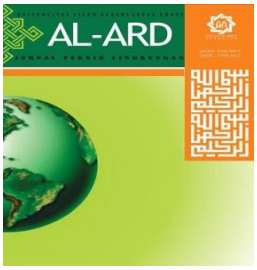

Al-Ard: Jurnal Teknik Lingkungan
Vol.11 No.2 – Maret 2026 (hal. 101-108)
http://jurnalsaintek.uinsa.ac.id/index.php/alard/index

Al-Ard:
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Preliminary Range-Finding Test of *Vetiver zizanioides* under Copper-Contaminated Water

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Abstract
Copper contamination from anthropogenic activities negatively impacts water quality and agriculture, with documented cases showing reduced crop yields and economic losses. This study conducted a preliminary screening to evaluate the survivability and toxicity signs of *V. zizanioides* under copper exposure as an initial assessment of its potential for phytoremediation. A *range finding test* was performed using five Cu concentration (100, 250, 500, 750, 1000 mg/L) and control. Following 40 days-propagation period, plants were exposed to Cu for 7-14 days. Daily observations included plant condition and height, while pH and temperature were periodically monitored to ensure that plant mortality resulted from toxicity rather than environmental factors. The results indicated that *V. zizanioides* tolerated copper concentration up to 100 mg/L. However at concentrations above 100 mg/L, typical toxicity symptoms, such as chlorosis and necrosis, appeared as early as the second day. Complete plant mortality was observed at the highest concentration, 1000 mg/L. Although this study did not assess metal removal performance, these findings highlight the resilience of *V. zizanioides* under copper stress and provide an important basis for future research to determine its suitability for phytoremediation or constructed wetlands.
Keywords: copper, range finding test, toxicity, *Vetiver*.

Abstrak
Kontaminasi tembaga akibat aktivitas antropogenik berdampak negatif terhadap kualitas air dan pertanian, dengan kasus terdokumentasi menunjukkan penurunan hasil panen dan kerugian ekonomi. Penelitian ini melakukan uji *screening preliminary* untuk mengevaluasi kelangsungan hidup dan respons toksisitas *V. zizanioides* terhadap paparan tembaga, yang berfungsi sebagai penilaian awal potensi penggunaannya dalam fitoremediasi. *Range finding test* (RFT) diterapkan menggunakan lima konsentrasi Cu (100, 250, 500, 750, 1000 mg/L) serta kontrol kontrol. Setelah periode propagasi selama 40 hari, tanaman dipaparkan terhadap Cu selama 7–14 hari. Pengamatan harian meliputi kondisi dan tinggi tanaman, sedangkan pH dan suhu diukur secara berkala untuk memastikan bahwa kematian tanaman disebabkan oleh toksisitas, bukan faktor lingkungan. Hasil RFT menunjukkan bahwa *V. zizanioides* mampu bertahan hingga konsentrasi tembaga 100 mg/L. Namun, pada konsentrasi di atas 100 mg/L, gejala toksisitas tembaga seperti klorosis dan nekrosis mulai terlihat sejak hari kedua. Kematian total tanaman terjadi pada konsentrasi tertinggi, yaitu 1000 mg/L. Meskipun penelitian ini tidak mengukur kemampuan penyerapan logam, temuan ini menunjukkan ketahanan *V.zizanioides* terhadap stress tembaga dan menjadi dasar bagi penelitian lanjutan untuk menilai kesesuaiannya dalam fitoremediasi atau lahan basah buatan.
Kata Kunci : tembaga, r*ange finding test*, toksisitas, *Vetiver*

1. INTRODUCTION

Heavy metal contamination deserves particular attention given its persistent, bioaccumulative nature, and potential for biomagnification throughout the food chain. Unlike organic pollutants, heavy metals are non-biodegradable and persist in aquatic environments for extended periods, often causing toxic effects even in areas geographically distant from the original sources (Kumar *et al*., 2019). Copper (Cu), one of the heavy metals commonly found in aquatic systems, is primarily introduced through anthropogenic activities such as urbanization, industrial development, and mining (Rehman *et al*. 2019; Shabbir *et al*. 2020). As a critical metal for the global energy transition, copper faces a projected demand growth of approximately 2.6% per year until 2035 (BHP 2024). Compared to other major metals

(aluminium, iron, zinc, lead and manganese), copper production is highly energy-intensive and has the greatest environmental impact per kilogram, including aquatic ecotoxicity, which is projected to increase by 2–4 times by 2060 (Elshkaki *et al*. 2016; OECD 2019).

