

Original Research

# Removal of Copper and Zinc from Aqueous Media Using White Rot Lignicolous Fungi

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## ABSTRACT

In Peru, white-rot lignicolous fungi represent a biotechnological resource with high potential for the bioremediation of environments contaminated with heavy metals, offering an ecological alternative to traditional physicochemical methods. This study aimed to evaluate the capacity of four strains *Ganoderma applanatum*, *Pycnoporus sanguineus*, *Schizophyllum commune*, and *Pleurotus ostreatus* to remove copper and zinc from aqueous media at three metal concentrations. The cultures were maintained for 14 days under controlled conditions, and mycelial growth, biomass production, and removal efficiency were determined by atomic absorption spectrometry. The results revealed significant differences among strains and concentrations. *S. commune* and *P. ostreatus* showed greater mycelial growth and biomass production, whereas *G. applanatum* and *P. sanguineus* stood out for their high metal adsorption capacity. Removal efficiencies reached values of 92.10–96.77% for copper and 91.88–99.05% for zinc, confirming the ability of these fungi to capture and retain heavy metals in aqueous systems. The findings demonstrate the potential of lignicolous fungi as a sustainable alternative for bioremediation, contributing to the development of

clean technologies applicable to the treatment of effluents contaminated with heavy metals. The use of lignicolous fungi in bioremediation processes could reduce operational costs, minimize secondary environmental impacts, and promote nature-based solutions, thereby strengthening sustainable environmental management strategies in the Peruvian context.

## 1. INTRODUCTION

Human activities and industrial expansion have significantly intensified the release of persistent organic pollutants and heavy metals into the environment, leading to soil and water degradation, ecosystem alterations, and considerable risks to human health (Zare et al., 2024; Majumdar, 2024). This phenomenon is closely associated with the growth of mining, metallurgical, electroplating, and manufacturing activities, as well as with the inadequate treatment of industrial effluents and acid mine drainage, which are recognized among the main sources of water pollution worldwide.

In countries with intensive extractive activities, such as Peru, the discharge of untreated or partially treated effluents represents a persistent environmental challenge, particularly in regions influenced by mining operations, where elevated concentrations of heavy metals are frequently reported in surface and groundwater.

In this context, the increasing release of heavy metals such as copper (Cu) and zinc (Zn) into aquatic environments represents a critical environmental threat. Due to their persistent and non-biodegradable nature, these elements tend to accumulate in the environment and become incorporated into trophic chains, promoting processes of bioaccumulation and biomagnification. As a consequence, they can alter ecological interactions, affect evolutionary processes, and contribute to the decline or disappearance of endemic species (Sanyal et al., 2015; Luna et al., 2020; Paria et al., 2021). Moreover, even at relatively low concentrations, Cu and Zn interfere with essential biological functions and can cause chronic toxic effects in both aquatic organisms and human health (Singh & Singh, 2024).

In response to this issue, conventional physicochemical effluent treatment methods present significant limitations, including high operational costs, reduced efficiency in complex contaminant mixtures, and the generation of secondary pollutants such as sludge (Chen et al., 2022; Gao et al., 2024; Harish et al., 2024). However, these approaches often fail to provide sustainable and cost-effective solutions, particularly in developing regions where technological and economic constraints persist.

These limitations have driven increasing interest in biological alternatives, which are characterized by lower environmental impact and greater adaptability. Among them, processes such as biosorption, biotransformation, and biomineralization have demonstrated considerable potential for the removal of heavy metals (Módenes et al., 2013; González-González et al., 2022). Nevertheless, their performance remains highly dependent on environmental conditions, biomass characteristics, and contaminant concentrations.

Among emerging biotechnological strategies, fungi have been widely recognized for their effectiveness in bioremediation due to their high tolerance to heavy metals, their capacity for mycosorption and bioleaching, and the presence of multiple functional groups (–OH, –NH, –COOH) on their cell surface that facilitate metal binding (Lei et al., 2018; Li et al., 2019; Amin et al., 2024; Zare et al., 2024).

Within this group, white-rot lignicolous fungi stand out due to their extracellular enzymatic system, composed of laccases, manganese peroxidases, and lignin peroxidases. This system not only enables the degradation of lignocellulosic compounds but also contributes to interactions with heavy metals through biosorption, bioaccumulation, and redox-mediated processes (Singh & Singh, 2024; Kato et al., 2024; Zehra & Meena, 2025; Singh et al., 2025).

Several studies have demonstrated their potential in heavy metal removal. For instance, strains of *Pleurotus ostreatus* have shown high tolerance to copper and significant removal efficiency in aqueous systems, along with the induction of ligninolytic enzymes under metal stress (Gao et al., 2024). Similarly, other white-rot fungi have achieved removal efficiencies exceeding 70% for metals such as lead, cadmium, and nickel under controlled conditions (Sharma et al., 2020). However, these studies are often limited to single-metal systems or controlled laboratory conditions that do not fully represent the complexity of real effluents.

Despite more than two decades of research in this field (Lu et al., 2020), critical gaps persist regarding the comparative evaluation of multiple lignicolous strains under factorial experimental designs that incorporate different metals and environmentally relevant concentrations. Furthermore, the rapid decline of ligninolytic activity in aqueous environments remains a significant limitation that has not been sufficiently addressed (Langer et al., 2021; Hultberg & Golovko, 2024).

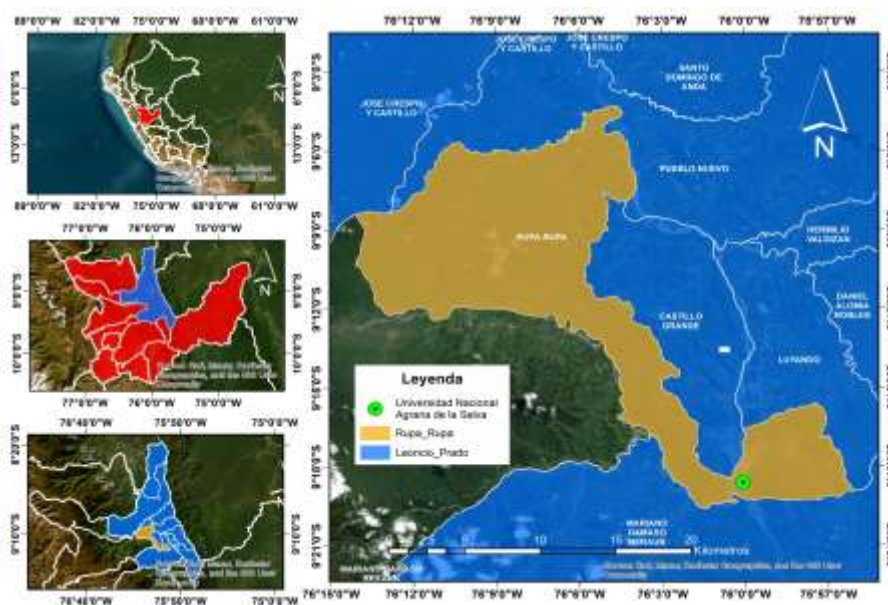
Within this framework, the present study aims to comparatively evaluate the removal capacity of four strains of white-rot lignicolous fungi for Cu and Zn in aqueous solutions simulating contaminated effluent scenarios. This research seeks to generate robust experimental evidence that supports the application of these microorganisms as sustainable, efficient, and cost-effective biotechnological alternatives for industrial wastewater treatment in Peru.

This study provides a novel comparative evaluation of native Peruvian lignicolous fungal strains under a factorial experimental design, assessing their removal capacity for Cu and Zn at environmentally relevant concentrations.

