STRESS SYNERGY BETWEEN ENVIRONMENTALLY REALISTIC LEVELS OF COPPER AND FROST IN THE EARTHWORM DENDROBAENA OCTAEDRA

Anne-Mette Bindesbøll, Martin Holmstrup, Christian Damgaard, and Mark Bayley*

†Department of Terrestrial Ecology, National Environmental Research Institute, P.O. Box 314, Vejløvej 25, Dk 8600 Silkeborg, Denmark
‡Department of Zoophysiology, Institute of Biological Sciences, University of Aarhus, Building 131, C.F. Möllers Allé, 8000 Aarhus C, Denmark

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Abstract—In their natural habitat, animals are exposed to a variety of stress factors, including extreme temperatures, low water availability, and toxic stress from chemical pollutants. In this study we examined the interaction between realistic environmental levels of soil–copper contamination and realistic winter temperatures on survival of the cosmopolitan freeze-tolerant earthworm Dendrobaena octaedra. These interactions were tested using a full factorial design with six copper concentrations between 0 and 200 mg Cu/kg dry soil and five temperatures from +2 to −8°C. A highly significant synergistic interaction existed that demonstrates that exposure to subzero temperatures significantly reduced copper tolerance and, conversely, that copper exposure significantly reduced freeze tolerance. Copper had no effect on glucose production, which is believed to be a major component of the cryoprotective system and the only known cryoprotectant in D. octaedra. This points to other mechanisms behind the observed synergy, possibly impaired osmoregulatory function of the cell membrane. The results support the working hypothesis that interactions between toxicants and dominant natural stress factors can alter the organisms’ tolerance to these individual stressors.

Keywords—Frost tolerance Copper Dendrobaena octaedra Synergy Risk assessment

INTRODUCTION

In natural environments it is not unusual for an organism to be exposed to several stressful factors, both physical and chemical, at the same time. These could include climatic stress or the exposure to chemicals of anthropogenic origin. In traditional ecotoxicological studies, organisms usually are exposed to a single chemical at increasing concentrations, while factors such as temperature and moisture content are held at a constant optimum. These traditional laboratory tests, therefore, can lead to an underestimate of the toxicity of the chemical in natural environments where the organism periodically will encounter several physical and chemical factors simultaneously. In the context of the current concerns over global climate changes, it is important to study the interactions between the toxic chemicals present in the environment and the climatic events likely to alter species biogeography.

Earthworms are important members of the soil fauna due to their ability to improve soil structure and their contribution to the breakdown of organic matter and release of plant nutrients [1]. Dendrobaena octaedra has a worldwide distribution. It is present in almost the entire European forest zone and tundra, and also is found in Russia (Siberia), North America, Canada, and Greenland [2–5]. Because of its wide geographical distribution, populations of this species frequently are exposed to chemicals of anthropogenic origin. Examples include heavy-metal pollution of surface soils from smelters [6] and brass mills [7] or from the use of copper fungicides [8]. Surface litter and humus are the principal metal sinks in the forest floor [9]. This means that D. octaedra, which is a litter-dwelling species, is likely to be more exposed to metals than burrowing species.

One of the climatic factors important in defining the geographical limits of ectotherm species is the winter temperature regime and many ectotherm species at some time in their life span will be exposed to subzero temperatures that may cause freezing of their body fluids. These cold-hardy animals have developed one of two strategies: Freeze-avoidance or freeze-tolerance [10]. Freeze-avoiding species die if frozen. They survive subzero temperatures by supercooling their body fluids or dehydrating until the melting point of their body fluids is lowered to the ambient temperature [11,12]. Freeze-tolerant species, on the other hand, can survive freezing of their extracellular body fluids. The earthworm D. octaedra is one of the only two known earthworm species that can survive this extracellular ice formation [13]. Although most other earthworm species migrate to deeper soil layers to avoid subzero temperatures, D. octaedra overwinters in the litter layer [14]. To facilitate this winter survival, D. octaedra accumulates glucose to high concentrations at the onset of the body fluid–freezing process [15]. This accumulation of glucose contributes to a slowing down of the freezing process, and to a reduction in the amount of ice formed. Furthermore, glucose is known to aid in the preservation of the structure and function of membranes and proteins during freezing-induced dehydration of tissues [16,17].

