amiard et al. 2006), as well as their activation as free radical scavengers and as a part of a general immune response (Marie et al. 2006). Moreover, metallothionein expression was found to be influenced by the season and size of dreissenids in Lake Balaton (Ács et al. 2016). Therefore, metallothionein expression in dreissenids indicates a more complex condition, in which the metal concentration is only one of the determinants, and may not be the most deterministic one. The picture of the metallothionein expression resembles the map of trophic conditions caused by increased organic matter production in the western basin. Obviously, the role of inlets in this process is pivotal by supporting the lake phytoplankton with nutrients. This can explain the enhanced level of metallothionein expression at the E5 site in the eastern basin, and at W3 in the western basin. Nevertheless, the present study showed a positive correlation between metallothionein expression and PC2 metal concentrations (Mn, Ba) in dreissenid soft tissues and shells. Although relationships between these parameters were not presented, it is hypothesized that, because both Mn and Ba in bivalves reflect phytoplankton activity (Vander Putten et al. 2000; Geeza et al. 2019), metallothionein synthesis might be connected with primary production and feeding. In contrast, concentrations of PC1 metals (heavy metals: Pb, Cu, Fe) in shells were negatively correlated with metallothionein expression. As PC1 metals had high scores in dreissenids (both soft tissues and shells) in spring, it can be hypothesized that the deposition of metals from the soft tissue to the shell can decrease the demand for metallothioneins, and hence synthesis may be reduced.

Conclusion

In a large shallow lake, metal redistribution among abiotic and biotic phases was prominent seasonally and depended on sampling location. Dreissenids, which are the key primary consumers in the littoral zone, filter SPM from the water, including resuspended particles from the upper layer of the sediments, and absorb metals into their body parts. As the external load of contaminants has been reduced over past decades, the internal element content, including the geochemical background, mostly accounts for metal partitioning cycles in the lake. It seems that dreissenids can intra-annually uptake and depurate metals through their interaction with the environment. This exchange takes part in a regulated way in the case of essential metals and passively in the case of non-essential ones. It is also suggested that the shell plays a double role in metal cycles as it can absorb metals in the biofilm layer covering its outer surface, and balance the soft tissue metal homeostasis on its inner surface. Despite the fact that the alkalic and oxic nature keeps metals in their stable chemical forms in abiotic phases, they can be dissolved and become bioavailable for dreissenids on the sediment surface, possibly due to microbial activity. Comparison of the basin, season, and phase correlations in metal distribution with and without the water (fluid) phase included, as well as the differences found between the western (high trophic status) and the eastern (low trophic status) basins demonstrate that phytoplankton activity strongly influences the metal distribution, particularly for Mn, Ba, and Zn. Even though metals can be grouped by their similar behaviour during interphase exchanges, each metal has a unique quality and quantity distribution pattern, reflecting specific associations and, in the case of essential metals, function in the biota. After continuous reduction of contaminant sources in the catchment area, the current metal content in Lake Balaton showed a slight reduction compared to previous data reported in the literature, and it is still sustained at a safe and non-toxic level.

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Data availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

Code availability All figures were prepared by using the MSOffice Excel and CorelDraw software applications.

Declarations

Conflict of interest The authors have no financial or proprietary interests in any material discussed in this article.

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Informed consent On the behalf of all authors, the corresponding author state that researchers participate in the study with their own free will.

Consent for publication On the behalf of all authors, the corresponding author state that researchers have seen and approved the submitted MS to be published.

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