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Changes in the body mass of *Megaphyllum kievense* (Diplopoda, Julidae) and the granulometric composition of leaf litter subject to different concentrations of copper

V. Brygadyrenko, V. Ivanyshyn

Department of Zoology and Ecology, Oles Honchar Dnipropetrovsk National University, Dnipropetrovsk, Ukraine

ABSTRACT: This article discusses the results of a 30-day experiment investigating the influence of copper which was introduced into the natural diet of *Megaphyllum kievense* (Lohmander, 1928) at concentrations of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mg Cu·g^{-1} dry leaf litter upon the body mass of the species. With copper contamination at a concentration 10^{-1} mg Cu·g^{-1} leaf litter the gain in body mass of *M. kievense* decreased by 69.8% (from 2.45 ± 1.28 to 0.74 ± 1.73 mg/individual per month). With an enrichment of the food substrate to 10^{-2}–10^{-7} mg Cu·g^{-1} litter the increase in body mass of the millipedes did not differ from the control value (it was 88–146% of the control). However, the gain in body mass for 10^{-8} mg Cu·g^{-1} dry leaf litter was twice higher than in the control. The results of the experiment do not permit us to claim that the food consumption of *M. kievense* changed in response to varying concentrations of copper in the litter samples. The mass of the largest litter fragments (> 2.05 mm) decreased by 8.5% as a result of consumption by *M. kievense* and that of the average size fragments (0.70–1.05 mm) increased by 6.0% due to the grinding of the plant remains by *M. kievense* and enrichment through their excretory process.

Keywords: heavy metals; litter invertebrates; polluted ecosystems; saprophages

The toxic effect of heavy metals upon saprophages has been studied in considerable detail (Hopkin 1990; Köhler, Alberti 1992; Köhler et al. 1996; Brygadyrenko, Ivanyshin 2014). The influence of copper upon macrofauna is more intensive under conditions of high concentration of industrial production (Hopkin, Martin 1982; Heikens et al. 2001). Annual discharges of copper as industrial waste contribute significantly to an increase in the concentration of this metal in leaf litter and in the upper soil horizons. This contamination is especially intensive in major metropolitan areas and near copper ore processing plants, as well as in agricultural plantations where fungicides containing copper are used (Sviden et al. 2001; McCay et al. 2013).

Excessive intake of copper in a diplopod leads to a slow accumulation of the pollutant in the body, partial loss of the cuticle during moult, and also to a more intense evacuation of the metal with excrements (Hopkin, Read 1992). For the purpose of conservation it is important to define the levels of this relatively persistent compound which do not reduce the trophic activity of animals (Hopkin 1990). Such experiments can be conducted only under laboratory conditions, where dozens of primary food parameters can be manipulated (Dangerfield 1993; Roy, Joy 2009; Kulbachko, Didur 2012; Svyrydchenko, Brygadyrenko 2014) and observations are conducted in the microclimatic conditions of the experiment (Striganova 1972; Dangerfield, Milner 1993), the microbiological capacities of the substrate and the microflora composition of diplopod intestines (Márialigeti et al. 1985; Zenova et al. 1996; Kaneko 1999; Maraun et al. 2003; Byzov 2006).

Laboratory experiments on the effect of copper within leaf litter on the feeding activity of diplopods have not been conducted up to now. Thus the objective of this article is to investigate three hypotheses: (1) the rate of increase in the body weight of a

diplopod will fall with increasing concentrations of copper in food, (2) the amount of food consumed will depend upon the concentration of copper in the food and (3) diplopods alter the granulometric composition of litter.

MATERIAL AND METHODS

Megaphyllum kievense (Lohmander 1928) (Diplopoda, Julida, Julidae), chosen as the experimental animal, is a widely distributed julid species in the forest-steppe and steppe zones of Ukraine, Romania and European part of Russia. The body length of a male is 16–18 mm, of a female 25–26 mm. The average weight of a male at the beginning of the experiment was 43.4 ± 4.3 mg and of a female was 69.2 ± 4.8 mg. The species is rarely numerous, which is likely why its ecology remains practically unstudied.

We have looked at 6 populations in the Dnipropetrovsk region. For this experiment the *M. kievense* specimens and the litter investigated in the laboratory experiment were collected on September 20, 2013 from one windbreak plantation (10 km south of Dnipropetrovsk, Central Ukraine) composed of the trees *Robinia pseudoacacia* L. (crown density 80%) and *Fraxinus lanceolata* Borkh. (crown density 10%) and a bushy understory of *Sambucus nigra* L. (crown density 10%).