The aim of the present study was to investigate the possible existence of a synergistic interaction between a common metal contaminant at realistic and sublethal soil concentrations and realistic winter temperatures in D. octaedra. Further, because of the importance of glucose production to winter survival in this species, the effects of copper on glucose synthesis were investigated.

MATERIALS AND METHODS

Animals

Animals used in this study were subadult and adult individuals of D. octaedra collected in the year 2000 around the
Arctic Station, Godhavn, Disko West Greenland, and since kept in culture at 15°C in the soil described below on a diet of cow dung. Animals used for experimentation were approximately three months old and had a fresh weight of between 100 and 320 mg.

Soil

Topsoil from an ecologically farmed Danish pea field (Foulum, Viborg) was used for the experiment. The soil was a loamy sand consisting of 35% coarse sand, 45% fine sand, 9.4% silt, 8.9% clay, and 1.7% organic matter. The pH–H₂O was approximately 6.8. Prior to use, the soil was dried for 24 h at 80°C and sieved through a 2-mm mesh. The soil was copper-skipped with CuCl₂·2H₂O (>99%, Merck, Damstadt, Germany), and the water content adjusted to 20% of dry weight (−pF = 2 and 50% water-holding capacity) and stored for 2 d before further use.

Experimental design

Copper and temperature were varied in a full factorial design with six Cu concentrations and five temperatures, giving a total of 30 treatments, including the control groups.

Animals were exposed to nominal soil concentrations of 0, 40, 80, 120, 160, and 200 mg Cu/kg dry weight for four weeks at 2°C before exposure to the experimental temperatures. Each group had three to 10 worms, depending on the treatment. Each worm was weighed and kept individually in a small container with 75 g of soil (wet wt) and 4 g of cow dung (wet wt) mixed into the soil. The cow-dung feed was produced by adding 400 ml of demineralized water to 150 g of dried and finely ground cow-dung. Animals in each treatment had the same average fresh weight. All containers were covered with lids having the same number of holes so that ventilation was equal between treatments. The pH was found to be equal (6.8) in all copper treatments after the addition of cow-dung. After four weeks at 2°C, 14 individuals from each Cu concentration were allowed to empty their gut for 48 h on filter paper kept at 5°C, rinsed, and then frozen at −80°C. Ten animals were used for copper analysis and four for glucose analysis. With the exception of the controls (+2°C), the remaining worms were placed in 8-ml tubes along with a few grams of the appropriate substrate. In each lid, two small needle holes were made for ventilation. The worms were exposed to −2, −4, −6, and −8°C in a freezer cabinet (WTB Binder Labortechnik, Tuttlingen, Germany). The freezer cabinet was programmed to lower the temperature from 0°C to −8°C at 0.042°C/h. When the temperature reached approximately −1.5°C, a small ice crystal was added to each tube to initiate freezing. As the temperature fell, the animals were removed to separate freezer cabinets at their intended experimental temperature. The animals remained at their experimental temperature for different periods such that each group remained at subzero temperatures for equal time.

Eight worms from each copper concentration were removed from the −2°C group and stored at −80°C for glucose analysis. Because Rasmussen and Holmstrup [13] showed that glucose production reached a maximum at −2°C, glucose was not measured at the lower temperatures.

When the final groups had reached their intended temperature (−8°C), the temperature was raised from −8 to 0°C within a 24-h period so that all groups reached 0°C at the same time. Prior to the assessment of survival rate, the temperature was raised from 0 to 2°C and the worms allowed 24 h to thaw.

The earthworms were considered to have survived if there was a reaction to tactile stimuli, normal locomotor activity, and no visible signs of freezing damage.

