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Reproductive Toxicity of the Fungicide Remilitine, Chromium Ions, and Their Mixture in the Earthworm Eisenia fetida

Aebeed S. Aebeed [1], Salaheddin A. Sharif [2], Abeer H. Amer [3*]
[1] Department of Zoology, Faculty of Arts and Science, University of Benghazi, Ghemines, Libya
[2] Department of Chemistry, Faculty of Arts and Science, University of Benghazi, Ghemines, Libya
[3] Department of Histology, Faculty of Medicine, University of Benghazi, Benghazi, Libya.

السمية التناسلية لمبيد الفطريات ريمليتين وأيونات الكروم وخليطهما في دودة الأرض *Eisenia fetida*

أعبيد أعبيد [1]، صلاح الدين الشريف [2]، عبير عامر [3*]
[1] قسم علم الحيوان، كلية الآداب والعلوم، جامعة بنغازي، قمينس، ليبيا
[2] قسم الكيمياء، كلية الآداب والعلوم، جامعة بنغازي، قمينس، ليبيا
[3] قسم علم الأنسجة، كلية الطب، جامعة بنغازي، بنغازي، ليبيا

[*]Corresponding author: abeer.amer@uob.edu.ly

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Abstract:
The widespread use of pesticides and heavy metals raises concerns about their long-term impacts on soil ecosystems. This study evaluated the sub-lethal reproductive effects of chromium (200 ppm) and the fungicide Remilitine (500 ppm), individually and in combination, on the earthworm *Eisenia fetida*. Adult worms were cultured in artificial soil and exposed for 70 days. Cocoon production was assessed at 28 and 70 days, while juvenile numbers were recorded at day 70. Statistical analysis revealed that Remilitine significantly reduced cocoon production compared to controls at 28 days, whereas chromium alone had no significant effect. By day 70, cocoon numbers in the chromium–Remilitine group were comparable to controls, indicating recovery. However, juvenile numbers were consistently reduced across all treated groups, with the mixture showing an antagonistic effect. These findings highlight the importance of evaluating mixture toxicity in soil organisms to better understand ecological risks of combined contaminants.

Keywords: *Eisenia fetida*, Mixture toxicity, Soil contamination, Chromium exposure, Remilitine fungicide.

الملخص
إن الاستخدام الواسع للمبيدات الحشرية والمعادن الثقيلة يثير مخاوف بشأن آثارها طويلة الأمد على النظم البيئية للتربة. هدفت هذه الدراسة إلى تقييم التأثيرات التناسلية تحت-القاتلة لكل من الكروم (200 جزء في المليون) والمبيد الفطري ريمليتين (500 جزء في المليون)، بشكل منفرد ومشترك، على دودة الأرض .تم تربية الديدان البالغة في تربة صناعية وتعريضها للمعاملات لمدة 70 يومًا. جرى تقييم إنتاج الشرنقات عند اليومين 28 و70، بينما تم تسجيل أعداد اليرقات عند اليوم 70. أظهرت التحليلات الإحصائية أن التعرض لريمليتين أدى إلى انخفاض ملحوظ في إنتاج الشرنقات مقارنةً بالشاهد عند اليوم 28، في حين لم يكن للكروم وحده تأثير معنوي. وبحلول اليوم 70، كانت أعداد الشرنقات في مجموعة الكروم–ريمليتين مماثلة للشاهد، مما يشير إلى حدوث تعافٍ جزئي. ومع ذلك، ظل عدد اليرقات منخفضًا في جميع المجموعات المعالجة، حيث أظهر الخليط تأثيرًا تضادياً. تؤكد هذه النتائج أهمية تقييم سمية الخلائط في الكائنات الحية في التربة لفهم أفضل للمخاطر البيئية الناجمة عن الملوثات المشتركة.

الكلمات المفتاحية: سمية الخلائط، تلوث التربة، التعرض للكروم، مبيد الفطريات ريمليتين

Introduction

Soil health is fundamental to ecosystem stability and agricultural productivity, yet it is increasingly threatened by anthropogenic contamination, particularly heavy metals and agrochemicals [1]. These pollutants degrade soil structure, impair fertility, and exert toxic effects on soil organisms. Metals present a dual challenge: while copper, zinc, and iron are essential micronutrients, others such as cadmium, lead, and chromium have no biological role and are toxic at elevated concentrations [2].