## 2. MATERIALS AND METHODS

### 2.1. Study area

The study was conducted in the Mycology and Propagation Technology Laboratory of the Universidad Nacional Agraria de la Selva, located in the Rupa Rupa district, Leoncio Prado province, Huánuco region (Figure 1). The geographic coordinates of the study area are  $76^{\circ}00'17''$  west longitude and  $9^{\circ}18'18''$  south latitude, at an altitude of 660 meters above sea level.



**Fig. 1:** Study area location.

## 2.2. White-rot lignicolous fungal strains

Four fungal strains were studied: *Ganoderma applanatum*, *Pleurotus ostreatus*, *Pycnoporus sanguineus*, and *Schizophyllum commune*. All specimens were collected from the Reserved Forest of the Universidad Nacional Agraria de la Selva and the Botanical Garden of Tingo María, located in the central Peruvian rainforest.

The samples were coded, photographed, and packaged *in situ*, and then transported to the laboratory, where they were dried for a period of 24 to 72 hours (depending on the strain) at a temperature of 60 °C (Robledo, 2006; Ryvardeen, 2007). Fungal identification was performed based on macroscopic characteristics and comparison with reference catalogs “Hongos de Allpahuayo-Mishana”; therefore, it should be considered as a preliminary identification (Mata, 1999; Mata et al., 2003).

## 2.3. Experimental design

Four fungal strains (*G. applanatum*, *P. sanguineus*, *S. commune*, and *P. ostreatus*), two contaminants (copper and zinc), and three concentrations per contaminant (10 mg L<sup>-1</sup>, 30 mg L<sup>-1</sup>, and 50 mg L<sup>-1</sup>) were studied. Additionally, three replicates were evaluated for each of the 24 treatments.

The evaluated concentrations (10, 30, and 50 mg L<sup>-1</sup>) were selected considering ranges reported for mining and metallurgical effluents, where Cu and Zn concentrations can vary widely depending on the degree of prior treatment. Studies on acid mine drainage and metallurgical effluents have documented metal concentrations ranging from 5 to >100 mg L<sup>-1</sup>, especially in partially treated or neutralized systems (Johnson & Hallberg, 2005; Fu & Wang, 2011).

Likewise, research on fungal biosorption frequently employs initial concentrations between 10 and 100 mg L<sup>-1</sup> to assess tolerance and removal efficiency under controlled conditions (Gadd, 2009; Kapoor & Viraraghavan, 1995).

In particular, concentrations of 10–50 mg L<sup>-1</sup> have been used in studies with ligninolytic fungi to determine removal capacity without causing complete growth inhibition (Anastopoulos & Kyzas, 2015; Baldrian, 2003). Therefore, the selected levels represent realistic scenarios of moderate to high contamination and allow for comparability with the international literature.

## 2.4. Preparation and plating of solid media

For the primary isolation of fungal strains, malt extract agar (50 g) and potato dextrose agar (39 g) were used to prepare 1.5 L of medium, each prepared separately. The components were dissolved under constant stirring and brought to a boil for 10 minutes until complete homogenization was achieved. Subsequently, the media were sterilized in an autoclave at 121 °C and 15 psi for 15 minutes.

After sterilization, the media were transferred to a laminar flow chamber previously disinfected with UV radiation for 15 minutes. Ceftriaxone (0.3 g per total prepared volume) was added as an antibacterial agent, and the mixture was homogenized prior to plating to minimize the risk of contamination.

## 2.5. Disinfection and establishment of axenic cultures

Fresh selected fruiting bodies were superficially cleaned and cut into fragments of approximately  $0.5 \times 0.5$  cm. Disinfection was carried out following the protocols of Córdova (2010) and Ríos-Ruiz and Ruiz-Rengifo (1993), using sodium hypochlorite (NaOCl) diluted 1:10 for 1 minute, followed by three consecutive rinses with sterile distilled water. The fragments were dried on sterile filter paper and placed at the center of 90 mm Petri dishes containing potato dextrose agar and malt extract agar.

The plates were sealed with parafilm and incubated at 27 °C in darkness until mycelial growth was observed. All procedures were conducted under aseptic conditions in a laminar flow hood.

## 2.6. Preparation and activation of fungal inoculum

Inoculum standardization is essential to ensure reproducibility in biosorption and biotransformation studies (Kapoor & Viraraghavan, 1995; Gadd, 2009). For strain activation, Sabouraud dextrose broth was used, a carbon-rich medium due to its high dextrose content, which promotes growth in the exponential phase and metabolic activation associated with increased expression of ligninolytic enzymatic systems (Baldrian, 2003).

For the preparation of 1 L of Sabouraud broth, 40 g of dextrose and 10 g of peptone were used, completing the volume with distilled water. The medium was sterilized in an autoclave (121 °C, 15 min), and once cooled, ceftriaxone ( $0.2 \text{ g L}^{-1}$ ) was added. The axenic mycelium was subcultured into flasks containing this medium and incubated for 5 days at room temperature to obtain cultures in the exponential phase.

Prior to inoculation in the Mineral Salts Minimal Medium (MSMM), the biomass was homogenized and quantified by dry weight following standard methodologies for fungal growth studies (Suh et al., 2001). This standardization allowed the initial density to be expressed in  $\text{g L}^{-1}$  and ensured uniformity among experimental treatments.

## 2.7. Mineral Salts Minimal Medium (MSMM)

The Mineral Salts Minimal Medium (MSMM) was prepared by dissolving 5.23 g of  $\text{K}_2\text{HPO}_4$ , 1.91 g of  $\text{KH}_2\text{PO}_4$ , 0.09 g of  $\text{MgSO}_4$ , and 1 g of  $(\text{NH}_4)_2\text{SO}_4$  in distilled water, adding 1 mL of trace element solution, and adjusting the final volume to 1000 mL. The trace element solution (1 L) consisted of 20 mg of boric acid, 30 mg of zinc sulfate, 1 mg of copper sulfate, 3 mg of sodium molybdate, 10 mg of iron sulfate, and 2.6 mg of magnesium sulfate, all dissolved in distilled water to complete the final volume. For the induction phase, the

MSMM was supplemented with glucose ( $0.25 \text{ g L}^{-1}$ ) and 0.1% yeast extract to promote metabolic activation of the microorganism.

## 2.8. pH control and culture conditions

The initial pH of the MSMM was adjusted to  $5.5 \pm 0.1$  using 0.1 M HCl or NaOH prior to sterilization. pH is a determining factor in the ionic speciation of  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  and in the efficiency of fungal biosorption (Fu & Wang, 2011; Anastopoulos & Kyzas, 2015). Values between 4.5 and 6.0 favor the interaction between functional groups on the cell wall ( $-\text{OH}$ ,  $-\text{COOH}$ ,  $-\text{NH}_2$ ) and metal cations, minimizing abiotic precipitation (Gadd, 2009).

## 2.9. Chemical source of metals and evaluated concentrations

Copper and zinc were added as copper sulfate pentahydrate ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ ) and zinc sulfate heptahydrate ( $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ), respectively, both of analytical grade. These salts were selected due to their high solubility and frequent use in biosorption studies (Kapoor & Viraraghavan, 1995; Fu & Wang, 2011).

## 2.10. Induction, incubation, and experimental controls

The metal-supplemented media were sterilized and distributed into flasks, which were then inoculated with 7.5 mL of previously activated and standardized culture. The flasks were incubated for 14 days, a period determined based on studies reporting that the active phase of biosorption and bioaccumulation occurs within the first two weeks (Baldrian, 2003; Gadd, 2009). Preliminary assays confirmed stabilization of biomass and residual metal concentration by day 14.

