NORTH-WESTERN JOURNAL OF ZOOLOGY 13 (1): 63-69
Article No.: e161507

http://biozoojournals.ro/nwjz/index.html

# Impact of anthropogenic activities on the concentration of trace elements in toe bones of the common toad (*Bufo bufo*)

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Received: 05. October 2015 / Accepted: 23. May 2016 / Available online: 27. July 2016 / Printed: June 2017

**Abstract.** Frogs and toads are frequently used as bioindicators of inorganic pollutants. Anthropogenic stress was assessed in three lakes from Hungary (Naplás, Frog Pond and Lak-völgyi Reservoir), by studying the concentration of trace elements in the toe bones of *Bufo bufo*. Lake Naplás was the most affected by anthropogenic effects, but Frog Pond was also characterized by higher anthropogenic activities than the Lak-völgyi Reservoir. The following trace elements were analysed in toe bones, water and soil samples: Al, Ba, Ca, Cu, Fe, Mg, Mn, P, Pb, V and Zn. In toe bones no significant differences were found in the Ca, P and Zn concentrations among the studied lakes. On the other hand, the Mg, Al, Fe, Ba, Pb and V concentrations were significantly higher in toe bones at Lake Naplás than at the two other lakes. The use of the bioaccumulation factor (BAF) showed that the potential of bioaccumulation from the soil was low for toads. For water, the BAFs values for Cu, Mn, V and Zn indicated that toads may accumulate these elements from water. Our results demonstrated that the accumulation rate of metals depends on several factors, including dietary and exposure time, stage of development – i.e. tadpole or adult. In summary, the analysis of toe bones demonstrated that toads may be useful indicator organisms; moreover, using modern analytical methods there was no need to kill these animals for bioaccumulation studies.

**Key words:** amphibians, bioindicators, bioaccumulation factor, spectrometry, ICP-OES, ICP-QMS.

## Introduction

Natural and artificial ponds are important aquatic habitats but the management and conservation of freshwater resources has rather been focused on running water and larger water bodies (Oertli et al. 2009). Due to their great abundance and importance for biodiversity, small ponds are equally significant from a socio-economic as well as from a conservation biology viewpoint (Oertli et al. 2009). Direct habitat degradation, large scale environmental changes and the contamination of these ponds are all major causes of amphibian population decline, as many species reproduce in small ponds, which have different abiotic and biotic conditions (Lind et al. 1996, Laurila & Aho 1997).

Amphibians are main components of both aquatic and terrestrial ecosystems (Unrine et al. 2007). This animal group, particularly toads and frogs, may be used as bioindicator to assess the quality of terrestrial and aquatic habitats, their use in this sense being different according to the various stages of their life cycles (Alford & Richards 1990, Simon et al. 2010, 2012, Qureshi et al. 2015). During the embryonic and larval period, amphibians may best indicate the contamination in water and in sediment (Rowe et al. 2001). As adults, they are directly exposed to the contamination of soil, water and air through their permeable skin (Blaustein et al. 2003) and they may accumulate trace elements rapidly from terrestrial habitats (Rowe et al. 2001).

*Bufo bufo* was chosen to be studied because of its stable conservation status and widespread distribution both in Europe and Hungary (Puky et al. 2005). This species occasionally breeds even in small urban ponds (Hitchings & Beebee 1998). The aim of our study was to assess if the concentration of trace elements of *Bufo bufo* toe bones reflects the environmental quality of different localities and local anthropogenic effects. The concentration of trace elements of toe bones was analysed at three lakes with different geochemical background and anthropic use. In different stages of their life cycles toads are exposed to different stressors from their habitat; thus, the local air quality and soil element

concentration background were also used to assess the results. In our study we used live toads and have therefore evaluated the usefulness of *Bufo bufo* as bioindicator of habitat quality without killing the test animals. Finally, using the bioaccumulation factor (BAF) we also tested from which substrate (water or soil) do the trace elements accumulate more in toads.

