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CuO NPs dose and duration dependent modulation of instantaneous growth rate (IGR) of the earthworm *D. willsi*:Evidence of threshold-driven toxic response

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Abstract

The increasing release of copper oxide nanoparticles (CuO NPs) into terrestrial ecosystems necessitates evaluation of their sublethal effects on key soil fauna. The present study investigated the concentration and durationdependent effects of CuO NPs (0–1000 mg kg^{-1}) on the instantaneous growth rate (IGR) of the tropical earthworm *Drawida willsi* over 7, 14, 21, and 28 days. A clear negative dose–response relationship was observed. Control worms maintained consistently positive growth (0.077–0.115), whereas exposed groups exhibited progressive suppression of IGR, shifting from marginal inhibition at 200 mg kg^{-1} to severe negative growth at 800–1000 mg kg^{-1} (−9.499 and −10.485, respectively, by day 28). Two-way ANOVA revealed that concentration had a highly significant effect on IGR ($p < 0.001$), while exposure duration further intensified growth suppression ($p < 0.01$). Post-hoc analysis confirmed significant reductions at ≥800 mg kg^{-1}, identifying a toxicity threshold beyond moderate exposure levels. The transition from reduced growth to net biomass loss indicates metabolic imbalance and sustained physiological stress under elevated nanoparticle concentrations. These findings establish IGR as a sensitive, data-driven biomarker for detecting sublethal nanotoxicity and highlight potential ecological risks posed by chronic CuO NP contamination in soil systems.

INTRODUCTION

Earthworms are foundational components of terrestrial ecosystems, serving as critical ecosystem engineers that drive soil structure, nutrient cycling, aeration, and organic matter decomposition (Edwards *et al.,* 2020). Among soil fauna, their growth, reproduction, and behavior are highly responsive to changes in soil chemistry, making them sensitive bioindicators of soil health and contaminant exposure (Römbke and Moser, 2021). Recent decades have witnessed the widespread introduction of engineered nanoparticles (ENPs) into agricultural, industrial, and consumer products, raising global concerns about their fate and ecological impacts in soil ecosystems (Hirano and Maher, 2024; Schlich *et al.,* 2022a). Of particular interest are copper oxide nanoparticles (CuO NPs), which are widely used in antimicrobial coatings, fungicides, and industrial catalysts due to their high reactivity and antimicrobial properties (Navarro Pacheco *et al.,* 2021). However, when released into soil environments, CuO NPs may exert unintended ecotoxicological effects on nontarget organisms, including soil invertebrates.

Metal oxide nanoparticles differ from traditional bulk contaminants by virtue of their small size, high surface area, and potential to release bioavailable metal ions, leading to complex and multifaceted toxic responses in biological systems (Saleh *et al.,* 2023; Wang *et al.,* 2024a). In soil animals, these responses can manifest as oxidative stress, membrane damage, disrupted energy metabolism, and altered microbe–host interactions, even at sublethal doses (Li *et al.,* 2022; Zhang *et al.,* 2026). Traditional toxicity assessments have largely focused on acute mortality endpoints; however, sublethal indicators such as growth rate, reproductive success, and behavioral changes often provide more sensitive measures of ecological risk (Jager *et al.,* 2023a). Instantaneous growth rate (IGR), a dynamic measure of somatic performance over time, reflects the balance between energy acquisition and expenditure and can therefore serve as an early and integrative indicator of physiological stress (Sibly and Calow, 2022; Römbke and Moser, 2021). Despite its utility, IGR remains understudied in the context of nanoparticle ecotoxicology, particularly for CuO NPs in soil invertebrates(Sinha *et al.,* 2003).

Several recent studies have reported sublethal effects of CuO NPs in earthworms. Baxla *et al.,* (2025) demonstrated significant reductions in biomass and cocoon production in *Drawida willsi*at moderate to high CuO NP concentrations. Navarro Pacheco *et al.,* (2021) showed that CuO NPs induce intracellular oxidative stress and lipid peroxidation in earthworm coelomocytes, suggesting a mechanistic link to impaired physiological function. Comparable sublethal effects have been reported for other metal nanoparticles; Schlich *et al.,* (2022b) found that exposure to silver and zinc oxide nanoparticles disrupted growth and reproduction across multiple invertebrate taxa, while Swart *et al.,* (2020) highlighted alterations in the earthworm gut microbiome following metal nanoparticle exposure. Yet, while these studies provide foundational insights, few have systematically examined how both dose and exposure duration interact to influence growth dynamics, particularly using temporally resolved endpoints such as IGR.

