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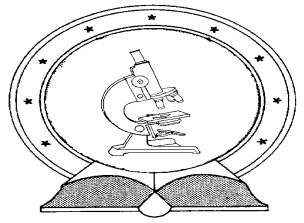

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ANALYSIS OF ENVIRONMENTAL LOAD BASED ON THE ELEMENTAL CONTENTS OF INDIVIDUALS

Egyetemi doktori (PhD) értekezés

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A doktori értekezés betétlapja

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Tanúsítom, hogy ***Simon Edina.*** *doktorjelölt 2006.- 2009. között a fent megnevezett Doktori Iskola* ***Kvantitatív és Terresztris Ökológia*** *programjának keretében irányításommal végezte munkáját. Az értekezésben foglalt eredményekhez a jelölt önálló alkotó tevékenységével meghatározóan hozzájárult. Az értekezés elfogadását javasolom.*
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Analysis of environmental load based on the elemental contents of individuals

Értekezés a doktori (Ph.D.) fokozat megszerzése érdekében
a Természettudomány tudományágban

Írta: Simon Edina okleveles biológus-ökológus

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Contents

Abbreviations used in text

AAS	Atomic absorption spectrometry
ANOVA	Analysis of variance
CDA	Canonocal Discriminant Analysis
CRM	Certified reference material
EIE	Easy ionisable element
ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectrometry
LOD	Limit od detection
USN	Ultrasonic nebulization

Papers included

1. **Simon, E.**, Braun, M. & Tóthmérész, B. (2010): Non-destructive method of frog (*Rana esculenta* L.) skeleton elemental analysis used during environmental assessment. *Water, Air and Soil Pollution* 209: 467-471. IF2009: 1.676.
2. Braun, M., **Simon, E.**, Tóthmérész, B., & Fábián I. (2010): How influences the quality of ethylene glycol the body mass and elemental composition of insect samples? *Environmental Science and Technology* – (manuscript). IF2009: 4.630.
3. Braun, M., **Simon, E.**, Tóthmérész B. & Fábián, I. (2010): Analytical methods for insect samples with optimization of ultrasonic nebulization coupled to axial inductively coupled plasma optical emission spectrometry. *Talanta* (manuscript). IF2009: 3.290.
4. Braun, M., **Simon, E.**, Fábián, I. & Tóthmérész, B. (2009): Ethylene glycol-based antifreeze trapping and ethanol preservation in the elemental analysis of insect samples. *Chemosphere* 77: 1447-1452. IF2009: 3.253
5. **Simon, E.**, Puky, M., Braun, M. & Tóthmérész B. (2009): Trace elemental analysis in *Bufo bufo* by toe bone from urban and rural sites in Hungary. *Environmental Monitoring and Assessment* – (under review). IF2009: 1356.

1. Introduction

1.1. Development of analytical method for environmental purposes

The use of indicator organisms as biological indicators in environmental pollution studies began to attract in the past decade (Clark, 1993; Dallinger, 1994; Herkovits and Helguero, 1998). Analytical chemistry is essential for environmental research. Available methods are relatively well developed and they allow the determination of elements in very low concentrations. In population level the elemental analysis may be a new opportunity in studies of environmental pollution. In certain cases the available amount of sample is small, e.g. the weights of invertebrates and the weights of some body parts of vertebrates. Previous studies have usually worked with large amount of material provided by averaging of individual samples. Investigation of elements in population level needs the analysis of individuals. With the use of microanalytical methods it is not necessary to kill vertebrates for the analytical samples. This approach may be usefull in sampling body parts of alive animals, e.g. toe bones (Simon et al., 2010) or bird feathers (Eens et al. 1999; Vallner et al. 1999).

In spite of the wide instrumental apparatus of analytical chemistry the multi-element method has not spread in the cases of animal samples in the environmental pollution studies yet. In most cases, the elemental contents of invertebrates were analysed by atomic absorption spectrometry (AAS) (Gräff et al., 1997; Boyd, 2002). In the case of vertebrates the elemental contents were analysed also by atomic

absorption spectrometry (AAS) (Puky and Oertel, 1997) and graphite furnace atomic absorption spectrometry (AAS) (Stolyar et al., 2008).

In some cases inductively coupled plasma optical emission spectrometry (ICP-OES) is an easier and more cost effective way of analysis to study animals (Dauwe et al., 2002). The limit of detection of axially viewed plasma with ultrasonic nebulization (USN) make this technique applicable for insect analysis (Braun et al., 2009). Spectral and matrix interferences have already been reported in some cases of complex samples using axially viewed ICP-OES techniques (Kola et al., 2002; Brenner and Zander, 2000). Ultrasonic nebulization may be affected by salt and acid effects which can often occur in the case of environmental samples (Dubuisson et al., 1998; Todoli et al., 1998; Todoli and Mermet, 1999). Therefore, such complex measuring system is usually verified by analysis of certified reference materials (CRM). During our studies insect CRM samples have not been available on commercially. Thus, in present study we followed the traditional way of method development such as optimization of plasma parameters, determination of limit of detection, and using standard addition.

By multi-elemental methods, we need to determine plasma parameters, in which the measuring system is stabile and robust e.g. certain change of parameters may cause small differences in the measured signal (Brenner and Zander, 2000). The magnesium 280.270 nm ion (Mg II) and 285.213 nm atomic (Mg I) line intensity ratios are suggested for the characterization of plasma robustness and analytical capacity (Brenner and Zander, 2000).

1.2. Insects as indicator organism

Insects play an important role in most terrestrial environments owing to their great abundance, biomass and diversity (Zödl and Wittman, 2003). Furthermore, they represent important links in metal-transport chains among trophic levels (Rabitsch, 1995; Lindqvist and Block, 1998). Therefore, trace-metal concentrations in insects have an important influence on the distribution of trace-elements in the biosphere (Lindqvist and Block, 1997). Invertebrates have also been widely used as bioindicators in environmental studies. The habitats of ground-living species are in close contact with soils (Nakamura et al., 2005), and these animals may accumulate inorganic and organic toxic compounds. The life cycle of invertebrates is usually short and their collection is easy making them suitable as indicators of concentration of environmental contaminants (Zödl and Wittman, 2003). Insects are also common in urban environments and they can be used to indicate the presence and concentrations of pollutants. The use of invertebrates, in particular insects, as biological indicators of environmental pollutants started to attract attention during the past decade. Accumulation of metals in different developmental stages of invertebrates has been studied under laboratory conditions in order to explore physiological mechanisms and toxicity issues (Devkota and Schmidt, 1999; Maryanski et al., 2002). Other researchers emphasise the usefulness of these animals for biomonitoring of metal pollutants in field studies (Lagisz et al., 2002; Nahmani and Rossi, 2003; Pearce and Venier, 2006).

Pitfall trapping is a simple and widespread method for sampling flightless or ground-living insects. This collection method is inexpensive, easy to use and operate round the-clock, resulting in large, species-rich

samples (Clark and Blom, 1992). Various substances have been used as killing agents in the traps (Southwood and Henderson, 2000; Schmidt et al., 2006; Jud and Schmidt-Entling, 2008; Thomas, 2008) e.g. ethanol fluid (Giles et al., 1973; Strojan, 1978; Pyatt et al., 1999; Nakamura and Taira, 2005) and formalin (Roberts and Johnson, 1978; Hunter et al., 1987; van den Berghe, 1992; Eichinger et al., 2007). However, formalin and picric acid are also used infrequently because of associated health hazards. Propylene glycol is a relatively new killing agent proposed for collecting insect specimens (Weeks and McIntyre, 1997), while ethylene glycol is often used, frequently in the form of automobile anti-freeze as an inexpensive filling fluid for traps (Tolbert, 1975; Suarez et al., 2000).

In ecological studies, the most common preservative fluids are formalin and ethanol for animal fixation. Storage fluid can cause changes in the wet and dry mass of animals (Geng, 1925; Wetzel et al., 2005). The degree of the changes varied with preservation time, species, the quality of water used for dilution (Donald and Paterson, 1977) and/or temperature as well as light conditions (Leuven et al., 1985). In general, 4 or 10 % formalin fluids were recommended because they caused relatively small morphological distortion, especially in soft-bodied animals (Black and Dodson, 2003). In the case of ethanol, 70 v/v % preservation solution is usually recommended (Englund and Polhemus, 2001; King and Porter, 2004; Akutsu et al., 2007). However, the effect of trapping fluid on the elemental composition of sampled insect material is less known.

1.3. Frogs and toads as indicator organism

Amphibian populations are declining globally which is caused by several factors such as habitat loss and fragmentation (Icochea et al.,

2002; Beebee and Griffiths, 2005), ultraviolet radiation and chemical pollution (Blaustein et al., 2003), climate change (Pounds, 2001) and epidemic disease, e.g. chytrig fungus (Pounds et al., 2006). These factors may also cause deformities and abnormalities in development (Blaustein and Johnson, 2003). Pond breeding amphibians are especially sensitive to contaminations because of their complex life cycles (Rowe et al., 1996, 1998). Effects of contamination may result in shorter body length, lower body mass, malformations of limbs or other organs (Sparling et al., 2000). The slow development, late metamorphosis, and small metamorph size result in increased risk of mortality, and exposure to predation (Rowe et al., 2001; Pahkala et al., 2002, 2003). Anurans are increasingly used as bioindicators of accumulation of contaminants in pollution studies (Loumbourdis et al., 2007). Elemental analysis, especially heavy metals in amphibians began to attract special attention (Herkovits and Helguero, 1998; Stolyar et al., 2008).

Toe clipping is a commonly used standard method to identify individuals, particularly frogs and toads (McCarthy and Parris, 2004). It is simple, safe and applicable for genetics (Noonan and Gaucher, 2006), histological examinations (Boyle et al., 2004) or amphibian skeletochronological age determination (Castanet and Smirina, 1990; Bruce et al., 2002). Toe clipping is an acceptable method, which does not cause any serious negative effects (Hartel and Nemes, 2006). McCarthy and Parris (2004) found that toe clipping reduces the recapture rate of the clipped individuals by 4-11%, which may be related to possible adverse effect of toe clipping.

Although a lot of elements play an important role in the building of skeleton, earlier studies about the elemental contents of bones are rare.

The composition of bones are 70% minerals, 20% collagen, 8% water and about 2% non-collagenous components (Klepinger, 1984). The concentration of elements in bones may be reported per g wet weight or dry weight but sometimes the elemental content of bone samples is given in μg or mg per bone samples in analytical studies (Elinder et al., 1994). The most important elements in the bones are calcium and phosphorous, which were the main components in the formation of hydroxiapatite (Janus et al., 2008). Ca/P and the Zn/Ca rate are also important factors to be evaluated in bones (Busetto et al., 2008). Our previous study (Simon et al., 2007) showed that the Zn concentration of toe bones are in a better correlation with the Zn concentration of the environment than those of several other organs, thus, it is an appropriate part of the body to study metal accumulation.

2. Objectives

In my dissertation, the main aims of studies were the following:

i. Development of an axial Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) with Ultrasonic Nebulizer (USN) method for the analysis of insect samples which is sensitive enough for measuring individual insects. During the development of the method the following aspects have been considered: the robustness of plasma parameters, the limit of detection, model solution experiment and standard addition.

ii. Study the effects of trapping and conservation fluids on body mass and elemental contents of insect samples.

iii. Study the influence of different quality of ethylene glycol as trapping liquid on the body mass and elemental contents of insect samples.

iv. Study the elemental composition of frog skeleton and gain information about which part of skeleton may be represented by the elemental composition of toe bones.

v. Study the effects of urbanization on elemental contents of toad toe bones.

3. Materials and methods

3.1. Optimization of USN axial ICP-OES method

During the method development IRIS Intrepid II XSP Duo type ICP-OES produced by Thermo, equipped with a CETAC 45 000 AT+ ultrasonic nebulization was used. The conditions of measurements have to be optimized since they are instrumental dependent. We used a seven-point calibration procedure (0.001, 0.005, 0.01, 0.05, 0.1, 0.5 and 1.0 mg L^{-1}) using multi-element calibration solution (Merck ICP multi-element standard solution IV). Other parameters used in this analysis are shown in Table 3.1.1. The analysis was performed using four or five atomic or ionic lines of the corresponding elements. In the case of alkaline metals (e.g. Li, Na and K) single lines were used. All of the selected lines were free of spectral interferences in insect and bone matrixes.

Table3.1.1. Instrumental conditions for USN-ICP-OES trace elemental analysis.

Background correction	Dynamic mode
Number of replicates	2
Pump speed, ml/min	2.77
Rinse time/s	40
Nebulizer flow, PSI	32
USN: heating stage/°C	140
USN: cooling stage/ °C	3
Analysis pump rate, ml/min	1.85
ICAP view	Axial
Low WL range, s	30
High WL range, s	10

To determine the robust plasma parameters the Mg II 280.270 nm / Mg I 285.213 nm intensity ratios were studied by measuring 0.1 mg L^{-1} magnesium containing solution based on Mermet (1991) recommendation. The excitation energy was set to 850, 950, 1150, 1250 and 1350 W, while the sample gas pressure to 15, 20, 25, 30, 35, 40 and 45 psi. These values were set in certain combinations of a randomized sequence. The limit of detection is important in the characterization of analytical methods. This definition (LOD) corresponds to that of the minimum detectable signal which still can be detected from the fluctuations of the background and which is than converted into a minimum concentration (Poussel and Mermet, 1996). Standard deviation was determined by 10 parallel measurements of blank solution for determining the limit of detection by means of 3 times standard deviation (Carré et al., 1997). Detection limits were determined by four different plasma conditions according to the robustness.