Several studies have extensively documented the long-term impacts of copper mining on water quality (Nascimento et al., 2023; Perlatti et al., 2021) and agriculture (Campos and Mardones 2023). Remediation techniques such as constructed wetland (CW), require careful consideration of plant selection. The choice of appropriate plant species is crucial for the performances, with ideal candidates characterized by high biomass productivity and adaptability to local nutrient and climatic conditions (Kataki *et al*., 2021; Panghal *et al*., 2024). *V. zizanioides* is one such species widely used in CW systems due to its high productivity, tolerance to a broad spectrum of pollutants, and ease of cultivation (Kataki *et al*., 2021).

Previous studies have highlighted the ressilience of *V. zizanioides* under Cu exposure, however, these investigation have generally focused on relatively low concentrations, typically below 100 mg/L (Halim et al., 2023; Putra et al., 2019; Suelee et al., 2017) with one notable studies reaching 118.92 mg/L. (Roongtanakiat et al., 2007). Furthermore, most of these studies were conducted in mixed-metal environments, where multiple contaminants may produce antagonistic or synergistic effects that obscure copper-specific toxicity responses (Khan et al., 2023). To address this gap, the present study was designed to assess the response of *V. zizanioides* across a range of copper-only concentrations, from 100 to 1000 mg/L. By isolating Cu exposure, this study allows for a precise identification of Cu-specific phytotocixity markers without interference from other metallic ellements.

As a preliminary step for future remediation studies, a *Range Finding Test* is essential to estimate the threshold concentration of contaminants that a plant species can tolerate (USEPA, 1996). According to Purwanti et al. (2020), lethal concentration, at which plants wilt or die, are considered beyonds the plant's tolerance capacity and should be excluded from phytoremediation stage. Determining the maximum non-lethal concentration provides the essensial baseline required to guide the design of future studies, including detailed phytoxicity assesment and the practical implementation of phytoremediation system or constructed wetlands.

2. RESEARCH METHOD

2.1 Propagation and Growth Measurement

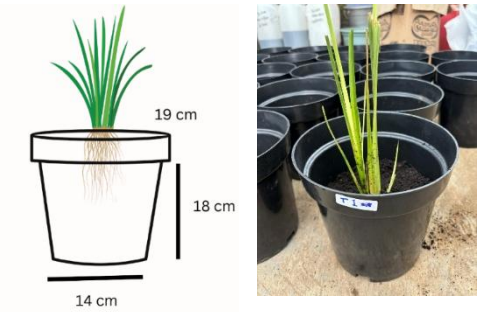


Figure 1. Propagation Reactors

Figure 1 shows the propagation stage conducted to multiply the plants required for the study and to obtain second-generation plants. *V. zizanioides* was cultivated in 4 L reactors using garden soil as the planting medium and was routinely watered every two days. In this stage, plant was observed daily for approximately 2 months, included plant height (measured from the base of the stem touching the ground to the top of the plants) (Purwanti *et al*., 2020).

2.2 Range Finding Test (RFT)

Synthetic Cu wastewater was prepared by dissolving Copper (II) Sulfate Pentahydrate ($CuSO_4.5H_2O$) in distilled water (Mojiri *et al*., 2017; Racha *et al*., 2022). The Cu concentrations used in the RFT ranged from 100 to 1000 mg/L (100, 250, 500, 750, 1000 mg/L), after lower concentration range 2-75 mg/L (2, 5, 10, 25, 50, 75 mg/L), which did not exhibit any symptoms of acute toxicity/ mortality in the test plants. The preliminary range included Cu concentrations associated with the maximum levels reported in global surface water bodies (Kumar *et al*., 2019). The concentration series followed a ratio <2.5, which is within the acceptable range of USEPA OPPTS 850.4400 (2-4 times) and OECD (<3.2).