Glucose analysis

Worms were removed from the −80°C freezer and freeze-dried immediately for 24 h. Glucose was measured using high-performance liquid chromatography (HPLC). The freeze-dried worms were pulverized individually in an Eppendorf tube using a glass rod. Approximately 3 mg of the pulverized tissue was added to a 600-µl Eppendorf tube. Cryoprotectants were extracted according to Bayley and Holmstrup [18] in 100-µl 40% ethanol with a rotating glass rod. Following homogenization, the samples were placed in an ultrasonic bath for 30 min. After warming to 80°C for 5 min in a heat block, the tubes were centrifuged for 10 min and the supernatant was removed to a 1,500-µl Eppendorf tube. The pellet then was rinsed again with 40% ethanol and centrifuged. The combined supernatants were left in the heat block at 60°C until dryness. This sample was redissolved in 1,000 µl. Five hundred µl of this solution was diluted to 1,500 µl with HPLC-grade water and filtered through a 0.45-µm filter before HPLC analysis. Twenty-five–µl samples were run in duplicate on a Shimadzu HPLC (Shimadzu Corporation, Kyoto, Japan) system described in detail by Holmstrup et al. [19]. Glucose concentrations were calculated using a D(+) glucose standard curve (Supelco-standard 4–7249 [Sigma-Aldrich, St. Louis, MO, USA]). In the present study, only an external standard was used, but recovery of internal standards using this methodology at our laboratory average 75% with a low variance (standard deviation 5%). Corrections for this were not applied because recovery rates may vary between individual samples. Concentrations reported in this paper, therefore, are slightly conservative.

Copper analysis

The worms were freeze-dried for 24 h and the whole worm was acid-digested using 3 ml of 14 M nitric acid at increasing temperatures (80–135°C). When all fluid had evaporated, 1 ml 14 M of nitric acid was added and again heated until dryness. The samples were redissolved in 0.1 M of nitric acid and analyzed using flame atomic absorption spectrometry (Perkin-Elmer 4100, Ueberlingen, Germany). Certified reference material (oyster tissue material from the National Institute of Standards and Technology, U.S. Department of Commerce and lobster hepatopancreas from National Research Council Canada) was analyzed to verify the efficiency of the digestion and atomic absorption spectrometry procedure, resulting in a measured concentration of approximately 95% of the certified values. All samples were analyzed in one run.

Statistical analysis

Traditionally, probability of mortality or survival has been modeled by constructing linear (additive) or log-linear (multiplicative) models after an appropriate transformation of the mortality data (e.g., the probit- or logit transformations). However, because mortality data are binary, it is natural to model the probability of dying as a function of the investigated factor(s) [20]. When two or more factors are considered, it is easier to model the inverse of the probability of mortality (i.e., the probability of survival) due to the rule of combined events.

The effect of subzero temperatures and copper and any
possible interaction were modeled using a modified sigmoid dose-response function.

\[ f(x; \theta, \theta_0) = \frac{1 + \exp(-\theta x)}{1 + \exp[\theta(x - \theta_0)]} \quad x \geq 0, \quad \theta \geq 0 \quad (1) \]

where \( f(x) \) is the expected probability of survival at a level of a single stress factor \( x, \theta_0 \) is the point of inflection, and \( \theta \) is the shape parameter of the function. For a more detailed description of the model, the reader is referred to Damgaard et al. \[21\].

It is very difficult to predict the target for the toxicity of both copper and subzero temperatures; therefore, the effects of subzero temperatures and copper on \( D. \ octaedra \) are assumed to be independent physically (they affect different cellular processes), and the effect of subzero temperatures, copper, and the interaction effect of combining the two factors are assumed to be multiplicative \[22\]. Consequently, the expected probability of survival \( p(st, [Cu]) \), when subjected to subzero temperatures and copper can be modeled as

\[ p(st, [Cu]) = (1 - \lambda)f(st; \theta_0) \times f([Cu]; \theta_{Cu}, \theta_{Cu0}) \quad (2) \]

where \( st \) is the subzero temperature, \([Cu]\) is the concentration of copper, and \( \lambda \in [0, 1] \) the residual or control mortality \[21\]. Note that the interaction effect of combining subzero temperatures and copper is assumed to be a dose-response function of the level of subzero temperatures multiplied by the concentration of copper.

