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**Comparative Analysis of Chernozem Microbiome Resistance to Toxic Heavy Metals
(Co²⁺, Cu²⁺, Zn²⁺, Ni²⁺) under Individual and Pairwise Exposure**

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Abstract: Anthropogenic polymetallic contamination threatens high-fertility chernozem ecosystems, disrupting microbiome-mediated nutrient cycling. This study evaluated the systemic functional stability of the native chernozem microbiome exposed to Cu^{2+} , Co^{2+} , Zn^{2+} , and Ni^{2+} . Resilience to individual stressors and 1:1 binary mixtures was quantified over 15 days using OD540 and CFU assays as thermodynamic proxies for active metabolic load. Logistic dose-response modeling established a strict acute toxicity hierarchy: Co^{2+} ($\text{EC}_{50}=130.8 \text{ mg/L}$) > Ni^{2+} ($\text{EC}_{50}=167.6 \text{ mg/L}$) > Zn^{2+} ($\text{EC}_{50}=290.0 \text{ mg/L}$) > Cu^{2+} ($\text{EC}_{50}=331.2 \text{ mg/L}$). Crucially, the Bliss Independence model demonstrated severe synergistic inhibition across all binary mixtures. At 400 mg/L total concentration, combinations yielded Synergistic Ratios up to 7.1, with viability reductions drastically exceeding additive predictions. These findings quantitatively prove that single-metal ecological risk assessments critically underestimate co-contamination hazards, emphasizing the urgent need to integrate synergistic factors into environmental safety standards and bioremediation strategies.

Introduction

Heavy metal contamination of the pedosphere represents a pervasive global environmental crisis that critically compromises ecosystem integrity and undermines food security [1]. Anthropogenic drivers, ranging from intensive mining operations and industrial emissions to the unregulated application of agrochemicals, have accelerated the accumulation of recalcitrant toxic elements specifically copper (Cu), zinc (Zn), nickel (Ni), and cobalt (Co) in terrestrial environments [2]. The interactions between autochthonous soil microorganisms and transition metals are governed by strict stoichiometric requirements and inviolable thermodynamic constraints[3]. Elements classified as essential transition metals, including copper, zinc, nickel, and cobalt, are biologically requisite primarily due to their unique, partially filled d-orbital electron configurations[4]. This specific atomic architecture allows them to seamlessly participate in complex redox chemistry, facilitate the transfer of electrons, and maintain the structural coordination geometry within the active sites of critical microbial enzymes [4,5]. However, the beneficial nature of these metals is strictly concentration-dependent, operating within an exceptionally narrow physiological window[6]. When local soil concentrations surpass the homeostatic efflux buffering capacity of the microbial cell, these transition metals trigger severe biochemical cascades that rapidly compromise cellular integrity [6]. Consequently, this biochemical stress fundamentally reshapes microbial community architectures, precipitating a collapse in

biodiversity and a subsequent loss of critical soil functionalities required for sustainable agriculture[7].

To mathematically quantify this transition, ecotoxicologists rely on robust metrics such as the half-maximal effective concentration as the half-maximal effective concentration (EC_{50}), which represents the toxicant level required to induce a 50% reduction in a specific biological parameter— such as microbial growth (optical density at 540 nm, OD₅₄₀) or biomass accumulation (colony-forming units, CFU, per g soil) relative to the control, and the No Observed Effect Concentration (NOEC), which denotes the highest tested concentration at which no statistically significant adverse effect is observed compared to the baseline [8]. Establishing universal thresholds is inherently complex, as bioavailability is heavily modulated by soil physicochemical properties, including cation exchange capacity (CEC), total organic carbon (TOC), and pH [8,9]. Nevertheless, empirical analyses reveal distinct threshold behaviors: Copper - exhibits background levels of 8.7-52.8 mg/kg; however, EC_{50} values for enzymatic endpoints (e.g., phosphatase) range widely from 580 mg/kg in sandy loams to 4850 mg/kg in organic-rich silt loams. For zinc regulatory limits are set at 200-300 mg/kg to preserve soil health, enzymatic EC_{50} thresholds span from 860 to over 26,000 mg/kg [9–11]. Nickel and Cobalt - demonstrate higher relative toxicity, with prominent growth inhibition for Ni noted at ~326 mg/kg and Co exhibiting even lower EC_{50} values [6,9,10].

Critically, high laboratory-derived EC_{50} values for acute enzymatic failure do not represent safe ecological limits [6,10]. Chronic accumulation at concentrations well below these points gradually erodes the functional redundancy and biodiversity of the soil microbiome [10]. Regulatory frameworks therefore establish maximum permissible limits, such as 100 mg/kg for Cu and Zn and <50 mg/kg for Ni and Co, to prevent the exponential escalation of toxicity observed as anthropogenic loading approaches acute tipping points[11]. Consequently, this study evaluates concentrations characteristic of moderately polluted soils, while extending the range to 1000 mg/L to account for extreme hotspots.

Soil microorganisms function as the primary ecological sentinels, representing the first biotic component to encounter and respond to the toxic effects of edaphic pollutants. The disruption of the soil microbiome is particularly critical in fertile soils, such as chernozems (black soils), which are cornerstones of global agricultural productivity[12]. Historically, the soil microbiome was often reduced to a few culturable genera: nitrogen-fixing *Azotobacter*, phosphate-solubilizing *Bacillus*, and cellulose-degrading *Streptomyces* [12–15]. However, in high-fertility chernozems, this "classic trio" represents only the tip of a much more

intricate functional architecture. The extraordinary resilience and productivity of these soils are driven by a multi-trophic, coordinated network of bacteria, archaea, and fungi that collectively manage the biogeochemical cycles of C, N, P, and S[13,16,17]. The structural integrity of chernozem's deep humus horizons relies on the systemic depolymerization of recalcitrant plant biopolymers. While *Streptomyces* aids late-stage decay, the initial attack on cellulose is orchestrated by specialized genera like *Cellulomonas* and *Cytophaga*, utilizing complex extracellular cellulosomes[18]. The degradation of lignin, the most stable aromatic polymer, is even more sophisticated, requiring oxidative enzymes (laccases and peroxidases) from taxa such as *Rhodococcus*, *Arthrobacter*, and *Planococcus* [14]. This synergistic cross-feeding between cellulolytic and ligninolytic consortia effectively regulates the "global soil carbon pump," maintaining the organic matrix that serves as the soil's primary toxicological buffer[14,19]. Nitrogen bioavailability in chernozems depends on an unbroken microbial chain. Beyond *Azotobacter*, a diverse assemblage of diazotrophs, including *Rhizobium* and the actinomycete *Frankia*[20], supply ammonia via symbiotic and associative fixation. This is seamlessly coupled with nitrification, the two-step oxidation of ammonia to nitrate, driven by specialized chemolithoautotrophs like *Nitrosomonas* and *Nitrospira* [20–22]. Phosphorus and sulfur cycles are deeply intertwined with the soil's mineral stability [15]. Phosphorus mobilization relies on "Phosphate-Solubilizing Microorganisms", including *Burkholderia*, *Aquabacterium*, and *Arthrobacter*, which secrete organic acids to chelate cations and release bound phosphates[15,23]. Simultaneously, the sulfur cycle, driven by oxidizers like *Thiobacillus* and reducers like *Desulfovibrio*, interacts with ferric minerals to influence nutrient availability[24].

While the organic matrix of chernozem acts as a natural chemostatic buffer, binding heavy metals and mitigating their immediate bioavailability, chronic pollution poses a significant risk of overwhelming these inherent retention mechanisms[17,23,25]. Once this pedogenic buffering capacity is saturated, the resulting toxicity precipitates a cascade of ecological failures: it not only compromises crop yields but also facilitates the trophic transfer and bioaccumulation of metals in the food chain, posing severe risks to human health[1,2,26].