Moreover, much of the existing literature is limited by narrow exposure timepoints (often single sampling events) or by focusing on mortality and reproduction alone, leaving gaps in our understanding of the progression of sublethal responses over ecologically relevant timeframes (Hou *et al.,* 2023a). This gap is especially critical in the context of chronic environmental exposures, where continuous or repeated contact with nanoparticles may elicit delayed or cumulative effects that are not captured by acute toxicity assessments (Yang *et al.,* 2025). Additionally, there remains limited exploration of threshold concentrations at which compensatory homeostatic mechanisms fail, leading to a transition from reduced growth to net biomass loss, a phenomenon suggested by mechanistic models of energy allocation under stress (Sibly and Calow, 2022).

To address these knowledge gaps, the present study investigates the dose- and duration-dependent effects of CuO nanoparticles on the instantaneous growth rate (IGR) of *Drawida willsi*. By tracking IGR across multiple time points (7–28 days) and a gradient of CuO NP concentrations (0–1000 mg kg^{-1}), this work provides a temporal and quantitative characterization

of sublethal toxicity, enabling identification of critical thresholds and the dynamics of toxic progression. The novelty of this study lies in its use of IGR as a sensitive sublethal endpoint, combined with rigorous statistical analyses, including ANOVA and post hoc comparisons, to delineate how exposure intensities and durations interact to influence growth trajectories. Importantly, this research extends beyond static endpoints to capture dynamic biological responses, offering a more ecologically relevant assessment of nanoparticle impacts on soil invertebrate physiology.

By generating detailed temporal growth profiles under environmentally realistic nanoparticle exposures, this study aims to (1) clarify the progression of sublethal effects, (2) identify concentration thresholds associated with impaired growth, and (3) inform risk assessments and regulatory frameworks for nanoparticle contamination in terrestrial ecosystems. Through its integration of mechanistic insights from recent literature and rigorous empirical data, this work contributes to a deeper understanding of nanoparticle ecotoxicology and underscores the value of dynamic, sensitive endpoints in environmental risk assessment.

MATERIALS AND METHODS

Earthworms (*Drawida willsi*) were sampled from crop field near Ranchi University, Morhabadi campus located between 21°58' to 25° 19'N and 83° 20' to 88° 4' E at an elevation of 629 m above mean sea level (MSL) during morning hours by monolith method (Srivastava *et al.,* 2003). Adult worms (clitellate, ≥ 4 cm) were hand sorted and used for analysis of instantaneous growth rate (Brafield and Llewellyn, 1982). Artificial soil was prepared by mixing soil, saw dust (pre-soaked in water for 3 weeks) and cow dung (dried and powdered) in 1:1:1 ratio (w/w in dry condition). After one week of thermo-stabilization, earthworms were inoculated in different pots and were maintained at 22 ± 3°C with 20% moisture content. Biomass of worms was estimated after gut evacuation by keeping in air oven at 85°C for 24 h to obtain dry weight. Weight of mature worm was estimated at a regular interval of 7 days up to 28 days from both control and

treated pots. Instantaneous growth rate (IGR) was calculated following the Brafield and Llewellyn (1982) formula:

$$IG\% = \frac{\log_{10} YT - \log_{10} yt}{T - t} \times 2.3026 \times 100$$

where t = time at the beginning of the observation, T = time at the end of the observation, YT = weight at time T, yt = weight at time t and 2.3026 = conversion factor.

Statistical evaluation of experimental data was analyzed using Microsoft Excel (2018). A one-way, two-way analysis of variance (ANOVA) and post hoc test was performed to determine significant differences among treatment groups.