It is important to note that all experimental units were inoculated with fungal biomass; therefore, abiotic controls (MSMM + metal without inoculum) were not included in the experimental design. Consequently, the study focuses on evaluating the overall removal efficiency in biological systems, without experimentally distinguishing between biological and physicochemical removal mechanisms such as precipitation or passive adsorption.

## 2.11. Determination of metals by FAAS

The residual concentration of Cu and Zn in the supernatant was determined by flame atomic absorption spectrophotometry (FAAS) using a SpectrAA 55B atomic absorption spectrophotometer (Varian Inc., USA), a widely validated technique for metal analysis in aqueous matrices (Welz & Sperling, 1999). Measurements were performed at 324.8 nm for Cu and 213.9 nm for Zn using an air–acetylene flame.

Calibration curves were constructed with coefficients of determination ( $R^2$ ) greater than 0.995. Analytical quality control included reagent blanks and triplicate measurements to ensure precision. Detection and quantification limits (LOD and LOQ) were determined following standard analytical procedures.

## **2.12. Analytical determinations**

### ***2.12.1. Evaluation of radial growth on solid media***

The radial growth of the fungal strains was evaluated on solid media consisting of potato dextrose agar (PDA) and malt extract agar (MEA). For each treatment, a 5 mm diameter mycelial disc was taken from the actively growing edge of a previously established culture and placed at the center of 90 mm diameter Petri dishes.

The plates were incubated at  $27 \pm 2$  °C under dark conditions. Colony diameter was measured daily for 15 days using a sterile millimeter ruler with a precision of 1 mm. In each plate, two perpendicular measurements (cross axes) were taken passing through the inoculation center, and the average of both was calculated to estimate daily radial growth.

Radial growth (mm) was determined as the distance from the point of inoculation to the active edge of the mycelium. When the colony reached the edge of the plate, growth was considered complete, and the maximum value corresponding to the internal diameter of the plate was recorded.

Additionally, macroscopic characteristics such as coloration, surface texture (cottony, waxy, or compact), presence of concentric zoning, mycelial density, and expansion pattern (dispersed or compact) were recorded. These observations were conducted descriptively and documented photographically to complement the quantitative analysis.

### ***2.12.2. Determination of fungal biomass***

After 14 days of incubation, the fungal biomass was recovered by centrifugation and filtration of the supernatant. The material was then dried in an oven until a constant weight was reached and quantified gravimetrically to determine the mycelial biomass corresponding to each copper and zinc concentration. Finally, an aliquot of the filtered supernatant was analyzed by spectrophotometry to assess the fungi's capacity to remove copper and zinc.

Cell density was determined from the dry weight of the mycelial biomass. To do this, the biomass was separated from the liquid medium by centrifugation at 3500 rpm for 15 minutes. It was then filtered using pre-weighed filter paper and placed in an oven at 80 °C until a constant weight was reached. The following equation was applied (López, 1998):

$$B = \frac{X_2 - X_1}{V} \quad \dots(1)$$

Where: B represents the biomass ( $\text{g L}^{-1}$ ),  $X_1$  refers to the weight of the filter paper, and  $X_2$  refers to the weight of the filter paper with biomass.

### 2.12.3. Determination of removal efficiency

Removal efficiency was determined based on the percentage correlation between the initial and final concentrations of copper and zinc in the aqueous medium contained in the flasks, using Equation 2, according to each applied treatment (Marcovecchio et al., 1991).

$$RE = \left( \frac{IC - FC}{IC} \right) \times 100 \quad \dots(2)$$

Where: RE represents the removal efficiency (%), IC refers to the initial concentration ( $\text{mg L}^{-1}$ ), and FC refers to the final concentration ( $\text{mg L}^{-1}$ ).

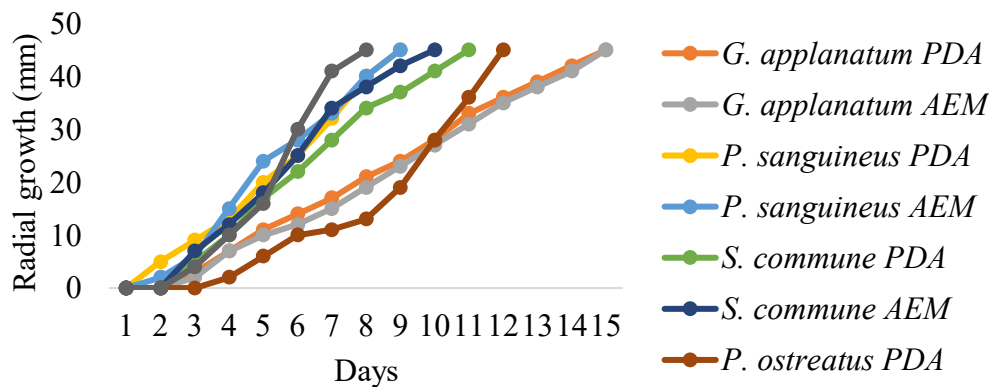
## 2.14. Data analysis

The data were analyzed using a completely randomized three-way ANOVA ( $4 \times 2 \times 3$ ), considering fungal strain (4 levels), metal type (2 levels), and concentration (3 levels) as factors, with three replicates per treatment ( $n = 72$ ). Main effects as well as two-way and three-way interactions were evaluated. Prior to analysis, assumptions of normality (Shapiro–Wilk test) and homogeneity of variances (Levene’s test) were verified. When significant differences were detected ( $p < 0.05$ ), Tukey’s multiple comparison test was applied.

## 3. RESULTS

### 3.1. Radial growth and macroscopic characterization of fungal strains

The radial growth of *Ganoderma applanatum*, *Pycnoporus sanguineus*, *Schizophyllum commune*, and *Pleurotus ostreatus* was evaluated on potato dextrose agar (PDA) and malt extract agar (MEA). The growth curves shown in Figure 2 exhibited a typical sigmoidal pattern, characterized by an initial lag phase, followed by an exponential phase, and finally a stationary phase, which is consistent with the dynamics of mycelial expansion in solid cultures.

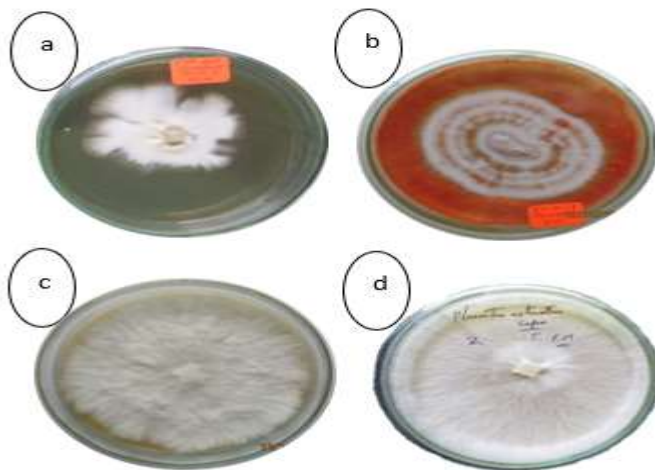


**Fig. 2:** Radial growth of fungal strains in two culture media.

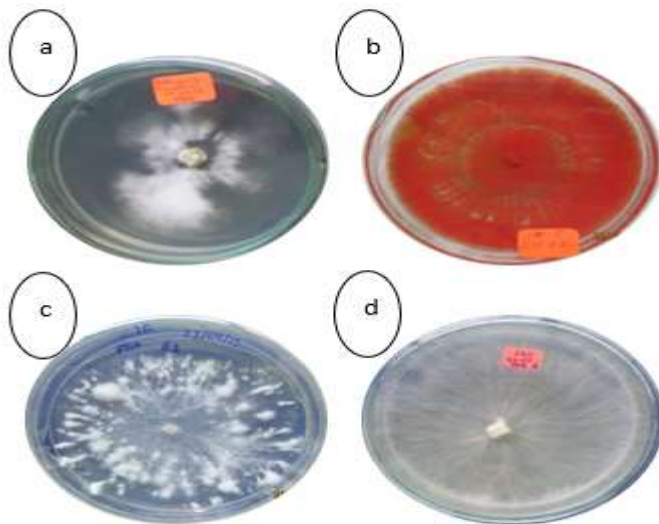
Two-way analysis of variance (species  $\times$  culture medium) revealed significant differences in radial growth attributable to the species factor ( $p < 0.001$ ), while the effect of the medium depended on the strain, showing a significant species  $\times$  medium interaction ( $p < 0.05$ ). This indicates that the response to the type of substrate is modulated by the intrinsic physiology of each fungus.

