



The effects of ethylene glycol and ethanol on the body mass and elemental composition of insects collected with pitfall traps

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ABSTRACT

Insects often used as accumulation indicators of hazardous elements. Pitfall traps with ethylene glycol as trapping fluid are frequently used to collect insects. We studied the effect of glycol and preservation with ethanol on the elemental composition of hand collected firebugs. Control samples were stored in a freezer and the following treatments were used: insect kept in trapping fluid for 2 weeks, and for 1 month, trapping fluid for 2 weeks plus 2 weeks in ethanol, and trapping fluid for 1 month plus ethanol for 1 month. Insects kept in trapping fluid gained mass with respect to control: 26% for the short trapping and 37% for the long trapping. Preservation in ethanol reversed this effect in each case. Trapping fluid did not alter the dry mass. A significant loss in dry mass only occurred in the long trapping plus long preservation treatment. We analysed the concentration of eight elements: Ca, Cu, K, Mg, Mn, Na, Sr and Zn. We found significant difference in the concentrations of elements among the four treatments in the case of all elements, except magnesium and zinc. Our results indicate the potential of both certain trapping fluids as well as preservation in ethanol influencing the concentration of certain elements in insects. Live trapping for collection and storage in under freezing conditions for preservation could be a more reliable method if quantitative analytical studies are to be performed, when invertebrates are used as indicators of the presence and concentrations of hazardous substances in the environment.

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1. Introduction

Insects play an important role in most terrestrial environments owing to their great abundance, biomass and diversity (Zödl and Wittmann, 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 Wittmann, 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).

Different methods are used to collect terrestrial invertebrates. Hand collecting is the most appropriate way to collect animals for elemental analysis, but this is not always effective nor representative (Leather, 2005). 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). The common pitfall trap is typically a small plastic or glass container, which is placed in a hole with the upper rim flush with the ground surface and contains an amount of killing-preserving agent. 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 solution (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

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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).

The trapping efficiencies of different fluids (ethanol, ethylene and propylene glycol, anti-freezes and formalin) can have an impact on the attractiveness, retention efficiency, and preservative effect on the catch (Holopainen, 1992; Weeks and McIntyre, 1997; Lemieux and Lindgren, 1999; Koivula et al., 2003). However, the effects of filling fluid on the elemental composition of sampled insect material is less known.

In ecological studies, the most common preservative solutions 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 solutions 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). In a few studies, insects used in elemental analysis were killed by freezing and kept frozen (Gräff et al., 1997; Eeva et al., 2004; Jelaska et al., 2007) because this preservation method is expected to lead to the most reliable analytical results. In some studies pitfall trapped animals were used for elemental analysis (Laskowski and Maryanski, 1993; Roth, 1993; Knutti et al., 1988).

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. In this study, we compare the effects of commercially available anti-freeze (a pitfall trapping fluid) and ethanol (used for insect preservation) on the body mass and elemental composition of insect samples under a variety of sampling conditions. We expected an increase in body mass because of the effects of the trapping fluid, and a decrease in body mass caused by ethanol preservation. We also expected that impurities in the trapping fluid may change the concentrations of elements in the firebugs.

2. Materials and methods

2.1. Experiments

Adult firebugs were hand collected during their reproductive period in a public park near the University of Debrecen, Hungary in May 2008. The sample contained altogether 250 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.

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 2 weeks. We simulated pitfall trapping using an ethylene glycol based anti-freeze as killing agent. The trapping fluid was GlycoShell, a common anti-freeze, diluted to 75% (v/v) following earlier practices (Magura et al., 2001, 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 2 weeks (mimicking a fre-

quent 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 2 weeks before analysis (to simulate short trapping plus short preservation). Ten other sample tubes were opened after 1 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 1 month (to simulate long trapping plus long preservation).