3.2. Firebugs

Firebugs (*Pyrrhocoris apterus* L.), a common species widely distributed in Europe were selected for our study. This bug can be found throughout the year (except in winter) in clusters under trees and shrubs or running on the ground. Adult firebugs are found on the bark of trees in large number even in urban areas. Firebugs are large enough (0.07 ± 0.01 g) to allow for elemental analysis on individual specimens. Adult firebugs were hand collected during their reproductive period in a public park near the University of Debrecen, Hungary in May and August 2008. The sample contained altogether 100, 250 and 450 individuals, which were placed in a large plastic vessel. The live insects were taken to the laboratory where they were transferred into polyethylene tubes in groups of 10 specimens. The firebugs were killed by freezing at -18 °C.

In the optimization experiments the samples were dried overnight at 105 °C, and the samples were reweighted to determine their dry mass. To the standard additional experiment about 100 individual were dried together. After drying homogenate was prepared by grind.

In the effects of trapping fluids and ethanol experiments five tubes were selected at random for each of the four treatments and for the control. The control samples were stored at -18 °C for two weeks. We simulated pitfall trapping using ethylene glycol based antifreeze as killing agent. The trapping fluid was GlycoShell, a common antifreeze, diluted to 75 % (v/v) following earlier practices (Magura et al., 2001; Magura et al., 2004). Twenty sample tubes containing 10 pre-frozen bugs were filled with 10 ml trapping fluid and stored in a dark and dry space at room temperature. Ten sample tubes were processed after two weeks (mimiking a frequent trap control regime). The contents of five sample tubes were

analysed. Bugs in the other five tubes were transferred into new tubes filled with 10 ml 70 % (v/v) ethanol and stored for a further two weeks before analysis (to simulate short trapping plus short preservation). Ten other sample tubes were opened after one month. Five of these sample tubes were analysed (infrequent checking of trap), while the bugs from the other five tubes were transferred into ethanol and analysed after one month (to simulate long trapping plus long preservation).

In the different grade of glycols experiments the polyethylene tubes with firebugs were divided into nine equal groups for the different treatments. Samples were soaked for two weeks and one month in ethylene glycols of four different grades: analytical grade, puriss, technical grade and common anti-freeze. Five tubes were selected randomly for each of the eight treatments and for the control experiments. Control samples (a) were stored in a freezer for two weeks. The tubes were filled with 10 mL glycol of different grades for the two-week (b_1, c_1, d_1, e_1) and the one-month (b_2, c_2, d_2, e_2) experiments: analytical (b), puriss (c), technical grade glycol (d) and common anti-freeze (e).

3.3. Frogs and toads

Rana esculenta is a common species in Europe, including Hungary. Frogs and water samples were collected during the summer of 2007. The sampling site was the Frog Pond (Békás tó) in Debrecen City, which is an urban pond with heavy traffic around and other anthropogenic effects (e.g. receiving thermal bath) in the centre of the city (47°33′N, 21°37′E). Landscape elements (in percentages) around the pond in a plot of 1 km by 1 km size were the following: forest: 39.7%, buildings: 59.9% and pond: 0.4%. Ten adult frogs were collected by hand net. On arrival at

laboratory, frogs were anesthetized with chloroform. After dissection the following large bones were processed: skull, spinal, femur, tibia-fibula, tarsal bones, metatarsus, humerus and digits from front and hind limbs. Reduced bones were found in the case of one frog specimen where the metatarsal and phalanges from hind limb were missing. In our study, the first phalange of the third digit was also analysed from each hind limb. The samples were stored at -18°C until preparation.

The specimens of frog *Bufo bufo* were collected during their reproductive period from three different ponds during spring of 2007. Two of these were rural sites: Garancsi Pond at Tinnye (43°37´N, 18°48´E) and Lake Naplás, a reservoir on the Szilas stream at eastern edge of Budapest (47°30´N, 19°14´E). The third sampling site was the Frog Pond which was an urban pond with heavy traffic around the pond and anthropogenic effects (thermal path and pharmaceutical factory) in the centre of the city Debrecen (47°33´N, 21°37´E). Characterization of the three ponds based on their different area was shown by Table 3.3.1.

Table3.3.1. Landscape elements (in percentages) around the ponds in a plot of 1km by 1km sizes.

Sites	pond (%)	forest (%)	field (%)	agricultural area (%)	buildings (%)
Frog Pond	0.4	39.7	–	–	59.9
Garancsi Pond	2	22.3	53.2	22.5	–
Naplás Pond	14.1	15.3	–	70.6	–

The adult frogs were collected by hand net in the urban sites. At the two rural sampling sites toe-bones were cut out from road-killed specimens during migration. Toe-clipping was made by stainless steel, surgical folding knife following Green (2001) recommendations. The

removed toes were stored in a freeze in plastic Eppendorf tubes to sample processing.

3.4. Sample preparation

In the cases of large bones of frogs samples were placed into a plastic sieve and flushed with 100ml of double deionised water. After this step, the large bones were placed into 5ml hydrogen peroxide for five days. The cleaning with hydrogen peroxide was important to clean the bones from conjunctive tissues. Femurs were chosen to test the effect of hydrogen peroxide on elemental contents of frogs. Similarly to the other bones, the right femurs were placed into 5ml 30% (m/m) hydrogen peroxide for five days, while the left femurs for eight days. The hydrogen peroxide contained remarkable concentration of Sn; thus, the elemental analysis the Sn concentration was not considered. This cleaning procedure was also applied in the case of phalanges. After the flush with double deionised water these smaller toe bones were placed into 0.5ml hydrogen peroxide for two days.

In the cases of frogs and toad after the hydrogen peroxide cleaning and in the cases of firebugs after treatments each sample was placed in a plastic sieve and flushed with 250 ml of double deionised water obtained from a Millipore Milli-Q system. Each sample was transferred individually into a 25 ml beaker. The wet mass of the samples were measured immediately with PRECISA 240A analytical balance. The samples were dried overnight at 105ºC, and the samples were reweighted to determine their dry mass. Toe bones were measured with a SATORIUS LE 26P micro analytical balance. The material was then digested using 2 ml 65 % (m/m) nitric acid (Scharlau) in the same container at 80 ºC for 4

h. High pressure digestion was not required because the applied method gave a clean colourless solution. Digested samples were diluted to 10 ml (firebugs), 20 ml (toe bones and firebugs), 50 ml (firebugs) and 100 ml (frog bones) using a 1 % (m/m) nitric acid.

During the standard addition experiment, the studied elements were added to the firebug homogenization in known amounts. We compared to the measured and expected elemental concentrations. To control the method in the model solution experiment standard solutions were digested and handled as sample.

3.5. Statistical analysis

Calculations were performed using SPSS/PC+ and R statistical software packages. Effects of urbanization on elemental contents in toe bones, the effects of four treatments on the body mass and elemental composition of firebugs specimens were evaluated by ANOVA. Homogeneity of variances was tested by the Levene test. One-Way ANOVA was used to compare urban and rural areas in the case of toe bones and different treatments and the control in the case of firebugs. In the case of significant results of comparison by ANOVA, Tukey's Multiple Comparison test was used for pairwise comparison of groups (Zar, 1996).

The effects of urbanization on elemental contents in toe bones and different treatment to elemental composition of firebugs evaluated by Canonical Discriminant Analysis (CDA). CDA allows for the comparison of the elemental compositions of the individual samples. It is used for identifying statistically meaningful variations in the composition by

taking into account all measured elements. CDA is a technique for classifying a set of observations into predefined classes. The model is based on a set of observations (sometimes referred to as the training set) for which the classes are known. Based on the training set, the technique constructs a set of linear functions of the predictors, known as discriminant functions (Green, 1971; Rushton and Eyre, 1992). The measured elements were the input variables and the treatments the predefined classes in the CDA. Concentration data were log(x+1) transformed.

Linear regression models were used to evaluate the relationships between the element contents and the weight of bones and in the case of standard addition experiment. The measured and estimated elemental contents of toe bones and the measured and expected elemental concentration in the model solution experiment were tested with t-test.

4. Results and discussion

4.1. Analytical methods for insect samples

4.1.1. Determination of robust plasma parameters

The robust plasma parameters, i.e. high power and low carrier gas flow rate were similar to results of Iglésias et al. (2004). In our study, the highest power was 1350 W while the gas flow was 0.65 lpm (Fig. 4.1.1.1.). The Mg II / Mg I ratio of the aerosol flow rate of reduction increased proportionately. The small amount of water present in the plasma dissociates and the released hydrogen increases the excitation temperature of the plasma channel lying in the centre (Brenner and Zander, 2000).

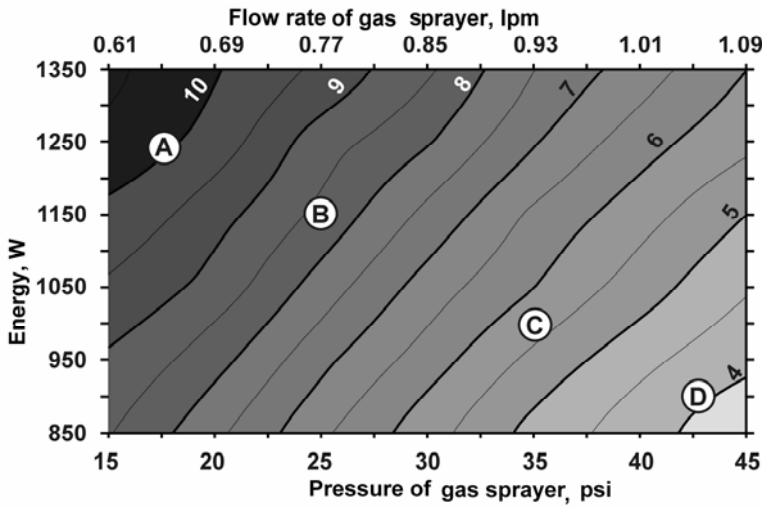

Fig.4.1.1.1. Mg II 280.270 nm / Mg I 285.213 ratios in function of nebulizate gas flow rate and excitation energy. The white circles marked plasma conditions which were set for determining LODs in further experiments.

The diameter of the injector has a very significant effect on the Mg II / Mg I ratio. The diameter of the injector in our instrument was 2 mm. Ivaldy and Tyson (1995) measured lower Mg II / Mg I ratio with narrow torch injector than Brenner et al. (1997, 1999) who used similar arrangement but a larger diameter (> 2 mm) torch injector.

Our results showed that map of robustness by axial plasma differed from that of pneumatic radial plasma system (Iglesias et al., 2004). In the case of pneumatic nebulization the surface is remarkable curved at ratio Mg II 280.270 nm / Mg I 285.213 > 8, which is relatively small bounded by the narrow parameters (Iglesias et al. 2004). In our case the surface of robust conditions was characterized by less curvature providing wider range of optimal conditions. By pneumatic nebulization wet aerosol enters the plasma, while in the case of USN dried aerosol goes into the plasma channel. Thus the cooling effect of water is less important and the plasma temperature gets higher (Stepan et al., 2001). The lifetime of torch is reduced remarkable at high levels of energy. It is possible to set lower energy combined by low sample gas flow rate to reach robust plasma conditions, which may elongate the lifetime of torches. The sample gas has got a significant effect on efficiency in the case of pneumatic nebulizers. This effect is negligible by USN, because the gas only carries the aerosol and do not take part in the aerosol forming process.

4.1.2. Selection of spectral lines

We selected 64 spectral lines of 17 elements from spectral atlas (Boumans, 1984) based on their relative intensities, detection limits and spectral properties.

The detection limits were studied at four different plasma conditions represented by ratio of Mg II 280.270 nm / Mg I 285.213 line intensities. The summarized results showed in Appendix Table 1. Detection limits were different under different plasma parameters. Similarly as it was reported by Brenner and Zander, (2000) in the case of easy ionisable elements (EIE), eg. sodium, potassium, lithium and strontium, the limits of detection were far from ideal under robust plasma condition. Low sample gas flow and at high excitation energy provided robust plasma and resulted low limit of detection in the case of other elements. For the insect elemental analysis from the four test settings, the second (B) was chosen. Compared to the (A) and (B) settings we found lower detection limits in the cases of most in (B) setting. The results of (B) setting parameters (1150 W, 25psi) recommended for measurements.

The following elements are major constitutes in insects body: Ca, K, Mg, Na (Engebretson and Mason, 1980; Braun et al., 2009). Although these elements were relatively high amount in insect these elements did not cause spectral interferences (Appendix Table 2).

4.1.3. Elemental composition of insects

Individual insect samples were analysed by ICP-OES-USN method which was optimized previously. Insect data from literature were collected in Appendix Table 4. In our earlier study, we reported the elemental composition of firebugs (Braun et al., 2009). Concentrations of major elements in mg.kg-1 (mean ± SE) were: Ca: 2170 ± 160, Cu: 17.5 ± 0.7, K: 2810 ± 50, Mg: 1810 ± 110, Mn: 22.4 ± 1.4, Na: 540 ± 20, Sr: 8.1 ± 0.5, Zn: 15.7 ± 1.8.