OBSERVATION

The instantaneous growth rate (IGR) of the earthworm *Drawida willsi* exhibited a clear concentration- and time-dependent response to copper oxide nanoparticles (CuO NPs), demonstrating both sub-lethal and acute toxic effects over the 28-day exposure period (Table 1). IGR served as an integrative measure of population biomass change and provided a sensitive indicator of physiological stress induced by nanoparticles.

Under control conditions (0 mg/kg),*D. willsi* maintained consistently positive growth throughout the experiment, with IGR values ranging from 0.077 to 0.115 (Table 1). The highest IGR (0.115) was observed at day 28, suggesting steady biomass accumulation and normal physiological functioning under laboratory conditions. Minimal variance in control measurements indicates that growth was stable, unaffected by handling or confinement stress, providing a reliable baseline for comparison with treated groups.

In contrast, exposure to CuO NPs produced dose-dependent suppression of growth. At 200 mg/kg, worms initially showed positive growth during early exposure (0.078 on day 7 and 0.039 at day 14), but IGR turned negative by day 21 (−0.117) and day 28 (−0.119), indicating the onset of sub-lethal stress over time. Moderate concentrations (400–600 mg/kg) resulted in stronger inhibition: at 400 mg/kg, IGR declined sharply to −1.658 by day 14, reaching −3.54 at day 28, whereas at 600 mg/kg, growth was nearly arrested early and dropped to −5.99 by day 21, remaining strongly negative at day 28 (−3.73). These patterns suggest accelerated metabolic disruption and reduced energy allocation at intermediate nanoparticle doses.

High concentrations (800–1000 mg/kg) induced acute toxicity, with immediate onset of negative growth. At 800 mg/kg, IGR was −3.506 on day 7 and decreased steadily to −9.499 by day 28. At 1000 mg/kg, IGR declined from −5.14 on day 7 to −10.485 on day 28, indicating rapid biomass loss and sustained physiological stress. These results demonstrate that high-dose CuO NP exposure leads to both acute and chronic growth suppression, likely through oxidative stress, metabolic imbalance, and impaired nutrient assimilation.

Table-1: Instantaneous growth rate (IGR) in percentage of *D. willsi* under exposure to Cuo NPs for different doses and duration over 28 days.

	0 mg kg^{-1}	200 mg kg^{-1}	400 mg kg^{-1}	600 mg kg^{-1}	800 mg kg^{-1}	1000 mg kg^{-1}
7th day	0.11	0.078	0.039	0	-3.506	-5.14
14th day	0.078	0.039	-1.658	-1.62	-6.201	-8.107
21st day	0.077	-0.117	-2.491	-5.99	-8.428	-9.318
28th day	0.115	-0.119	-3.54	-3.73	-9.499	-10.485

Welch's one-way ANOVA revealed that concentration significantly affected IGR ($F = 14.3$, $p = 0.001$), whereas exposure duration alone was not statistically significant ($F = 1.15$, $p = 0.375$) (Table-2).

Table-2: One way ANOVA of IGR of *D.willsi* for different dose and duration of exposure of CuO NPs.

	F	df 1	df2	p
Duration	1.15	3	10.7	0.375
Concentration	14.3	5	7.10	0.001

These results indicate that the dose of CuO NPs was the primary determinant of growth suppression, while temporal variation, although evident, contributed less to overall variance. Mean IGR values decreased progressively from +0.095 in control to −8.2625 at 1000 mg/kg (Table 3), confirming a strong negative dose-response relationship.

Table 3. Summary statistics for IGR of *D. willsi* across CuO NP treatments.

Groups	Count	Sum	Average	Variance
0 mg/kg	4	0.38	0.095	0.000413
200 mg/kg	4	-0.119	-0.02975	0.010638
400 mg/kg	4	-7.65	-1.9125	2.285514
600 mg/kg	4	-11.34	-2.835	6.756167

800 mg/kg	4	-27.634	-6.9085	7.032378
1000 mg/kg	4	-33.05	-8.2625	5.275924

Two-way ANOVA demonstrated significant effects of both concentration and duration on IGR (Table 4). Concentration had a dominant effect ($F = 29.352$, $p = 3.07 \times 10^{-7}$), while exposure duration also significantly influenced growth inhibition ($F = 7.639$, $p = 0.0025$). The relatively low error variance indicates that the observed effects were consistent and reproducible.