2.2. Chemical analysis

At the end of the trapping and preservation cycles the content of each sample tube was placed in a plastic sieve and flushed with 250 mL of double deionised water obtained from a Millipore Milli-Q system. Four specimens were selected at random from each sample tube for analysis. Each firebug was transferred individually into a 25 mL beaker. The wet body mass of the bugs were measured immediately. The samples were dried overnight at 105 °C, and the samples were reweighed to determine their dry mass. The material was then digested using 2 mL 65% (m/m) nitric acid (Scharlau) in the same container at 80 °C for 4 h. High pressure digestion was not required because the applied method gave a clean colourless solution. Digested samples were diluted to 20 mL using a 1% (m/m) nitric acid.

Analysis of the elements was performed by ICP-OES IRIS Intrepid II XSP equipped with a CETAC 45 000 AT⁺ ultrasonic nebulizer. 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) with multi-element calibration solution (Merck ICP multi-element standard solution IV). The analysis was performed using two or three atomic or ionic lines of the corresponding elements. In the case of alkaline metals (e.g. Li, Na and K) we used single lines. The selected lines were free of spectral interferences in these sample matrices. The limit of quantification values (LOQ) are given in mg L⁻¹; Ba: 0.006, Ca: 0.008, Cd: 0.001, Co: 0.004, Cr: 0.002, Cu: 0.003, K: 0.003, Li: 0.002, Mg: 0.001, Mn: 0.001, Na: 0.002, Ni: 0.004, Pb: 0.013, Sr: 0.002 and Zn: 0.003. Other parameters used in this analysis are shown in Table 1.

2.3. Statistical analysis

The average body mass values and elemental concentrations of the four individuals per tube were used in the statistical analyses. Thus, five independent replicate samples were analysed per treatment. Effects of the four treatments on the elemental composition of specimens were evaluated by ANOVA and Canonical Discriminant Analysis (CDA). Calculations were performed using the SPSS/PC + statistical software package. One-Way ANOVA was used to compare the four treatments and the control. Homogeneity of variances was tested by the Levene test. In the case of significant differences between treatments, Tukey's Multiple Comparison test was used to determine between which treatments the differences occurred (Zar, 1996). 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.

Table 1
Instrumental conditions for USN-ICP-OES trace elemental analysis.

Wavelengths/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:	221.458{152}, 221.458{153}, 224.700{150}
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}
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

3. Results

3.1. 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. 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 signifi-

cantly 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. 1).

3.2. 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 Table 2. 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 (Table 3). The concentration of copper did not differ 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 (Table 3). The concentrations of calcium, sodium and strontium were significantly higher in the treatment samples, while potassium was significantly lower compared to the control treatment (Tables 2 and 3). Manganese concentrations were higher in samples in the long trapping plus long preservation treatments compared to the other treatments (Tables 2 and 3).

Trap duration (short trapping vs. long trapping, see Table 2) 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 (Table 3).

3.3. 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

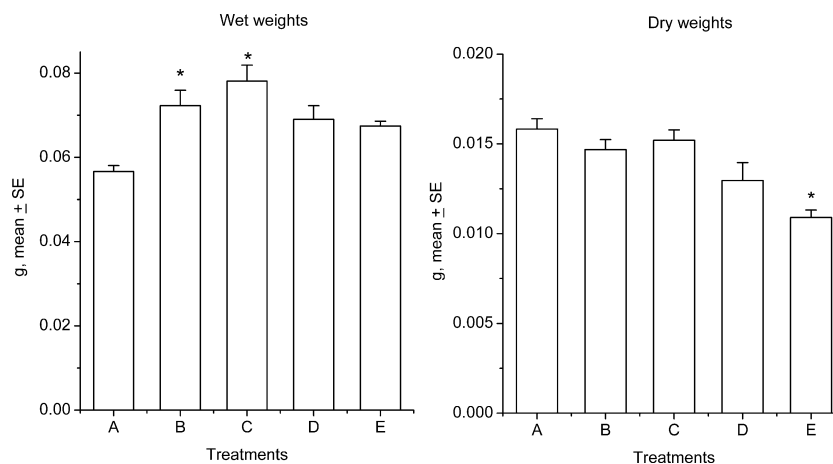


Fig. 1. Effect of treatments on wet and dry body mass of firebugs. (A = control treatment, B = short trapping treatment, C = long trapping treatment, D = short trapping plus short ethanol preservation treatment, and E = long trapping plus long ethanol preservation treatment). Asterisks indicate significant differences ($p < 0.05$).