Sample pre-treatment and the analysis process were tested by model solutions prepared of standards with known concentrations. Known amount of model solutions were handled the same way as an insect sample. The investigated elements were: Ba, Cd, Co, Cu, Fe, Mn, Ni, Pb, Sr and Zn. Expected and measured concentration of elements were compared using t-test. In the cases of all elements, significant difference was not found between the expected and measured concentrations ($p > 0.05$) (Appendix Table 5).

The effect of matrix and spectral interferences were tested by standard addition experiments. Homogenized firebug samples were processed using normal calibration method. The weight of homogenized firebug was 0.02 g similarly to the average dry body mass of bugs. During the standard addition the investigated elements were added in known amount to firebug homogenised and they were run throughout the analytical process. Using linear regression model from standard addition could be calculated the elemental concentration of firebugs (Table 4.1.3.1). Concentrations obtained by the two methods were compared with t-test. Significant differences were not found at level $p = 0.05$. The selected lines are summarized in Appendix Table 3. In the cases of other lines matrix and other interferences were found. The measurements have not been effected by matrix or other disturbing effects in the case of insect sample solutions.

Table4.1.3.1. Summary statistics of elemental concentration (mean ± SD, mg L^{-1}) of firebug homogenisation. Noticed: A = elemental concentration of homogenisation by classical method, B = elemental concentration of homogenisation by standard addition.

Elements	A	B	t value	df	p
Ba	0.012 ± 0.001	0.010 ± 0.001	1.750	2	0.222
Cd	0.001 ± 0.0001	0.001 ± 0.0002	1.184	1	0.447
Co	0.001 ± 0.0002	0.001 ± 0.001	-1.035	2	0.409
Cu	0.065 ± 0.002	0.066 ± 0.002	3.812	2	0.062
Fe	0.470 ± 0.018	0.472 ± 0.007	-0.163	2	0.886
Mn	0.060 ± 0.012	0.053 ± 0.024	1. 085	2	0.391
Ni	0.013 ± 0.001	0.012 ± 0.001	2.882	2	0.102
Pb	0.054 ± 0.003	0.030 ± 0.0002	11.439	1	0.056
Sr	0.027 ± 0.001	0.021 ± 0.008	1.200	1	0.442
Zn	0.192 ± 0.001	0.019 ± 0.001	6.000	1	0.105

4.1.4. Conclusion

The aim of our study was to develop an analytical method using inductively coupled plasma optical emission spectrometry (Iris Intrepid II Duo, Thermo) with ultrasonic nebulization for micro analytical purposes. Robust plasma parameters are more or less instrument dependent. Setting the robust conditions we followed recommendations of Mermet (1991) using the Mg II/ Mg I intensity ratios. The suggested range of Mg II 280.270 nm / Mg I 285.213 > 8 was relatively wide in case of USN. Initially our measurement method used 64 lines of 17 elements. We selected lines based on their limit of detection and considering the results of standard addition experiments. Our results showed that limit of detection depend on excitation energy and sample gas velocity as it was reported similarly in earlier studies (Novotny et al., 1996). Robust plasma

conditions resulted small limit of detections in case most of the elements excepting easy ionisable ones.

The other aspect of the line selection was the result of model solution experiment. Significant differences were not found between the measured and expected concentrations in the case of high sensitive lines characterized by low LOD. Similar results were found in the case of standard addition experiments. Neither spectral interferences nor matrix effects were detected by the selected lines. The developed method is suitable for the analysis of small weight samples which allows to study elemental composition of individual insects.

4.2. Effects of trapping and conservation fluid on body mass and elemental contents

4.2.1. Effects of trapping and preservation fluid on firebug body mass

No evidence of statistically significant heterogeneity in the variances of wet ($df_1 = 4$, $df_2 = 20$, $p = 0.300$) or dry body mass ($df_1 = 4$, $df_2 = 20$, $p = 0.172$) were found. Significant differences were found in the wet body mass of firebugs between the treatments ($p = 0.015$ (short trapping) and $p = 0.001$ (long trapping), $N = 25$ in each cases). The body mass of the bugs increased in the trapping fluid. The increase was about 26 % in the short and 37 % in the long trapping treatments, respectively. After preservation in ethanol, the differences disappeared; the wet body mass of ethanol-preserved samples was not significantly different from the wet body mass of the controls ($p = 0.068$ for short preservation and $p = 0.135$ for long preservation, $N = 25$ in each cases) (Fig. 4.2.1).

Dry body mass measured after the short ($p = 0.777$, $N = 25$) and long ($p = 0.968$, $N = 25$) trapping experiments did not differ significantly from the controls. Dry body mass of the bugs showed a significant decrease compared to the control (31 %) only when long trapping and long preservation was used ($p = 0.001$, $N = 25$). In contrast, short preservation with ethanol did not change the dry mass ($p > 0.064$, $N = 25$, see Fig. 4.2.1.1.).

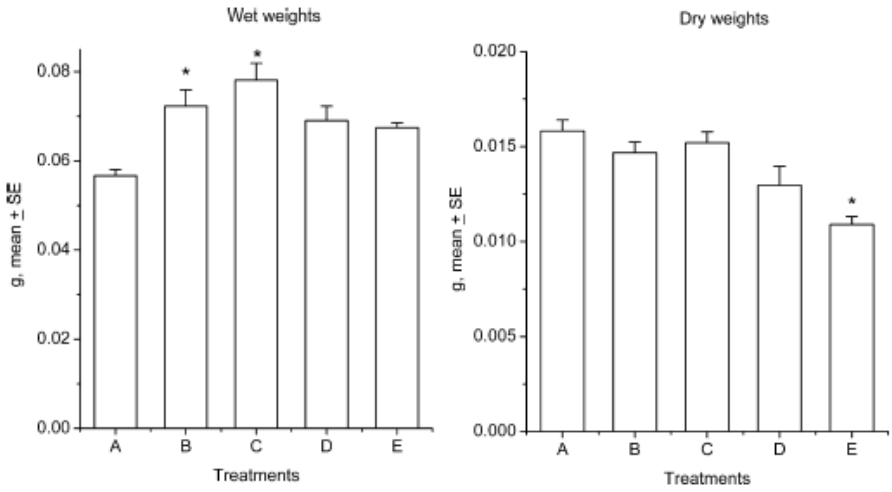

Fig.4.2.1.1. Effect of treatments on wet and dry body mass of firebugs. (A = control treatment, B = short trapping treatment, C = long trapping treatment, D = short trapping plus short ethanol preservation treatment, E = long trapping plus long ethanol preservation treatment). Asterisks indicate significant differences ($p < 0.05$).

4.2.2. Effect of trapping and preservation on elemental composition of insects

The concentrations of barium (<7.6 mg kg^{-1}), cadmium (<1.3 mg kg^{-1}), cobalt (<5.1 mg kg^{-1}), chromium (<2.5 mg kg^{-1}), lithium (<2.5 mg kg^{-1}), nickel (<5.0 mg kg^{-1}) and lead (<16.5 mg kg^{-1}) were below the limit of quantification in the bugs. Descriptive statistics of the concentrations of the quantifiable elements measured in the differently treated firebugs are given in Appendix Table 6. No evidence of statistically significant heterogeneity in the variances of all elements analysed were detected ($df_1 = 4$, $df_2 = 20$, $p > 0.05$). The concentrations of magnesium and zinc did not differ significantly between the treatments (Appendix Table 6). The concentration of copper did not different significantly between the control and treated samples ($p > 0.05$), but was significantly higher in samples in

the long trapping plus long preservation treatment compared to the other treatments (Appendix Table 7). The concentrations of calcium, sodium and strontium were significantly higher in the treatment samples, while potassium was significantly lower compared to the control treatment (Appendix Table 6 and 7). Manganese concentrations were higher in samples in the long trapping plus long preservation treatments compared to the other treatments (Appendix Table 6 and 7).

Trap duration (short trapping vs. long trapping, see Appendix Table 6) did not cause any significant differences in the elemental composition of the samples. Neither were there significant differences in the elemental composition of the samples between the short trapping vs. the short trapping plus short preservation treatments. Long preservation caused an increase in the concentration of copper in the samples compared to the long trapping treatment (p = 0.005), but no other differences in the concentrations of elements were found between these two treatments. Only the concentration of copper (p = 0.021) differed significantly between the short trapping plus short ethanol preservation vs. the long trapping plus long ethanol preservation treatments (Appendix Table 6).

4.2.3. Discriminant analysis

On the basis of the concentrations of the measured elements, four canonical discriminant functions were used in the analysis. The first two canonical discriminant functions were significant ($p < 0.01$). Using the calculated discriminant functions, 88% of the cases were correctly classified. In the case of control samples the separation was 100% from

the treatments. The first discriminant function showed a significant negative correlation with the concentration of potassium, which indicates that the concentration of this element decreased due to the treatments (Appendix Table 8). Significant negative correlations were found in the pairwise comparison between the second discriminant function and the concentrations of calcium, manganese and copper (Appendix Table 8). These negative correlations indicated that the concentration of these elements increased with the trapping plus ethanol preservation treatment. A biplot of the two significant discriminant functions shows the separation of treatment groups (Fig. 4.2.3.1.).

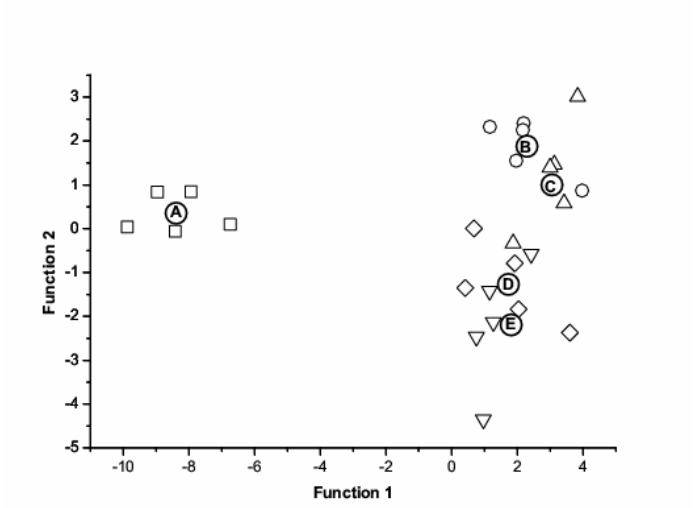

Fig.4.2.3.1. Canonical discriminant analysis of five treatments based on element concentrations ($\mu g \cdot g^{-1}$). (A = group centroid of the control, B = group centroid of the short trapping treatment, C = group centroid of the long trapping treatment, D = group centroid of the short trapping plus short ethanol preservation treatment, E = group centroid of the long trapping plus long preservation treatment).

The first discriminant function indicates the differences between the control and treated samples. The differences among the treatments are

mainly explained by the second discriminant function. Results of the discriminant analysis show that trapping and preservation lead to changes in the chemical composition of the specimens.

4.2.4. Elemental composition of the trapping and preservation fluids

Impurities in the antifreeze and ethanol fluids were also analysed. The concentration of each element in the ethanol solution was below detection limits. However, the ethylene glycol based antifreeze solution contained measurable impurities in the following concentrations (mean ± SE): calcium: 276 ± 52 $mg \cdot L^{-1}$, sodium: 190 ± 6 $mg \cdot L^{-1}$, magnesium: 46.2 ± 3.8 $mg \cdot L^{-1}$, potassium: 26.1 ± 4.1 $mg \cdot L^{-1}$, zinc: 27.5 ± 2.5 $mg \cdot L^{-1}$ and strontium: 0.8 ± 0.1 $mg \cdot L^{-1}$.

4.2.5. Conclusion

The number of publications investigating elemental compositions of invertebrates is increasing, yet only limited effort has been made to understand the effects of the collection methods and preservation on the element concentrations of the specimens. In earlier studies elemental analyses have been conducted on animals captured with ethanol (Giles et al., 1973; Strojan, 1978) or formalin (Williamson, 1979; Clausen, 1984; Hunter et al., 1987; Zödl and Wittman, 2003). In at least some of these studies it was been assumed that the trapping fluid does not affect the elemental concentrations in insects (Clausen, 1984; Hunter et al., 1987).

This assumption has, however, not been tested empirically (Hendrickx et al., 2003), until now.

Several publications have studied the effects of formalin and ethanol on the wet and dry body mass of organisms. Gaston et al. (1996) found no significant differences in wet body mass, but found minor variations in the dry body mass of macro-benthic species in formalin and ethanol. In contrast, Donald and Paterson (1977) and Wetzel et al. (2005) reported remarkable differences in wet and dry body mass studying macro-invertebrates both ethanol and formalin. Our result showed that the trapping fluid (antifreeze) increased the wet body mass of firebugs, possibly due to the diffusion of the trapping fluid into the tissues of the bugs, while the preservation fluid (ethanol) reversed this effect. Ethanol most likely removes antifreeze and lipids from insect tissues during storage. Our study also indicated that ethanol preservation resulted in the loss of firebug dry body mass. Similar results were reported by Leuven et al. (1985) for dry body mass of insects. This decrease in body mass may cause significant differences in the concentrations of some elements when frozen and treated samples are compared (e.g. manganese).