**Materials and methods**

Location of study sites and samples collection

Adult *Bufo bufo* specimens were collected during their reproductive period in March 2008 from three localities. The first lake was Lake Naplás (47°30'N, 19°14'E), which was most affected by environmental load, situated in the eastern part of Budapest, the Hungarian capital, with a busy road along its southern edge. The second lake was Békás Pond (47°33'N, 21°37'E), a typical urban pond with considerable traffic around and other anthropogenic effects (thermal bath and pharmaceutical factory outflow) in Debrecen. The third lake was the Lak-völgyi Reservoir, which is situated in the Bükk Mountains (48°03'N, 20°20'E). The habitat characterization of the lakes was based on the Geochemical Atlas of Europe (Salimen et al. 2006). At two lakes (Lake Naplás: N = 14, Lak-völgyi Reservoir: N = 6) toe bone digits were collected from fresh road-killed specimens on land, at Frog Pond (N = 24), adult toads were caught by hand net from water. From each individual one digit was collected from the right back limbs. The sampling period was two days in the cases of each lake during spring, 2008. Toe clipping was made with stainless steel scissors following Green's (2001) recommendations. At each studied lake soil and water samples were also collected.

Laboratory analysis

The clipped toes samples were stored in a freezer in plastic Eppendorf tubes until processing. Toe bones were flushed with 100mL of doubled-distilled water, then cleaned from conjunctive tissue with 2mL 30% (m/m) hydrogen peroxide. After this procedure, the samples were flushed with deionised water again and dried overnight at 105°C. The dry weight of each digit was measured with a SARTORIUS LE 26P micro analytical balance. The average weight of samples was 0.47 ± 0.15 mg (Lake Naplás), 0.87 ± 0.34 mg (Frog Pond) and 0.87 ± 0.33 mg (Lak-völgyi Reservoir) (mean ± SD). The dried samples were digested with 2 mL 65% nitric acid at 80 °C for 4 hours. The sample digestion procedure was under open system. Digested samples were diluted to 20mL using 1 % nitric acid (m/m).

For all three lakes, 50-100 g soil was collected from two different localities. From each locality 10 sub-samples were collected and pooled. Samples were dried at room temperature. After drying, stones, plant roots and residues were removed with plastic tweezers. Samples were sieved through a 2 mm plastic sieve then were homogenised with agate mortar and stored in plastic tubes until pre-treatment. For elemental analysis soil sample weighting 0.2 g was digested using 4.5 ml 65% (m/m) nitric acid and 0.5 ml 30% (m/m) hydrogen peroxide in a microwave digestion unit (Milestone 1200 Mega) for 5 min at 300W and 5 min at 600W. Digested samples were diluted to 25 ml with deionised water. The applied digestion method gave a clean colourless solution.

From all study lakes surface water samples were collected in 1.0 L plastic bottles; bottles were rinsed out with deionised water three times before sampling. From each study lake two pooled water samples were collected from two localities in the centre of the lakes. From each locality 10 sub-samples were collected and pooled. Samples were acidified with 65% (w/w) nitric acid. Samples were filtered with Pall 0.45 µm filter paper. Deionised water was used as blank sample for elemental analysis.

Ca, Mg and P concentration determination was performed by inductively coupled plasma optical emission spectrometry (ICP-OES) IRIS Intrepid II XSP. Certified reference material (NCS ZC 81001 pork muscle approved by China National Analysis Centre) was used. During measurements we found less than 1% difference from the certified reference material. A six-point calibration procedure (0.001, 0.005, 0.01, 0.05 and 1.0 mg $L^{-1}$) was used with multi-elemental calibration solutions (Merck ICP multielement standard solution IV). A X7 type (Thermo Elemental, Winsford, UK) (nowadays XSeries[1] Thermo Fisher Scientific, Germany) inductively coupled plasma quadrupole mass spectrometer (ICP-QMS) was used to detect the concentration of other elements. The instrument was operated with a Peltier cooled impact bead spray chamber, single piece quartz torch (1.5 mm i.d. injector) and a conventional glass concentric nebulizer. The following isotopes were measured during the research: $^{27}$Al (LOD: 0.090 mg/L), $^{51}$V (LOD: 0.4 µg/L), $^{55}$Mn (LOD: 0.005 mg/L), $^{56}$Fe (LOD: 0.020 mg/L), $^{65}$Cu (LOD: 0.002 mg/L), $^{66}$Zn (LOD: 0.005 mg/L), $^{137}$Ba (LOD: 0.4 µg/L) and $^{208}$Pb (LOD: 0.1 µg/L). As internal standards, $^{72}$Ge, $^{103}$Rh and $^{209}$Bi isotopes were applied in the interpolation. The limits of detection were calculated from 3*, the standard deviation of the blank. The concentration of $^{52}$Cr (<0.4 mg/L), $^{59}$Co (<0.003 mg/L), $^{60}$Ni (<0.0003 mg/L), $^{75}$As (<0.007 mg/L), $^{80}$Se (<0.2 µg/L), $^{95}$Mo (<0.2 mg/L), $^{111}$Cd (<0.001 mg/L), $^{139}$La (<0.1 µg/L) and $^{140}$Ce (<0.1 µg/L) was below detection limit. An eight-point calibration curve (curve fitting was 2$^{nd}$ order generally, sometimes linear) was used with multi-elemental calibration solutions, prepared from Scharlau Chemie and Merck 1000mg/L standard solutions.