Equation 2 was fitted to the mortality data of \( D. \ octaedra \) exposed to various levels of subzero-temperature stress and concentrations of the heavy-metal copper by the maximum likelihood approach. The number of survivors \( X \), out of \( n \) individuals exposed to a given degree of subzero temperatures and copper concentrations was assumed to be binomial-distributed with a probability calculated by Equation 2. The likelihood function is

\[ L = \prod R p(st, [Cu])^X (1 - p(st, [Cu]))^{n - X} \quad (3) \]

where \( R \) is the number of trials. The maximum likelihood estimates were found using the NMaximize routine in Mathematica \[23\], but any software that optimizes nonlinear functions might be used instead.

The fit of the model was tested by checking whether a null-model, where each of 30 treatment combinations were assigned a specific probability, fitted the mortality data significantly better than Equation 2.

A linear regression model was used to test the correlation between copper in the soil and the subsequent concentration in earthworm tissue. A visual inspection of the residuals suggested that the data should be log transformed. After this transformation, the residuals approximately were distributed normally and the variation homogeneous.

Differences in glucose concentrations between treatments were analyzed using analysis of variance after analysis for variance heterogeneity.

RESULTS

Effect of subzero temperatures and copper on survival

A highly significant interaction occurred between copper toxicity and the severity of frost exposure \( (p = 0.0004) \). This means that there is a significant increase in copper toxicity with decreasing temperatures and, conversely, a significant reduction in frost tolerance with copper exposure. This synergistic interaction between subzero temperatures and copper is illustrated in Figure 1 by the probability isoclines of mortality when both stresses are present. Survival of \( D. \ octaedra \) exposed to 70 mg Cu/kg soil dry weight will be reduced by 5% when the worms are exposed to \(-2^\circ C\) and by 20% when exposed to \(-6^\circ C\). At a higher but still realistic copper concentration of 170 mg Cu/kg soil dry weight, 10% of the worms will die after exposure to \(-1.5^\circ C\) and this increases to 50% when exposed to \(-7^\circ C\). If the results are considered in terms of the concentrations of copper resulting in a 10% mortality (Fig. 2), it becomes evident that exposure even to mild subzero temperatures radically changes the toxicity estimate of this metal and the Cu-concentration that is lethal to 10% of the worms estimate drops from 200 mg Cu/kg dry soil at \(-1^\circ C\) to only 100 mg Cu/kg dry soil at \(-3^\circ C\).

The mathematical model used to predict the effect of subzero temperatures and copper on mortality is sufficient to give an adequate description of the data. A null-model, where each of 30 treatment combinations were assigned a specific probability, did not fit the mortality data significantly better \( (p = \)
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Table 1. Survival (%) of Dendrobaena octaedra after four weeks of exposure to copper at 2°C followed by 10 d at subzero temperatures in the same soil. The numbers in parentheses are the values calculated from the 0-model

<table>
<thead>
<tr>
<th>Soil copper concn. mg Cu/kg dry wt soil</th>
<th>Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40</td>
</tr>
<tr>
<td>2°C</td>
<td>100%</td>
</tr>
<tr>
<td>-2°C</td>
<td>(99.9%)</td>
</tr>
<tr>
<td>-4°C</td>
<td>(97.5%)</td>
</tr>
<tr>
<td>-6°C</td>
<td>87.5%</td>
</tr>
<tr>
<td>-8°C</td>
<td>87.5%</td>
</tr>
</tbody>
</table>

DISCUSSION

This study has revealed a highly significant interaction between environmentally realistic levels of soil–copper contamination and winter temperatures that are encountered frequently by this earthworm species in the climatic zones it inhabits. The implications of this result are twofold. First, that the biogeography of this species, in all probability, will be affected by the presence of copper in its environment. Second, that traditional laboratory estimation of copper toxicity, which is performed at constant benign temperatures, will underestimate the toxicity of this metal in the field because earthworms inevitably will encounter subzero temperatures during the winter [13,14]. Holmstrup et al. [24] previously have observed a trend indicating a synergistic interaction between copper and subzero temperatures on the viability of cocoons from D. octaedra. However, this trend was only significant at −8°C. Comparison with the present study is difficult because the cocoons were exposed to copper in aqueous solution and cocoons of D. octaedra are not freeze-tolerant like adult worms, but dehydrate at subzero temperatures to an extent that their melting point equilibrates with the environmental temperature [15]. Considerable evidence suggests that there are many common physiological adaptations in response to drought and subzero temperatures because both involve dehydration [12,25,26]. Indeed, copper previously has been shown to affect summer drought tolerance in the euedaphic collembolan Folsomia candida [27] and in the earthworm Aporrectodea caliginosa [28]. However, the design in these studies only allowed the inclusion of a single contamination level at 300 and 150 mg Cu/kg dry soil, respectively.