In consistence with our findings, pitfall trap collected isopods, carabids and ants showed significantly higher concentrations of zinc, cadmium and lead compared to hand collected samples (Rabitsch, 1995; Zödl and Wittmann, 2003); they used formalin as killing agent. In this context, it is worthwhile to note that the concentrations of some metals were lower in formalin-fixed human tissues (Koizumi et al., 1994) than in reference samples. This was attributed to the dilution of the metallic compounds in the preserving fluid. Similar phenomena may alter the analytical results of pitfall trapped insects.

Analytical grade glycol has only rarely used for trapping experiments (Lövei, 1984). Antifreeze is often used because it is cheaper than chemical grade ethylene glycol. However, insect tissues may adsorb impurities in these fluids. We conclude that commercial antifreeze cause a significant change in the elemental composition of the collected insect material. Certain elements (e.g. potassium) leached out of insect tissues, while others (e.g. calcium, strontium, manganese, and sodium) may enrich these tissues due to adsorption or other chemical interactions.

Our results demonstrated that the use of commercial antifreeze as a trapping fluid and the preservation of invertebrates in ethanol may lead to biased elemental concentrations, particularly in macro-elements (calcium, sodium and potassium). Firebugs have soft exoskeleteon, which may cause the rapid leaching out or enriching of elements to insect tissues from trapping fluid. The effects of trapping fluid and preservation may depend on the structure and hardness of exoskeleton of insects. Our results suggest that careful consideration is required in the analyses of trapped specimens. Live trapping for collection and frozen fixation for preservation is recommended as a more reliable method if the aim of elemental analysis is the precise measurement of environmental pollutants in insect samples.

4.3. Effect of ethylene glycol quality on the body mass and elemental composition of insects

4.3.1. Effects of ethylene glycol on firebugs mass

The wet and dry masses of the insects are important data in studies related to biomass (Donald and Paterson, 1977; Gaston et al.,1996; Wetzel et al., 2005)). Dry weight is generally accepted reference for the basic elemental analysis (Elinder et al., 1995). Wet body masses only of hand-collected animals can be directly measured. Wet body masses of trapped insects may be different.

Initial wet body masses of firebugs in the set of experiment were compared using Levene-test and ANOVA. Variances ($df_1 = 8$, $df_2 = 36$, $p = 0.071$) and averages ($F= 2.130$, $p = 0.061$) of wet body masses did not differ significantly. Average of wet body mass was 42.9 $\pm$ 2 mg. This is the control value, which was compared to the treatments.

Two factors were studied in the experiment one was the quality of glycol (four levels) and the other was the duration of trapping (two levels). Wet body masses after treatments were compared by two-ways ANOVA. Variances of treatments did not differe significantly ($df_1 = 7$, $df_2 = 32$, $p = 0.586$). The effect of different quality glycols was not significant ($F = 0.673$, $p = 0.575$). The duration of trapping had significant effect on wet body masses ($F =4.404$, $p = 0.044$). Wet body masses after one month treatments (65.3 $\pm$ 3 mg) were larger than after two week treatments (61.7$\pm$ 2 mg). Interaction between the two factors

was not significant (F = 0.385, p = 0.764). The glycol caused 43.8 % after two weeks and 52.2 % after one month treatment increase in wet body mass compared the treatments to control. In our earlier study, the increase was 26% and 37 % in the corresponding treatments when 75 % (v/v) diluted GlycoShell was used (Braun et al., 2009).

Dry mass of untreated control was 13.3 $\pm$ 1.7 mg. Due to the effects of treatments dry body masses increased significantly. Investigating the two factors duration of trapping was not significant (F = 1.153, p = 0.291). Quality of glycol had significant effect on dry body mass (F = 3.805, p = 0.019). Interaction between two factors was not significant (F = 0.265, p = 0.850). Analytical grade (28.2 $\pm$ 3.7 mg), puriss (30.0 $\pm$ 3.1 mg) and technical grade (29.1 $\pm$ 3.1 mg) glycols had similar effect on dry body mass; their pooled average was 29.1 $\pm$ 1.7 mg. Using common anti-freeze the dry body mass was lower (23.5 $\pm$ 3.1 mg). Using different grade glycols the increase of dry body mass was 18.8 %. In the case of common anti-freeze it was 6.7 %. The glycol diffused into the insect body and may not evaporate completely. Heat induced polymerisation of ethylene glycol may cause significant increase of weight during drying at 105 ºC (Viciosa et al., 2008). This effect was detectable in all cases, although by common antifreeze was less than others. According to earlier studies, other trapping fluids also caused remarkable differences in wet and dry body masses (Donald and Paterson, 1977; Gaston et al.,1996; Wetzel et al., 2005). Ethanol and formalin had reverse effect they reduced the body mass (Donald and Paterson, 1977; Wetzel et al., 2005).

Dry body mass measured after treatments changed significantly. The uneffected wet and dry weight is not measurable in field experiments.

Prior to treatments the wet body mass was measured and the water content (60.53 %) of manually collected control samples was determined. Using these measurements unaffected dry body masses of treated samples were calculated. Concentration elements were based on wet body masses and measured and calculated dry body masses.

4.3.2. Characterization of different grades of glycol

The summary of the elemental compositions of different grades of glycols before and after various treatments are given in Appendix Table 9. The following elements were below the quantification limits: barium (<0.006 mgL^{-1}), cadmium (<0.001 mgL^{-1}), cobalt (<0.004 mgL^{-1}), chromium (<0.002 mgL^{-1}), copper (<0.003 mgL^{-1}) lithium (<0.002 mgL^{-1}), nickel (<0.004 mgL^{-1}) and strontium (<0.002 mgL^{-1}).

The sulphate ash content was used for characterising the trapping fluids. It was calculated on the basis of the measured elemental contents. In the case of analytical, puriss and technical grade glycol the sulphate ash was not significantly different ($F = 1.881$, $p = 0.232$). Its pooled average was 5.6 ± 1.4 mg L^{-1} which was consistent with the certificates (< 50 mg L^{-1}.). Sulphate ash content of common anti-freeze was very high (2730 ± 602 mg L^{-1}).

Characterising materials leached out of insect bodies sulphate ash content of trapping fluid were determined after treatments. In the case of common anti-freeze the concentration of sulfate ash was extremely high and covered the difference between two week and one month treatments. Comparison by ANOVA was not significant ($F = 2.638$, $p = 0.120$).

Evaluation of different glycols were carried out by two way ANOVA. Factors similarly to previous comparisons were the quality of glycols and duration of treatments. Variances did not differ significantly by Levene-test (F=2.431, $df_1 = 8$, $df_2 = 30$, $p = 0.069$). Effect of glycol quality was not significant (F = 0.462, $p = 0.634$). Duration of treatments had significant effect on sulphate ash content (F = 84.885, $p < 0.001$). Sulphate ash in trapping fluids increased to 40.3 $\pm$ 6.1 mg L^{-1} during two weeks and to 54.9 $\pm$ 5.5 mg L^{-1} during one month treatment (Fig.4.3.2.1.).

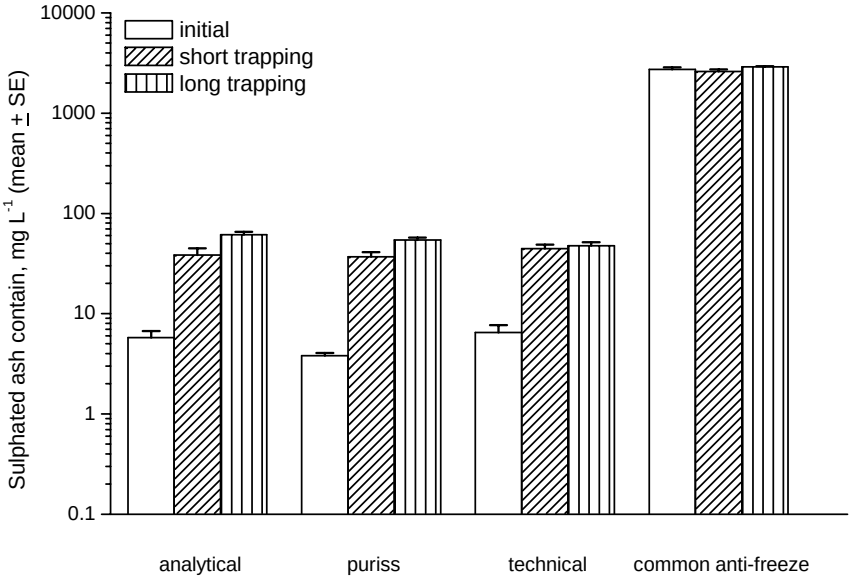

Fig.4.3.2.1. Sulphate ash contain (mg L^{-1}) in different grade of glycol according to the initial, two-week and one-month treatment.

4.3.3. Effects of glycol on elemental composition of insects

The elemental concentrations in biological samples may be reported per mg wet mass, dry mass or mg element per individual

(Elinder et al., 1994). Treatments with different grade of glycol caused remarkable changes in wet and dry body masses. Its effects on elemental concentrations were showed expressing the concentrations in mg kg^{-1} on the basis of initial wet body mass and measured and calculated dry body mass. The descriptive statistics of the concentrations of the quantifiable elements are given in Appendix Table 10. Concentrations of cadmium (<0.4 mg kg^{-1}), cobalt (<1.5 mg kg^{-1}), lithium (<0.8 mg kg^{-1}), nickel (<1.5 mg kg^{-1}) and lead (<5.0 mg kg^{-1}) in the firebugs were below the quantification limit of the procedure (Braun et al., 2009).

Using ANOVA significant differences were found in the concentration based on the initial wet body mass in the cases of potassium and sodium ($p < 0.01$) (see Appendix Table 11). Similar results were shown when the elemental concentrations were based on calculated dry body mass (Appendix Table 12). In this case also the concentration of potassium and sodium differed significantly ($p < 0.01$). By potassium using Tukey Multiple Comparison tests the control samples differed significantly of two-week technical, one-month puriss, one-month technical and one-month common anti-freeze treated samples when the concentrations were calculated on the basis of initial wet and calculated dry body masses ($p < 0.05$). Concentrations of sodium based on initial wet and calculated dry body masses were significantly lower than the control in the case of two-week and one-month common anti-freeze, and one-month puriss treatments ($p < 0.05$). If the elemental concentrations were based on measured dry body mass (which were increased due to the polymerization of glycol), significant differences were found in the cases of each element ($p < 0.001$) (Appendix Table 13). Concentrations of control samples were different in all cases ($p < 0.01$).

Duration of treatments did not cause any significant differences on elemental composition neither in the two-week nor in the one-month trapping independently of reference bases ($p > 0.05$). Our results show that trapping with ethylene glycol treatments caused differences between the control and treated samples. Using glycols significant decrease in elemental concentrations was observed by the insect material. This effect was particularly caused by the increase of body mass (Table 4.3.3.1.). In comparison with hand collected and pitfall collected isopods and carabids similar difference has been reported which were explained by defecation (Zödl and Wittmann, 2003).

Table 4.3.3.1. Statistically significant differences was found between control and different treatments according to the different reference base.

Elements	initial wet body mass	measured dry body mass	calculated dry body mass
Ba	-	●	-
Ca	-	●	-
Cu	-	●	-
Fe	-	●	-
K	●	●	●
Mg	-	●	-
Mn	-	●	-
Na	●	●	●
Sr	-	●	-
Zn	-	●	-

Noticed: ● means the significant differences, - means that significant difference was not detected.

Our results demonstrated the rank of quality of glycol based on sulfate ash contents was as expected: analytical, puriss, technical grade and common anti-freeze. The common anti-freeze contained relative high

levels of impurities (Ca, Fe, K and Na). In the cases of these elements the puriss and technical grade's glycol are recommended as trapping fluids for insect collection. Using puriss grade is cost effective and its quality seemed to be appropriate.

We found that the potassium and sodium leached out of insect tissues. Our results also indicated that the undiluted trapping fluids caused an increase in body mass (Braun et al., 2009). In contrast with formaldehyde the ethylene glycol increased the mass, and in that way caused concentration decreasing in the treated insect samples (Hendrickx et al., 2003). Concentration of glycol in trapping fluid may change in field conditions by evaporation of water. The rate of evaporation depends on the concentration of trapping fluids which increasing the body mass and biasing the results of elemental analysis of insects. Effects of different diluted trapping fluids on the body mass should study in the future.

4.4. Frog skeleton elemental analysis

There was no significant difference between the five and eight days long hydrogen peroxide cleaning for the studied elemental concentrations except the manganese. In the case of manganese content we found weak significant decrease in the concentration (Table 4.4.1). Thus, we recommend using the five days cleaning for the large bones. In the cases of phalanges two days was always sufficient for the hydrogen peroxide cleaning.

Table 4.4.1. Effect of hydrogen-peroxide cleaning on elemental contents of frog femur (mean ± SE, mg bone^{-1}) according to two different periods (5 and 8 days).

Elements	5 days	8 days	*P*
Ba	0.004 ± 0.001	0.004 ± 0.001	n.s.
Ca	15.2 ± 1.8	16.8 ± 2.2	n.s.
Mg	0.18 ± 0.02	0.19 ± 0.03	n.s.
Mn	0.004 ± 0.001	0.003 ± 0.001	*
Na	0.10 ± 0.03	0.11 ± 0.03	n.s.
P	5.8 ± 0.8	5.1 ± 0.7	n.s.
S	0.07 ± 0.01	0.07 ± 0.01	n.s.
Zn	0.014 ± 0.002	0.016 ± 0.002	n.s.