Both essential and non essential metals disrupt intracellular homeostasis and induce oxidative stress when present above physiological thresholds [3,4]. Their persistence, bioaccumulation, and biomagnification further exacerbate ecological and human health risks [5]. Chromium, in particular, is a widespread contaminant; hexavalent chromium [Cr(VI)] is highly mobile and toxic, associated with nephrotoxicity, hepatotoxicity, and neurotoxicity in animals [3,6].

In parallel, extensive pesticide use introduces complex chemical mixtures into soils. Remilitine, a fungicide containing cymoxanil (8%) and mancozeb (64%), is widely applied against potato late blight [7,8]. Although designed to target fungal pathogens, such compounds can adversely affect non target soil fauna. The co occurrence of heavy metals and pesticides in agricultural soils creates scenarios of combined pollution, where interactions may be synergistic, additive, or antagonistic [9].

Earthworms, particularly Eisenia fetida, are vital ecosystem engineers and globally recognized bioindicators in soil ecotoxicology [10,11]. Their close contact with soil, sensitivity to contaminants, and role in nutrient cycling make them ideal model organisms. Sub lethal endpoints such as cocoon and juvenile production are especially sensitive markers of chronic stress [12]. Previous studies have shown that mixtures of metals and Remilitine can severely inhibit earthworm reproduction [13–15]. However, the reproductive toxicity of chromium–Remilitine mixtures remains incompletely understood. Given the prevalence of both contaminants in agricultural environments, assessing their interactive effects on earthworm reproduction is crucial for ecological risk evaluation.

Material and methods

Test Organism and Culturing Conditions

The earthworm species Eisenia fetida was selected due to its standardized use in ecotoxicological testing and high reproductive rate. Specimens were obtained from a research supplier in the Czech Republic and maintained as a laboratory stock culture prior to experimentation. Adult worms were cultured in glass aquaria containing artificial soil prepared according to OECD Guideline 222 [16]. The culture medium was maintained at 20 ± 2 °C under a 16:8 h light:dark cycle. Soil moisture was adjusted to approximately 60% of the water holding capacity (WHC) using deionized water and monitored weekly. Visible fungal growth was manually removed. Worms were fed weekly with 5 g of powdered barley grains, and the substrate was replaced every eight weeks to ensure soil quality. Experimental worms were sexually mature clitellate individuals weighing 7–9 g.

Test Substances and Soil Preparation

The contaminants tested were chromium (Cr), supplied as potassium dichromate ($K_2Cr_2O_7$), and the fungicide Remilitine (containing 8% cymoxanil and 64% mancozeb). Stock solutions were prepared in deionized water. Artificial soil was composed of 70% quartz sand, 20% kaolin clay, 10% sphagnum peat, and calcium carbonate ($CaCO_3$) to adjust pH to 5–6.5. For toxicity testing, 250 g (dry weight equivalent) of soil was placed in glass vessels (15 × 12 × 20 cm). Treatments included: (1) chromium alone (200 mg/kg dry soil), (2) Remilitine alone (500 mg/kg dry soil), (3) chromium–Remilitine mixture (200 + 500 mg/kg dry soil), and (4) control (deionized water). Soil moisture was re adjusted to 60% WHC after spiking.

Experimental Design and Procedure

The experiment followed a completely randomized design with three replicates per treatment. Ten pre weighed adult worms were introduced into each container, which was covered with muslin cloth to allow aeration while minimizing moisture loss. Containers were maintained under the same conditions as the stock culture (20 ± 2 °C, 16:8 h L:D). Worms were fed weekly with 5 g of powdered barley grains. Soil moisture was checked weekly and adjusted with deionized water. Cocoon production was recorded on days 28 and 70 by manual sorting. At day 70, surviving adults were counted and weighed, and juveniles were collected and counted.

Data Analysis

Data are presented as mean ± SD. Statistical analyses were performed using SPSS. Normality was assessed with the Shapiro–Wilk test and homogeneity of variance with Levene's test. One way ANOVA followed by Tukey's post hoc test was used to compare cocoon and juvenile counts across treatments. Independent t tests were applied for pairwise comparisons.