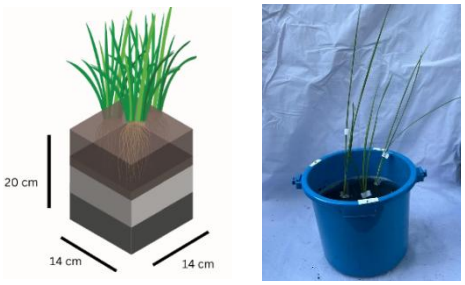


Figure 2. *Range Finding Test Reactors*

The number of plants used in the RFT was 3 (the recommended number of plants is 3-5) (EPA, 2012). The growth medium consisted of a gravel and sand mixture (1:2) in a plastic reactor with a capacity of approximately 4 L, as shown in Figure 2. Plants were initially exposed for 7 days, if no changes occurred during the exposure period, the testing was extended by 24 hours – 14 days (Arliyani *et al*., 2023). At each RFT concentration, plants conditions, including changes in size, chlorosis, necrosis, or other abnormalities, were recorded. If plants died or wilted within the RFT timeframe, the corresponding concentration was considered beyond the tolerance limit of the plants (Purwanti et al., 2020).

The survival function was estimated utilizing the Kaplan-Meir method, with analysis performed using DataTab. Furthermore, to ascertain the influence of copper concentration on overall survivability, a log-rank test was applied, with statistic significance defined as p-value < 0.05 (Imron *et al*., 2023).

3. RESULT AND DISCUSSION

3.1 Propagation and Growth Measurement

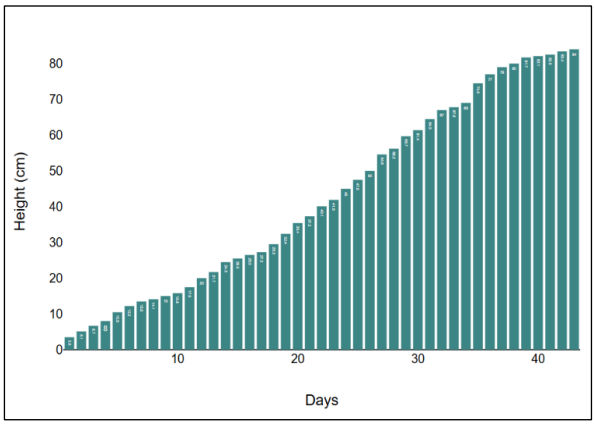
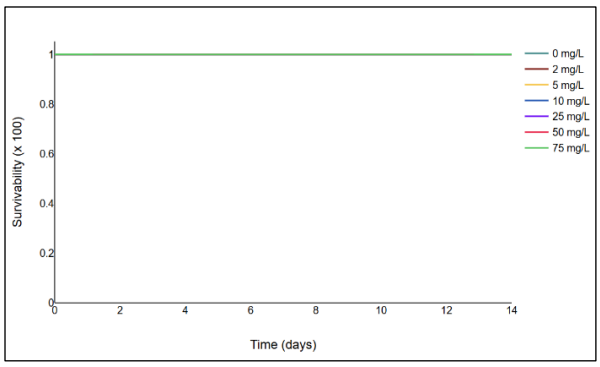