*S. commune* exhibited the highest radial growth rate, reaching 45 mm by day 11 on PDA and by day 9 on MEA, corresponding to an average rate of approximately  $4\text{--}5\text{ mm day}^{-1}$  during the exponential phase. In contrast, *G. applanatum* showed the slowest growth, failing to cover the entire plate within the same experimental period. *P. sanguineus* and *P. ostreatus* displayed intermediate rates, with vigorous development on both media.

In comparative terms, Figures 3 and 4 show that PDA favored a faster initial mycelial expansion in fast-growing strains, whereas MEA promoted greater continuity and stability in growth, particularly in *P. sanguineus* and *S. commune*. These differences suggest variations in the utilization of complex carbon sources and metabolic efficiency among species.



**Fig. 3:** Radial growth of fungal strains on malt extract agar (MEA) medium: a) *Ganoderma applanatum*. b) *Pycnoporus sanguineus*. c) *Schizophyllum commune*. d) *Pleurotus ostreatus*.



**Fig. 4:** Radial growth of fungal strains on potato dextrose agar (PDA) medium a) *Ganoderma applanatum*. b) *Pycnoporus sanguineus*. c) *Schizophyllum commune*. d) *Pleurotus ostreatus*.

From a macroscopic perspective, clear differences in colony morphology were observed. *S. commune* displayed dispersed growth on PDA and more compact mycelium on MEA. *P. sanguineus* initially showed a waxy texture on both media, becoming cottony on MEA and later developing concentric lines with pink to reddish hues. *G. applanatum* developed waxy mycelium on PDA and a more homogeneous and vigorous growth on MEA. *P. ostreatus* exhibited uniform and consistent growth on both media.

The observed differences in radial growth and mycelial morphology have direct implications for subsequent biotechnological performance. Strains with higher mycelial expansion rates, such as *S. commune*

and *P. sanguineus*, exhibit a larger active surface area and higher hyphal density in a shorter time, potentially increasing the availability of cell wall functional groups ( $-\text{OH}$ ,  $-\text{COOH}$ ,  $-\text{NH}_2$ ) involved in metal biosorption processes.

Likewise, the metabolic stability observed on MEA suggests a greater capacity for physiological maintenance, a condition relevant for coping with metal stress in the Mineral Salts Minimal Medium supplemented with Cu and Zn. In this regard, prior growth behavior serves as an indirect indicator of physiological vigor and potential tolerance to the  $10\text{--}50\text{ mg L}^{-1}$  concentrations evaluated in the removal assay.

Therefore, the characterization of radial growth not only allowed verification of the viability and purity of the strains but also provided a basis for their selection and differential performance in the  $4 \times 2 \times 3$  factorial design applied in the bioremediation stage.

### 3.2. Fungal biomass

Table 1 presents the results of the Shapiro–Wilk normality test for the fungal biomass variable ( $\text{g L}^{-1}$ ). The W value of 0.94 indicated an acceptable fit to the normal distribution ( $p > 0.05$ ), allowing the application of the three-way ANOVA.

**Table 1:** Shapiro–Wilk normality test for the fungal biomass variable.

Variable	Mean ( $\text{g L}^{-1}$ )	S.D ( $\text{g L}^{-1}$ )	W*
Fungal biomass	1.93	0.84	0.94

S.D: Standard deviation.

The analysis of variance (Table 2) showed a coefficient of variation of 30.34%, indicating moderate variability among treatments. The adjusted coefficient of determination ( $R^2_{\text{adj}} = 0.51$ ) suggests that the model explained 51% of the observed variability in fungal biomass.

**Table 2:** Analysis of variance for the fungal biomass variable.

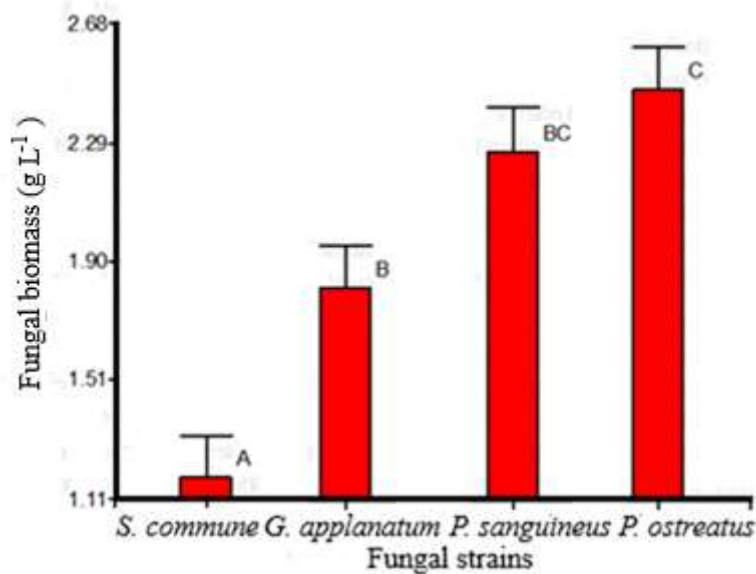
Source of variation	gl	S.S	M.S	p
Fungus	3	17.43	5.81	<0.0001
Metal	1	0.05	0.05	0.7088
Concentration	2	1.25	0.62	0.1746
Fungus*Metal	3	0.67	0.22	0.5879
Fungus*Concentration	6	5.57	0.93	0.0244

Metal*Concentration	2	3.87	1.93	0.0064
Fungus*Metal*Concentration	6	4.22	2.04	0.0780
Error	48	16.52	0.34	
Total	71	49.57		

S.S: Sum of squares. M.S: Mean squares.

Significant differences were detected among strains ( $p < 0.05$ ), while the factors metal and concentration showed no significant effect on biomass ( $p > 0.05$ ). No significant interactions were observed between fungus  $\times$  metal or fungus  $\times$  metal  $\times$  concentration, indicating that the growth response did not vary significantly across the evaluated levels of Cu and Zn.

Figure 5 shows numerical differences in average biomass among fungal strains, with *P. ostreatus* presenting higher mean values and *S. commune* lower values. However, Tukey's multiple comparison analysis (Table 3) did not reveal statistically significant differences among specific strain–metal–concentration combinations, as all treatments shared the same statistical grouping.