A critical methodological deficiency in current ecological risk assessment (ERA) frameworks is the predominant reliance on single-metal toxicity models. In *situ* pedospheric environments are infrequently compromised by single contaminants; rather, they are typically characterized by polymetallic mixtures originating from complex anthropogenic sources such as mining tailing dams [27], industrial emissions [28], or wastewater

discharges [29]. The interaction between co-occurring metals (e.g., Cu^{2+} and Zn^{2+}) introduces unpredictable variables where combined effects can be synergistic (amplified toxicity), antagonistic, or additive. Fan et al. [30] demonstrated such complex non-linear hormetic responses in soil bacteria under combined Cd and Pb stress, highlighting the unpredictability of binary exposures. However, the majority of existing ecotoxicological inquiries remain restricted to isolated, laboratory-adapted bacterial strains, such as *Pseudomonas putida* [31] or *Bacillus subtilis* [32], or focus exclusively on single-metal exposures [33]. Even within model organisms like *P. putida*, the specificity of metal regulators is complex; a protein initially characterized as a copper-responsive regulator (CsoR) was found to be genetically linked to a downstream Ni/Co efflux protein (RcnA), highlighting the intricate and sometimes ambiguous nature of metal-specific responses [34]. While these reductionist approaches have been fundamental in elucidating basic resistance mechanisms, such as the role of efflux pumps or specific operons like the *ars* system in *B. subtilis* [35], they often fail to capture the complexity of microbial responses in natural edaphic environments.

Therefore, advancing the field of soil ecotoxicology requires a paradigm shift towards more holistic and environmentally realistic experimental designs. Future inquiries must move beyond simplified binary systems and prioritize the investigation of native bacterial consortia under conditions that mimic the complexity of polluted soils. This reductionist approach leaves a substantial epistemic gap regarding the resilience thresholds of autochthonous (native), unadapted microbial consortia to complex binary stress. Consequently, deciphering these synergistic interactions is not merely academic but is a prerequisite for developing realistic bioremediation strategies and recalibrating environmental safety standards. Identifying the remedial pathway to ecosystem recovery depends entirely on deciphering these complex biogeochemical interactions [36]. Consequently, investigating the combined effects of co-occurring metals is fundamentally crucial for developing realistic bioremediation strategies and updating environmental safety standards to reflect polymetallic risks. It is hypothesized that the high organic content of chernozem may provide a unique physicochemical resilience profile, yet the saturation limits of this resistance under dual-metal stress remain completely unknown. While substantial research has documented the impacts of individual heavy metals on soil microbiomes, significant knowledge gaps persist regarding resistance thresholds and adaptive mechanisms, particularly in native chernozem communities unadapted to chronic pollution [2,37]. Contemporary ecological theory suggests that the resilience of such

"pristine" communities may be governed by the "Rare Biosphere" - a vast reservoir of low-abundance taxa (<0.1% relative abundance) that act as a genetic insurance policy[38]. In unpolluted soils, these rare taxa often harbor dormant metal-resistance genes (MRGs) that remain metabolically silent until a stochastic environmental disturbance, such as acute polymetallic influx, triggers their clonal amplification[39,40]. However, the efficacy of this genetic reservoir in chernozems is often masked by the soil's superior geochemical buffering. High concentrations of humic acids and clay minerals (montmorillonite/illite) strictly govern the chemical speciation of cations, effectively reducing their immediate bioavailability[41]. This leads to a dangerous "geochemical masking" effect, where functional indicators, such as dehydrogenase activity, a highly sensitive proxy for active microbial biomass, show no significant inhibition until a critical tipping point is reached.

The majority of ecotoxicological investigations have disproportionately prioritized metal-adapted populations or hyper-tolerant microbial isolates, thereby creating a substantial epistemic gap regarding the acute vulnerability of pristine, unadapted microbiomes [28,42–44]. Critically, *in situ* pedospheric contamination originating from diverse anthropogenic vectors such as agricultural runoff, industrial emissions, or mining activities is invariably characterized by polymetallic mixtures rather than isolated elements [28,29,33,45,46]. This chemical complexity generates stochastic interaction effects, where combined exposures manifest as synergistic (amplified toxicity), antagonistic, or additive outcomes that cannot be reliably extrapolated from single-metal toxicity data[47]. Fundamentally, this interactive toxicity is mechanistically governed by specific microbial polyresistance pathways [48,49]. Microbial resilience to polymetallic toxicity is mechanistically underpinned by two distinct genetic pathways: (i) co-resistance, where distinct resistance determinants such as those for copper and zinc are genetically coupled within the same mobile genetic element (MGE), typically a plasmid; or (ii) cross-resistance, wherein a singular physiological detoxification apparatus, such as a multi-substrate efflux pump, confers simultaneous tolerance to structurally distinct metal ions [27,50]. A classic example of cross-resistance is the CzcABC efflux pump system found in *Cupriavidus metallidurans*, which actively exports Co^{2+} , Zn^{2+} , and Cd^{2+} , allowing the organism to thrive in highly toxic environments[37,49,51]. This is critically important because these MGEs frequently also carry antibiotic resistance genes (ARGs), leading to the dangerous co-selection of antibiotic resistance under heavy metal stress[37,50–52]. Empirically, binary combinations such as Cu^{2+} and Zn^{2+} have demonstrated profound synergistic inhibition of microbial biomass and enzymatic kinetics in contaminated soils, while Ni^{2+} interactions with Zn^{2+} or Cu^{2+} induce complex alterations

in soil biological properties that can either exacerbate or attenuate overall toxicity outcomes[52,53]. These findings underscore that single-metal resistance profiles serve as insufficient proxies for predicting microbiome resilience in polymetallic scenarios, where co-occurring ions exert complex non-additive effects through competitive sorption at binding sites, the induction of cross-tolerance mechanisms, or the collective amplification of oxidative damage[54]. In chernozem soils, where organic matter influences metal speciation, these interactions remain poorly quantified, hindering reliable ecological risk assessments and bioremediation efforts[55–57].

Consequently, a significant epistemic gap persists. While resistance mechanisms in acclimated populations from highly contaminated environments (such as mining spoils) are well-documented, the native synergistic tolerance of unadapted microbiomes in fertile chernozems remains largely unexplored. It remains undetermined whether the substantial organic matter and physicochemical buffering capacity of chernozem can effectively mitigate synergistic toxicity, or if polymetallic contamination overwhelms native microbial defenses at concentrations conventionally regarded as 'safe' for individual metals. While modern ecotoxicology frequently employs molecular sequencing to track taxonomic shifts under stress, such approaches often obscure the macroscopic functional resilience of the microbial system. DNA-based methods can be confounded by the persistence of extracellular or dormant DNA, failing to reflect immediate metabolic activity. Therefore, this study deliberately adopts a systemic functional perspective. Rather than analyzing structural community composition, we prioritize the evaluation of the collective metabolic potential and systemic functional stability of the unadapted chernozem microbiome. In this context, classical microbiological metrics— specifically CFU and biomass accumulation kinetics (OD540)—are utilized not merely as viability assays, but as integral thermodynamic proxies for the community's capacity to maintain active cell division and biogeochemical cycling under acute polymetallic stress.

Therefore, the primary objective of this study is to quantify the ecotoxicological resistance limits and non-linear growth kinetics of the native chernozem microbiome under individual and combined heavy metal stress (Cu^{2+} , Co^{2+} , Zn^{2+} , Ni^{2+}). This study is designed to:

1. Determine the dose-response kinetics and exact resistance thresholds of the microbial community under single-metal exposure.
2. Quantify the collective metabolic inhibition induced by their pairwise binary mixtures to simulate environmentally realistic co-contamination.

3. Mathematically classify these polymetallic interactions (synergistic, additive, or antagonistic) to identify the critical tipping points of systemic microbiome collapse.

Results

Determination of Maximum Tolerated Concentrations and Dose-Response

Kinetics

The integrated analysis of optical density (OD₅₄₀) and colony-forming unit (CFU) assays reveals that the native chernozem microbiome exhibits a distinct non-linear, dose-dependent response to heavy metal stress (Fig. 1 and Fig. 2). The comparative topology of the growth curves identifies a critical biogeochemical threshold effect:

Phase I (Resistance Plateau). At low concentration ranges (0-100 mg/L), the microbial community demonstrates high resilience. Biomass reduction is minimal, suggesting that the chemostatic buffering capacity of the chernozem organic matrix effectively reduces metal bioavailability, protecting the microbiome from acute toxicity.