In the present study, copper had no influence on glucose

Table 2. Maximum likelihood estimates of the survival data model

<table>
<thead>
<tr>
<th>Model parameter*</th>
<th>Max. likelihood estimate</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ = (residual mortality)</td>
<td>0</td>
</tr>
<tr>
<td>$b_\alpha$ (shape parameter at $x_{0,\alpha}$)</td>
<td>0.408</td>
</tr>
<tr>
<td>$s_{0,\alpha}$ (inflexion point of subzero temperature dose-response curve)</td>
<td>−12.19</td>
</tr>
<tr>
<td>$b_{st}[Cu]$ (shape parameter at $X_{st}[Cu]$)</td>
<td>0</td>
</tr>
<tr>
<td>$X_{st}[Cu]$ (inflexion point of copper dose-response curve)</td>
<td>−NE</td>
</tr>
<tr>
<td>$b_x[Cu]$ (shape parameter at $x_{0,x}[Cu]$)</td>
<td>0.00163</td>
</tr>
<tr>
<td>$s_{x}[Cu]$ (inflexion point at interaction dose-response curve)</td>
<td>1,582.65</td>
</tr>
</tbody>
</table>

* $st$ is subzero temperatures and [Cu] is copper.
* Not estimable.
* Transformed value to fit the model: $(−t + 2)\cdot [Cu]$(mg/kg) = 1,582.65.

0.38) (Table 1). The maximum likelihood estimates are listed in Table 2. The shape parameter of copper ($b_{st}$) is zero because we have assessed only sublethal copper concentrations. This means that the inflection point ($x_{0,\alpha}$) of copper cannot be estimated and that a full dose-response for copper alone cannot be obtained from this data.

The model in Equation 2 was expanded with a covariance function of size to test whether the size of the animal interacted with the observed mortality. We could find no evidence of any effects of size ($p > 0.9$), possibly due to the rather limited size range of animals used in this study.

Copper content in worms

Earthworm internal body copper burden increased linearly with exposure to elevated soil–copper concentrations. A significant positive correlation existed between copper of earthworm tissue and copper content in substrate ($r^2 = 0.80$, $p < 0.0001$; Fig. 3). All 10 worms exposed to control soil had approximately the same Cu content in their tissue (11 mg/kg). At the highest Cu–soil concentration, the mean tissue Cu concentration was 94 mg/kg. The variation in the internal copper concentrations in the earthworm increased with increasing copper exposure.

Glucose content in worms

Copper had no effect on glucose production ($p > 0.3$), but there was a highly significant temperature effect in all copper concentrations ($p < 0.001$). The mean glucose concentration of the control worms (2°C), independent of copper treatments, was 14.1 ± 3.2 mg/g worm dry weight (mean ± standard deviation). Worms frozen at −2°C synthesized, on average, 137 ± 18.2 mg glucose/g worm dry weight.
synthesis, which was chosen for measurement due to its significance in frost tolerance [13,29]. Hence, copper must have had other effects that underlie the interaction between copper and subzero temperatures on worm survival seen in this study. One of the alternative targets for copper toxicity that might explain this interaction between toxic and climatic stress is the cell membrane. Copper concentrations in the range used in the present study have been shown to deteriorate the membrane stability of lysosomes from coelomocytes of the earthworm *Lumbricus rubellus*, measured as the ability of these cell components to retain a neutral red dye [30]. The phase behavior and physical properties of membrane phospholipids fatty acids are extremely sensitive to temperature changes [31]. As the temperature falls, ectothermic organisms increasingly will introduce unsaturated phospholipids into membranes, thus maintaining optimal membrane fluidity at low temperatures [32]. Interaction between copper and the membrane enzymes involved in the control of membrane fluidity possibly could have a significant impact on the ability of the organism to survive changes in temperature. Alternatively, copper may interfere with the ability of the earthworm cells to regulate their volume, which has been shown in the marine flagellates [33] and which would be of particular importance during the rehydration that occurs during thaw [34]. In many of the worms that failed to survive subzero temperatures at higher copper concentrations, there was evidence of edema and internal bleeding that could point to such effects.