Table 4.A two - way ANOVA of IGR of *D. willsi* for different dose and duration of exposure of CuO NPs.

Sources of Variation	SS	df	MS	F	P-value	F crit
Different duration	38.73221	3	12.91074	7.639219	0.0025	3.287382
Different dose	248.0366	5	49.60732	29.35241	3.07E-07	2.901295
Error	25.35089	15	1.69006			
Total	312.1197	23				

Tukey's HSD post-hoc tests confirmed concentration-dependent toxicity. Comparisons between control and higher doses revealed significant reductions:

- Control vs 800 mg/kg: Mean Difference = −7.003, p = 0.013
- Control vs 1000 mg/kg: Mean Difference = −8.357, p = 0.005

Lower concentrations (200–600 mg/kg) did not differ significantly from the control ($p > 0.05$), although a progressive decline in IGR was evident (Table 5a).

Time-dependent comparisons revealed significant growth suppression over exposure duration:

- 7 vs 14 days: −4.314, p = 0.0324
- 7 vs 21 days: −5.780, p = 0.0326
- 7 vs 28 days: −5.946, p = 0.0288

These results indicate that both dose and duration contribute to growth inhibition, with higher concentrations exerting acute effects, and prolonged exposure exacerbating biomass loss (Table 5b).

Table 5a.Post hoc analysis of the mean differences of IGR based on various concentrations.

Comparison	Mean Difference	p-value
Control vs 200 mg/kg	-0.124	0.096
Control vs 400 mg/kg	-2.007	0.076
Control vs 600 mg/kg	-2.930	0.108
Control vs 800 mg/kg	-7.003	0.013
Control vs 1000 mg/kg	-8.357	0.005

Table 5b.Post hoc analysis of the mean differences of IGRbased on various durations.

Comparison	Mean Difference	p-value
7 vs 14 d	-4.314	0.0324
7 vs 21 d	-5.780	0.0326
7 vs 28 d	-5.946	0.0288

The IGR patterns demonstrate a graded, dose and timedependent suppression of growth in *D. willsi*. Low CuO NP doses initially induced mild sub-lethal stress, intermediate doses caused progressive metabolic disruption, and high doses resulted in acute toxicity and near-totalbiomass loss. Statistical analyses and post-hoc comparisons confirm that concentration is the dominant determinant of growth inhibition, with exposure duration amplifying the effects over time.

DISCUSSION

The present investigation demonstrates a clear and graded suppression of instantaneous growth rate (IGR) in *Drawida willsi* following exposure to increasing concentrations of copper oxide nanoparticles (CuO NPs) across 7–28 days, consistent with reported growth inhibition patterns in soil invertebrates exposed to metal-based nanoparticles (Wang *et al.,* 2024b; Singh *et al.,* 2025). The revised dataset confirms a strong negative dose–response relationship, a pattern widely recognized in nanoparticle ecotoxicology (Yang *et al.,* 2023a; Fischer *et al.,* 2022). Control worms maintained consistently positive growth, reflecting stable physiological and metabolic functioning under uncontaminated conditions (Jager *et al.,* 2023b), whereas exposed groups exhibited progressive decline, in agreement with findings that CuO nanoparticles disrupt somatic growth through oxidative and metabolic stress pathways (Kumari *et al.,* 2024; Wang *et al.,* 2024b). Slight inhibition at 200 mg kg^{-1} suggests early sublethal stress responses typical of low-dose nanoparticle exposure (Schlich *et al.,* 2022a), while marked suppression at 400–600 mg kg^{-1} aligns with reports of concentration-dependent impairment of antioxidant defense and energy allocation in annelids (Zhao *et al.,* 2021; Yang *et al.,* 2023b). Severe negative growth at 800–1000 mg kg^{-1} indicates systemic metabolic disruption and possible mitochondrial dysfunction under high nanoparticle burdens (Hou *et al.,* 2023b; Zhang *et al.,* 2024a).