Table 2
Summary statistics of elemental concentrations (dry mass $\mu\text{g g}^{-1}$) 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 \pm 160 ^a	3450 \pm 270 ^b	3770 \pm 250 ^b	3960 \pm 560 ^b	5140 \pm 430 ^b
Cu	17.5 \pm 0.7 ^a	16.2 \pm 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 \pm 50 ^a	770 \pm 190 ^b	500 \pm 40 ^b	430 \pm 100 ^b	480 \pm 60 ^b
Mg	1810 \pm 110 ^a	1460 \pm 180 ^a	1240 \pm 50 ^a	1700 \pm 200 ^a	1520 \pm 110 ^a
Mn	22.4 \pm 1.4 ^a	25.3 \pm 2.8 ^a	27.3 \pm 3.4 ^a	31.6 \pm 3.8 ^a	44.1 \pm 7.9 ^b
Na	540 \pm 20 ^a	2090 \pm 250 ^b	1920 \pm 180 ^b	1770 \pm 340 ^b	1840 \pm 210 ^b
Sr	8.1 \pm 0.5 ^a	16.0 \pm 1.1 ^b	16.9 \pm 1.3 ^b	16.1 \pm 1.6 ^b	20.4 \pm 1.1 ^b
Zn	15.7 \pm 1.8 ^a	19.8 \pm 3.8 ^a	21.1 \pm 2.3 ^a	17.4 \pm 1.6 ^a	28.0 \pm 3.7 ^a

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

Table 3
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			

($p < 0.01$). 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 (Table 4). Significant negative correlations were found in the pairwise comparison between the second discriminant function and the concentrations of calcium, manganese and copper (Table 4). 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. 2). 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.

3.4. Elemental composition of the trapping and preservation fluids

Impurities in the anti-freeze and ethanol solutions were also analysed. The concentration of each element in the ethanol solu-

Table 4
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
Eigenvalue	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
<i>Elements of Structure Matrix</i>				
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

tion was below detection limits. However, the ethylene glycol based anti-freeze solution contained measurable impurities in

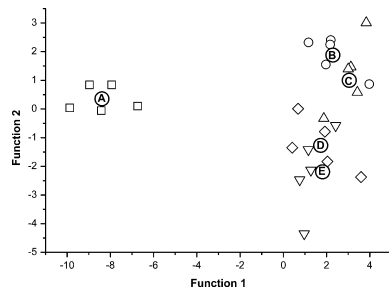


Fig. 2. Canonical discriminant analysis of five treatments based on element concentrations ($\mu\text{g g}^{-1}$). (A = group centroid of the control, B = group centroid of the short trapping treatment, C = group centroid of the long trapping treatment, D = group centroid of the short trapping plus short ethanol preservation treatment, and E = group centroid of the long trapping plus long preservation treatment).

the following concentrations (mean \pm SE): calcium: $276 \pm 52 \text{ mgL}^{-1}$, sodium: $190 \pm 6 \text{ mgL}^{-1}$, magnesium: $46.2 \pm 3.8 \text{ mgL}^{-1}$, potassium: $26.1 \pm 4.1 \text{ mgL}^{-1}$, zinc: $27.5 \pm 2.5 \text{ mgL}^{-1}$ and strontium: $0.8 \pm 0.1 \text{ mgL}^{-1}$.

4. Discussion

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; Strojjan, 1978) or formalin (Williamson, 1979; Clausen, 1984; Hunter et al., 1987; Zödl and Wittmann, 2003). In at least some of these studies it was 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, 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 (anti-freeze) 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 anti-freeze 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 consistency 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 solution. Similar phenomena may alter the analytical results of pitfall trapped insects.

Analytical grade glycol has only rarely used for trapping experiments (Lövei, 1984). Anti-freeze 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 anti-freeze 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 anti-freeze 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 exoskeleton, 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.

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