In the case of large bones we found strong correlation between the elemental contents and dry weights: P ($r^2 = 0.96$, $p < 0.001$, df = 9), Ca ($r^2 = 0.95$, $p < 0.001$, df = 9). Similar relationships were found in the cases of other elements: Mg ($r^2 = 0.97$, $p < 0.001$, df = 9), S ($r^2 = 0.91$, $p < 0.001$, df = 9), Na ($r^2 = 0.89$, $p < 0.001$, df = 9), Mn ($r^2 = 0.73$, $p < 0.01$, df = 9), Ba ($r^2 = 0.63$, $p < 0.01$, df = 9) and Zn ($r^2 = 0.57$, $p < 0.05$, df = 9). The

elemental concentrations of bones were significantly correlated with bones weight in the case of each element ($p < 0.05$). The significant relationships between the weight of bones and elemental contents mean that the elemental contents of bones were commensurable to their weights. Our result showed that strong correlation was between the main components of bone (P, Ca, Mg, S and Na) and weights. In the case of Ba and Zn the relatively lower correlation may resulted in by their smaller concentration in bones.

There was no significant difference between the elemental contents of phalanges from right and left hind limbs ($p>0.05$). In the case of phalanges, the following elemental contents were found: Ca: 0.4 ± 0.1 mg bone^{-1}, P: 0.3 ± 0.1 mg bone^{-1}, Mg: 4.0 ± 1.0 µg bones^{-1} and Zn: 0.4 ± 0.01 µg bones^{-1}. Because of the weights of phalanges were very small the following elements were not measurable with this technique: Ba, Mn, Na and S; i.e. the concentration of Ba (<0.006 mg L^{-1}), Mn (<0.001 mg L^{-1}), Na (<0.002 mg L^{-1}) and S (<0.01 mg L^{-1}) was below the detection limit.

The aim of our study was to work out a method by which the elemental contents of frog bones could be estimated effectively based on phalanges. We found that the elemental contents of large bones significantly correlated with bones weight. Thus, we can estimate the elemental contents of phalanges based on this correlation. Measured elemental contents of phalanges were compared to the estimated elemental contents based on large bones to test the usefulness of the method. The measured and the estimated elemental contents of phalanges were not different significantly based on the tibiafibula, metatarsalis bones and from and hind limb digits (Table 4.4.2). We found significant

differences for the skull, spinal, femur and humerus between the measured and estimated elemental contents.

Table 4.4.2. Significant differences between the measured and estimated elemental contents of phalanges by elemental contents of large bones. (digits I = from front limb, digits II = from hind limb).

	skull	spinal	femur	humerus	tibia-fibula	tarsals	metatarsus	digits I	digits II
Ca	n.s.	**	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.
Mg	n.s.	*	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
P	*	*	*	*	n.s.	*	n.s.	n.s.	n.s.
Zn	*	**	**	**	n.s.	*	n.s.	n.s.	n.s.

n.s., Not significant. $^{**}P < 0.01$; $^{*}P < 0.05$.

In the case of other large bones the weakly significant differences were caused by the high concentration of elements in the large bones. Our results indicated that the elemental contents of phalanges represented reliably the elemental contents of tibiafibula, metatarsalis bones and digits. Thus, elemental contents of these bones were represented by the smaller phalanges. Frogs by phalange elemental analysis may be useful indicator organism for environmental pollution assessment.

Our results show that the main elements were calcium, phosphorous and magnesium in the bones, similar to the study of Oudadesse et al. (2004). The concentration of sodium and barium were also present in the large bones similarly to others findings (Klepinger, 1984). The concentration of strontium was below detection limits in our study similarly to Alexander et al. (1955). Similar result was reported by other studies in the case of zinc contents in the large bones (Flyaks and Borkin, 2004). Pavel and Kucera (1986) studied the elemental contents in the body of *Rana esculenta* by atomic absorption spectrophotometry

(AAS). In contrast with their work, the concentrations of Cu, Cr and Pb were below detection limits in our study. They found that a few elemental contents (Fe and Mn) in frogs were significantly affected the ecological characteristics and the pollution of the localities. Puky and Oertel (1997) reported that *Rana esculenta* can accumulate metals in higher concentration than *Bombina bombina.*

With ionchromatography the following water compounds were analysed: F^-: 0.4 mg L^{-1}, Cl^-: 4.0 mg L^{-1}, NO_2^-: <0.01 mg L^{-1}, NO_3^-: 0.7 mg L^{-1}, PO_4^{3-}: <0.01 mg L^{-1}, SO_4^{2-}: 0.5 mg L^{-1}. The results of titration of water were the following: HCO_3^-: 119.8 mg L^{-1}, CO_3^{2-}: 117.8 mg L^{-1}. The following trace elements were analysed in the water: Ca: 32.8 mg L^{-1}, K: 1.5 mg L^{-1}, Mg: 18.7 mg L^{-1}, Na: 63.2 mg L^{-1}, Fe and Mn: < 0.05 mg L^{-1}, Zn: <0.001mg L^{-1}, Ba: 0.3 mg L^{-1} and Sr: 0.1mg L^{-1}. The Cd and Pb concentration in the water were below the detection limit (<0.001 mg L^{-1}) similarly to earlier studies (Stolyar et al., 2008). The pond was receiving thermal water, which resulted in the high Ba and Sr concentration (Wang et al., 2001). All the above water chemistry analyses indicated that the Frog Pond was rich in carbonates and hydrogen-carbonates.

In this study we demonstrated that the elemental contents of bones can be estimated using phalanges; thus, there is no need to kill specimens for extracting large bones (e.g. tibiafibula, metatarsalis bones and digits) for environmental quality assessment studies. The elemental analysis of phalanges had some advantages. The method may apply alive frog, the analytical analysis has small chemical reagent demand, and a high number of samples can be analysed by this method. In spite of the small size of the phalanges, the elemental concentrations can be measured reliably. Our result showed that the elemental analysis based on phalanges

add a further way of use of these bones for environmental quality assessment. Thus, frogs by their phalanges may be useful biological indicators of contamination in the pollution studies. With further technical development or using highly sensitive analytical technique (ICP-MS) the microelements (Ba and Mn) may also be measured in the phalanges.

4.5. Effects of urbanization on elemental contents of toe bones

In our study to the elemental content of bone samples was expressed toe bone per mg or μg elements to the analysis of bone elemental contents. The dry weights (mg, mean ± SE) of toe bones were 0.9 ± 0.1 from the urban pond (N = 11), 0.8 ± 0.1 (N = 21) and 1.9 ± 0.5 (N = 5) from the rural ponds, respectively. In the toe bones the calcium and phosphorus occurred in the highest amount (Fig. 4.5.1.). The percent rates of these elements of the rural and urban sites were the following: urban: Ca-20.5%, P-14.6%, rural: Ca-30.4% and 29.5%, P-22.4% and 21.7%.

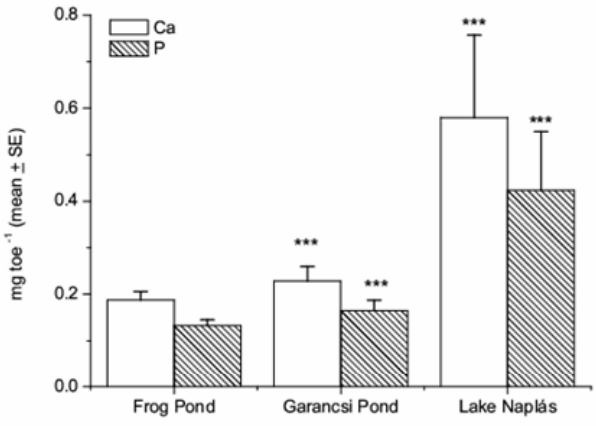

Fig.4.5.1. Calcium and phosphorus contents of *Bufo bufo* toe bones at urban (Frog Pond) and rural (Garancsi Pond and Lake Naplás) sites. Asterisks indicate significant differences ($p < 0.001$).

The microelement contents of toe bones were shown in Fig. 4.5.2. In the case of these elements the percent rate was small, urban: B-0.7%, Mg-0.3%, and Zn-0.06%, rural: B-1.1% and 0.4%, Mg-0.4%, and Zn-0.05%. Contrast with other studies the concentration of strontium, sodium and barium were below detection limits in the frog bones (Alexander et al. 1995; Busetto et al. 2008).

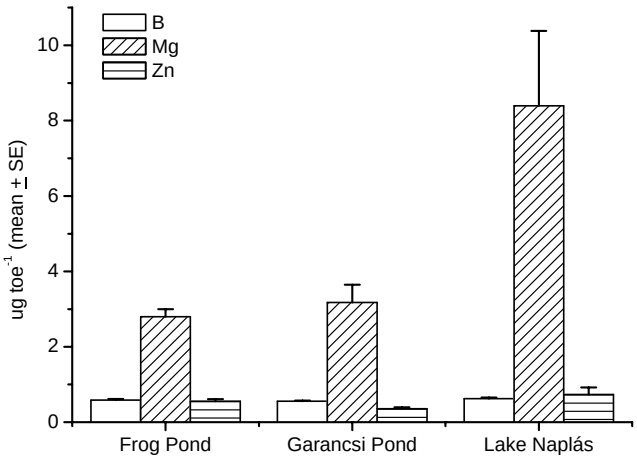

Fig.4.5.2. Microelement contents Bufo bufo at urban (Frog Pond) and rural (Garancsi Pond and Lake Naplás) sites.

Based on concentration of elements the canonical discriminant analysis was indicated significant differences ($p < 0.001$) in the first discriminant function. Biplot of discriminant scores shows the separation of urban and two rural sites based on the element concentrations of toe bones (Fig. 4.5.3.). The frogs of the urban pond were separated completely. There were 91.7% of samples classified correctly.

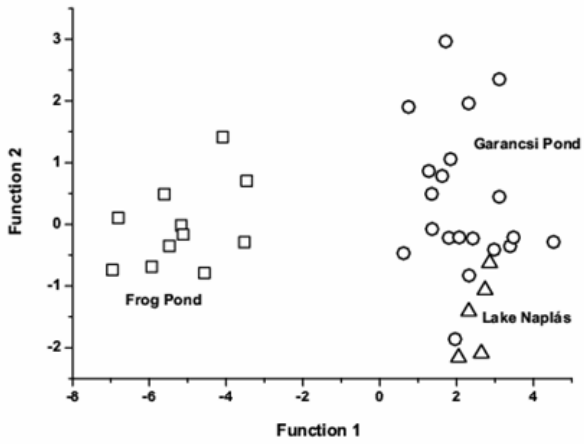

Fig.4.5.3. Canonical discriminant analysis of the urban and rural sites based on element concentrations in *Bufo bufo* toe bones (μg g^{-1} dry weight).

The summary statistics of analysis are given in Appendix Table 14. A significant positive correlation was found between the first discriminant function and the measured elements (Ca, P). This correlation indicated that these element concentrations were significantly higher in the rural sites. In the case of B and Zn the correlation were positive with the second functions so these elements were smaller in toe bones in the rural sites, than urban ones. The Mg concentration was negatively correlated with the second functions.

The concentration of calcium, phosphorus and magnesium was significantly higher in the toe bones from the rural sites ($p<0.001$) but no significant differences were found between these sites ($p>0.05$). On the other hand, the concentration of boron markedly differed only between the rural ponds ($p<0.05$). The zinc concentration was the highest at the urban site which significantly differed from only one of the rural sites

($p<0.05$). Detailed results of the statistical analysis were shown in Appendix Table 15.

Similarly to an earlier study (Oudadesse et al. 2004), our results show that the main elements are calcium, phosphorous and magnesium in the bones. Smaller calcium and phosphorus concentrations cause smaller hydoxyapatite content in the bones. Although, physiological differences were not found in the toe bones, the decreasing hydroxiapatite content may cause more porous structure in the bones (Janus et al. 2008). In contrast with other analyses (Klepinger 1984; Alexander et al. 1995; Busetto et al. 2008), the concentration of strontium, sodium and barium were below the detection limits in our study.

Zinc is the one of the microelements which is retained in terrestrial metamorphosis (Unrine et al. 2007), as such, zinc may also be monitored in adult amphibians. Our results show significantly higher zinc concentration at the urban site than at one of the rural sites. It is in agreement with the literature, similar differences were also reported by Flyaks et al. (2004), Simon et al. (2007) and Stolyar et al. (2008), while Pavel and Kucera (1986) did not find much difference in the concentration of zinc in the body of *Rana esculenta* at urban and rural sites.

Our results show that anthropogenic effects cause differences in the elemental contents of *B. bufo* toe bones. Although physiological differences and morphological deformities were not found in the toe bones, the concentrations of the main elements in the bone were markedly lower at the urban site than at the rural ones. In spite of the small size of the toe bones, the element concentrations may be measured reliably.

Thus, toad toe bones may be useful biological indicators for the assessment of contamination in pollution studies.