Bioaccumulation factor

Aquatic and terrestrial organisms may accumulate chemical compounds from their environment directly or indirectly (Ivanciuc et al. 2006; Van Gestel et al. 2011). The bioaccumulation is characterized with a bioaccumulation factor (BAF) defined as a ratio of concentration of chemical compounds in the whole body or specific tissue of an organism and the concentration in the surrounding environment (Ivanciuc et al. 2006; Stolyar et al. 2008):

$BAF = C_{org} / C_m$

where $C_{org}$ means the concentration of chemical compounds in the whole body or tissue of organism based on dry weight and $C_m$ is the concentration of the chemical compounds in the surrounding environment. The weight of an organism can be expressed based on a wet weight (WW), dry weight (DW), or lipid weight (LW) (Gobas & Morrison 2000). In our study the organism's weight was expressed as mg $kg^{-1}$ dry weight, the concentration of soil as mg $kg^{-1}$ dry weight, and the concentration of water as $\mu gL^{-1}$ (Gobas & Morrison 2000). Because of their life cycle, toads contacted both aquatic and terrestrial environments (Rowe et al. 2001); thus, BAFs values were calculated both for the aquatic and the terrestrial environment, respectively.

Statistical analysis

SPSS Statistics for Windows, Version 17.0 software package was used for the statistical analysis. The effects of the lakes on the element contents of toe bones were evaluated by canonical discriminant analysis (CDA) and by ANOVA. The canonical discriminant analysis is a multivariate technique for classifying a set of observations into classes. We realised the classification based on the trace element concentration in toe bones. The normality of distribution was tested with the Shapiro-Wilk test and the homogeneity of variances was tested with Levene's test for assumptions of ANOVA. Significance level was $\alpha$=0.05. In case of significant differences in ANOVA, the Tukey Multiple Comparison test was used to determine among which lakes the differences occurred (Zar 1996).

## Results

Concentration of trace elements in toe bones

Based on the concentration of trace elements in toe bones, the canonical discriminant analysis showed marginally significant differences in the first and second discriminant functions, respectively ($P$ < 0.001). In the first function, the percentage of variance was 68.2, while in the second 31.8. Canonical correlation was 0.888 in the first and 0.797 in the second discriminant function. The results of CDA showed that the lakes separated well from each other (Fig. 1). There were positive correlations between the first discriminant function and all measured elements, except Mn (Ba: r = 0.549, Mg: r = 0.352, Al: r = 0.342, Pb: r = 0.319, V: r = 0.300, Fe: r = 0.289, Cu: r = 0.211, Ca: r = 0.151, Zn: r = 0.129, P: r = 0.053), indicating significantly higher trace element concentrations in *Bufo bufo* toe bones originating from Lake Naplás, the lake under the greatest anthropic pressure. However, Mn (r = -0.402) was negatively correlated with the second function, indicating higher Mn concentration in toe bones from the Lak-völgyi Reservoir than in those from the Frog Pond.

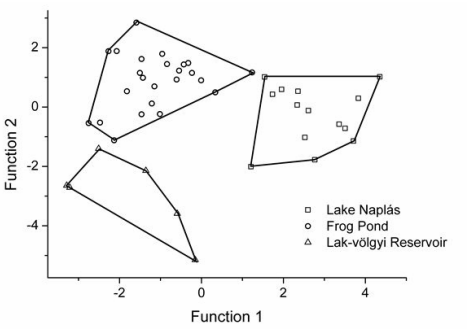


**Figure 1.** Canonical discriminant analysis of lakes based on the elemental concentrations of *B. bufo* toe bones (µg $g^{-1}$).