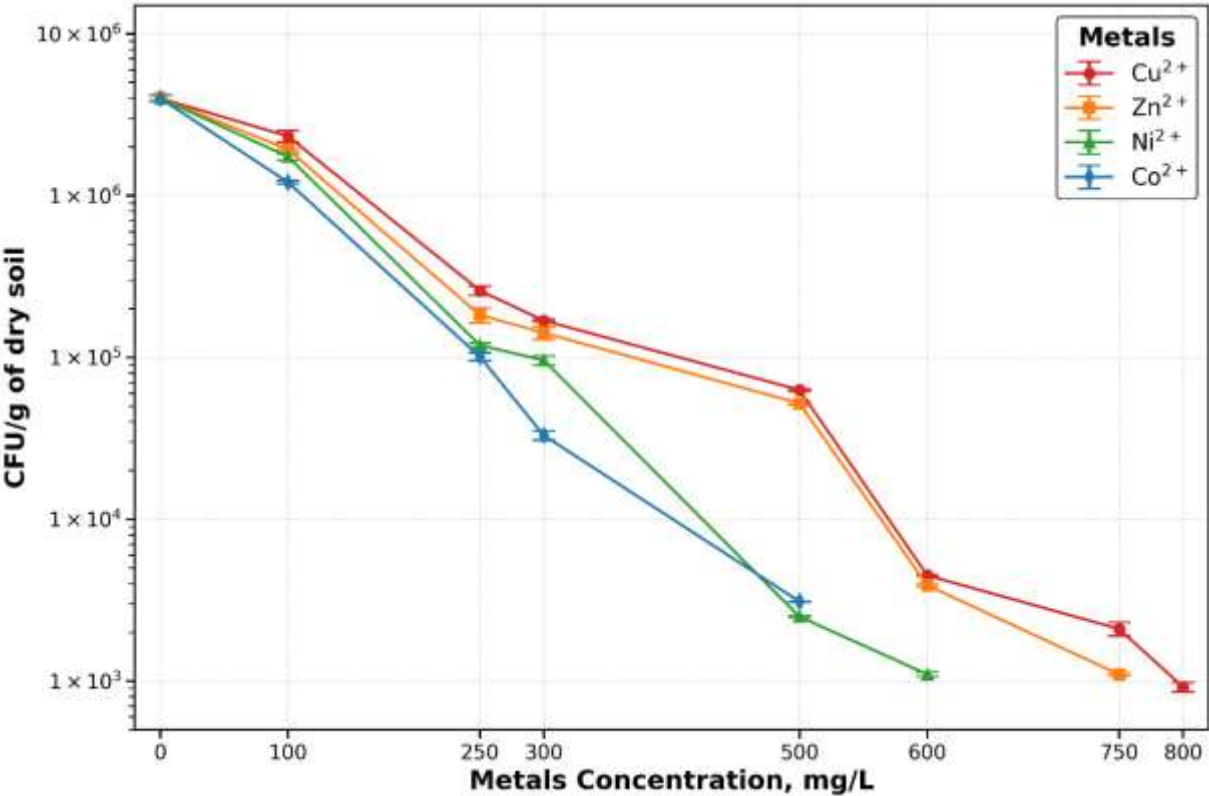
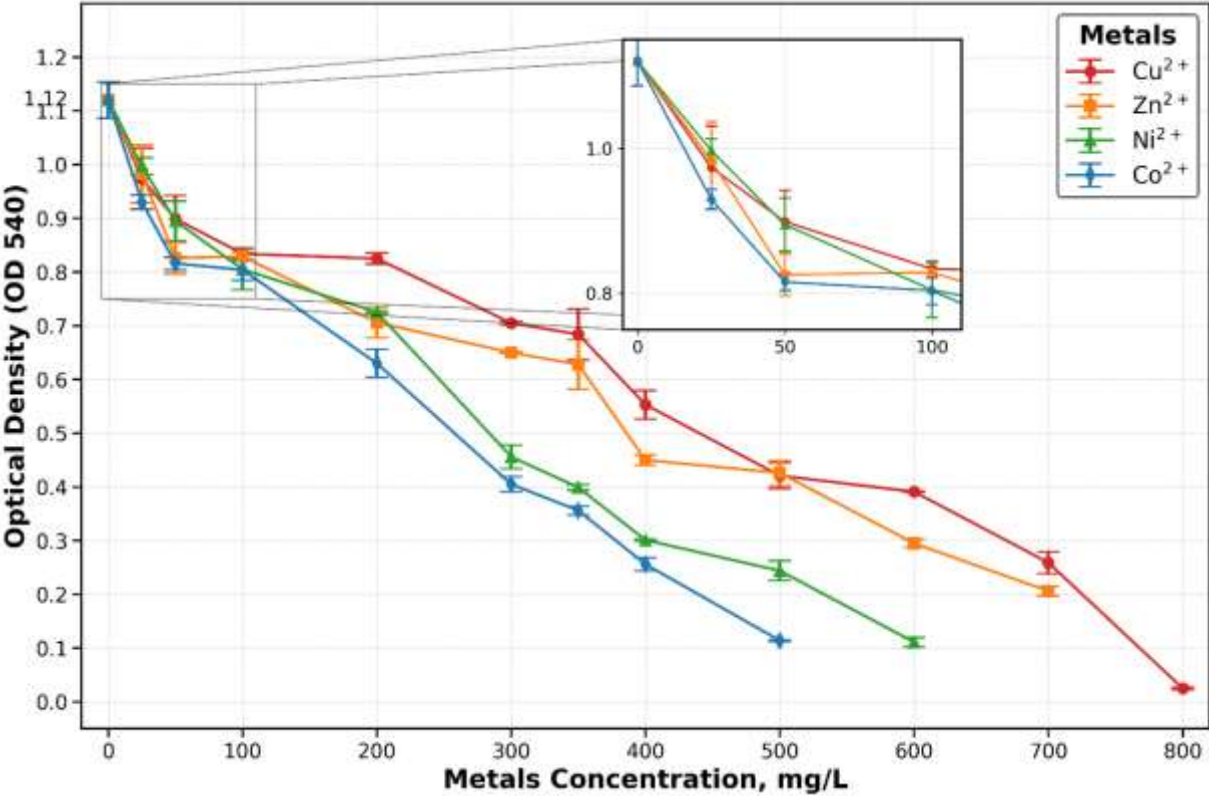
Phase II (Critical Stability Threshold). Once specific concentration thresholds are exceeded, a precipitous exponential decline in both biomass and viability is observed, indicating the saturation of extracellular buffers and the failure of intracellular resistance mechanisms.

Cobalt exhibited the highest cytotoxic potential among the tested cations. The "tipping point" for Co-induced inhibition was notably low (50-100 mg/L), followed by a rapid collapse in viability. While the control samples maintained high viability ($(4.00 \pm 0.3) \times 10^6$ CFU/g), exposure to Cobalt caused severe metabolic suppression. By 500 mg/L, the inhibitory effect became extreme, and at 600 mg/L, microbial growth was effectively arrested ($OD_{540} \approx 0$), confirming Cobalt as the primary stressor in this series.

Nickel demonstrated intermediate toxicity, significantly exceeding the inhibitory effects of Copper and Zinc but remaining less potent than Cobalt. The dose-response curve for Nickel shows a steeper decline than Cu/Zn, with a significant reduction in optical density observed at 600 mg/L and complete cessation of bacterial proliferation at 750 mg/L.