During the autumn the grass stand in the plantation was dominated by *Chelidonium majus* L. (density 40%). Apart from these species, the dry remains of *Galium aparine* L., which dominates in the studied ecosystem (density 85%) during the first half of summer, were found in the litter compound at the time of the experiment with an insignificant number of young plants of this species (density 2%). The litter was thick (45 ± 11 mm), single-layered, and composed mostly of the leaves of *R. pseudoacacia* L. Corresponding to the crown density, the litter composition was *R. pseudoacacia* (80%) and *F. lanceolata* (10%) and *S. nigra* (up to 10%). The proportion of grass in the litter was not more than 2–3%, composed of the following species: *Ch. majus* and *G. aparine*. This is because in our region grass decomposes several times more rapidly than leaf litter.

The *M. kievense* specimens were kept in a 20 litre plastic container holding 12 kg of litter for 14 days at a temperature of +22°C for acclimatization to laboratory conditions. The litter was sifted through a sieve with 15 mm apertures to extract the larger fragments of plant remains, after which the smaller fragments were extracted using a sieve with 3 mm apertures. The remaining leaf litter was dried in the laboratory over 7 days and sifted again to remove any remaining soil. When weighing out the dry litter we made sure that the litter placed in the containers was as homogeneous as possible in granulometric composition.

The total experiment used 126 containers (a plastic cup 0.25 l in volume) and 216 millipedes. This distribution of containers and millipedes was chosen in order to avoid an unmanageably high number of replicates in the containers with millipedes and equally to provide a sufficiently high number of replicates in containers without millipedes. Two grams of dry litter (weighed to the nearest 1 mg) were placed in each container, moistened from a pipette with a solution of $CuSO_4 \cdot 5H_2O$ (concentrations of the metal of 10^{-1}–10^{-8} mg·g^{-1} dry litter) and at 10^0 mg·g^{-1} with an equivalent amount of distilled water. For each concentration of copper there were 14 containers: in 6 of them there were 4 *M. kievense* specimens in each and 8 containers without millipedes were the control. The containers representing different variants of the experiment were arranged randomly on the laboratory table and positioned so as not to be exposed to direct sunlight from the window (northern side of the building). Containers were covered with sheets of black paper for preventing the excess loss of moisture and ingress of foreign substances. Once in 2–3 days, the containers were sprayed with distilled water from an atomizer from a height of 50–60 cm to compensate for moisture evaporation.

Before the beginning of the experiment, the *M. kievense* specimens were weighed in groups composed of 2 males and 2 females on electronic scales to the nearest 1 mg (average weight –56.3 ± 6.4 mg). Thirty days after the beginning of the experiment, the animals were taken out of the containers and weighed. During the month of the experiment, 4 out of 216 *M. kievense* specimens died (1 in the variant with 10^{-8} mg Cu·g^{-1}, 1 in the 10^{-5} mg Cu·g^{-1} treatment, 1 in the 10^{-8} mg Cu·g^{-1} and 1 in the control). For the 4 containers with dead specimens the initial weight of animals was multiplied by the coefficient 0.75. This was because all four millipedes were weighed collectively as was their food and excrements; each millipede and its food and excrements were not weighed individually.

After taking the millipedes out of the containers, the litter was air-dried using a convection heater (to prevent microbial decomposition of the litter), weighed and transferred as carefully as possible to the series of laboratory sieves (as detailed above) for granulometric analysis. The mass of every frag-

Table 1. Changes in the body mass gain of *M. kievense* (mg/specimen) in a laboratory experiment on the diet of leaf litter with different concentrations of copper (n = 6)

Concentration of Cu (mg·g^{-1} litter)	Median	$x \pm S_x$	Min–Max	F, $F_{0.05}$ = 2.15, df_1 = 8, df_2 = 45	P
10^{-1}	1.00	0.74 ± 1.73[a]	–1.5–2.9		
10^{-2}	2.38	2.29 ± 1.35[b]	0.0–3.8		
10^{-3}	3.38	3.29 ± 1.58[b]	1.3–5.5		
10^{-4}	3.88	3.58 ± 1.43[b]	1.8–5.0		
10^{-5}	3.38	3.42 ± 1.99[b]	0.8–6.3	4.39	0.0006
10^{-6}	2.50	2.15 ± 2.07[b]	–1.0–4.5		
10^{-7}	3.00	3.25 ± 0.87[b]	2.5–4.3		
10^{-8}	6.00	5.23 ± 2.19[c]	2.3–7.7		
Control	3.00	2.45 ± 1.28[b]	1.0–4.0		

[a–c] differences in body weight of *M. kievense* represented by different letters are statistically significant, $P < 0.05$ (Tukey test)

ment was determined to the nearest 1 mg. The sifted samples of litter were analysed using a microscope with photographic attachment.