Summary

The main aim of my dissertation was to develop method for analysis of small amount of animal samples which is suitable for individual analysis of insects and analysis of toe bones of amphibians. Elemental composition of insects and the toe bone of anurans were determined in population level which gives a more detailed study of environmental loads. In my dissertation the main scientific results are the following:

Measured parameters of ICP-OES-USN were optimized for insect sample analysis. The robust plasma parameters were determined based on ratio of Mg II 280.270 nm / Mg I 285.213. Selection of spectral lines was based on intensities, limit of detections and results of model solution and standard addition experiments. Therefore, 36 spectral lines of 16 elements are recommended for analysis of insect samples. In the cases of other lines of elements, their limit of detections and their tolerance of interference were higher. This method is suitable for small weight insect samples and it provides a new opportunity studying variability of elemental contents of insects in population level in environmental studies.

Based on our result trapping and conservation fluids were significant effect on elemental composition of insect samples. These fluids changed the wet and dry body mass therefore they biased the determination of elemental concentrations. On the other hand impurities of the common anti-freeze solution were enriched in insect tissues.

Similar effect on body mass was found, when high and low quality ethylene glycols were used as trapping fluid. The analytical, puriss and technical glycols did not contain measurable impurities.

Micro analytical methods give a new opportunity using anurans as bioindicators. A small toe bones provide appropriate amount of sample estimating elemental composition of bones. Our results showed that elemental composition of toe bones correlated by large part of skeleton .

Anthropogenic activity of urbanized areas has effect on the elemental composition of toad toe bones. Concentration of major elements Ca, P, Mg was higher in the toe bones in rural sites, while concentration of Zn was the highest in urban sites.

The developed method is appropriate for elemental analysis of small weight invertebrates and small body parts of vertebrates. In the cases of invertebrates the live trapping collection and storage under freezing conditions could be a more reliable method for quantitative analytical studies. Analysis of anurans toe bones is a new fault-free method which could apply without serious damage of living frogs. It may open further ways in environmental load assessments.

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Appendix

Table 1. Different Mg II 280.270 nm / Mg I 285.213 ratio measured detection limits.

Parameters	**A**	**B**	**C**	**D**	Parameters	**A**	**B**	**C**	**D**
Energy (W)	1250	1150	1000	900	Energy (W)	1250	1150	1000	900
Gas (lpm)	0.65	0.77	0.93	1.05	Gas (lpm)	0.65	0.77	0.93	1.05
Mg II /Mg I	~10	~8.5	~5.5	~4	Mg II / Mg I	~10	~8.5	~5.5	~4
Wavelenghts/nm		LoD µg L^{-1}			Wavelenghts/nm		LoD µg L^{-1}		
Ba230.424{145}	0.45	0.43	1.64	1.58	K766.491{44}	1.44	0.72	0.48	0.19
Ba230.424{146}	0.34	0.31	0.3	1.66	Li670.784{50}	0.05	0.06	0.03	0.01
Ba233.527{143}	0.26	0.3	0.74	1.4	Mg279.079{120}	1.94	2.41	1.56	3
Ba233.527{144}	0.42	1.07	1.64	2.01	Mg285.213{117}	0.09	0.11	0.25	0.11
Ba455.403{74}	0.07	0.13	0.03	0.03	Mg285.213{118}	0.19	0.16	0.14	0.19
Ca183.801{182}	0.43	0.51	1.61	3.32	Mn257.6610{130}	0.1	0.1	0.04	0.16
Ca183.801{183}	0.95	2.33	1.98	6.7	Mn259.3373{129}	0.06	0.08	0.2	0.14
Ca184.066{182}	0.55	0.3	0.75	1.82	Mn260.569{128}	0.11	0.23	0.38	0.9
Ca315.887{106}	0.29	0.56	1.67	4.24	Mn260.569{129}	0.07	0.08	0.22	0.76
Ca317.933{105}	0.17	0.08	0.33	1.23	Mn293.306{114}	0.59	0.58	0.83	1.37
Cd214.438{156}	0.06	0.06	0.08	0.34	Mn293.931{114}	0.11	0.45	0.32	0.6
Cd214.438{157}	0.05	0.06	0.18	0.29	Mn294.921{114}	0.09	0.22	0.31	0.45
Cd226.502{148}	0.1	0.1	0.32	0.45	Na588.995{57}	0.25	0.15	0.13	0.14
Cd228.802{146}	0.15	0.21	0.14	0.44	Na589.592{57}	0.21	0.29	0.18	0.11
Cd228.802{147}	0.03	0.1	0.12	0.11	Ni216.556{155}	0.09	0.07	0.34	0.27
Co228.616{146}	0.9	1.12	2.18	3.76	Ni218.461{154}	0.46	0.6	1	2.07
Co228.616{147}	0.23	0.19	0.61	0.89	Ni221.647{151}	0.04	0.17	0.29	1.03
Co230.786{145}	0.85	1.22	2.01	1.99	Ni231.604{145}	0.45	1.17	1.7	2.98
Co237.862{141}	1.22	1.27	1.76	6.19	Pb216.999{154}	6.22	3.62	2.09	5.29
Cr206.149{163}	0.08	0.08	0.25	0.31	Pb216.999{155}	1.38	0.51	0.83	1.27
Cr267.716{125}	0.46	0.54	0.38	1.43	Pb220.353{152}	0.42	0.76	0.51	0.85
Cr283.563{118}	0.22	0.28	0.28	1.07	Pb220.353{153}	1.09	1.31	2.01	4.88
Cu219.958{152}	0.81	0.78	0.26	1.24	Sr346.446{97}	0.4	0.33	0.43	1.92
Cu219.958{153}	0.83	0.42	0.41	0.43	Sr407.77{82}	0.06	0.07	0.02	0.02
Cu221.458{152}	2.25	0.73	1.97	4.09	Y_224.306{150}	0.12	0.23	0.45	1.26
Cu221.458{153}	1.01	1.04	1.43	0.87	Y_242.220{138}	1.32	1.72	1.9	4.92
Cu224.700{149}	0.34	0.21	0.62	0.81	Y_332.789{101}	0.16	0.18	0.13	0.22
Cu224.700{150}	0.45	0.34	0.79	0.75	Y_437.494{77}	0.09	0.08	0.08	0.11
Cu324.754{103}	0.17	0.11	0.22	0.33	Y_488.369{69}	0.26	0.25	0.09	0.16
Fe238.204{141}	0.64	1	1.54	2.38	Zn202.548{165}	0.77	0.16	0.34	0.7
Fe239.562{140}	0.58	0.62	0.67	1.55	Zn202.548{166}	0.12	0.07	0.08	0.35
Fe240.488{139}	1.21	0.69	1.39	1.93	Zn206.200{162}	0.1	0.1	1.45	2.64
Fe259.837{129}	0.61	0.87	0.95	0.62	Zn213.856{156}	0.27	0.09	0.14	0.64
Fe259.940{129}	0.53	0.41	1	0.52	Zn213.856{157}	0.11	0.08	0.01	0.06

Table 2. Intensity, state and spectral interferences of the analysed spectral lines. Noticed: n.d. means, spectral interference was not detected.

	Wavelengths/ order	Intensity	State	Spectral interferences
Ba	230.424 {145}	1.500.000	II	Ni230.300, Co230.397, Co230.418
	230.424 {146}	1.500.000	II	Ni230.300, Co230.397, Co230.418
	233.527 {143}	1.800.000	II	Fe233.280, Co230.599, Ni233.749
	233.527 {144}	1.800.000	II	Fe233.280, Co230.599, Ni233.749
	455.403 {74}	1.500.000	II	n.d.
Ca	183.801 {182}	120.000	II	n.d.
	183.801 {183}	120.000	II	n.d.
	184.066 {182}	180.000	II	n.d.
	315.887 {106}	180.000	II	Co315.878
	317.933 {105}	300.000	II	Mn317.850, Y317.941
Cd	214.438 {156}	1.000.000	II	n.d.
	214.438 {157}	1.000.000	II	n.d.
	226.502 {148}	1.500.000	II	n.d.
	228.802 {147}	2.000.000	I	Co228.616, As228.812, Ni228.998
Co	228.616 {146}	1.000.000	II	Co228.781, Cd228.802, As228.812
	228.616 {147}	1.000.000	II	n.d.
	230.786 {145}	600.000	II	Cd230.661,Co230.902
	237.862 {141}	700.000	II	Co237.722, Fe237.928, Co238.048, Fe238.076
Cr	206.149 {163}	400.000	II	Zn206.200
	267.716 {125}	1.000.000	II	Cr267.879
	283.563 {118}	1.500.000	II	Fe283.546, Mn283.631
Cu	219.958 {152}	500.000	I	Cu219.975
	219.958 {153}	500.000	I	Cu219.975
	221.458 {152}	150.000	I	Mn221.385, Ni221.647
	224.700 {149}	1.000.000	II	Ag224.641, Pb224.688
	224.700 {150}	1.000.000	II	Ag224.641, Pb224.688
	324.754 {103}	5.000.000	I	Co324.718, Mn324.852
Fe	238.204 {141}	1.800.000	II	Co238.048, Fe238.076, As238.118
	239.562 {140}	900.000	II	Co239.390, Ni239.452, Co239.739
	240.488 {139}	600.000	II	Fe240.443, Fe240.666, Co240.725
	259.837 {129}	600.000	II	Mn259.890, Fe259.940, Fe259.957
	259.940 {129}	2.000.000	II	Fe239.837, Mn259.890, Fe259.957

Table 2 continued

	Wavelengths/ order	Intensity	State	Spectral interferences
Ni	216.556 {155}	250.000	II	Cu216.509, As216.552, Fe216.677
	218.461 {154}	80.000	II	Cu216.509, As216.552, Fe216.677
	221.647 {151}	600.000	II	Cu221.458, Cu221.810
	231.604 {145}	600.000	II	Ni231.366, Co231.405, Co231.498
Pb	216.999 {154}	90.000	I	n.d.
	216.999 {155}	90.000	I	n.d.
	220.353 {152}	120.000	II	n.d.
	220.353 {153}	120.000	II	n.d.
Sr	346.446 {97}	400.000	II	Co346.280, Co346.580, Fe346.586
	407.77 {82}	30.000.000	II	Y407.738, Cr407768
Zn	202.548 {165}	1.000.000	II	Cu202.434, Mg202. 582
	202.548 {166}	1.000.000	II	Cu202.434, Mg202. 582
	206.200 {162}	900.000	II	n.d.
	213.856 {156}	3.000.000	I	Cu213.853
	213.856 {157}	3.000.000	I	Cu213.853
Na	588.995 {57}	900.000	I	Co589.048
Na	589.592{57}	500.000	I	Y589.394
Li	670.784{50}	12000000	I	n.d
K	766.491{44}	900.000	I	n.d.

Table 3**.** Selected spectral lines to insect samples analysis.

Wavelenghts/nm	**Ba**: 230.424{145}. 230.424{146}. 233.527{144}
	Ca: 183.801{182}.183.801{183}. 315.887{106}
	Cd: 214.438{156}. 228.802{147}
	Co: 228.616{146}. 228.616{147}. 230.786{145}
	Cr: 206.149{163}. 283. 563{118}
	Cu 224.700{149} 224.700{150} 324.754 {103}
	Fe: 238.204{141}. 239.562{140}. 240.488{139}
	K: 766.491{44}
	Li: 670.784{50}
	Mg: 279.079{120}. 285.213{117}
	Mn: 257.610{130}. 260.569{128}. 294.921{114}
	Na: 588.995{57}
	Ni: 216.556{155}. 218.461{154}. 221.647{151}
	Pb: 216.999{154}. 220.353{153}
	Sr: 346.446{97}. 407.77{82}
	Zn: 202.548{166}. 213.856{157}

Table 4. Different elemental concentrations in insect samples.