Figure 3. *V. zizanioides* Growth Observation

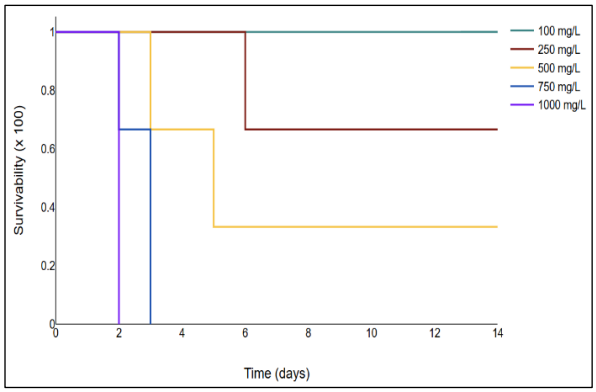
Figure 3 shows the average growth rate of *V. zizanioides*, approximately 1.9 cm/day; within 40 days of observation, plants reached ±84 cm. The observed rate aligns with reported literature, typically ranging from 1.8 cm/day (Kumar, 2025) to 4 cm/day (Mahmoudpour *et al*., 2021), with variation influenced by environment conditions, where the fastest growth rates are reported at 25 C.

3.2 Range Finding Test

It should be noted that copper is one of the eight essensial micronutrients for plants growth, primarily due to its role in the formation of metalloproteins and enzymes that act as catalysts, facilitating various physiological and biochemical processes within the plant (Shabbir *et al*., 2020).



(a)



(b)

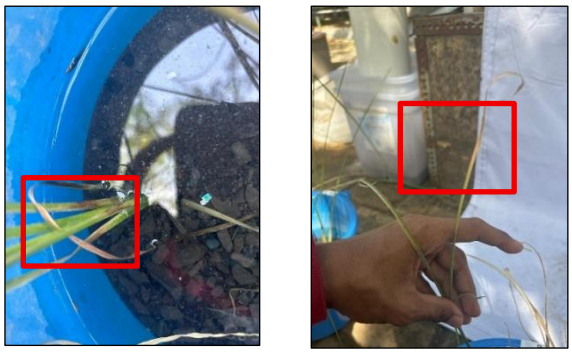
Figure 4. *V. zizanioides* Survivability during Range Finding Test: (a) 0-75 mg/L; (b) 100-1000 mg/L

Based on RFT observations, it was found that *V. zizanioides* could tolerate Cu up to 100 mg/L (pH 4). Figure 4, referring to the Kaplan–Meier graph, shows a survival rate of 100% at concentrations < 100 mg/L, while at concentrations > 100 mg/L, survival rates ranged from 0–66%. The log-rank test indicated that concentration level had a significant effect on plant survival (p = 0.002). This study found that a high Cu concentration (1000 mg/L) induced plant death after 2 days of exposure, with survival rate is 0 %, while exposure to 250 mg/L Cu showed a 66 % survival rate, with the first mortality observed on day 6. A study by Trung Minh *et al*. (2023), showed that *V. zizanioides* did not die at a Cu concentration of 46 mg/L (mixed metal concentration), whereas other plants (*Pistia stratioites, Lemna sp, Tape grass*) showed toxicity at concentration ranging from 10–25 mg/L (Enochs *et al*., 2023; Tang, Awa, & Hadibarata, 2020; Trung Minh *et al*., 2023). However, it should be noted that heavy metals

toxicity varies depending on concentration and the plant species or genotype involved (Cruz et al. 2022). Common Cu toxicity symptoms include: (1) leaf chlorosis (causing white or cream-coloured spots); (2) necrosis at the tips or edges of leaves (Shabbir *et al*., 2020); (3) root system darkening; (4) reduced plant growth (diameter, length, area, biomass) (José Rodrigues Cruz *et al*., 2022), which were observed in this study at concentration > 100 mg/L.

Study Halim *et al*. (2023) on Cu toxicity in *V. zizanioides* showed a daily change in plant colour from green to brown, caused by the inhibition of plant metabolism by heavy metals, which reduced chlorophyll content. Chlorosis caused by Cu can occur through inhibition of pigment formation and disruption of chlorophyll synthesis in the photosynthetic system (Kumar *et al*. 2021).