Copper and Zinc displayed a distinct, more protracted inhibition profile. Unlike the sharp collapse seen with Cobalt, these metals induced a gradual, linear decrease in biomass accumulation. Even at relatively low concentrations (100 mg/L), a statistically significant reduction in viable cell counts was recorded: Zn^{2+} reduced the population to $(1.93 \pm 0.19) \times 10^6$ CFU/g, and Cu^{2+} to $(2.33 \pm 0.15) \times 10^6$ CFU/g, representing a ~50% decline

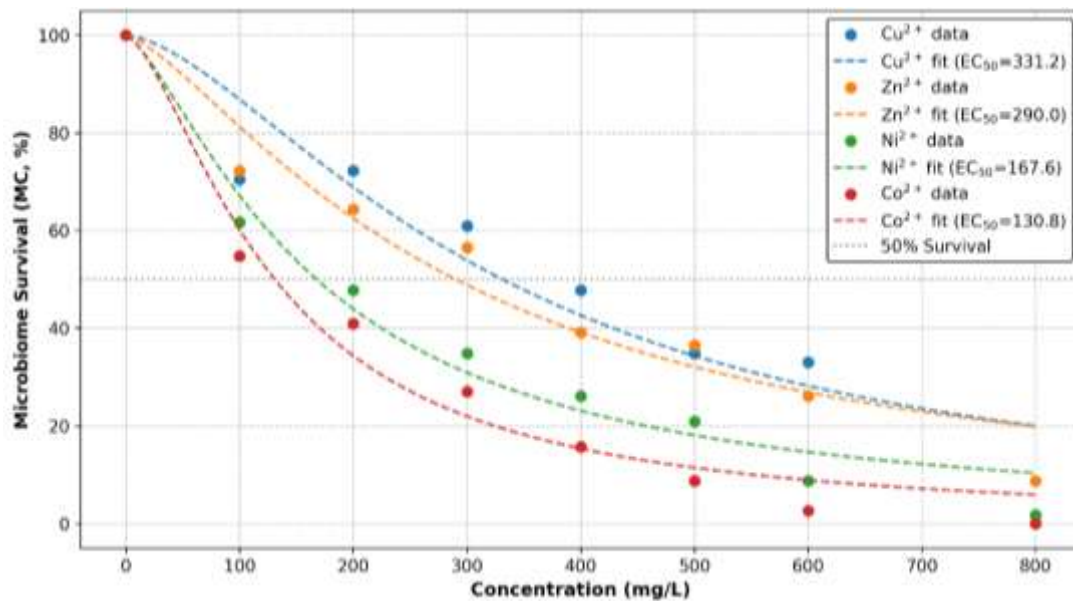
281 compared to the control $((4.00 \pm 0.3) \times 10^6$ CFU/g). However, the community retained partial
282 viability at higher doses, with complete growth inhibition only achieved at 800-1000 mg/L.



Based on the comparative magnitude of inhibition (OD₅₄₀ reduction) and lethality (CFU reduction), the toxicity hierarchy for the native chernozem microbiome was established as: Co > Ni > Zn > Cu.

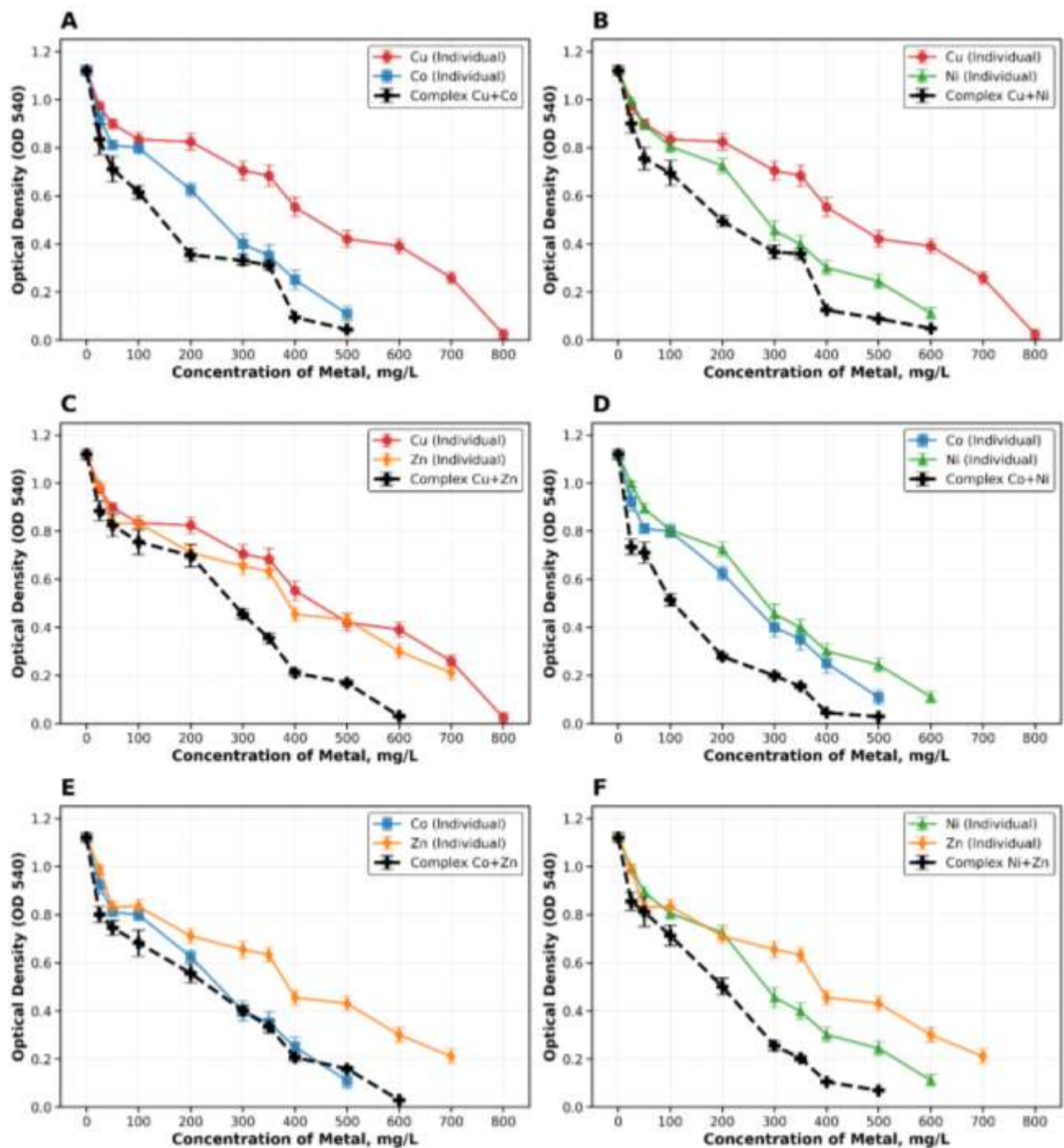
This order reflects the specific vulnerability of the unadapted chernozem community, where Cobalt toxicity manifests earliest (lowest threshold) and most intensely, while Copper allows for partial survival up to the highest concentrations.

This hierarchy was strictly validated by the computed EC₅₀ values derived from the logistic dose-response models (Figure 3). Cobalt demonstrated the highest acute toxicity with an EC₅₀ of 130.8 mg/L, followed closely by Nickel (EC₅₀ = 167.6 mg/L). In contrast, Zinc and Copper exhibited significantly higher thresholds, requiring 290.0 mg/L and 331.2 mg/L, respectively, to induce a 50% reduction in microbiome viability.



Assessment of Polyresistance: Synergistic Toxicity of Binary Combinations

To evaluate the microbiome's resilience under environmentally relevant conditions, the inhibitory kinetics of six binary metal combinations were quantified (Fig. 3 A-F). The experimental design maintained a 1:1 mass ratio for all pairs, where the abscissa represents the cumulative metal concentration. In this experimental design, the total metal concentration indicated on the x-axis for the binary 'Complex' (black dashed line) represents sum of the two constituent metals (e.g., a total concentration of 400 mg/L corresponds to 200 mg/L of one metal plus 200 mg/L of another).