Significant differences were found in the trace element concentrations of *Bufo bufo* toe bones between all lakes in the cases of all trace elements studied, except Ca, P and Zn (Ca: F = 2.552, $P$ = 0.090; P: F = 0.247, $P$ = 0.782; Zn: F = 1.345, $P$ = 0.272) (Table 1). Mg, Al, Fe, Ba and Pb were present in significantly higher concentrations in *Bufo bufo* toe bones from Lake Naplás, than at Frog Pond or Lak-völgyi Reservoir (Mg: F = 10.311, $P$ < 0.001; Al: F = 9.378, $P$ < 0.001; Fe: F = 6.774, $P$ < 0.01; Ba: F = 24.108, $P$ < 0.001; Pb: F = 8.454, $P$ < 0.01). Mn concentration was significantly higher in *Bufo bufo* toe bones from Lake Naplás than in those from the Frog Pond ($P$ < 0.001), but the difference was not significant between Lake Naplás and Lak-völgyi Reservoir ($P$ = 0.753). Cu concentration was the lowest in *Bufo bufo* toe bones from the Lak-völgyi reservoir but this concentration did not differ significantly from those

**Table 1.** Elemental concentrations in *Bufo bufo* toe bones (mean ± standard error) in lakes. Different superscript letters indicate significant differences among lakes ($P$ > 0.05).

| Elements | Lake Naplás (N = 14) | Frog Pond (N = 24) | Lak-vögyi Reservoir (N = 6) |
|---|---|---|---|
| Al (µg $g^{-1}$) | 2787 ± 234$^a$ | 1849 ± 152$^b$ | 1636 ± 240$^b$ |
| Ba (µg $g^{-1}$) | 214 ± 14$^a$ | 114 ± 9$^b$ | 97 ± 9$^b$ |
| Ca (mg $g^{-1}$) | 252 ± 10 | 226 ± 12 | 238 ± 5 |
| Cu (µg $g^{-1}$) | 108 ± 41$^a$ | 57 ± 12$^{ab}$ | 32 ± 5$^b$ |
| Fe (µg $g^{-1}$) | 3199 ± 2041$^a$ | 2041 ± 195$^b$ | 1944 ± 304$^b$ |
| Mg (mg $g^{-1}$) | 9.3 ± 0.6$^a$ | 6.3 ± 0.5$^b$ | 6.2 ± 0.6$^b$ |
| Mn (µg $g^{-1}$) | 229 ± 23$^a$ | 119 ± 10$^b$ | 205 ± 38$^a$ |
| P (mg $g^{-1}$) | 325 ± 19 | 235 ± 25 | 249 ± 22 |
| Pb (µg $g^{-1}$) | 17.5 ± 2.0$^a$ | 10.2 ± 1.1$^b$ | 11.4 ± 3.4$^b$ |
| V (µg $g^{-1}$) | 6.1 ± 1.2$^a$ | 3.5 ± 0.3$^b$ | 3.5 ± 0.5$^{ab}$ |
| Zn (µg $g^{-1}$) | 541 ± 58 | 470 ± 35 | 427 ± 29 |

**Table 2.** Micro element concentration (mean ± half range, N = 2) in the water and topsoil of the studied lakes. Different superscript letters indicate significant differences among lakes ($P > 0.05$).

| | Surrounding environment | | | | | |
|---|---|---|---|---|---|---|
| | Water μg $L^{-1}$ | | | Topsoil, g $kg^{-1}$ | | |
| Elements | Lake Naplás | Frog Pond | Lak-völgyi Reservoir | Lake Naplás | Frog Pond | Lak-völgyi Reservoir |
| Al | 97.4 ± 38.4[a] | 17.5 ± 6.2[b] | 10.0± 2.1[b] | 4.5 ± 0.01[a] | 3.5 ± 0.1[a] | 4.1 ± 0.4[a] |
| V | 2.5 ± 0.1[a] | 0.8 ± 0.01[b] | 0.3 ± 0.02[c] | 2.0 ± 0.2[a] | 0.8 ± 0.02[b] | 1.9 ± 0.03[a] |
| Mn | 7.1 ± 2.0[a] | 6.9 ± 0.4[a] | 6.8 ± 0.6[a] | 0.2 ± 0.01[a] | 0.2 ± 0.04[a] | 0.2 ± 0.1[a] |
| Fe | 94.3 ± 19.0[a] | 63.7 ± 26.4[ab] | 23.2 ± 10.6[b] | 10.0 ± 0.6[a] | 4.0 ± 0.05[b] | 8.3 ± 1.4[a] |
| Cu | 3.7 ± 1.4[a] | 8.3 ± 4.9[a] | 4.4 ± 2.4[a] | 1.8 ± 0.01[ab] | 0.9 ± 0.03[b] | 3.2 ± 0.6[a] |
| Zn | 5.3 ± 5.1[a] | 13.7 ± 0.1[a] | 7.0 ± 5.6[a] | 8.3 ± 1.9[a] | 3.8 ± 1.0[a] | 6.0 ± 0.6[a] |
| Ba | 56.1 ± 2.5[a] | 119.5 ± 0.1[b] | 15.3 ± 0.6[c] | 5.9 ± 0.9[a] | 2.6 ± 0.01[b] | 5.3 ± 0.05[a] |
| Pb | 5.4 ± 1.6[a] | 3.3 ± 3.1[a] | 2.3 ± 2.1[a] | 1.9 ± 0.01[a] | 0.8 ± 0.04[b] | 1.9 ± 0.3[a] |