Statistical analysis of the data was conducted using a set of Statistica 7 (SPSS, Tulsa, USA). The differences between the samples were assessed using ANOVA and Tukey's test. The differences between the samples were considered statistically significant at $P < 0.05$. The diagrams show the median, 25–75% quartiles, maximum and minimum values and, in certain cases, extremes.

RESULTS

Under the influence of increasing concentrations of copper in their diet *M. kievense* showed a statistically significant decrease in body mass gain in the course of the experiment (Table 1). At a concentration of 10^{-1} mg Cu·g^{-1} in the litter the millipedes rapidly decreased (to 30.2% in relation to the control) their feeding and motor activities, some of them lost weight. It is interesting that, in comparison with the control, there was not observed an increase in the death rate of *M. kievense* specimens during the experiment even at such a high concentration of copper (10^{-1} mg·g^{-1} litter) which is absent under natural conditions. With a slight enrichment of the litter with copper salts (10^{-8} mg·g^{-1} litter) the gain in mass was 213.6% compared to the control. With enrichments of the litter of 10^{-2}–10^{-7} mg Cu·g^{-1} the gain in body mass of the millipedes did not differ significantly from the control indicators (ranging from 87.8 to 146.1% of the control).

According to the results of the experiment we cannot reliably claim that the food consumption of *M. kievense* significantly changed depending upon the concentration of copper in the litter samples. For the concentrations of 10^{-1}, 10^{-4}, 10^{-6}, 10^{-7} mg·g^{-1} and the control the differences in the mass of litter, which was not decomposed in the course of the experiment by microorganisms or millipedes, were not significant (Table 2). At concentrations of 10^{-2}, 10^{-3}, 10^{-5} and 10^{-8} mg·g^{-1} in containers holding

Table 2. Changes in the litter mass in a laboratory experiment on the influence of copper upon the rate of food consumption by *M. kievense*

Concentration of Cu (mg·g^{-1} litter)	Changes in litter mass during the experiment in containers		F, $F_{0.05}$ = 4.74, df_1 = 1, df_2 = 12	P
	with *M. kievense* (%), $x \pm S_x$ (n = 6)	without *M. kievense* (%), $x \pm S_x$ (n = 8)		
10^{-1}	53.3 ± 0.67	52.9 ± 1.00	0.86	0.37
10^{-2}	53.9 ± 0.82	52.8 ± 1.03	4.36	0.06
10^{-3}	54.5 ± 1.24	53.1 ± 0.87	6.33	0.03
10^{-4}	52.9 ± 1.63	52.6 ± 0.81	0.23	0.63
10^{-5}	53.7 ± 1.18	52.6 ± 0.86	4.00	0.07
10^{-6}	54.1 ± 0.69	53.9 ± 1.41	0.09	0.77
10^{-7}	54.3 ± 1.12	54.1 ± 2.14	0.04	0.85
10^{-8}	54.2 ± 0.84	52.9 ± 0.93	7.76	0.02
Control	53.5 ± 1.10	52.8 ± 0.78	2.11	0.17
F	1.43; $F_{0.05}$ = 2.15, df_1 = 8, df_2 = 45	1.73; $F_{0.05}$ = 2.09, df_1 = 8, df_2 = 63	–	–
P	0.21	0.11	–	–

Table 3. Changes in the granulometric composition of litter in the laboratory experiment on food consumption of *M. kievense*