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Universal Synergistic Potentiation

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The comparative analysis of individual versus binary exposure profiles reveals a consistent and profound synergistic toxicity across all tested combinations. In every scenario (Fig. 4 A-F), the microbial biomass accumulation (OD₅₄₀) in the presence of the binary complex was significantly suppressed compared to the theoretical additive predictions derived from single-metal isotherms. This indicates that the co-presence of metal cations induces a non-linear potentiation of cytotoxicity, effectively overwhelming the diverse

resistance mechanisms (e.g., efflux pumps, sequestration) that function adequately under single-stressor conditions.

Cobalt-Driven Severe Inhibition

Combinations involving Cobalt (Co^{2+}) exhibited the most catastrophic synergistic effects, confirming its role as a critical driver of community collapse.

$\text{Co}^{2+} + \text{Ni}^{2+}$ (Fig. 4D): This pairing induced the most severe inhibition. While the community retained partial viability at 400 mg/L under individual Ni exposure, the binary mixture at the same total concentration (200 mg Ni + 200 mg Co) resulted in near-total lethality ($\text{OD}_{540} \approx 0.045 \pm 0.04$). This represents a functional extinction of the microbiome at a dosage where individual metals would still permit survival.

$\text{Cu}^{2+} + \text{Co}^{2+}$ (Fig. 4A): A dramatic divergence from additivity was observed. At a total concentration of 400 mg/L, the OD_{540} plummeted to 0.095 ± 0.03 marking a ~91% reduction relative to the control. This "tipping point" occurs at a concentration significantly lower than the MTC for Copper alone, illustrating that the presence of Cobalt effectively nullifies the community's natural tolerance to Copper.

Amplification of Toxicity in "Moderate" Stressors

Crucially, synergistic amplification was not limited to the most toxic elements. Even pairings of metals with higher individual MTCs (Cu, Zn) resulted in significantly enhanced toxicity profiles:

$\text{Ni}^{2+} + \text{Zn}^{2+}$ (Fig. 4F): Although Zinc is generally the least toxic element in this series, its combination with Nickel resulted in a sharp decline in biomass. At 400 mg/L, the mixture suppressed growth to $\text{OD} \approx 0.105 \pm 0.02$, whereas individual Zinc exposure at this concentration typically sustains high biomass levels.

$\text{Cu}^{2+} + \text{Zn}^{2+}$ (Fig. 4C): This combination, often found in agricultural soils due to agrochemical application, showed a clear "suppression gap." The binary curve consistently tracked below the lowest individual component, indicating that even "essential" micronutrients become lethal stressors when acting in concert.

Implications for Risk Assessment

The data provides unequivocal evidence that the toxicity of heavy metals in the chernozem microbiome is fundamentally non-additive. The interaction between co-occurring ions results in a "mutually assured destruction" of cellular defenses, likely due to the simultaneous saturation of distinct detoxification pathways or the amplification of oxidative stress. Consequently, Ecological Risk Assessments (ERA) based solely on single-metal MTCs will critically underestimate the environmental hazard, as the "safe"

concentration for a mixture is definitively lower than the sum of its parts.

Quantitative Analysis of Synergistic Inhibition Indices

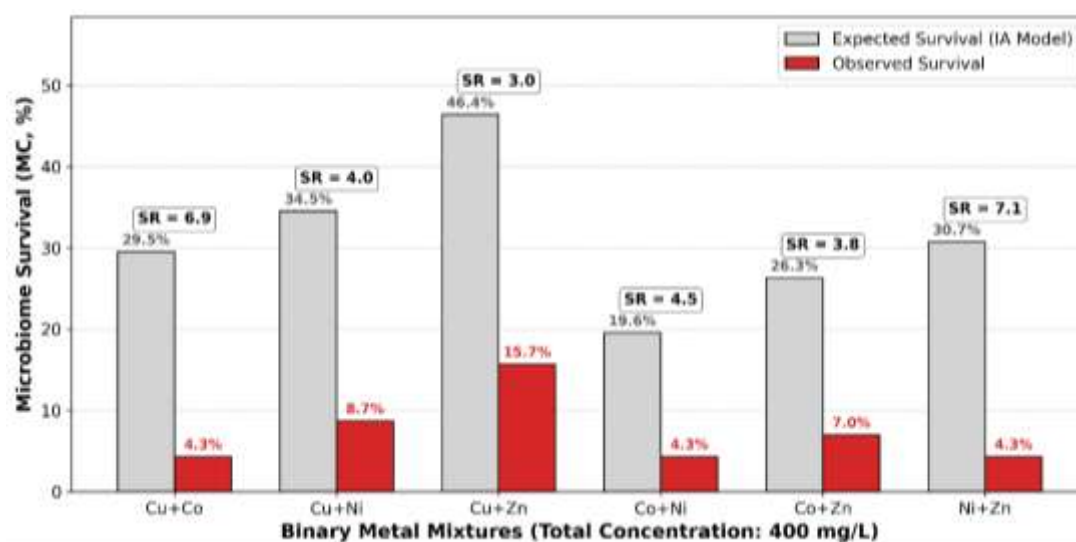
To standardize the assessment of toxicity across distinct metal species and rigorously quantify the magnitude of inhibition, the Multiplicity Coefficient (MC) was calculated for all exposure scenarios. This index normalizes microbial survival relative to the unexposed control (100%), permitting a direct numerical comparison of "biological cost" between single and binary stress regimes (Table 1).

Table 1. Comparative survival rates (Multiplicity Coefficient, %) of the chernozem microbiome under individual and binary heavy metal exposure (OD₅₄₀ normalized to control).

Concentration (mg/L)	Cu ²⁺	Zn ²⁺	Ni ²⁺	Co ²⁺	Cu+Co	Cu+Ni	Cu+Zn	Co+Ni	Co+Zn	Ni+Zn
100	70.4	72.2	61.7	54.8	43.5	47.8	60.9	39.1	45.2	41.7
200	72.2	64.3	47.8	40.9	26.1	34.8	45.2	21.7	27.8	26.1
300	60.9	56.5	34.8	27.0	13.0	17.4	26.1	8.7	15.7	13.0
400	47.8	39.1	26.1	15.7	4.3	8.7	15.7	4.3	7.0	4.3
500	34.8	36.5	20.9	8.7	1.7	4.3	8.7	1.7	2.6	1.7
600	33.0	26.1	8.7	2.6	0.9	1.7	4.3	0.9	0.9	0.9
800	1.7	8.7	1.7	0.0	0.0	0.0	0.9	0.0	0.0	0.0

The primary ecotoxicological significance of Table 1 lies in the systematic deviation of binary mixtures from the expected additive toxicity. To rigorously quantify this non-linear potentiation, the Bliss Independence (IA) model was applied to calculate the expected additive survival (MC_{exp}) and the specific Synergistic Ratio (SR).

This synergistic amplification is most conspicuously illustrated at the 400 mg/L total concentration threshold—the critical tipping point for systemic community collapse (Figure 4). Across all tested configurations, the empirically observed survival (MC_{obs}) was significantly suppressed compared to the theoretical additive predictions derived from single-metal isotherms ($SR > 1$).



The most extreme divergence from additivity was observed in the $\text{Ni}^{2+} + \text{Zn}^{2+}$ mixture (SR = 7.1), where the independent action model predicted a 30.7% survival rate, yet the observed viability collapsed to just 4.3%. Similarly, the highly toxic $\text{Co}^{2+} + \text{Ni}^{2+}$ pairing yielded an SR of 4.5 (19.5% expected vs. 4.3% observed). This profound reduction in viability serves as indisputable numerical evidence that the interaction is strictly synergistic rather than additive.

Mechanistically, these SR values indicate that cellular defense systems (e.g., specific efflux pumping or intracellular sequestration) capable of managing 200 mg/L of a single metal are completely overwhelmed when simultaneously forced to mitigate 200 mg/L of a secondary stressor with a distinct cellular target. This confirms that polymetallic contamination fundamentally lowers the "lethal threshold" of the chernozem microbiome by a factor of up to 7 compared to single-metal scenarios. Thus, even if the concentrations of individual metals in soils are within levels conventionally regarded as safe, simultaneous exposure to diverse metal cations induces catastrophic metabolic failure, critically compromising the soil microbiome.