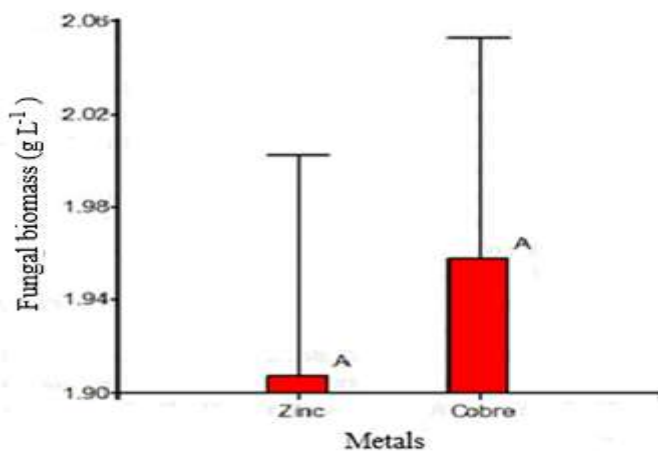
**Fig. 5:** Tukey test for the effect of fungal strains on fungal biomass ( $\text{g L}^{-1}$ ).

**Table 3:** Tukey test for the effect of treatments on the fungal biomass variable.

Fungal strains	Metal type	Concentration ( $\text{mg L}^{-1}$ )	Fungal biomass ( $\text{g L}^{-1}$ )
<i>S. commune</i>	Zinc	30	0.85a
<i>G. applanatum</i>	Zinc	50	0.85a
<i>S. commune</i>	Copper	50	0.98a
<i>S. commune</i>	Copper	30	1.02a
<i>P. sanguineus</i>	Copper	30	1.29a

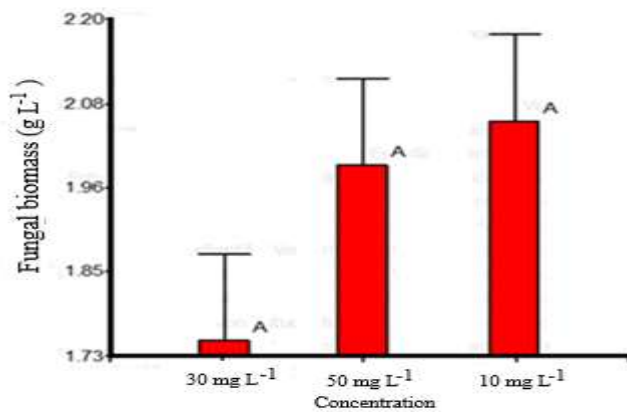
<i>S. commune</i>	Zinc	50	1.29a
<i>S. commune</i>	Copper	10	1.33a
<i>S. commune</i>	Zinc	10	1.64a
<i>P. sanguineus</i>	Zinc	50	1.82a
<i>G. applanatum</i>	Copper	50	1.82a
<i>G. applanatum</i>	Copper	30	1.87a
<i>P. sanguineus</i>	Copper	10	2.00a
<i>G. applanatum</i>	Zinc	30	2.05a
<i>G. applanatum</i>	Zinc	10	2.09a
<i>P. ostreatus</i>	Zinc	10	2.09a
<i>P. ostreatus</i>	Copper	30	2.09a
<i>G. applanatum</i>	Copper	10	2.22a
<i>P. ostreatus</i>	Copper	10	2.31a
<i>P. sanguineus</i>	Zinc	30	2.40a
<i>P. ostreatus</i>	Zinc	30	2.44a
<i>P. ostreatus</i>	Zinc	50	2.62a
<i>P. sanguineus</i>	Zinc	10	2.75a
<i>P. ostreatus</i>	Copper	50	3.24a
<i>P. sanguineus</i>	Copper	50	3.33a

Figure 6 indicates that biomass in the presence of copper and zinc was statistically similar, confirming the absence of a significant metal effect, as supported by the ANOVA results.



**Fig. 6:** Tukey test for the effect of metal type on fungal biomass (g L<sup>-1</sup>).

Similarly, Figure 7 shows that the concentrations of 10, 30, and 50 mg L<sup>-1</sup> did not produce significant differences in biomass, indicating that no significant differences in biomass were detected across the evaluated concentration levels, suggesting that fungal growth was not significantly affected within the tested range.



**Fig. 7:** Tukey test for the effect of metal concentration on fungal biomass (g L<sup>-1</sup>).

Although the combinations *P. ostreatus*–copper–50 mg L<sup>-1</sup> and *P. sanguineus*–copper–50 mg L<sup>-1</sup> showed higher numerical biomass values, these differences were not statistically significant according to Tukey’s test. Therefore, no specific treatment can be considered superior, and the results suggest a general physiological tolerance of the strains to the evaluated conditions rather than a treatment-specific effect.

Overall, while the ANOVA detected significant effects for the factor ‘fungus’, the lack of significant differences at the treatment level indicates that biomass remained statistically similar across specific combinations of fungus, metal, and concentration.

### 3.3. Removal efficiency

Table 4 presents the results of the Shapiro–Wilk normality test for the removal efficiency variable (%). The statistic  $W = 0.96$  ( $p > 0.05$ ) confirmed the fulfillment of the normality assumption, allowing the valid application of the three-way ANOVA.

**Table 4:** Shapiro–Wilk normality test for the removal efficiency variable.

Variable	Mean (%)	S.D (g L <sup>-1</sup> )	W*
Removal efficiency	95.48	2.15	0.96

S.D: Standard deviation.

The analysis of variance (Table 5) showed a coefficient of variation of 0.64%, indicating high experimental precision and minimal dispersion among replicates. Likewise, the adjusted coefficient of determination ( $R^2_{adj} = 0.92$ ) indicated that the model explained 92% of the observed variability, confirming a high explanatory capacity of the evaluated factors and their interactions.

**Table 5:** Analysis of variance for the removal efficiency variable.

Source of variation	gl	S.S	M.S	p
Fungus	3	29.98	9.99	<0.0001
Metal	1	88.13	88.13	<0.0001
Concentration	2	79.44	39.72	<0.0001
Fungus*Metal	3	16.70	5.57	<0.0001
Fungus*Concentration	6	43.21	7.20	<0.0001
Metal*Concentration	2	28.78	14.39	<0.0001
Fungus*Metal*Concentration	6	25.47	4.25	<0.0001
Error	48	17.72	0.37	
Total	71	329.4		

S.S: Sum of squares. M.S: Mean squares.

Highly significant effects ( $p < 0.0001$ ) were detected for the main factors fungus, metal, and concentration as well as for all two-way interactions and the three-way interaction (fungus  $\times$  metal  $\times$  concentration). This indicates that removal efficiency does not depend solely on each factor individually, but rather on the specific combination of fungal strain, metal type, and concentration level, revealing a differential behavior of the biological system under each experimental condition.

Figure 8 shows that *P. ostreatus* (95.94%), *S. commune* (95.84%), and *P. sanguineus* (95.76%) exhibited statistically similar efficiencies, all higher than *G. applanatum* (94.37%), suggesting a greater average removal capacity in the first three strains.

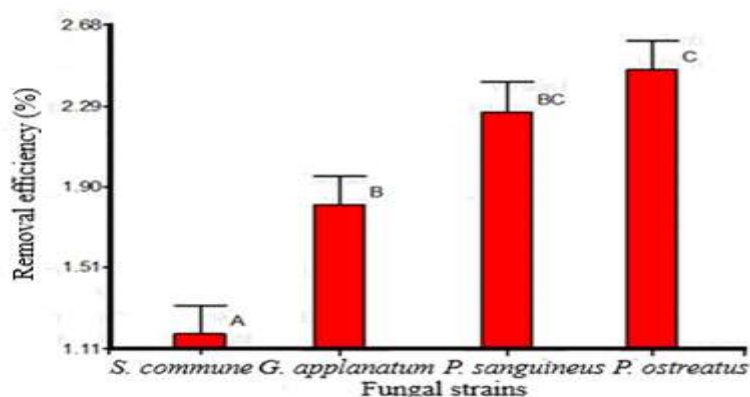
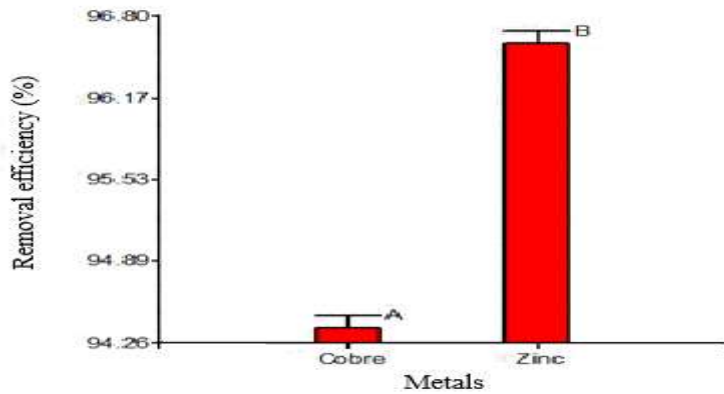
**Fig. 8:** Tukey test for the effect of fungal strains on removal efficiency (%).