		Species	Analysed elements							References
			Cd	Cu	Fe	Mn	Ni	Pb	Zn	
Terrestrial invertebrates	Ground beetles	*Agonum assimile*	9.2 ± 5.1	32.4 ± 7.8	59.6 ± 45.8	–	–	59.6 ± 45.8	140 ± 59	Straalen et al., 2001
		Agonum obscurum	2.3 ± 3.3	5.80 ± 8.20	438 ± 620	–	–	35.2 ± 48.5	78.8 ± 77.2	Straalen et al., 2001
		Pterostichus niger	5.2 ± 1.2	13.1 ± 1.5	160 ± 19	–	–	12.4 ± 4.5	126 ± 14	Straalen et al., 2001
		P. oblongopunctatus	7.4 ± 3.2	15.6 ± 2.7	210 ± 23	–	–	41.2 ± 32.9	210 ± 23	Straalen et al., 2001
			16.6 ± 6.0	–	–	–	–	–	8.64 ± 2.99	Mozdzer et al., 2003
			15.5 ± 6.9	–	–	–	–	–	3.87 ± 5.14	Mozdzer et al., 2003
			0.9 ± 0.4	–	–	–	–	–	78.2 ± 13.9	Lagisz et al., 2005
			5.8 ± 0.4	38.3 ± 3.5	–	–	–	6.5 ± 0.9	201 ± 11.8	Stone et al., 2002
			3.8 ± 1.9	43.6 ± 5.6	–	–	–	1.8 ± 0.6	112 ± 23.1	Stone et al., 2002
			10.8 ± 2.3	43.2 ± 10.8	–	–	–	1.9 ± 0.2	131 ± 13.9	Stone et al., 2002
			4.6 ± 0.7	38.1 ± 4.9	–	–	–	1.3 ± 0.4	114 ± 10.6	Stone et al., 2002
			1.3 ± 0.3	44.6 ± 3.9	–	–	–	0.2 ± 0.1	79 ± 7.6	Stone et al., 2002
			5.4 ± 0.4	46.4 ± 5.6	–	–	–	8.7 ± 3	130 ± 12	Stone et al., 2002
			3.6 ± 0.6	53.6 ± 3.0	–	–	–	1.4. ± 0.1	118 ± 14	Stone et al., 2002
			2.6 ± 0.6	45.8 ± 4.4	–	–	–	1.5 ± 0.2	86 ± 5.2	Stone et al., 2002
			2.3 ± 0.4	52.9 ± 4.2	–	–	–	0.8 ± 0.2	81 ± 2.3	Stone et al., 2002
			1.2 ± 0.3	39.3 ± 4.0	–	–	–	0.4 ± 0.1	86 ± 11.2	Stone et al., 2002
		Poecilus cupreus	57 ± 4	–	–	–	–	–	0.4 ± 0.1	Kramarz, 1999
		Carabus convexus	0.4 ± 0.4	21.6 ± 7.20	135 ± 64.7	36.4 ± 19.1	–	0.3 ± 0..1	120 ± 19.2	Jelaska et al., 2007
		Carabus coriaceus	3.3 ± 2.8	33.3 ± 23.8	158 ± 84.1	67.4 ± 28.8	–	0.3 ± 0.2	148 ± 63.5	Jelaska et al., 2007
		Carabus violaceus	4.9 ± 1.9	27.1 ± 6.8	220 ± 162	112 ± 154	–	0.3 ± 0.2	193 ± 25.6	Jelaska et al., 2007
		Harpalus rubripes	*58 ± 104*	12.2 ± 3.6	–	–	–	–	128.6 ± 8.2	Zödl & Wittmann, 2003
			49 ± 52	19.7 ± 9.0	–	–	–	–	117.4 ± 10.7	Zödl & Wittmann, 2003
		Calathus fuscipes	*205 ± 117*	25.4 ± 12.8	–	–	–	*140 ± 76*	127.5 ± 15.9	Zödl & Wittmann, 2003
			151 ± 212	19.3 ± 14.3	–	–	–	*201 ± 95*	131.2 ± 15.7	Zödl & Wittmann, 2003
	Ants	*Formica aquilonia*	10.1 ± 0.5	42.9 ± 2.9	–	–	8.8 ± 0.7	1.8 ± 0.2	550 ± 13.7	Eeva et al., 2004
			49.7 ± 8.7	20.5 ± 0.8	–	–	3.7 ± 0.2	1.0 ± 0.1	507 ± 15.7	Eeva et al., 2004
	Bugs	*Pyrrhocoris apterus*	–	17.5 ± 0.7	–	22.4 ± 1.4	–	–	15.7 ± 1.8	Braun et al., 2009

Table 5. Summary statistics of different elemental concentrations in model solution experiment.

Elements	Expected concentration μ L^{-1}	Average of measured concentration μ L^{-1}	t value	df	*P*
Ba	0.05	0.050 ± 0.004	0.000	2	1.000
	0.10	0.102 ± 0.006	0.569	2	0.626
	0.15	0.157 ± 0.011	1.096	2	0.387
	0.20	0.209 ± 0.015	1.058	2	0.401
	0.25	0.258 ± 0.019	0.727	2	0.543
Cd	0.05	0.046 ± 0.002	-1.667	1	0.344
	0.10	0.103 ± 0.005	0.714	1	0.605
	0.15	0.157 ± 0.008	1.182	1	0.447
	0.20	0.209 ± 0.008	1.462	1	0.382
	0.25	0.258 ± 0.013	0.889	1	0.537
Co	0.05	0.046 ± 0.003	-2.309	2	0.147
	0.10	0.100 ± 0.003	0.000	2	1.000
	0.15	0.154 ± 0.007	1.057	2	0.401
	0.20	0.204 ± 0.009	0.808	2	0.504
	0.25	0.250 ± 0.011	0..000	2	1.000
Cu	0.05	0.046 ± 0.002	-3.051	2	0.093
	0.10	0.103 ± 0.002	2.000	2	0.184
	0.15	0.151 ± 0.002	1.109	2	0.383
	0.20	0.207 ± 0.004	3.355	2	0.079
	0.25	0.255 ± 0.004	2.440	2	0.135
Fe	0.05	0.054 ± 0.002	3.250	2	0.083
	0.10	0.097 ± 0.002	-2.774	2	0.109
	0.15	0.145 ± 0.003	-3.200	2	0.085
	0.20	0.196 ± 0.003	-2.000	2	0.184
	0.25	0.248 ± 0.004	-0.961	2	0.438
Mn	0.05	0.044 ± 0.008	-1.205	2	0.351
	0.10	0.093 ± 0.017	-0.743	2	0.535
	0.15	0.140 ± 0.022	-0.800	2	0.508
	0.20	0.187 ± 0.025	-0.885	2	0.469
	0.25	0.230 ± 0.024	-1.490	2	0.275
Ni	0.05	0.049 ± 0.001	-4.000	2	0.057
	0.10	0.100 ± 0.001	1.000	2	0.423
	0.15	0.154 ± 0.004	3.000	2	0.058
	0.20	0.200 ± 0.001	1.000	2	0.423
	0.25	0.250 ± 0.001	1.000	2	0.423
Pb	0.05	0.054 ± 0.001	4.000	1	0.156
	0.10	0.113 ± 0.001	13.000	1	0.049
	0.15	0.151 ± 0.004	0.200	1	0.874
	0.20	0.198 ± 0.001	-5.000	1	0.126
	0.25	0.263 ± 0.001	13.000	1	0.049
Sr	0.05	0.047 ± 0.003	1.500	1	0.374
	0.10	0.100 ± 0.004	0.200	1	0.874
	0.15	0.153 ± 0.010	0.429	1	0.742
	0.20	0.203 ± 0.012	0.412	1	0.751
	0.25	0.248 ± 0.016	0.182	1	0.886
Zn	0.05	0.054 ± 0.001	7.000	1	0.090
	0.10	0.114 ± 0.002	9.000	1	0.070
	0.15	0.161 ± 0.003	5.500	1	0.114
	0.20	0.206 ± 0.003	3.000	1	0.205
	0.25	0.259 ± 0.003	4.500	1	0.139

Table 6. Summary statistics of elemental concentrations (dry mass μg g^{-1}) in firebugs expressed as the means and SE of five tubes. Different letters indicate significant differences ($p < 0.05$).

Elements	control	short trapping	long trapping	short trapping plus short preservation	long trapping plus long preservation
Ca	2170 ± 160^{a}	3450 ± 270^{b}	3770 ± 250^{b}	3960 ± 560^{b}	5140 ± 430^{b}
Cu	17.5 ± 0.7^{a}	16.2 ± 1.9^{a}	$15.1 \pm 1.3^{a,b}$	$16.4 \pm 1.2^{a,b}$	$25.9 \pm 3.1^{a,c}$
K	2810 ± 50^{a}	770 ± 190^{b}	500 ± 40^{b}	430 ± 100^{b}	480 ± 60^{b}
Mg	1810 ± 110^{a}	1460 ± 180^{a}	1240 ± 50^{a}	1700 ± 200^{a}	1520 ± 110^{a}
Mn	22.4 ± 1.4^{a}	25.3 ± 2.8^{a}	27.3 ± 3.4^{a}	31.6 ± 3.8^{a}	44.1 ± 7.9^{b}
Na	540 ± 20^{a}	2090 ± 250^{b}	1920 ± 180^{b}	1770 ± 340^{b}	1840 ± 210^{b}
Sr	8.1 ± 0.5^{a}	16.0 ± 1.1^{b}	16.9 ± 1.3^{b}	16.1 ± 1.6^{b}	20.4 ± 1.1^{b}
Zn	15.7 ± 1.8^{a}	19.8 ± 3.8^{a}	21.1 ± 2.3^{a}	17.4 ± 1.6^{a}	28.0 ± 3.7^{a}

Note: Different superscript letters indicate significant differences between the treatments.

Table 7. Results of One-Way ANOVA for each elemental concentration to test for differences between the treatments.

		Sum of Squares	Df	Mean Square	F	P
Ca	Between Groups	1.9	4	0.5	12.59	< 0.001
	Within Groups	0.8	20	0.04		
	Total	2.7	24			
Cu	Between Groups	0.9	4	0.2	5.25	0.005
	Within Groups	0.9	20	0.04		
	Total	1.7	24			
K	Between Groups	12.9	4	3.2	22.78	< 0.001
	Within Groups	2.8	20	0.1		
	Total	15.8	24			
Mg	Between Groups	0.4	4	0.1	2.23	0.102
	Within Groups	0.9	20	0.05		
	Total	1.4	24			
Mn	Between Groups	1.1	4	0.3	3.70	0.021
	Within Groups	1.5	20	0.1		
	Total	2.6	24			
Na	Between Groups	6.2	4	1.5	18.44	< 0.001
	Within Groups	1.7	20	0.1		
	Total	7.9	24			
Sr	Between Groups	2.1	4	0.5	22.98	< 0.001
	Within Groups	0.5	20	0.02		
	Total	2.6	24			
Zn	Between Groups	0.6	4	0.2	2.16	0.111
	Within Groups	1.4	20	0.1		
	Total	2.0	24			

Table 8. Summary statistics of discriminant analysis. Significant pooled within-groups correlations between variables and standardized canonical discriminant functions are in bold face.

	Discriminant functions			
	I	**II**	**III**	**IV**
Eigenevalue	22.9	2.8	1.3	0.2
Percentage of Variance	84.1	10.3	4.9	0.6
Cumulative Percentage	84.1	94.5	99.4	100.0
Canonical Correlation	1.0	0.9	0.8	0.4
Wilks' Lambda	0.00	0.10	0.37	0.86
Chi-square	96.6	41.0	17.6	2.7
df	32	21	12	5
Significance	<0.001	<0.01	0.128	0.742
Elements of Structure Matrix				
K	**-0.456**	0.403	0.287	0.046
Ca	0.322	**-0.529**	0.487	-0.222
Mn	0.092	**-0.443**	0.214	-0.143
Cu	-0.012	**-0.494**	0.545	0.229
Sr	0.443	-0.392	**0.533**	-0.040
Zn	0.074	-0.251	**0.395**	-0.320
Na	0.365	0.108	0.278	**0.383**
Mg	-0.090	-0.043	-0.105	**0.254**

Table 9. The concentrations (mean ± SE) of various elements in different grades of ethylene glycol before and after two-week and one-month treatments.

		Treatments	Ca, mg L^{-1}	Fe, mg L^{-1}	K, mg L^{-1}	Mg, mg L^{-1}	Mn, mg L^{-1}	Na, mg L^{-1}	Zn, mg L^{-1}
Quality of trapping fluids	analytical	initial	0.45 ± 0.09^{a}	0.19 ± 0.06	0.12 ± 0.06^{a}	0.06 ± 0.01^{a}	0.003 ± 0.001	0.7 ± 0.1^{a}	0.31 ± 0.03
		two-week	1.37 ± 0.29^{ab}	0.15 ± 0.02	8.49 ± 1.32^{b}	0.95 ± 0.28^{ab}	0.009 ± 0.001	2.9 ± 0.4^{b}	0.31 ± 0.03
		one-month	2.36 ± 0.36^{b}	0.28 ± 0.03	13.44 ± 1.33^{c}	1.80 ± 0.21^{b}	0.015 ± 0.002	4.0 ± 0.2^{b}	0.37 ± 0.03
	puriss	initial	0.32 ± 0.02^{a}	0.06 ± 0.01	0.16 ± 0.03^{a}	0.07 ± 0.01^{a}	0.001 ± 0.001	0.5 ± 0.03^{a}	0.05 ± 0.002
		two-week	1.06 ± 0.08^{ab}	0.16 ± 0.04	9.01 ± 1.26^{b}	0.75 ± 0.06^{ab}	0.008 ± 0.001	2.8 ± 0.4^{b}	0.16 ± 0.05
		one-month	1.69 ± 0.23^{b}	0.15 ± 0.03	12.93 ± 1.06^{b}	1.36 ± 0.15^{a}	0.009 ± 0.001	3.9 ± 0.2^{b}	0.20 ± 0.02
	technical	initial	0.59 ± 0.20	0.06 ± 0.01	0.24 ± 0.06^{a}	0.17 ± 0.01	0.006 ± 0.003	0.09 ± 0.1^{a}	0.04 ± 0.02
		two-week	1.15 ± 0.14	0.49 ± 0.20	10.25 ± 1.11^{b}	0.93 ± 0.17	0.014 ± 0.002	3.6 ± 0.4^{b}	0.10 ± 0.01
		one-month	1.12 ± 0.09	0.29 ± 0.04	11.28 ± 0.96^{b}	1.16 ± 0.22	0.012 ± 0.001	3.7 ± 0.2^{b}	0.13 ± 0.01
	common anti-freeze	initial	15.81 ± 1.32	1.33 ± 0.17	197 ± 6	3.02 ± 0.09^{a}	0.024 ± 0.002	717 ± 43	1.18 ± 0.18
		two-week	9.99 ± 0.94	1.90 ± 0.86	190 ± 14	1.43 ± 0.23^{ab}	0.027 ± 0.010	691 ± 35	1.05 ± 0.13
		one-month	16.92 ± 4.02	1.99 ± 0.60	232 ± 7	4.62 ± 1.67^{ac}	0.038 ± 0.006	740 ± 8	1.56 ± 0.35

Note: Different superscript letters indicate significant differences between the treatments. Homogenise groups are indicated by the same letter (e.g. 0.45 $\pm$ 0.09a and 1.37 $\pm$ 0.29a,b means that this averages did not differ significantly and latter did not differ of 2.36 $\pm$ 0.36b).