Discussion

The determination of Maximum Tolerated Concentrations (MTC) serves as a fundamental metric for quantifying the "ecological stability" of soil ecosystems under anthropogenic load [30,56].

The results support the hypothesis that the chernozem microbiome exhibits differentiated resistance to heavy metals, which can be quantified by MC. The established order of toxicity ($\text{Co} > \text{Ni} > \text{Zn} > \text{Cu}$) is consistent with well-known data on the toxicity of these metals to bacteria, where cobalt and nickel are often more toxic than copper and

zinc[58].

The experimental design employed metal concentrations up to 1000 mg/L. While these absolute values significantly exceed typical background levels in uncontaminated soils, it is critical to distinguish between *total soil concentration* (mg/kg) and the biologically active, water-soluble fraction in the soil pore water (mg/L). Chernozem soils possess an exceptional heavy metal buffering capacity due to their high cation exchange capacity (CEC), substantial total organic carbon (TOC), and slightly alkaline pH. These pedogenic properties effectively mask metal toxicity through aggressive complexation (e.g., with humic acids) and precipitation into insoluble phases. Consequently, to achieve a concentration of 400–500 mg/L of freely bioavailable toxic ions in the soil solution, the total accumulated metal load in the bulk soil (mg/kg) must be astronomically high—characteristic of extreme anthropogenic hotspots, such as mining tailing spills or chronic industrial discharge areas.

Therefore, evaluating these high concentration ranges in a liquid medium is highly ecologically relevant: it specifically models the exact thermodynamic tipping points where the soil's natural buffering mechanisms are completely saturated. Only under these saturation conditions is the unadapted native microbiome exposed to acute, unshielded metallic stress, allowing for the precise measurement of its inherent physiological resistance limits.

Understanding the stability of metal compounds in the environment and the pathways of their transformation by microorganisms is key to investigating the patterns of metal toxicity toward soil microbiomes. Thermodynamic calculations of stability fields of metal compounds can provide a mechanistic explanation for the observed differences in metal toxicity[59,60]. The experimentally derived toxicity series, correlates strictly with the thermodynamic stability fields of metal species in the specific physicochemical environment of chernozem (pH 6.8-7.4, oxidizing conditions).

According to the Pourbaix (Eh-pH) diagrams and hydrolysis constants (pK_h), each metal exhibits distinct behavior that dictates its bioavailability[61]. The extreme toxicity of cobalt is explained by its exceptionally wide stability domain in the soluble ionic form (Co^{2+}). On the Pourbaix diagram, the equilibrium line for the transition $Co^{2+} \rightleftharpoons Co(OH)_2$ (s) lies at $pH \approx 8.5-9.0$. In the chernozem pH range (6.5-7.5), cobalt remains strictly in the phase of the thermodynamically stable aquacation $[Co(H_2O)_6]^{2+}$. Oxidation to the insoluble form $Co(OH)_3$ requires a high redox potential ($Eh > +0.5$ V) combined with high alkalinity, which is untypical for the bulk soil phase. Consequently, Co^{2+} does not undergo abiotic

precipitation and exerts maximum partial pressure on microbial membranes, acting as a persistent stressor. Similar to cobalt, Ni^{2+} is resistant to hydrolysis. The solubility product constant for nickel hydroxide ($K_{\text{sp}} \text{Ni}(\text{OH})_2 \approx 2 \times 10^{-15}$) indicates that precipitation begins only at $\text{pH} > 7.7$. Although nickel forms complexes with soil fulvic acids, their stability constants ($\log K \approx 3.0-4.0$) are significantly lower than those of copper. This leaves a significant fraction of nickel in a free, cationic form capable of penetrating the cell via magnesium transport systems (e.g., CorA), causing the observed inhibition. Zinc begins to hydrolyze at $\text{pH} \sim 7.0-7.5$ ($\text{Zn}^{2+} \rightarrow \text{Zn}(\text{OH})^+$), which is close to the physiological pH of chernozem. Unlike Co^{2+} and Ni^{2+} , zinc is prone to forming insoluble carbonates (ZnCO_3) and phosphates in soil solution. This abiotic sequestration reduces the effective concentration of the toxic agent acting on the cell, providing a partial thermodynamic "shield" for the microbiome. Copper showed the highest resistance threshold ($\text{MTC} > 800 \text{ mg/L}$), which seems paradoxical given its high bactericidal potential in pure cultures. However, in chernozem, this is strictly governed by the Irving-Williams series of stability constants for metal complexes: $\text{Mn} < \text{Fe} < \text{Co} < \text{Ni} < \text{Cu} > \text{Zn}$ [62]. Copper forms the most stable complexes with organic ligands of chernozem (humic acids)[25]. The stability constant for Cu-humate ($\log K \approx 8.0-9.0$) is orders of magnitude higher than for Co^{2+} or Ni^{2+} . Furthermore, Cu^{2+} begins to precipitate as hydroxides $\text{Cu}(\text{OH})_2$ and hydroxycarbonates (malachite-like phases) already at $\text{pH} \approx 6.0$. Thermodynamically, the reaction $\text{Cu}^{2+} + \text{Soil}_{\text{Matrix}} \rightarrow \text{Cu-Complex}$ is strongly exergonic ($\Delta G \ll 0$). This leads to rapid "chemical immobilization" of the metal. The microbiome survives not because Cu is less toxic, but because the free ion activity is reduced to near-zero values by the soil matrix until the buffer capacity is fully saturated.

Another side of mechanistic explanation lies in the attack on different but vital cellular targets. For example, copper (Cu^{2+}) is known for its ability to generate reactive oxygen species (oxidative stress), while cobalt (Co^{2+}) and nickel (Ni^{2+}) can competitively replace other ions (e.g. Mg^{2+} or Fe^{2+}) in the active sites of enzymes [9,11,47].

For example, if thermodynamically advantageous detoxification pathways (chelation, precipitation, reduction) for cobalt are less affordable or energy-intensive for chernozem microorganisms compared to other metals, this may explain its higher toxicity [58].

The most critical finding of this study, the strong synergistic toxicity of binary combinations, can be described through the theory of coupled metabolic load[63]. In a single-metal scenario, the microbial community allocates a specific portion of its ATP pool to detoxification (e.g., operation of cation diffusion facilitators or P-type ATPases).

However, in a polymetallic environment, thermodynamic calculations suggest a non-linear increase in the energy required for survival. When treated simultaneously (Fig 3 A-F), the cell is forced to deal with both oxidative stress and enzymatic inhibition, leading to a rapid collapse that is not observed with individual treatments. The entropy of the system increases, and the efficiency of enzymatic reactions drops, requiring the cell to synthesize *de novo* enzymes, which is energetically expensive.

The observed threshold effect on the growth curves (Figure 1) is an important aspect [29,64]. From a thermodynamic perspective, the initial plateau corresponds to the capacity of spontaneous neutralization. It suggests that the chernozem microbiome has internal buffering mechanisms that can effectively neutralize low and moderate concentrations of metals[32,46]. However, after exceeding a certain threshold, these defense mechanisms become saturated or overloaded, leading to a rapid and catastrophic decline in the viability of microorganisms[46]. This indicates a nonlinear dose-dependent response, where the initial “flat” part of the curve reflects the capacity of the internal stability of the system, and a sharp decline indicates the point of failure of the system[55]. This understanding is critical for the further search for resistant strains to heavy metals.

The limitations of this study must be contextualized within its methodological philosophy. While *in vitro* assays using nutrient-rich media do not fully replicate the complex bioavailability dynamics of the in situ soil matrix, they provide a highly controlled environment necessary to isolate and quantify exact toxicological tipping points. Furthermore, while the absence of molecular sequencing (e.g., 16S rRNA) precludes the identification of specific taxonomic shifts or the depletion of specific functional groups (such as fungal components), this constraint is aligned with the study's core objective.