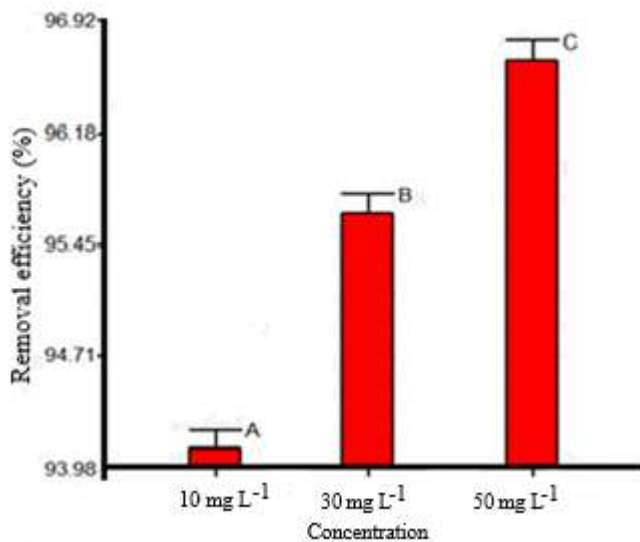
Figure 9 showed that the type of metal significantly influenced removal efficiency, with zinc being removed more efficiently than copper. This difference may be associated with differences in the physicochemical

behavior of each metal in solution; however, no mechanistic analyses were performed in this study to confirm the underlying processes.



**Fig. 9:** Tukey test for the effect of metal type on removal efficiency (%).

Figure 10 showed that the highest efficiencies were achieved at 50 mg L<sup>-1</sup>, suggesting an effect of the concentration gradient. A higher availability of the metal increases the driving force for mass transfer, suggesting a concentration-gradient effect, in which a higher initial concentration may enhance the overall removal capacity until approaching saturation conditions; however, the specific mechanisms involved were not evaluated.



**Fig. 10:** Tukey test for the effect of metal concentration on removal efficiency (%).

The analysis of the three-way interaction (Table 6) revealed that the highest efficiencies (>98%) were obtained in the combinations *S. commune*–zinc–50 mg L<sup>-1</sup> (98.92%) and *P. ostreatus*–zinc–50 mg L<sup>-1</sup> (99.05%), both belonging to the top statistical group. These results confirm the high removal capacity for zinc at elevated

concentrations and position these strains as promising candidates for bioremediation applications in matrices with high metal loads.

**Table 6:** Tukey test for the effect of treatments on removal efficiency.

Fungal strains	Metal type	Concentration (mg L <sup>-1</sup> )	Removal efficiency (%)
<i>G. applanatum</i>	Zinc	10	91.88a
<i>G. applanatum</i>	Copper	10	92.10ab
<i>P. sanguineus</i>	Copper	10	92.11ab
<i>P. ostreatus</i>	Copper	30	92.80abc
<i>P. ostreatus</i>	Copper	50	93.98bcd
<i>S. commune</i>	Copper	50	94.03cd
<i>S. commune</i>	Copper	30	94.15cd
<i>G. applanatum</i>	Copper	30	94.22cde
<i>P. sanguineus</i>	Zinc	10	94.44cdef
<i>S. commune</i>	Copper	10	95.04defg
<i>P. sanguineus</i>	Copper	30	95.15defg
<i>S. commune</i>	Zinc	10	95.43defg
<i>P. ostreatus</i>	Zinc	10	95.54defgh
<i>G. applanatum</i>	Copper	50	95.78defghi
<i>G. applanatum</i>	Zinc	30	96.09efghij
<i>G. applanatum</i>	Zinc	50	96.12efghij
<i>P. ostreatus</i>	Copper	10	96.33efghij
<i>P. sanguineus</i>	Copper	50	96.77efghij
<i>P. sanguineus</i>	Zinc	30	97.44hijkl
<i>S. commune</i>	Zinc	30	97.49ijkl
<i>P. ostreatus</i>	Zinc	30	97.96jkl
<i>P. sanguineus</i>	Zinc	50	98.65kl
<i>S. commune</i>	Zinc	50	98.92l
<i>P. ostreatus</i>	Zinc	50	99.05l

However, despite the high efficiencies observed, the absence of abiotic controls prevents completely ruling out the contribution of physicochemical processes, such as metal precipitation or passive adsorption. Therefore, the observed decrease in metal concentration may be attributed to both biological and non-biological mechanisms.

The study was based on a single endpoint measurement after 14 days of incubation, without intermediate temporal evaluation. This limitation prevents the assessment of removal rates and the identification of potential multi-phase behavior. Therefore, the dynamics of metal removal over time cannot be determined. Future studies

should incorporate kinetic modeling, mechanistic analyses, and experiments using real wastewater matrices to better understand the removal processes and validate large-scale applicability.

Tables 7 and 8 present the residual concentrations (Cf) of copper and zinc determined by FAAS. For both metals, although the residual concentration increases with higher initial concentrations, the final values remain well below the initial levels, confirming the high removal capacity of the evaluated strains.

For copper, final concentrations ranged from 0.37 to 0.79 mg L<sup>-1</sup> at 10 mg L<sup>-1</sup>; from 1.46 to 2.16 mg L<sup>-1</sup> at 30 mg L<sup>-1</sup>; and from 1.62 to 3.01 mg L<sup>-1</sup> at 50 mg L<sup>-1</sup>. At the lowest concentration, *P. ostreatus* recorded the lowest residual value (0.37 mg L<sup>-1</sup>), while at 50 mg L<sup>-1</sup>, *P. sanguineus* showed the lowest final concentration (1.62 mg L<sup>-1</sup>), suggesting greater tolerance and performance under higher metal loads.

In contrast, *P. ostreatus* and *S. commune* exhibited values close to 3 mg L<sup>-1</sup> at 50 mg L<sup>-1</sup>, which may indicate a decrease in removal efficiency at higher concentrations, possibly associated with system limitations under these conditions.