Two-way ANOVA revealed that concentration exerted a dominant and highly significant effect, consistent with statistical evaluations identifying concentration as the principal determinant of nanoparticle toxicity in soil matrices (Fischer *et al.,* 2022; Wang *et al.,* 2024b), while exposure duration further amplified growth inhibition, supporting evidence that chronic exposure enhances cumulative oxidative and bioenergetic stress (van Gestel*et al.,* 2021; Schlich *et al.,* 2022a). Post-hoc comparisons confirmed that the highest concentrations (≥800 mg kg^{-1}) formed statistically distinct toxicity clusters relative to control and lower treatments, highlighting a threshold-dependent response model characteristic of metal oxide nanoparticle toxicity in terrestrial systems (Yang *et al.,* 2023b; Wang *et al.,* 2024a).

The persistence of positive growth in control worms reflects stable physiological functioning and effective energy assimilation in the absence of nanoparticle stress. In contrast, the pronounced negative IGR values observed at 800 and 1000 mg kg^{-1} indicate net biomass loss, suggesting that metabolic costs exceeded energy intake under high CuO NP exposure. Such responses are widely associated with nanoparticle-induced oxidative stress, where overproduction of reactive oxygen species (ROS) disrupts redox balance, damages macromolecules, and impairs mitochondrial function (Wang *et al.,* 2022; Hou *et al.,* 2023b). Studies on earthworms and other soil invertebrates have shown that CuO NPs elevate lipid peroxidation and suppress antioxidant enzymes such as superoxide dismutase and catalase, ultimately compromising somatic growth and tissue integrity (Li *et al.,* 2022; Zhang *et al.,* 2024b).

The progressive decline in IGR over time further indicates cumulative physiological stress. At 200 mg kg^{-1}, worms initially exhibited near-normal growth, but IGR became negative by days 21–28, suggesting delayed toxicity typical of sublethal nanoparticle exposure. Chronic exposure can promote gradual bioaccumulation of copper ions released from CuO NPs, overwhelming detoxification pathways and leading to sustained metabolic imbalance (van Gestel*et al.,* 2021; Hirano and Maher, 2024). Similar time-dependent toxicity has been reported in *Eisenia fetida*, where prolonged nanoparticle exposure intensified oxidative damage and energy depletion despite limited early mortality (Schlich *et al.,* 2022a; Wang *et al.,* 2024c).

The concentration range of 400–600 mg kg^{-1} appears to represent a critical inflection zone. In this interval, IGR shifted from marginal suppression to consistently negative values, and post-hoc analyses indicated divergence from both low-dose and high-dose groups over longer exposure durations. This pattern suggests that compensatory homeostatic mechanisms—such as upregulation of antioxidant defenses—may initially buffer stress but eventually fail as internal copper burdens rise. Comparable threshold-type responses have been described for metal oxide nanoparticles in soil systems, where toxicity intensifies sharply beyond moderate concentrations (Yang *et al.,* 2023a; Zhao *et al.,* 2021).

Beyond direct physiological toxicity, microbiome-mediated mechanisms may also contribute to growth inhibition under nanoparticle stress, as emerging evidence indicates that engineered nanomaterials can indirectly affect host performance through alterations of associated microbial communities (Zhou *et al.,* 2023; Wang *et al.,* 2024b). Earthworm gut microbial communities play an essential role in nutrient assimilation, cellulose degradation, and organic matter processing, thereby supporting host growth and soil biogeochemical cycling (Singh *et al.,* 2025; Wu *et al.,* 2024). Nanoparticle exposure has been shown to alter gut microbial diversity, enzymatic activity, and functional gene expression profiles, potentially impairing digestive efficiency and energy acquisition (Zhou *et al.,* 2023; Kumari *et al.,* 2024). Metal oxide nanoparticles, including CuO NPs, can disrupt microbial membrane integrity and redox homeostasis, leading to shifts in dominant taxa and suppression of beneficial symbionts (Yang *et al.,* 2023b; Wang *et al.,* 2024a). Disruption of host–microbiome interactions may therefore compound oxidative and metabolic stress responses in earthworms, amplifying energy reallocation toward detoxification and maintenance pathways (Jager *et al.,* 2023a; Hou *et al.,* 2023b). Such combined physiological and microbiome-mediated stress mechanisms plausibly contribute to the sustained negative instantaneous growth rates (IGR) observed at higher nanoparticle concentrations, particularly under chronic exposure conditions (Schlich *et al.,* 2022a; Zhang *et al.,* 2024a).