Size of litter fragments	Mass of litter fragments in containers		F, $F_{0.05} = 3.92$, $df_1 = 1$, $df_2 = 124$	P
	with *M. kievense* (%), $x \pm S_x$ ($n = 53$)	without *M. kievense* (%), $x \pm S_x$ ($n = 73$)		
> 2.05	61.04 ± 6.98	69.51 ± 3.64	78.30	$7.5 \cdot 10^{-15}$
1.55–2.05	8.11 ± 0.97	7.71 ± 1.06	4.56	0.030
1.05–1.55	8.83 ± 1.44	7.34 ± 1.10	43.42	$1.1 \cdot 10^{-9}$
0.70–1.05	11.64 ± 3.90	5.68 ± 1.27	149.52	$4.8 \cdot 10^{-23}$
0.35–0.70	7.15 ± 2.07	6.33 ± 0.83	9.31	0.003
0.20–0.35	3.24 ± 0.71	3.44 ± 0.41	3.97	0.048

M. kievense a statistically significant rise in the rate of litter decomposition was observed. The results thus do not show any clearly defined patterns.

It is interesting to note that statistically significant differences in the mass of decomposed litter were observed neither in the containers where *M. kievense* were present nor in the containers in which individuals were absent (Table 2).

As there were not observed any statistically significant differences in the speed of food consumption at different concentrations of copper, we became interested in the impact of individuals of the studied species upon the granulometric composition of litter (Table 3). The mass of the largest fragments (> 2.05 mm) of litter subject to feeding by *M. kievense* decreased by 8.49%, the mass of the fragments measuring 1.55–2.05 mm increased by 0.39%, that of the 1.05–1.55 mm fragments increased by 1.49%, that of the 0.70–1.05 mm fragments increased by 5.97%, that of the 0.35–0.70 mm fragments increased by 0.82%, while that of the 0.20–0.35 mm fragments decreased by 0.20%. Thus, there was a redistribution in the granulometric composition of the litter fragments: the greater part of the largest feeding fragments of plant remains in the containers with *M. kievense* was transformed into 0.70–1.05 mm fragments, mostly represented in the form of slightly crumbled plant remains and diplopod excrements.

For further analysis of the experiments, the containers with *M. kievense* ($n = 53$) were divided depending upon the resulting change in body mass into 6 categories: in the first variant the weight of millipedes remained the same – from –1.0 to 1.0 (from decrease in the body mass of an individual by 1 mg to increase in mass by 1 mg, $n = 10$), in the other five variants the body mass increased by 1.1–2.0 ($n = 9$), 2.1–3.0 ($n = 14$), 3.1–4.0 ($n = 8$), 4.1–5.0 ($n = 7$) and 5.1–7.0 mg ($n = 5$). No statistically significant decrease in the mass of the largest litter fragments (>2.05 and 1.55 to 2.05 mm), depending upon changes in the body mass of the millipedes in the course of the experiment, was observed (Fig. 1a, b).

The mass of average size fragments (0.70–1.05 and 1.05–1.55 mm) showed a statistically significant increase in the containers where *M. kievense* fed more intensively. This showed in the results in the form of accumulated small food remains and in the form of excrements (Fig. 1c, d). The content of small fragments (0.20–0.35 and 0.35–0.70 mm) in the variants of experiment with intensive and slower feeding by *M. kievense* did not significantly differ (Fig. 1e, f).

DISCUSSION

The toxic impact of metals upon the soil organisms can have both short-term and chronic effects (Carter 1983; Eijsackers et al. 2005). In our 30-day experiment, in which acute impacts were not evaluated because only 2% of the specimens died in this period, the primary effect of the experiment upon the animals' body mass was caused by long-term metabolic changes (relating to anabolic and catabolic processes). It is possible that the statistically significant impact of copper upon the body mass of *M. kievense* in the containers with concentrations of 10^{-1} mg·g^{-1} litter is connected with high tolerance of the diplopods to this particular metal (Morgan et al. 1986; Köhler, Alberti 1992; Köhler et al. 1992, 1995). Based on our study of the territorial distribution of this species (we have looked at 6 populations in the Dnipropetrovsk region) we cannot draw any conclusions concerning the tolerance of individuals to areas most heavily polluted with metals (Brygadyrenko 2006; Brygadyrenko, Komarov 2008).