In this research, the chernozem microbiome is intentionally treated as a single, macroscopic functional-structural entity rather than a conglomerate of individual species. This holistic approach was deliberately prioritized over species-level diversity analysis to evaluate Systemic Functional Stability. Total viable microbial counts (CFU) and biomass accumulation kinetics (OD) serve as direct, real-time indicators of the ecosystem's capacity to maintain essential functions. Unlike genomic data, which can overestimate community resilience due to relic DNA, biomass kinetics provide a direct measurement of the active ATP-driven metabolic load. By utilizing the Multiplicity Coefficient (MC), we transform these classical metrics into an Integral Response Detection system. This allows for the precise identification of the 'systemic collapse point'—the exact thermodynamic threshold where the community's collective defense mechanisms and buffering capacity

catastrophically fail under single or dual-metal stress.

Comparison of the obtained data with literature sources allows to contextualize the identified patterns. Literature on isolated strains (*Pseudomonas putida* USM4 and *Brevundimonas vesicularis* USM1) confirms that Co(II) is one of the most toxic metals, while Fe(III) is the least toxic [65]. This observation aligns with the critical review by Mahey et al. (2020) [58], who highlighted that cobalt, despite being a micronutrient, exhibits a significantly narrower threshold between essentiality and toxicity compared to zinc or copper. Furthermore, Wierzba (2015) [32] demonstrated similar toxicity rankings for *Bacillus subtilis* and *Stenotrophomonas maltophilia*, confirming that sensitivity to Cobalt and Nickel is a conserved trait across diverse soil taxa. However, quantitative differences are notable when comparing our community-level data with industrial isolates. For instance, Cai et al. (2019) [29] reported significantly higher absolute resistance thresholds for Ni and Zn in bacteria isolated from electroplating wastewater. This contrast highlights the vulnerability of the unadapted chernozem microbiome compared to acclimated industrial strains. Our results for the chernozem microbial community show that the community as a whole can tolerate significantly higher cobalt concentrations, as well as slightly higher nickel and copper concentrations, than the individual isolates investigated by Hovorukha et al [54]. This may indicate the collective resistance of the chernozem microbial community, where the interaction of different species and the presence of more tolerant strains provides higher overall resistance. In addition, high chernozem microbial diversity may mean a greater likelihood of pre-existing resistant strains, contributing to overall community resilience[49,66]. These differences in resilience between soil types highlight the role of the organic matrix and microbial diversity in shaping the ecosystem's adaptive potential[66,67].

The detection of polyresistant forms in chernozem capable of surviving complex polymetallic contamination highlights their significant potential for bioremediation[68]. This turns chernozem not just into an object of research, but into a valuable natural "reservoir" of bioremediation potential[69]. Due to its internal diversity and buffer capacity, chernozem can contain or rapidly develop microorganisms that are effective agents for cleaning up contaminated areas.

In summary, this study demonstrates that traditional single-metal ecotoxicological models critically underestimate the risks associated with polymetallic contamination. While the chernozem microbiome exhibits substantial resilience at moderate concentrations—driven by the soil's organic buffering capacity and native functional redundancy—it undergoes a critical non-linear decline in functional stability once these specific

thermodynamic thresholds are exceeded. The established acute toxicity hierarchy ($\text{Co}^{2+} > \text{Ni}^{2+} > \text{Zn}^{2+} > \text{Cu}^{2+}$) is fundamentally exacerbated under co-contamination. As mathematically validated by the Bliss Independence model, binary mixtures invariably induce severe synergistic inhibition, significantly lowering the critical stability threshold of the microbial community. These findings highlight an urgent need to integrate synergistic interaction factors into ecological risk assessments and future bioremediation strategies, as concentrations conventionally regarded as safe for individual metals fail to protect the pedosphere against the compounding metabolic load of complex metallic mixtures.

Materials and Methods

Preparation of Cu^{2+} , Co^{2+} , Zn^{2+} and Ni^{2+} solutions

Stock solutions of Cu(II), Co(II), Zn(II), and Ni(II) were prepared at a standardized metal cation concentration of 25 g/L using analytical grade reagents. The source salts included: copper(II) sulfate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$), cobalt(II) chloride hexahydrate ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$), zinc(II) sulfate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$), and nickel(II) chloride hexahydrate ($\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$) (Chemlaborreaktiv LLC, Kyiv, Ukraine). To achieve the target cation concentration, precise stoichiometric quantities of salts (29.46 g, 30.30 g, 32.94 g, and 31.92 g, respectively) were dissolved in distilled water within 300 mL volumetric flasks. The resulting stock solutions were sterilized by autoclaving under standard protocol (121 °C, 1 atm, 20 min) prior to use.

Inoculum preparation.

Chernozem soil samples were retrieved from the arable horizon (0-20 cm depth) of an agricultural field located in the Kyiv region, Ukraine (50.113756° N, 30.580236° E). Following collection, the soil aggregates were mechanically homogenized under laboratory conditions. To prepare the microbial inoculum, a 1 g aliquot was aseptically transferred into 9 mL of sterile physiological saline (0.85% w/v NaCl) and subjected to vigorous mechanical agitation for 5 minutes to ensure the desorption of indigenous microorganisms from soil particles. The resulting supernatant (1:10 dilution) served as the primary suspension for subsequent serial dilutions.

Determination of Maximum Tolerated Concentrations on a solid nutrient medium

Sterile HM Peptone B Agar (Meat extract B Agar M806; HiMedia Laboratories, Mumbai, India) was prepared and tempered to 45-50°C to prevent thermal shock and metal precipitation. Aliquots of the sterile heavy metal stock solutions were aseptically

supplemented into the molten agar to achieve a final concentration gradient of: 0 (control), 100, 250, 300, 500, 600, 750, 800, and 1000 mg/L. The medium was homogenized to ensure uniform metal distribution and dispensed into sterile Petri dishes (20 mL per plate). Upon solidification, a 0.1 mL aliquot of the selected soil suspension dilution was inoculated onto the agar surface using the spread-plate technique with a sterile glass spatula. All assays were performed in triplicates. The plates were incubated at $30 \pm 2^\circ\text{C}$ for 15 days to allow for the development of slow-growing autochthonous taxa. Following incubation, survivability was quantified by enumerating the CFU.

Determination of Maximum Tolerated Concentrations on a Liquid Nutrient Medium for Single Metals and Pairwise Combinations

Nutrient Broth (NB; M002, HiMedia Laboratories, Mumbai, India) was prepared as the basal liquid medium following the manufacturer's protocol. To assess microbial growth kinetics under metallic stress, sterile metal stock solutions (25 g/L cation basis) were aseptically supplemented into the NB medium to establish a broad concentration gradient ranging from 0 to 1000 mg/L. The specific test concentrations were: 0 (control), 25, 50, 100, 200, 300, 350, 400, 500, 600, 700, 800, 900, and 1000 mg/L.

The experimental design included two exposure scenarios: (i) Single-Metal Exposure: the medium was amended with individual metal ions (Cu^{2+} , Co^{2+} , Zn^{2+} , or Ni^{2+}) to the target final concentration. (ii) Pairwise Binary Exposure: six combinations were tested ($\text{Cu}^{2+}+\text{Co}^{2+}$, $\text{Cu}^{2+}+\text{Zn}^{2+}$, $\text{Cu}^{2+}+\text{Ni}^{2+}$, $\text{Co}^{2+}+\text{Zn}^{2+}$, $\text{Co}^{2+}+\text{Ni}^{2+}$, $\text{Zn}^{2+}+\text{Ni}^{2+}$). In these mixtures, metals were combined at a 1:1 mass ratio, such that the stated concentration represents the sum of the two constituents (e.g., a 100 mg/L binary treatment consisted of 50 mg/L of one metal and 50 mg/L of another).