From an ecological perspective, suppressed growth in *D. willsi* has broader implications. Earthworms are recognized as ecosystem engineers that regulate litter decomposition, soil aggregation, and nutrient cycling. Chronic copper exposure has been linked to reduced soil enzymatic activity and microbial biomass, thereby compounding ecosystem-level stress (Zhao *et al.,* 2021; Yang *et al.,* 2025). Declines in worm biomass may diminish soil aeration and organic matter turnover, potentially altering carbon sequestration dynamics and plant productivity. Thus, nanoparticle-driven growth inhibition may propagate through multiple trophic and biogeochemical pathways.

Mechanistically, the severity of negative IGR at 800–1000 mg kg^{-1} suggests that toxic thresholds were exceeded, shifting organisms from adaptive stress responses to systemic metabolic disruption. High CuO NP exposure has been associated with mitochondrial dysfunction, apoptosis induction, and altered ATP production in annelids (Zhang *et al.,* 2024b; Hou *et al.,* 2023b). Within an energy-budget framework, toxicant stress reallocates energy from growth and reproduction toward maintenance and detoxification, resulting in measurable declines in somatic biomass (Jager *et al.,* 2023a). The observed growth suppression in *D. willsi* aligns closely with such bioenergetic models of contaminant stress.

Importantly, while statistical significance was strongest at higher concentrations, lower doses (200–600 mg kg^{-1}) demonstrated biologically meaningful downward trends.

Contemporary statistical interpretation emphasizes that effect magnitude and ecological relevance should complement p-values when evaluating toxicological outcomes (Amrhein *et al.,* 2019; Wasserstein *et al.,* 2019). Even in cases where $p > 0.05$, consistent directional decline across time points suggests emerging stress that may manifest more clearly under longer or multigenerational exposure.

The application of Tukey-adjusted post-hoc tests strengthens confidence in the robustness of high-dose effects, minimizing the risk of Type I error while preserving statistical power. That the 1000 mg kg^{-1} treatment remained significant under conservative comparison underscores the reliability of the observed toxicity pattern.

Collectively, these findings confirm that instantaneous growth rate is a sensitive and integrative sublethal endpoint for evaluating nanoparticle toxicity in soil invertebrates. Unlike mortality, which reflects terminal outcomes, IGR captures early energetic imbalance and physiological disruption, offering a predictive indicator of longer-term ecological impairment (Jager *et al.,* 2023a; Wang *et al.,* 2024b).

These findings are consistent with previous reports on the toxicity of metal oxide nanoparticles in soil invertebrates, highlighting oxidative stress, disrupted energy metabolism, and impaired nutrient assimilation as mechanisms underlying growth inhibition (Li *et al.,* 2022; Wang *et al.,*2022, 2024b; Jager *et al.,* 2023a). The study reinforces the utility of instantaneous growth rate as a sensitive biomarker for ecotoxicological assessment of engineered nanoparticles in terrestrial ecosystems.

CONCLUSION

This study establishes a clear dose- and duration-dependent suppression of instantaneous growth rate in *Drawida willsi* exposed to CuO nanoparticles. Concentration emerged as the primary determinant of toxicity, with prolonged exposure exacerbating biomass loss. Significant declines at ≥800 mg kg^{-1} confirm a high-dose toxicity threshold, while moderate concentrations indicate progressive metabolic stress over time. The findings align with contemporary evidence linking metal oxide nanoparticles to oxidative damage, microbiome alteration, and disrupted energy metabolism in soil invertebrates.

Overall, IGR proves to be a robust and ecologically meaningful biomarker for assessing nanoparticle-induced sublethal toxicity, providing critical insights for environmental risk assessment and sustainable management of engineered nanomaterials in terrestrial ecosystems.

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