The high rate of microbiological decomposition of the studied litter samples (Table 2) did not allow us to conclude that *M. kievense* had a significant impact upon the mass of the food substrate. Simi-

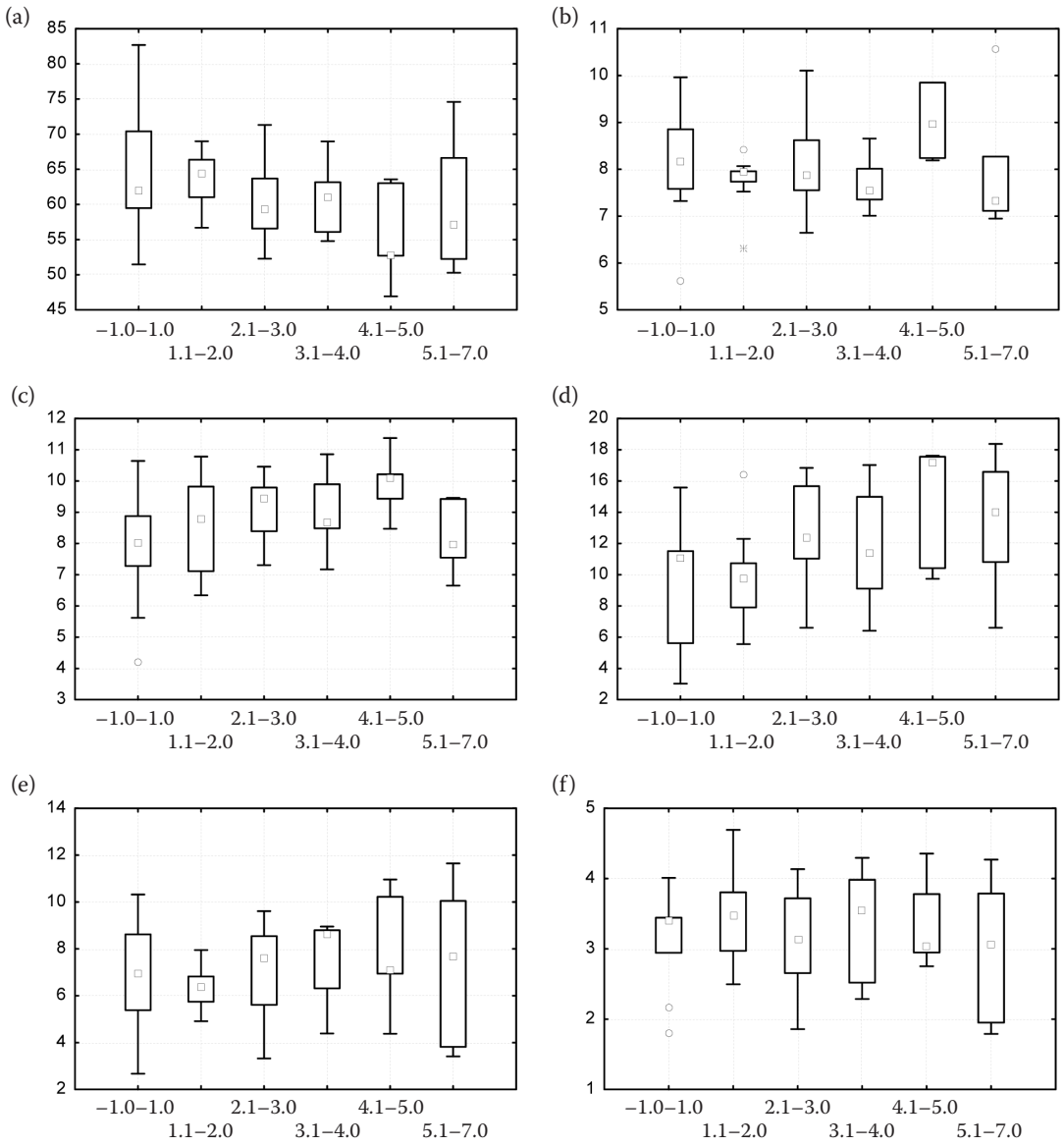

Fig. 1. Changes in the mass of litter fragments of different sizes as a result of the trophic influence of *M. kievense*: (a) fragments > 2.05 mm (F = 2.04, P = 0.09); (b) –1.55–2.05 mm (F = 1.99, P = 0.10); (c) –1.05–1.55 mm (F = 2.45, P = 0.04); (d) –0.70–1.05 mm (F = 2.50, P = 0.04); (e) –0.35–0.70 mm (F = 0.44, P = 0.82); (f) –0.20–0.35 mm (F = 0.42, P = 0.83)
abscissa – change in a millipede's body weight in a particular container (mg·day^{-1}), ordinate – the ratio of the mass of litter fragments of different sizes to the total litter mass in a particular container (n = 126) after conclusion of the experiment (%); 25–75% quartiles are shown as rectangles; lines show maximum and minimum values and, in certain cases, extremes shown as asterisks and circles