**Table 3.** Bioaccumulation Factor (BAF) in toe bones of the frog *B. bufo.*

| | BAF values based on water | | | BAF values based on topsoil | | |
|---|---|---|---|---|---|---|
| | Lake Naplás | Frog Pond | Lakvölgyi Reservoir | Lake Naplás | Frog Pond | Lakvölgyi Reservoir |
| Elements | N = 14 | N = 24 | N = 6 | N = 14 | N = 24 | N = 6 |
| Al | 0.10 | 0.52 | 0.90 | 0.62 | 0.53 | 0.4 |
| Ba | 0.12 | 0.05 | 0.40 | 0.04 | 0.04 | 0.02 |
| Cu | 1.70 | 0.68 | 1.15 | 0.06 | 0.06 | 0.01 |
| Fe | 0.10 | 0.14 | 0.40 | 0.32 | 0.51 | 0.23 |
| Mn | 1.00 | 0.92 | 1.02 | 1.15 | 0.6 | 1.03 |
| Pb | 0.83 | 1.19 | 1.76 | 0.01 | 0.01 | 0.01 |
| V | 1.39 | 3.62 | 9.76 | 0.003 | 0.004 | 0.002 |
| Zn | 1.49 | 0.56 | 1.10 | 0.06 | 0.12 | 0.07 |

from the Frog Pond, unlike the ones from Lake Naplás ($P = 0.040$). Multiple comparison tests showed significant differences between the trace element concentrations of *Bufo bufo* toe bones from the Frog Pond and the Lak-völgyi Reservoir in only one case: for Mn ($P = 0.016$) (Table 1). The highest average V concentration was found in *Bufo bufo* toe bones from Lake Naplás.

Concentration of trace elements of water and soil

Significantly higher Al and V concentration was found in the surface water of Lake Naplás, than in the Frog Pond and Lakvölgyi Reservoir (Table 2). The highest Ba concentration was found in the surface water of the Frog Pond. For Fe, higher concentration was found in the Lake Naplás, than in the Lak-völgyi Reservoir, but no significant difference was found between Frog Pond and Lake Naplás (Table 2). In the case of soil samples, the highest V, Ba and Pb concentrations were found in the Lake Naplás and Lak-völgyi Reservoir. Significantly higher Cu concentration was found in the soil from Lak-völgyi Reservoir and the soil from Frog Pond, but no significant difference was found between Lake Naplás and Lak-völgyi Reservoir (Table 2).

Bioaccumulation factor

Regarding the bioaccumulation factors (BAF), remarkable differences were found between the surrounding environment, water and soil (Table 3). BAF values were less than 1 for all elements when it was calculated for soil, except in the case of Mn: the BAF values for Mn were higher than 1 in the case of toads collected from Lake Naplás Reservoir and Frog Pond (Table 3). Thus, low level of accumulation was found from soil for Mn. Based on the microelement concentration of water samples, BAF values exceeding 1 were found for Cu, Mn, V and Zn in toad toe bones from Lake Naplás. For Pb and V, based on the BAF values, low level of accumulation was found in toad toe bones which were collected from the Frog Pond. Similar results were found in the case of the Lak-völgyi Reservoir where the BAF values were higher than 1 for Cu, Mn, Pb, V and Zn (Table 3).

**Discussion**

Many authors studied frogs and toads as biological indicators in environmental pollution studies (Burger & Snodgrass 1998, Flyaks & Borkin 2004, Stolyar et al. 2008). In comparison with the different organs, bones have a high storage capacity of metals because they are mineralized tissues (Linder & Grillitsch 2000). Flyaks and Borkin (2004) reported that the Fe, Zn, Mn, Cu, Pb, Ni and Cd concentration of the femur was the highest at a lake located near chemical and metallurgical factories. Similarly to our findings, Stolyar et al. (2008) also demonstrated that higher anthropogenic activities may cause a rise in the element content of amphibians. However, the actual values may differ. The effects of vanadium to wildlife are little known (Hopkins et al. 1998). Yamaguchi et al. (1989) reported that the low dose of V may play a nutritional role in the structure of bone, but in the bone tissue V may also accumulate in the form of vanadate, which may replace phosphate from apatite, the bone mineral (Facchini et al. 2006).