**Table 7:** Final copper (Cu) concentration as a function of fungal strain and initial concentration.

Fungal strain	Initial concentration (mg L <sup>-1</sup> )	Mean (mg L <sup>-1</sup> ) ± SD	Min	Max
<i>G. applanatum</i>	10	0.79 ± 0.09	0.69	0.86
	30	1.73 ± 0.20	1.50	1.88
	50	2.11 ± 0.06	2.06	2.18
<i>P. sanguineus</i>	10	0.79 ± 0.16	0.61	0.91
	30	1.46 ± 0.14	1.30	1.56
	50	1.62 ± 0.10	1.52	1.71
<i>S. commune</i>	10	0.50 ± 0.08	0.41	0.56
	30	1.76 ± 0.08	1.68	1.84
	50	2.98 ± 0.11	2.89	3.11
<i>P. ostreatus</i>	10	0.37 ± 0.08	0.29	0.45
	30	2.16 ± 0.07	2.08	2.21
	50	3.01 ± 0.13	2.87	3.10

In the case of zinc, residual concentrations were generally lower than those recorded for copper. At 50 mg L<sup>-1</sup>, *P. ostreatus* (0.47 mg L<sup>-1</sup>) and *S. commune* (0.54 mg L<sup>-1</sup>) showed the lowest final concentrations, while *G. applanatum* presented the highest value (1.94 mg L<sup>-1</sup>). This pattern is consistent with the statistical analysis, which demonstrated a higher overall removal efficiency for zinc.

The increase in residual concentration with rising initial concentration, while maintaining efficiencies above 94%. This behavior is consistent with concentration-dependent removal patterns reported in the literature; however, no specific mechanism can be confirmed based on the present data.

**Table 8:** Final zinc (Zn) concentration as a function of fungal strain and initial concentration.

Fungal strain	Initial concentration (mg L <sup>-1</sup> )	Mean (mg L <sup>-1</sup> ) ± SD	Min	Max
<i>G. applanatum</i>	10	0.81 ± 0.08	0.74	0.89
	30	1.17 ± 0.32	0.88	1.52
	50	1.94 ± 0.15	1.77	2.06
<i>P. sanguineus</i>	10	0.56 ± 0.09	0.47	0.64
	30	0.77 ± 0.07	0.70	0.83
	50	0.68 ± 0.23	0.41	0.82
<i>S. commune</i>	10	0.46 ± 0.01	0.44	0.47
	30	0.75 ± 0.09	0.65	0.83
	50	0.54 ± 0.22	0.30	0.70
<i>P. ostreatus</i>	10	0.45 ± 0.02	0.43	0.47
	30	0.61 ± 0.07	0.54	0.68
	50	0.47 ± 0.16	0.36	0.66

Overall, the results demonstrate that all four strains exhibit a high removal capacity for both copper and zinc, with differential responses depending on the type of metal and the initial concentration. This is consistent with the significant interactions detected in the three-way ANOVA and reinforces the biotechnological potential of the evaluated system.

The high removal efficiencies of Cu and Zn observed in this study confirm the potential of the evaluated strains as bioremediation agents in contaminated aqueous systems. However, the statistical analysis highlights a key aspect that goes beyond simple percentage comparisons: the three-way ANOVA showed that all main effects and all interactions (strain × metal, strain × concentration, metal × concentration, and strain × metal × concentration) were highly significant ( $p < 0.0001$ ). This implies that removal efficiency does not depend solely on the strain or the metal individually, but on the specific combination of both and the concentration level tested.

The strain × metal interaction indicates that each fungus responds differently to Cu and Zn. For example, while *P. ostreatus* and *S. commune* showed higher efficiencies against Zn at 50 mg L<sup>-1</sup>, *Ganoderma applanatum* exhibited relatively lower values under the same treatment, highlighting differential affinities among species.

This behavior suggests that the composition and density of active sites in the cell wall, as well as the specific physiology of each strain, influence the selective uptake of metal cations.

#### 4. DISCUSSION

Radial growth results of *G. applanatum*, *P. sanguineus*, *S. commune*, and *P. ostreatus* showed significant differences among species and culture media, confirming the physiological and metabolic variability characteristic of white-rot lignicolous fungi. These differences are associated with their capacity to utilize carbon and nitrogen sources and regulate extracellular enzymes such as laccases and manganese peroxidases, which are essential for ligninolytic metabolism (Zehra & Meena, 2025).

Under metal-free conditions, the highest radial growth in nutrient-rich media such as PDA and MEA agrees with Imtiaj et al. (2008), who reported optimal mycelial expansion for *S. commune* in media with easily assimilable carbon sources. Similarly, Sastre-Ahuatzi et al. (2007) demonstrated that glucose- or potato-based media promote rapid activation of primary metabolism in basidiomycetes, resulting in higher radial growth rates. In this context, *P. ostreatus* exhibited fast and uniform growth, consistent with Krupodorova et al. (2024), reflecting high metabolic plasticity and efficient adaptation to simple substrates. In contrast, *G. applanatum* showed slower growth, likely due to energy allocation toward secondary metabolism and ligninolytic enzyme production, as reported for *Ganoderma* species (Kato et al., 2024).

In the presence of Cu and Zn, significant interactions among species, metal type, and concentration indicate that mycelial response depends on the specific combination of factors. This behavior is consistent with Baldrian (2003), who reported that high copper concentrations may inhibit fungal growth through oxidative stress, while moderate levels can induce laccase activity. Likewise, Gadd (2009) explained that Cu and Zn exhibit dual roles as essential micronutrients and toxic elements, affecting membrane integrity, proteins, and redox balance at high concentrations. Zinc generally shows lower redox activity, whereas copper can promote reactive oxygen species formation, explaining its stronger inhibitory effect. In agreement with Fomina & Gadd (2014), the results suggest metal-specific tolerance mechanisms such as extracellular chelation, cell wall immobilization, and intracellular compartmentalization.

It is important to note that radial growth was used here as an indicator of physiological tolerance rather than removal capacity. Thus, while these results confirm the ability of strains to maintain growth under metal stress, they do not directly demonstrate bioremediation performance.

In this context, fungal biomass provides a more direct link between tolerance and potential removal capacity. Fungal strains are widely recognized for their resistance and adaptability to heavy metals (Lei et al., 2018; Li et al., 2019; Amin et al., 2024; Zare et al., 2024). In the present study, biomass remained stable or even increased under several fungus–metal–concentration combinations, indicating that growth was not significantly limited within the evaluated range. The highest biomass values were recorded for *P. ostreatus*, *P. sanguineus*, and *G. applanatum*, particularly at intermediate metal concentrations, suggesting the absence of critical inhibition of primary metabolism. Similar responses have been reported in ligninolytic fungi, where moderate metal levels can induce adaptive metabolic activity, including increased enzyme production (Baldrian, 2003).

Although structural characterization was not performed, fungal cell walls composed of chitin, glucans, and proteins contain functional groups ( $-\text{COOH}$ ,  $-\text{OH}$ ,  $-\text{NH}_2$ ) have been reported in the literature as potential sites for metal binding; however, these interactions were not directly evaluated in this study (Fomina & Gadd, 2014; Gadd, 2009). In this sense, biomass plays a key role, as it determines the number of available binding sites (Fomina & Gadd, 2014). Additionally, fungi have been reported to combine mechanisms such as surface biosorption, intracellular bioaccumulation, and extracellular precipitation; however, these processes should be considered as potential mechanisms, since they were not directly evaluated here (Gadd, 2009). For *P. ostreatus*, high biomass likely reflects its metabolic plasticity and enzymatic capacity (Baldrian, 2003), which has also been associated with enhanced metal removal (Araújo et al., 2023; Acosta et al., 2024).

These observations are consistent with removal behaviors described in the literature, which are often explained through biosorption-related frameworks. However, such mechanisms were not directly assessed in this study. Biosorption typically involves processes such as physical adsorption, ion exchange, complexation, and precipitation, and is influenced by factors including pH, temperature, biomass loading, and initial metal concentration (Magdum & Itankar, 2026).

Consistent with these observations, removal efficiency results demonstrated significant differences between metals and their interactions with strain and concentration. The higher removal of Zn compared to Cu ( $p < 0.0001$ ) may be associated with their chemical behavior:  $Zn^{2+}$  forms less stable coordination complexes, while  $Cu^{2+}$  has higher affinity for nitrogen-containing groups and greater competition for active sites (Lei et al., 2018; Li et al., 2019). This chemical difference supports the observed pattern of higher Zn removal efficiency.