Results and discussion
Results
Cocoon production:

Analysis of variance revealed significant differences in cocoon numbers between control and Remilitine-treated worms ($F = 3.33$, $p < 0.05$). Control worms produced significantly more cocoons than those exposed to Remilitine. At 28 days, the mean ± SD cocoon numbers were 46.67 ± 2.08 for controls and 33.0 ± 4.35 for Remilitine-treated worms. After 70 days, cocoon production increased in both groups, but treated worms still produced fewer cocoons (123.67 ± 7.0 for controls vs. 87.67 ± 5.77 for Remilitine; Fig. 1). Chromium exposure did not significantly affect cocoon production compared to controls ($F = 4.07$, $p > 0.05$ at 28 days; $F = 0.40$, $p > 0.05$ at 70 days). Mean ± SD values were 37.00 ± 2.00 (control) vs. 35.67 ± 8.62 (chromium) at 28 days, and 114.67 ± 15.88 (control) vs. 86.00 ± 19.92 (chromium) at 70 days (Fig. 2). For the chromium–Remilitine mixture, cocoon production was significantly reduced at 28 days ($F = 8.47$, $p < 0.05$), with mean ± SD values of 46.67 ± 2.08 (control) vs. 37.00 ± 9.29 (mixture). However, by 70 days, no significant difference was observed ($F = 4.43$, $p > 0.05$), with values of 123.67 ± 7.10 (control) vs. 121.33 ± 6.60 (mixture; Fig. 3).

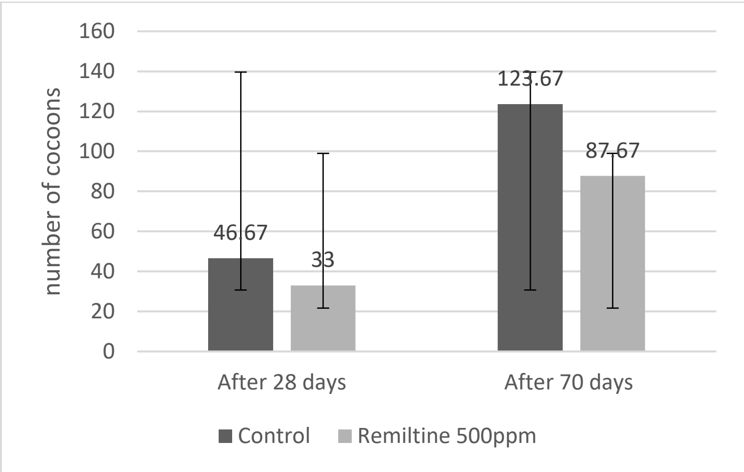

Figure 1 Cocoons per worm in control and Remilitine-treated soils at 28- and 70-days post-treatment. *Significantly different from controls at $p < 0.05$, *$p < 0.01$.

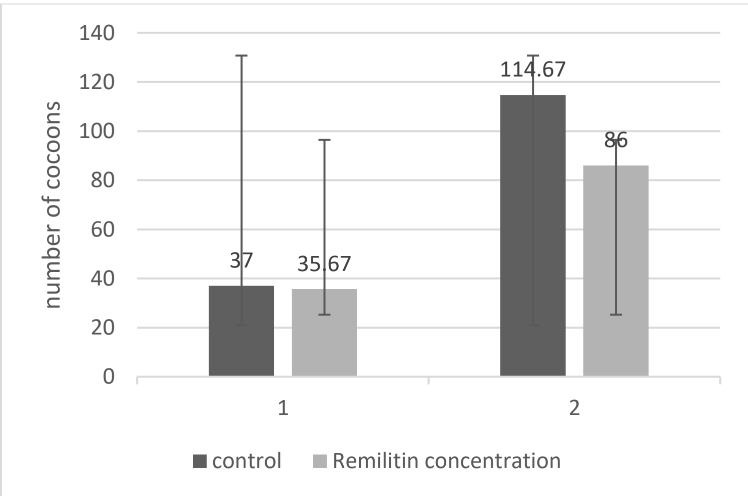

Figure 2 Cocoons per worm in control and chromium-treated soils at 28 and 70 days post-treatment.