lar effects of a multidirectional influence of Julidae have also been observed by other authors while analysing food consumption in the laboratory (Souza et al. 2014). It is probable that, having a relatively slow metabolic rate and a prolonged period of ontogenesis like other diplopods (Wooten, Crawford 1975; Striganova, Prishutova 1990), *M. kievense* can undergo diapauses of different depth and duration in adverse conditions. The induction of such diapauses can be observed among different Diplopoda species caused by inappropriate diet (presence of leaf remains of plants that are poisonous to a particular species, reproduction of fungi and bacteria in certain conditions that decompose the litter), unfavourable temperature and humidity, the influence of parasites and diseases, or by technogenic pollution of the environment (Gere 1956; Baker 1980; Devi, Prabhoo 1990; Boccardo, Penteado 1995; Couteaux et al. 2002; Brygadyrenko 2004). Ecdysis did not take place during the experiment: possibly this was because of seasonal factors. It is likely that

under the conditions of our experiment random factors were more significant for the diplopods' metabolic condition, at least in particular variants of the experiment. Thorough mixing, homogenization and sifting of the primary mass of the litter before the experiment allowed us to exclude the possibility that the results of research could have been affected by lack of homogeneity of the primary food compound. However, during the 30-day laboratory experiment (despite the thorough control of temperature and humidity in the laboratory, randomization of the containers with different concentrations of metal on the laboratory table and thorough maintenance of an equal level of humidity of the substrate in all variants of the experiment) there could have possibly been insignificant accidental differences in the litter humidity among the containers. This probably led to stimulation of reproduction of different groups of microorganisms, which had a significant effect on the results of the experiment (Hopkin, Read 1992; Byzov et al. 1996; Ashwini, Sridhar 2005; Byzov 2006).

It would be interesting to examine in greater detail the results of the impact of *M. kievense* upon the granulometric composition of litter (Kheirallah 1990; Köhler et al. 1991; Koukoura et al. 2003; Brygadyrenko, Ivanyshyn 2014). The increase in the mass of the average size fragments (0.70–1.55 mm) in the containers where more intensive feeding by *M. kievense* was observed shows the intensive impact of this species upon litter decomposition. If we take into account the fact that in the *Robinia pseudoacacia* L. windbreak where we collected the millipedes for the experiment, the density of the species was 10–80 indd·m^{-2} (personal observations for this site conducted over three years), the role they play in the destruction of plant remains must be quite intensive.

CONCLUSIONS

The results of our 30-day laboratory experiment on the effect of *M. kievense* upon the decomposition of plant remains at different levels of copper pollution showed a statistically significant reduction in the rate of increase in the animals' body mass (by 69.8%) in the variant with 10^{-1} mg Cu·g^{-1} litter. At lower concentrations of the metal in litter no statistically significant changes in body weight gain were recorded relative to the control.

The impact upon the mass of food substrate in different variants of the experiment was different, which may well be connected with a factor not controlled by us in this experiment, the infestation of *M. kievense* individuals with parasites and their exposure to infectious diseases (we plan to publish shortly on this subject).

The increase in the share of average size litter fragments (0.70–1.55 mm) in the containers is connected with accumulations of millipede excrements and fragments of leaves, the plant remains being macerated by the millipedes prior to being eaten.

Out of the three hypotheses formulated in the article's introduction, the first and the third (concerning the slowing of the *M. kievense* anabolism with an increase in copper concentration and the impact of *M. kievense* upon the granulometric composition of litter) were upheld while the second (that the amount of food consumed depends upon the concentration of copper it contains) needs additional experiments (based on the results of this experiment it could be neither supported nor rejected).

Further study of the impact of heavy metals upon the food consumption of saprophages would lead to a better understanding of the peculiarity of their role in different types of anthropogenically transformed ecosystems and, perhaps, help to work out practical measures to restore the numbers of certain rare species of invertebrate animals.

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Corresponding author:

Dr. Viktor V. Brygadyrenko, Oles Honchar Dnipropetrovsk National University, Gagarin Ave, 72, Dnipropetrovsk, 49010, Ukraine; e-mail: brigad@ua.fm