Figure 5 shows chlorosis symptoms on day 2 of Cu exposure (250 mg/L), while *necrosis* symptoms appeared on day 5. As reported by Mir *et al.* (2021), excessive Cu accumulation damages the internal structure of chloroplast, leading to alterations ih the thylakoid membrane. Thus, leaf chlorosis serves as an early indicator of copper toxicity.



(a) (b)

Figure 5. Toxicity Symptoms: (a) *Necrosis;* (b) *Chlorosis*

Study by Banerjee et al. (2016) showed *V. zizanioides* cultivated in mine-medium exhibited a notable reduction in chlorophyll content, as well in shoots and root length, along with a significant increase in malondialdehyde (MDA) level, indicating an imbalance in reactive oxygen species (ROS) due to stress. ROS are naturally produced are typically neutralized by antioxidants. The role of Cu in plants can be twofold: (1) it is beneficial in maintaining stable ROS levels within plant metabolism, and (2) it becomes harmful when excessive ROS production leads to oxidative stress, damaging essential components such as DNA, proteins, and lipids (Shabbir et al., 2020). According to Cruz *et al.* (2022), Cu triggers oxidative stress due to its redox properties, catalyzing reactions that produce reactive oxygen species such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH•), thereby promoting lipid peroxidation. The formation of these radicals is driven by mechanisms such as Fenton-type reactions, depletion of reduced glutathione, and substitution of iron in Fe-clusters, and is further intensified by the Haber–Weiss reaction, which generates additional superoxide and hydroxyl radicals.

Comparing to other studies, Suelee (2016) reported that *V. zizanioides* exhibited symptons like chlorosis due to malnutrion (controls) or over nourishment, as well as a leaf browning wilting, indicative of necrosis, under prolonged exposure to Cu concentration up to 10 mg/L. However, this study was conducted in mixed metal solution (with Fe, Pb, Mn and Zn). Putra et al. (2019), using *V. zizanioides* to remove Cu and Fe at concentrations of 30 mg/L and 12 mg/L in wastewater, observed phytomorphological changes such as wilting and drying (burn-like symptoms). Chlorosis (yellowing) occurred on the 3rd day of exposure, while necrosis appeared on the 5th day. Similarly, Halim et al. (2023) reported symptoms in *V. zizaniodes*, including leaf browning due to over-nourishment (high concentration of Cu and Fe, conc. of 30 mg/L and 10 mg/L) and wilting as an indication of necrosis. As Cu levels increase, plant leaves show sign of damage, particularly necrotic areas at the tips and edges. Concurrently, the overall size of the leaves, including their area, diameter, and length, tends to diminish. In case of severe Cu poisoning, leaves may initially wilt before turning necrotic (Xu et al. 2024).

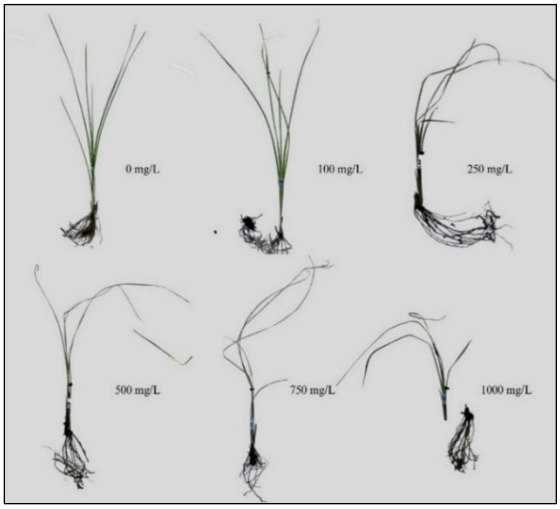


Figure 6. Toxicity of *V. zizanioides* Post-Harvesting After 14 Days of Copper Exposures

Figures 6 show severe damage in *V. zizanioides* when Cu concentrations hit > 100 mg/L, with root darkening observed at 250–750 mg/L and structural detachment occurring at 1000 mg/L. According to Gautam and Agrawal (2017), darkening of root structures (from cream to dark brown) occurs through suberization mechanism, whereby metal movement is blocked by suberized walls in the Casparian strip, limiting metal transport and causing accumulation in the cortex, which subsequently leads to discolouration. Visible symptoms of root damage do to Cu exposure vary, including damage to the root cuticle, reduction in root hair growth, and severe deformation of root structure (Kumar *et al*., 2021).