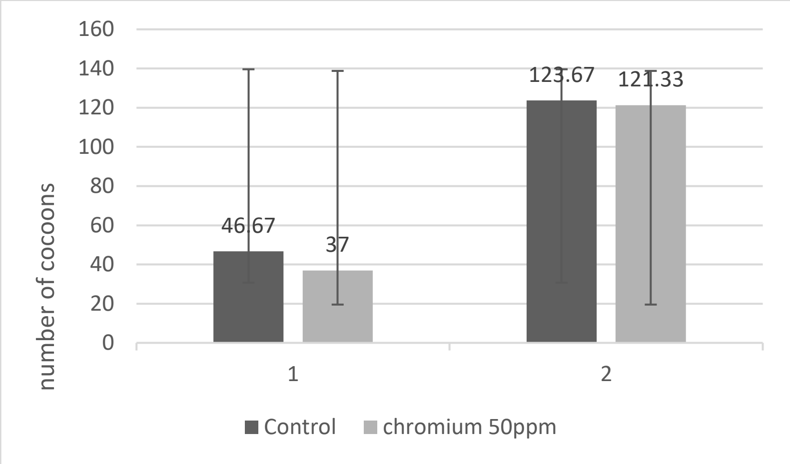

Figure 3 Cocoons per worm in control and chromium–Remilitine-treated soils at 28 and 70 days post-treatment. *Significantly different from controls at $p < 0.05$, *$p < 0.01$..

Juvenile number:
Remilitine exposure reduced juvenile numbers, although ANOVA indicated no statistically significant difference ($F = 3.0$, $p > 0.05$; Fig. 4). Chromium exposure significantly decreased juvenile numbers compared to controls ($F = 8.06$, $p < 0.05$). At 70 days, mean ± SD values were 94.00 ± 35.53 (control) vs. 60.33 ± 9.07 (chromium; Fig. 5). The chromium–Remilitine mixture also significantly reduced juvenile numbers ($F = 10.93$, $p < 0.05$). At 70 days, mean ± SD values were 94.00 ± 35.53 (control) vs. 57.67 ± 10.69 (mixture; Fig. 6).

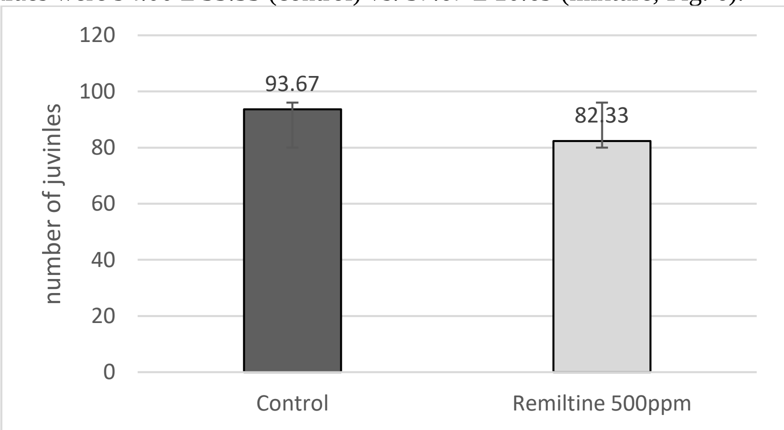

Figure 4 Juveniles per worm in control and Remilitine-treated soils. *Significantly different from controls at $p < 0.05$, *$p < 0.01$.

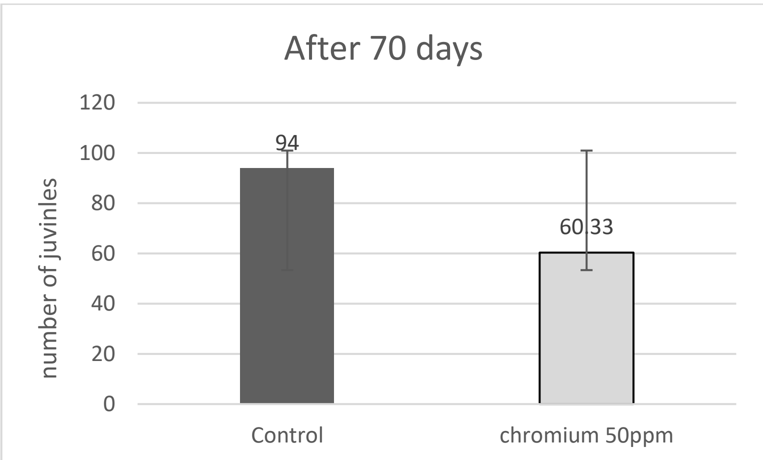

Figure 5 Juveniles per worm in control and chromium-treated soils. *Significantly different from controls at $p < 0.05$, *$p < 0.01$.