The metal  $\times$  concentration interaction and the three-way interaction further indicate that increasing initial concentration does not affect all strains uniformly. In several cases, removal efficiency increased at  $50 \text{ mg L}^{-1}$ , suggesting a concentration-gradient effect in which a higher driving force may enhance overall removal performance under higher concentration gradients; however, the specific processes involved were not determined (Lei et al., 2018; Amin et al., 2024).

Although comparisons with other studies (Albert et al., 2018; Dey et al., 2020; Chen et al., 2019) should consider differences in experimental conditions such as pH, temperature, and contact time, the factorial design used here allows robust internal comparisons. Importantly, ANOVA results indicated that neither metal type nor concentration significantly affected biomass within the  $10\text{--}50 \text{ mg L}^{-1}$  range, highlighting the physiological tolerance of all strains under metal stress (Harish et al., 2024).

While previous studies report additional metabolic capabilities for *S. commune*, such as nanoparticle biosynthesis (Bragta et al., 2025) and degradation of recalcitrant compounds (Augustine & Abraham, 2025), these processes were not directly evaluated in this study and should be interpreted as potential rather than demonstrated mechanisms.

From an applied perspective, these findings provide preliminary evidence of the potential of lignicolous fungi for heavy metal removal under controlled laboratory conditions. However, it is important to note that the experiments were conducted using synthetic aqueous solutions, which do not fully replicate the complexity of real industrial or mining effluents. Factors such as mixed-metal interactions, organic matter content, pH variability, and scaling conditions may significantly influence removal performance. Therefore, further studies using real wastewater matrices and pilot-scale systems are required to validate the practical applicability of these fungal strains in mining-impacted environments in Peru.

Overall, the results demonstrate a clear relationship between radial growth, biomass production, and removal efficiency: radial growth reflects physiological tolerance, biomass determines the availability of binding sites, and removal efficiency results from the interaction between biological capacity and metal chemistry. This integrative approach confirms that metal removal is a multifactorial process dependent on strain, metal type, and concentration, reinforcing the biotechnological potential of lignicolous fungi (Lei et al., 2018; Li et al., 2019; Amin et al., 2024).

A limitation of this study is that the mechanisms of metal removal were not directly evaluated. Although processes such as biosorption, bioaccumulation, and enzymatic transformation are widely reported in ligninolytic fungi, no specific analyses were conducted in this work to confirm their occurrence. The present results demonstrate a reduction in metal concentration in the aqueous phase; however, the exact removal pathways cannot be determined. Future studies should include mechanistic approaches such as metal quantification in fungal biomass, FTIR or SEM-EDS analyses, enzymatic activity assays, and adsorption isotherms to better elucidate the processes involved.

Although the results demonstrated high removal efficiencies and stable biomass production across treatments, several methodological limitations should be considered when interpreting the findings. One of the main limitations of this study is the absence of abiotic controls, which restricts the ability to quantitatively differentiate between biological removal mechanisms—such as biosorption, bioaccumulation, and enzymatic transformation—and physicochemical processes including precipitation or passive adsorption. Consequently, while the observed metal removal is strongly associated with fungal activity, a minor contribution of non-biological processes cannot be completely excluded.

Additionally, the mechanisms underlying metal removal were not directly evaluated. Although ligninolytic fungi are widely reported to remove metals through multiple pathways, no specific analyses were conducted in this study to confirm the occurrence of these processes. Therefore, while a significant reduction in metal concentration in the aqueous phase was observed, the exact removal pathways remain undetermined.

Another limitation lies in the taxonomic identification of the fungal strains. The identification was based on macroscopic characteristics and comparison with reference catalogs, which should be considered preliminary.

Although the selected taxa correspond to well-documented genera with distinctive morphological traits (e.g., *Ganoderma*, *Pleurotus*, *Pycnoporus*, and *Schizophyllum*), the possibility of misidentification at the species level cannot be entirely ruled out due to the absence of molecular confirmation (e.g., ITS rDNA sequencing).

Despite these limitations, the consistency of the experimental responses and the statistically significant effects detected in the factorial analysis support the validity of the observed trends. These findings provide a reliable basis for further investigation into the bioremediation potential of these fungi.

Future research should incorporate abiotic controls, continuous monitoring of pH, and molecular identification techniques to improve both mechanistic understanding and taxonomic resolution. In addition, mechanistic approaches such as metal quantification in fungal biomass, FTIR or SEM-EDS analyses, enzymatic activity assays, and adsorption isotherms are recommended to elucidate the specific pathways involved in metal removal and to strengthen the applicability of these fungal systems in environmental bioremediation.

One of the main limitations of this study is that all experiments were conducted under controlled laboratory conditions using synthetic aqueous solutions. While this approach allows for precise evaluation of fungal responses to specific metals and concentrations, it does not fully represent the physicochemical complexity of real wastewater systems. In natural and industrial settings, the presence of multiple contaminants, fluctuating environmental conditions, and potential inhibitory compounds may alter fungal performance. Consequently, the results should be interpreted as indicative of potential rather than direct evidence of field applicability.

## 5. CONCLUSIONS

This study demonstrates the feasibility of using lignicolous fungi (*G. applanatum*, *P. sanguineus*, *S. commune*, and *P. ostreatus*) for the bioremediation of copper- and zinc-contaminated water. All strains achieved high removal efficiencies (>91%), with a maximum of 99.05%, indicating strong metal retention under the tested conditions. No single strain showed absolute superiority, as all exhibited comparable performance, though *S. commune* and *P. ostreatus* displayed faster mycelial growth, suggesting higher physiological tolerance.

Fungal biomass was highest in *P. ostreatus*, *P. sanguineus*, and *G. applanatum*, particularly at intermediate metal concentrations, though variability among replicates (CV = 30.34%) highlights the influence of individual

adaptation, colonization heterogeneity, and nutrient fluctuations. Future studies should standardize inocula, increase replicates, and control physicochemical conditions more strictly.

While removal was confirmed via FAAS, this study did not directly assess mechanisms such as biosorption, bioaccumulation, or enzymatic activity. Nonetheless, the results provide robust evidence of the strains' capacity to reduce metal concentrations, establishing a foundation for mechanistic studies, kinetic evaluations, and process scale-up in bioremediation applications.

It is important to highlight that the absence of kinetic data represents a limitation of this study, as the use of a single endpoint measurement does not allow evaluation of removal dynamics over time. Future research should include kinetic analyses to better understand the rate and mechanisms of metal removal.

A kinetic analysis is recommended for future studies to better understand the removal dynamics.

## 5. PATENTS

There are no patents resulting from this work.

**Author Contributions:** For research articles with multiple authors, include a brief paragraph outlining each author's contributions using the following format: “Conceptualization, A.L.R.T, L.R.R, J.E.G.C; methodology, A.L.R.T and L.R.R; software, J.M.A.S and C.V.C.L; validation, J.M.A.S and J.E.G.C.; formal analysis, J.M.A.S and J.E.G.C.; investigation, A.L.R.T, L.R.R, J.M.A.S, C.V.C.L and J.E.G.C; data curation, J.M.A.S and J.E.G.C.; writing—original draft preparation, J.M.A.S, J.E.G.C, C.V.C.L; writing—review and editing, A.L.R.T, L.R.R and J.E.G.C; visualization, L.R.R, J.M.A.S and J.E.G.C.; supervision, L.R.R and J.E.G.C. All authors have read and agreed to the published version of the manuscript.”

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