BAFs are usually determined for various aquatic species such as mussels (Geyer et al. 1982), fish (Veith et al. 1979) and frogs (Stolyar et al. 2008). The measured BAF values of elemental concentrations in toe bones and the elemental concentration of the surrounding soil was less than 1, indicating no bioaccumulation. Our findings suggest that toads can accumulate the Mn in small amount from the soil. Likely, toads can accumulate a higher number of microelements from water, as BAF values were higher than 1 for Cu, Mn, Pb, V and Zn. For amphibians, water is one major source for bioaccumulation, and many studies reported that amphibian larvae are highly susceptible to bioaccumulation (Burger & Snodgrass 1998, James & Little 2003, Unrine et al. 2007, Zhang et al. 2007). During the larval period, elements may accumulate which can be later eliminated or retained and redistributed throughout the metamorph tissues and organs (Snodgrass et al. 2003). Our results indicate that these accumulated elements may be detected also in adult amphibians.

Our results also indicated that the different dietary route of exposure between tadpole and toad may also cause differences between BAF values. Toads at tadpole stage are more susceptible to toxic effects than during the adult stage (Xu & Oldham 1997). Tadpoles in general are adapted for feeding on periphyton, detritus and aquatic plants (Diaz-Paniagua 1989), while *Bufo* tadpoles are relatively unselective suspension feeders (Seale & Beckvar 1980). The diet of *Bufo bufo* tadpoles consists of algae, planktonic crustaceans and debris (Diaz-Paniagua 1989). Similar to the tadpoles, *Bufo* adults are unselective feeders and feed on ground dwelling nocturnal arthropods (Clarke 1974). The most frequent food items of *Bufo* toads are beetles (Coleoptera), ants (Hymenoptera) and earwigs (Dermoptera) (Krakauer 1968, Evans & Lampo 1996). Earlier studies demonstrated that the metal adsorption of tadpoles was more significant than that of toads due to a specific ion exchange mechanism in algae, bacteria and higher plants, which are the basis of their diet and which are shown to be good heavy metal accumulators (Schneider et al. 2001, Novák et al. 2014, Bácsi et al. 2015). Carabids and ants, essential in the diet of adult toads, are poor accumulators which may be due to effective mechanism of metal elimination (Avgin & Luff 2010; Wilczek et al. 2003). Besides differences in their diet, the exposure time of larvae stage in water and the adult stage in water and on land may also cause differences in BAF values. Zhang et al. (2007) demonstrated that tadpoles can moderately increase their enzyme activities in polluted environments during the increasing exposure time.

Our study demonstrated that the trace element concentrations of *Bufo bufo* toe bones are useful indicators to assess the quality of terrestrial and aquatic environment. However, it should be considered that for some elements there are inconsistencies in the correlation of concentrations between toe bones and the environment (water and soil), which may be caused by the dietary route of exposure and the differences in exposure time in aquatic and terrestrial habitats in tadpole and toads. Thus, the accumulation rate of metals depended on dietary and exposure time, and development stage – i.e. tadpole and adult stages. Using modern analytical methods there was no need to kill these animals for bioaccumulation studies. Toe clipping is a commonly used method, which is simple, safe and applicable for genetics (Noonan & Gaucher 2006), histological study (Hyatt et al. 2007), age determination (Takashi & Masafumi 2009) and environmental pollution study (Simon et al. 2012). Our results demonstrated that the developed technique based on the trace element concentration of toe bones was useful for the assessment of contamination.

**Acknowledgments.** We are grateful to the Hertelendi Laboratory of Environmental Studies (Hungarian Academic of Science, ATOMKI, Debrecen) and ANALAB Ltd. for providing the instrumental background for our studies. B. Kovács was supported by the János Bolyai Research Scholarship of the Hungarian Academy of Sciences. The work is supported by the TÁMOP 4.2.1./B-09/1/KONV-2010-0007 and TÁMOP 274 4.2.2/B-10/1-2010-0024 projects. The project is implemented through the New Hungary Development Plan, co-financed by the European Social Fund and the European Regional Development Fund. E. Simon was supported the TÁMOP-4.2.2.A-11/1/KONV-2012-0043.

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