The copper concentrations used in this experiment are within the range that can be found in natural soils, which can vary from 2 to 250 mg/kg, depending on the soil type and the mother rock [35]. Near a brass mill in southeast Sweden, *D. octaedra* were found in a coniferous forest contaminated with copper. The earthworm density increased with increasing distance to the mill [9]. At the most-contaminated sites closest to the mill, no individuals were found, which may indicate that synergistic interactions like those seen in this study exist in the field. The internal copper concentrations in this experiment range from a mean of 11 to 94 mg/kg dry weight in worms exposed to control soil and soil containing 200 mg Cu/kg dry weight, respectively. Bengtsson et al. [9] measured internal copper concentrations in *D. octaedra* collected in the field ranging from approximately 100 to 300 mg/kg dry weight, which would suggest, in the light of our data, that these field animals would be vulnerable to subzero winter temperatures. The concentrations in the worms in the present experiment were very similar to those measured by Weeks and Svendsen [30] in *Lumbricus rubellus* under similar experimental conditions. Also, in a laboratory experiment performed by Bengtsson et al. [36], the earthworm *Dendrobaena rubida* (= *Dendrodrilus rubidus*) had internal copper concentrations in the muscle of approximately 57 mg/kg dry weight when exposed to 100 mg Cu/kg soil at pH 6.5 for 21 d. These observations are in good agreement with our results. According to Spurgeon and Hopkin [37], the worm *Aporrectodea caliginosa* excretes copper at a fast rate after transfer to clean soil with a half-life of less than 1 d. This suggests that the internal copper concentrations measured in this study are lower than they would be in field populations confronting a similar contamination, because the worms in the present study were depurated for 48 h on wet filter paper before freeze drying and analysis to avoid copper-contaminated gut contents affecting the measured concentrations. Soil pH also has an effect on copper uptake. The uptake is higher at lower pH [36] and, because *D. octaedra* often is found in acidic soils [38], the copper uptake in the present study may well be lower than the uptake seen in natural populations.

According to Streit and Jäggy [39], the earthworm *Octolasion cyanenum* regulates tissue copper concentrations from approximately 40 mg/kg to 100 mg/kg. The increased variation in the tissue copper concentrations with increasing copper exposure seen in the present study is likely to be caused by differences in individual efficiencies of the copper regulatory mechanisms.

Earthworms living in copper-contaminated soil obviously will be exposed periodically to subzero temperatures and copper at the same time. The experimental design in the present study was chosen to simulate natural conditions as far as possible. The worms were first acclimated in copper-contaminated soil and then exposed to subzero temperatures and copper in the soil at the same time. It could be speculated that the exposure to the subzero temperatures and copper should be separated to enable the study of the synergistic interaction between stressors, without the confusing interaction between bioavailability and temperature [40]. However, the earthworms exposed to subzero temperatures do not move and, because the water surrounding them at subzero temperatures is frozen, they probably would not accumulate copper during the exposure to frost.

CONCLUSION

In conclusion, our data indicate that the assessment of the toxicity copper by the traditional laboratory studies where test organisms are exposed to only one stress factor and otherwise optimal conditions (e.g., temperature and humidity), will underestimate the impact of the pollutant on the survival of field populations, which regularly will encounter stressful climatic conditions. Our data also indicate that the presence of environmental contaminants such as copper will alter the climatic tolerance limits of *D. octaedra* and, thereby, its geographical distribution.

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