The final reaction volume in each culture tube was standardized to 10 mL. Metal-free NB served as the abiotic negative control and the biotic positive control (0 mg/L). Each tube was inoculated with a 1% (v/v) aliquot (100 μL) of the prepared soil suspension. All assays were conducted in triplicates. The cultures were incubated under static conditions at $30 \pm 2^\circ\text{C}$ for 15 days. Following incubation, total biomass accumulation was quantified via optical density measurements at 540 nm (OD_{540}) using a KFK-2MP photoelectric photometer, calibrated against a sterile medium blank.

Data analysis

The number of CFU/g of dry soil of metal-resistant microorganisms isolated from chernozem on NA with a gradient of Co^{2+} , Ni^{2+} , Zn^{2+} , Cu^{2+} from 0 to 1000 mg/L, in 1 mL of suspension was calculated by the formula:

$$M=(a \times 10^n)/V$$

where: M is the number of cells in 1 mL; *a* is the average number of colonies; 10^n is the dilution factor; V is the volume of the inoculum, mL.

The number of microorganisms in the samples was recalculated to the total number of microorganisms in 1 g of a completely dry sample according to the formula:

$$X=M \times k$$

where: X is the number of colony-forming units in 1 g of a completely dry sample; M is the number of cells in 1 mL of the suspension of the microorganisms under study; k is the humidity coefficient of the sample.

The humidity coefficient was determined by the formula:

$$k=100/(100-H)$$

where: *H* is the moisture content of the sample.

The moisture content (*H*) in the samples was determined by weight method. Samples were dried in an oven at 105 °C until constant weight. Moisture was determined by the formula:

$$H=A/(m-A) \times 100\%$$

where: A is the mass of the evaporated moisture; m is the mass of the sample.

To quantify the toxicity of metal ions and compare their inhibitory effects, a Multiplicity Coefficient (MC) was calculated. The calculation was carried out for each metal concentration relative to the control sample (without metal).

The index was calculated according to the following formula:

$$MC(\%)=N_{Cmet}/N_0 \times 100$$

Where: MC(%) is the inhibition index, expressed as a percentage (%). It shows the percentage of surviving cells compared to the control. N_{Cmet} is the titer of viable cells (OD₅₄₀) grown in a medium with a certain concentration of the corresponding metal. N_0 is the titer of viable cells in a control sample grown on a medium without the addition of metals. This value is taken as 100% survival.

To rigorously quantify and classify the interactive effects of binary metal mixtures, the Bliss Independence (Independent Action, IA) model was employed. The IA model is mechanistically appropriate for this study, as the tested transition metals possess distinct primary cellular targets and modes of action (e.g., Cu²⁺ predominantly induces oxidative stress, whereas Co²⁺ and Ni²⁺ competitively displace essential cofactors in metalloenzymes).

The model predicts the expected fractional survival of a microbial community exposed to a mixture of independently acting toxicants. Adapting this model for the empirically

derived Multiplicity Coefficient (MC, expressed as a percentage of control survival), the expected additive survival (MC_{exp}) for a binary mixture of metal A and metal B was calculated as:

$$MC_{exp}(\%) = \frac{MC_A \times MC_B}{100}$$

Where: MC_A and MC_B represent the observed survival percentages of the microbiome exposed to the individual metals.

Because the experimental design utilized a 1:1 mass ratio based on total metal concentration, the expected additive survival for a binary complex at a total concentration x was derived using the individual survival rates at the concentration of its constituent parts ($x/2$). For instance, the theoretical MC_{exp} for a Cu+Zn mixture at 400 mg/L was calculated using the individual MC values of Cu^{2+} and Zn^{2+} at 200 mg/L.

To quantify the magnitude and direction of the interactive effect, a Synergistic Ratio (SR) was defined as:

$$SR = \frac{MC_{exp}}{MC_{obs}}$$

Where: MC_{obs} is the empirically observed survival of the binary mixture. The interactions were classified according to the following criteria: $SR > 1$ indicates synergistic toxicity (the mixture is more toxic than predicted by independent action), $SR = 1$ indicates strict additivity, and $SR < 1$ indicates antagonism.

Dose-Response Modeling and EC50 Calculation

To quantitatively evaluate the susceptibility of the chernozem microbiome to individual heavy metals and standardize the toxicological endpoints, the half-maximal effective concentration (EC50) was calculated. Dose-response curves were modeled by fitting the Multiplicity Coefficient (MC) data to a standard logistic function (Hill equation) using non-linear least squares regression:

$$MC(x) = \frac{100}{1 + (x/EC_{50})^h}$$

Where: $MC(x)$ is the percentage of surviving cells at metal concentration x , 100 represents the baseline control survival (100%), EC_{50} is the concentration inducing a 50% reduction in viability, and h is the Hill slope characterizing the steepness of the inhibition curve.

Data analysis and visualization were performed using the Python programming language (Version 3.10, Python Software Foundation, Wilmington, DE, USA). Statistical

processing was conducted using the NumPy library (Version 1.23.5) and SciPy (Version 1.10.1). Graphical representations were generated using Matplotlib (Version 3.7.1) and Seaborn (Version 0.12.2). All experimental data points in the resulting graphical plots are presented as the arithmetic mean accompanied by corresponding standard deviation (Mean $\pm$ SD) error bars. All computational workflows were executed in the Google Colaboratory cloud environment (Google LLC, Mountain View, CA, USA), ensuring reproducibility and accessibility of the code.

Data Availability

Data are available on request from the corresponding author.

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Author Contributions

I.K. conceptualized the experimental design, conducted the microbiological investigations, performed formal analysis, and wrote the original draft of the manuscript. **V.H.** provided supervision, performed data curation, and contributed significantly to the critical review and editing of the manuscript. **E.M.** performed project administration, conducted spatial validation, review of manuscript. **O.T.** served as the principal investigator, formulated the

overarching fundamental vision, developed the core methodology, and critically reviewed the final manuscript. All authors have read and agreed to the published version of the manuscript

Additional information

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Figure Legends

Figure 1. The inhibitory effects of Cu^{2+} , Zn^{2+} , Ni^{2+} , and Co^{2+} on biomass accumulation of chernozem microorganisms.

Figure 2. The inhibitory effects of Cu^{2+} , Zn^{2+} , Ni^{2+} , and Co^{2+} on CFU counts of chernozem microorganisms.

Figure 3. Dose-response kinetics and EC50 determination for the native chernozem microbiome under single-metal exposure. The empirically observed Multiplicity Coefficient (MC) data points (circles) were fitted to a standard logistic function (dashed lines) to model the concentration-dependent decline in systemic microbial viability. The intersection with the 50% survival threshold (dotted horizontal line) yields the calculated half-maximal effective concentrations (EC50). The quantitative models confirm a strict acute toxicity hierarchy of Co^{2+} (130.8 mg/L) > Ni^{2+} (167.6 mg/L) > Zn^{2+} (290.0 mg/L) > Cu^{2+} (331.2 mg/L).

Figure 4. Comparative Analysis of Resistance to Individual and Binary Metal Stress (Dual-Metal Stress Resistance).((A) Cu and Co vs Cu+Co ; (B) Cu and Ni vs Cu+Ni ; (C) Cu and Zn vs Cu+Zn ; (D) Co and Ni vs Co+Ni ; (E) Co and Zn vs Co+Zn F. Ni and Zn vs Ni+Zn)

Figure 4. Quantitative validation of synergistic toxicity in binary metal mixtures at the critical 400 mg/L threshold. The clustered bar chart compares the theoretical additive survival (MC_{exp} , grey bars) predicted by the Bliss Independence (IA) model against the empirically observed fractional survival (MC_{obs} , red bars). Additive predictions were

908 derived from individual metal toxicities at 200 mg/L (representing a 1:1 mass ratio for the
909 400 mg/L mixture). The Synergistic Ratio ($SR = MC_{exp}/MC_{obs}$) is indicated above each
910 pair. For all combinations, observed viability was significantly suppressed compared to the
911 IA prediction ($SR > 1$), confirming profound synergistic inhibition across the unadapted
912 chernozem microbiome.
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