Table 10. Summary statistics (mean ± SE) of element concentrations (different reference base mass, mg kg^{-1}) in firebugs according to different treatments. (A = elemental concentration reported per mg initial wet weight before treatments, B = elemental concentration reported per mg measured dry weight, C = elemental concentration reported per mg calculated dry weight).

		Treatments	Reference base	Ba mg kg^{-1}	Ca mg kg^{-1}	Cu mg kg^{-1}	Fe mg kg^{-1}	K mg kg^{-1}	Mg mg kg^{-1}	Mn mg kg^{-1}	Na mg kg^{-1}	Sr mg kg^{-1}	Zn mg kg^{-1}
Quality of trapping fluids		control	A	1.8 ± 0.1	746 ± 39	9.3 ± 0.2	98 ± 8	875 ± 72	508 ± 26	19.8 ± 1.2	266 ± 16	4.5 ± 0.2	70 ± 3
			B	4.6 ± 0.3	1879 ± 57	23.5 ± 0.5	246 ± 14	2207 ± 166	1282 ± 57	50.1 ± 3.2	671 ± 38	11.5 ± 0.4	177 ± 8
	analytical	two-week	A	1.3 ± 0.1	567 ± 46	7.4 ± 0.2	60 ± 7	684 ± 42	435 ± 16	15.2 ± 1.5	200 ± 9	3.9 ± 0.2	55 ± 3
			B	2.0 ± 0.2	887 ± 87	11.5 ± 0.7	93 ±10	1070 ± 87	678 ± 40	23.8 ± 3.1	312 ± 20	6.0 ± 0.4	87 ± 8
			C	3.2 ± 0.3	1431 ± 116	18.6 ± 0.5	151 ± 17	1726 ± 107	1097 ± 40	38.2 ± 3.8	504 ± 23	9.7 ± 0.5	139 ± 8
		one-month	A	1.8 ± 0.4	746 ± 69	7.8 ± 0.3	88 ± 28	649 ± 33	461 ± 26	17.8 ± 2.0	180 ± 9	4.4 ± 0.3	62 ± 5
			B	2.6 ± 0.5	943 ± 108	11.7 ± 1.2	126 ± 36	964 ± 100	679 ± 52	26.1 ± 2.8	267 ± 26	6.5 ± 0.5	91 ± 10
			C	4.4 ± 0.9	1620 ± 173	19.8 ± 0.7	222 ± 70	1636 ± 84	1163 ± 67	44.9 ± 5.2	454 ± 23	11.1 ± 0.7	155 ± 13
	puriss	two-week	A	1.2 ± 0.1	530 ± 38	10.8 ± 2.7	51 ± 2	653 ± 35	421 ± 12	15.1 ± 0.4	190 ± 8	3.8 ± 0.2	54 ± 3
			B	1.9 ± 0.1	862 ± 87	17.2 ± 3.8	82 ± 4	1048 ± 48	679 ± 31	24.3 ± 1.2	305 ± 12	6.2 ± 0.5	87 ± 5
			C	3.0 ± 0.1	1338 ± 97	27.3 ± 6.8	128 ± 5	1646 ± 88	1062 ± 30	38.0 ± 1.0	479 ± 20	9.7 ± 0.4	136 ± 9
		one-month	A	1.4 ± 0.1	609 ± 26	7.2 ± 0.4	57 ± 5	574 ± 42	414 ± 20	15.8 ± 0.9	174 ± 8	4.0 ± 0.1	57 ± 5
			B	2.2 ± 0.2	923 ± 68	11.0 ± 0.8	86 ± 9	867 ± 71	626 ± 47	24.0 ± 2.1	263 ± 18	6.1 ± 0.4	85 ± 7
			C	3.6 ± 0.2	1537 ± 67	18.3 ± 1.0	143 ± 12	1449 ± 106	1043 ± 51	39.8 ± 2.4	439 ± 21	10.2 ± 0.2	143 ± 12
	technical	two-week	A	1.4 ± 0.1	541 ± 43	7.4 ± 0.2	69 ± 19	580 ± 38	388 ± 11	15.4 ± 0.6	186 ± 6	4.1 ± 0.3	49 ± 2
			B	2.4 ± 0.2	953 ± 86	13.0 ± 0.7	119 ± 29	1018 ± 68	682 ± 34	27.0 ± 1.4	327 ± 18	7.1 ± 0.4	86 ± 5
			C	3.4 ± 0.4	1364 ± 108	18.6 ± 0.6	174 ± 47	1462 ± 96	977 ± 27	38.8 ± 1.6	469 ± 16	10.3 ± 0.7	123 ± 6
		one-month	A	1.4 ± 0.1	605 ± 20	7.5 ± 0.3	56 ± 4	593 ± 41	416 ± 15	14.4 ± 0.5	181 ± 8	4.1 ± 0.2	52 ± 2
			B	2.0 ± 0.1	878 ± 47	10.9 ± 0.6	81 ± 8	858 ± 61	604 ± 32	21.0 ± 1.2	262 ± 14	5.9 ± 0.4	75 ± 4
			C	3.5 ± 0.2	1526 ± 51	19.0 ± 0.8	141 ± 11	1495 ± 102	1049 ± 38	36.4 ± 1.2	456 ± 20	10.3 ± 0.4	130 ± 5
	common anti-freeze	two-week	A	1.3 ± 0.1	626 ± 13	7.6 ± 0.1	60 ± 3	694 ± 57	463 ± 23	15.8 ± 1.2	149 ± 27	4.0 ± 0.1	58 ± 5
			B	2.4 ± 0.2	1163 ± 25	14.2 ± 0.2	111 ± 5	1284 ± 89	857 ± 33	29.2 ± 2.1	279 ± 54	7.4 ± 0.1	107 ± 9
			C	3.3 ± 0.3	1580 ± 33	19.2 ± 0.4	150 ± 8	1751 ± 143	1167 ± 59	39.8 ± 3.0	376 ± 67	10.0 ± 0.3	146 ± 14
		one-month	A	1.8 ± 0.3	718 ± 118	7.6 ± 0.6	82 ± 19	588 ± 66	481 ± 79	16.8 ± 1.7	168 ± 22	4.7 ± 0.5	61 ± 7
			B	2.8 ± 0.3	1128 ± 105	12.3 ± 1.1	127 ± 19	935 ± 56	756 ± 71	26.7 ± 1.3	275 ± 26	7.5 ± 0.3	97 ± 6
			C	4.6 ± 0.9	1811 ± 297	19.2 ± 1.5	207 ± 47	1482 ± 166	1214 ± 200	42.3 ± 4.3	441 ± 53	12.0 ± 1.3	154 ± 18

Table 11. Results of univariate ANOVA. Elemental concentration reported mg per initial wet mass before the treatments.

		Sum of Squares	d*f*	Mean Square	*F*	*P*
Ba	Between Groups	0.3	8	0.04	1.89	0.199
	Within Groups	0.8	36	0.02		
	Total	1.2	44			
Ca	Between Groups	0.5	8	0.06	1.95	0.093
	Within Groups	1.2	36	0.03		
	Total	1.7	44			
Cu	Between Groups	0.4	8	0.05	2.04	0.082
	Within Groups	0.9	36	0.03		
	Total	1.3	44			
Fe	Between Groups	1.6	8	0.20	2.08	0.069
	Within Groups	3.5	36	0.10		
	Total	5.1	44			
K	Between Groups	0.7	8	0.09	3.25	< 0.01
	Within Groups	1.0	36	0.03		
	Total	1.7	44			
Mg	Between Groups	0.3	8	0.03	1.66	0.141
	Within Groups	0.7	36	0.02		
	Total	0.9	44			
Mn	Between Groups	0.3	8	0.04	1.69	0.136
	Within Groups	0.9	36	0.02		
	Total	1.2	44			
Na	Between Groups	1.2	8	0.15	3.86	< 0.01
	Within Groups	1.4	36	0.04		
	Total	2.5	44			
Sr	Between Groups	0.1	8	0.02	1.56	0.170
	Within Groups	0.4	36	0.01		
	Total	0.5	44			
Zn	Between Groups	0.4	8	0.05	2.10	0.061
	Within Groups	0.9	36	0.03		
	Total	1.4	44			

Table 12. Results of univariate ANOVA. Elemental concentration reported mg per calculated dry body mass.

		Sum of Squares	d*f*	Mean Square	*F*	*P*
Ba	Between Groups	0.6	8	0.07	1.98	0.191
	Within Groups	1.3	36	0.04		
	Total	1.9	44			
Ca	Between Groups	0.5	8	0.06	2.02	0.078
	Within Groups	1.2	36	0.03		
	Total	1.7	44			
Cu	Between Groups	0.5	8	0.06	2.07	0.072
	Within Groups	1.0	36	0.03		
	Total	1.5	44			
Fe	Between Groups	1.7	8	0.21	2.13	0.066
	Within Groups	3.5	36	0.10		
	Total	5.2	44			
K	Between Groups	0.7	8	0.09	3.35	< 0.01
	Within Groups	1.0	36	0.03		
	Total	1.7	44			
Mg	Between Groups	0.3	8	0.03	1.72	0.127
	Within Groups	0.7	36	0.02		
	Total	0.9	44			
Mn	Between Groups	0.4	8	0.04	1.68	0.137
	Within Groups	1.0	36	0.03		
	Total	1.3	44			
Na	Between Groups	1.1	8	0.14	3.85	< 0.01
	Within Groups	1.3	36	0.04		
	Total	2.5	44			
Sr	Between Groups	0.2	8	0.02	1.67	0.140
	Within Groups	0.5	36	0.01		
	Total	0.6	44			
Zn	Between Groups	0.4	8	0.06	2.08	0.064
	Within Groups	1.0	36	0.03		
	Total	1.4	44			

Table 13. Results of univariate ANOVA. Elemental concentration reported mg per measured dry body mass.

		Sum of Squares	*df*	Mean Square	*F*	*P*
Ba	Between Groups	1.6	8	0.20	8.27	< 0.001
	Within Groups	0.9	36	0.02		
	Total	2.5	44			
Ca	Between Groups	2.6	8	0.32	9.21	< 0.001
	Within Groups	1.2	36	0.03		
	Total	3.8	44			
Cu	Between Groups	2.2	8	0.27	8.17	< 0.001
	Within Groups	1.2	36	0.03		
	Total	3.4	44			
Fe	Between Groups	4.7	8	0.58	7.00	< 0.001
	Within Groups	3.0	36	0.08		
	Total	7.7	44			
K	Between Groups	3.4	8	0.42	14.81	< 0.001
	Within Groups	1.0	36	0.03		
	Total	4.4	44			
Mg	Between Groups	2.1	8	0.27	14.32	< 0.001
	Within Groups	0.7	36	0.02		
	Total	2.8	44			
Mn	Between Groups	2.4	8	0.29	9.98	< 0.001
	Within Groups	1.1	36	0.03		
	Total	3.4	44			
Na	Between Groups	3.6	8	0.45	10.41	< 0.001
	Within Groups	1.6	36	0.04		
	Total	5.2	44			
Sr	Between Groups	1.4	8	0.17	13.18	< 0.001
	Within Groups	0.5	36	0.01		
	Total	1.9	44			
Zn	Between Groups	2.5	8	0.31	11.55	< 0.001
	Within Groups	1.0	36	0.03		
	Total	3.5	44			

Table 14. Summary of canonical discriminant functions for element concentrations in the toe bones of frogs (μg g^{-1} dry weight). Largest absolute correlation between each variable and any discriminant function are marked with bold letters.

	Discriminant functions	
	I	**II**
Eigenevalue	12.8	0.4
Percentage of Variance	96.8	3.2
Cumulative Percentage	96.8	100.0
Canonical Correlation	1.0	0.5
Wilks' Lambda	0.05	0.71
Chi-square	90.6	10.6
df	12	5
Significance	p<0.001	0.059
Elements of Structure Matrix		
P	**0.575**	0,306
Ca	**0.518**	0,338
B	0,020	**0.841**
Zn	-0,144	**0.707**
Mg	0,146	**-0.214**

Table 15. Results of analysis of variance (ANOVA) of elements concentrations in toe bones (μg g^{-1} dry weight).

Elements		Sum of Squares	df	Mean Square	F	Sig.
B	Between Groups	3.419	2	1.710	4.947	*
	Within Groups	11.404	33	0.346		
	Total	14.823	35			
Ca	Between Groups	1.220	2	0.610	57.448	***
	Within Groups	0.350	33	0.011		
	Total	1.571	35			
Mg	Between Groups	0.545	2	0.273	4.829	*
	Within Groups	1.863	33	0.056		
	Total	2.409	35			
P	Between Groups	1.378	2	0.689	70.236	***
	Within Groups	0.324	33	0.010		
	Total	1.701	35			
Zn	Between Groups	0.787	2	0.393	7.799	**
	Within Groups	1.664	33	0.050		
	Total	2.451	35			

n.s., Not significant, ***P<0.001; **P<0.01; P<0.05.