Cu toxicity initially affects plant roots before spreading to above-ground parts, disrupting numerous physiological functions. The inhibition of root growth is often associated with damage to the epidermis and exodermis. Previous research indicated that copper ions influences root development by altering the proliferation rate of root meristem cells through the regulation of hormones such as melatonin, auxin, and abscisic acid (Chen et al., 2022). A similar finding was reported by Gautam and Agrawal (2017), where *V. zizanioides* roots darkened under higher ratios of red-mud containing various heavy metals, including Cu.

Table 1. Wet Biomass and Growth Rate After 14 Days Exposure

Treatment (mg/L)	Sample Size	Biomass Before (gr)	Biomass After (gr)	Biomass Change (%)	Growth Rate (cm/day)
Control	3	6.40 (±0.96)	8.14 (±3.23)	+ 24.48 (30.93)	0.8 (±0.9)
100		6.39 (±0.56)	6.67 (±1.76)	+ 3.2 (18.95)	0.6 (±0.9)
250		6.45(±0.68)	5.96 (±1.62)	- 8.55 (18.88)	0.0 (±0.1)
500		5.99 (±1.89)	5.73 (±3.58)	- 11.35 (33.31)	0.1 (±0.0)
750		6.38 (±1.20)	5.32 (±2.88)	- 20.06 (27.86)	0.0 (±0.0)
1000		6.38 (±1.13)	5.55 (±0.83)	- 12.75 (2.65)	0.1 (±0.1)

Furthermore, heavy metals stress in plants can lead to reduction in root elongation and root hairs, that may result in decreased in water absorption and nutrient transport to the upper parts of the plants (Gautam and Agrawal 2017). Similar results were observed in this study, as shown in Table 1, where a decrease in wet biomass occurred at Cu concentrations > 100 mg/L. Athough no statistically significant correlation was found between Cu concentration and post-biomass using Pearson analysis (p= 0.12, r= -0.38), a significant negative correlation was observed between Cu concentration and growth rate (p= 0.021; r =-0.54), indicating that increasing Cu concentration reduces the growth rate of *V. zizanioides*. Cu toxicity generally impairs key physiological process in plants, including photosynthesis, antioxidant mechanisms, and mineral metabolism, ultimately leading to reduced plant growth (Chen et al., 2022). One-way ANOVA revealed that differences in biomass change and growth rate across treatments were not statistically significant (p>0.05), although visual toxicity symptoms were clearly evident.

V. zizanioides has a high adaptability to diverse environmental conditions, with temperature tolerance ranging from -15 °C to 55 °C and a pH range of 3-11 (Barcellos-Silva *et al*., 2025). Based on observations, temperature and pH remained within the acceptable range for *V.zizanioides.*

4. CONCLUSION

This study evaluated the potential of *V. zizanioides* for remediating copper-contaminated water using a range-finding test (RFT) as a preliminary assessment. The plant showed tolerance up to 100 mg/L, while concentrations > 100 mg/L induced toxicity symptoms, including chlorosis, necrosis, and

root damage (darkening and detachment), observed as early as the second day. These findings indicate the plant's resilience under moderate Cu exposure but sensitivity at higher concentrations.

Although *V. zizanioides* is adaptable to diverse environmental conditions, the interaction between high Cu levels and acidic conditions may increase metal bioavailability and stress. The RFT identified a response transition between 100–250 mg/L, representing an operational tolerance limit rather than a precise threshold. This range provides an important baseline for designing future phytoremediation studies. Further studies using narrower concentration ranges and additional parameters, such as Cu removal efficiency and uptake factors, are required to accurately evaluate phytoremediation performance.

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