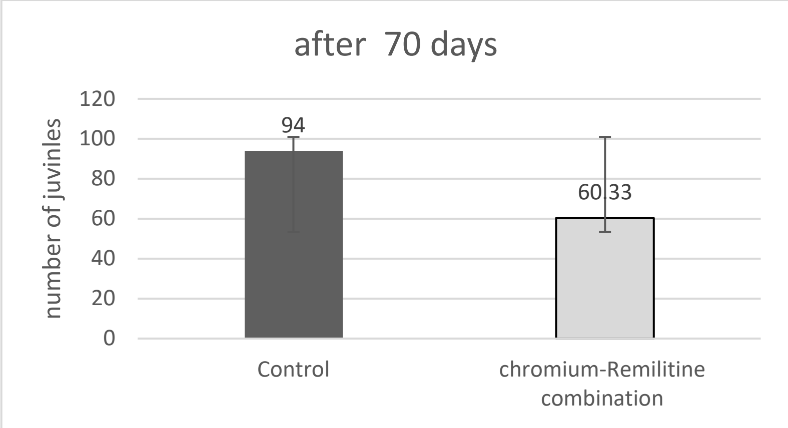

Figure 6 Juveniles per worm in control and chromium–Remilitine-treated soils. *Significantly different from controls at $p < 0.05$, **$p < 0.01$*.

Discussion:

Earthworms are widely used as indicator species to assess the ecological effects of pollutants. A variety of substrates, including artificial soils such as those specified in OECD guidelines, test designs, and endpoints have been standardized to evaluate adverse effects [16]. The compost worms *Eisenia andrei* and *E. fetida* are most frequently used in laboratory studies due to their ease of culturing and high reproductive potential, unlike field species such as *Lumbricus rubellus* and *Aporrectodea caliginosa*, which are more difficult to rear.

The objectives of this study align with previous findings [13–15], which emphasized that mortality is an insensitive endpoint, whereas changes in body weight and reproduction provide more meaningful measures of sub-lethal toxicity. While reductions in body weight have been reported in earthworms exposed to heavy metals [17], other studies found no significant effect [18]. Spurgeon and Hopkin clarified that worms in contaminated soils often exhibit slower growth or reduced maximum weight compared to those in uncontaminated environments [12].

In the present study, Remilitine fungicide significantly reduced cocoon and juvenile production, confirming its strong reproductive toxicity. Chromium alone had little effect on cocoon numbers but reduced juvenile viability. The chromium–Remilitine mixture showed antagonistic effects: cocoon production recovered by day 70, but juvenile numbers remained significantly reduced. These findings are consistent with earlier studies showing that copper–Remilitine and lead–Remilitine mixtures negatively affected reproduction in *E. fetida* [13–15, 19-20].

Other researchers have also demonstrated that juvenile survival is a particularly sensitive endpoint. Choo and Baker reported that juvenile numbers per cocoon are reliable indicators of pesticide toxicity [21], while Aebeed et al. found juvenile *E. fetida* more sensitive to copper–Remilitine contamination than adults [14]. Our results confirm that Remilitine alone exerts stronger toxicity on juvenile production, whereas the chromium–Remilitine mixture produces antagonistic interactions, reducing juvenile numbers but not cocoon output.

Conclusion

This study investigated the combined toxic effects of the fungicide Remilitine and chromium on the reproduction of Eisenia fetida. Remilitine alone significantly reduced cocoon and juvenile production, while chromium had limited effects on cocoon numbers but reduced juvenile viability. The chromium–Remilitine mixture exhibited antagonistic interactions: cocoon production recovered over time, but juvenile numbers remained suppressed. These findings highlight the complexity of mixture toxicity and suggest that competitive adsorption between Remilitine and chromium ions in soil and at biomembrane interfaces may influence bioavailability. Overall, reproductive endpoints in earthworms provide sensitive measures of chronic soil contamination and are essential for ecological risk assessment of combined agrochemical–metal exposures.

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Statements & Declarations

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Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

Authors' Contributions

All authors contributed to the study conception and design, data collection, analysis, and manuscript preparation. All authors read and approved the final manuscript.

Ethics Approval

This study was conducted in accordance with OECD (2004) guidelines for soil toxicity testing using *Eisenia fetida*. As the research involved only invertebrate organisms, no formal approval from an animal ethics committee was required.

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