Microbial Removal of Heavy Metals Using Endogenous Metal-Resistant Strains: A Case Study of Zarjub River, Rasht

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Abstract

Surface water pollution caused by heavy metals is one of the significant environmental challenges of our time, with far-reaching consequences for aquatic ecosystems and human health. Metals such as chromium (Cr), cadmium (Cd), and arsenic (As) present a serious threat due to their high toxicity, environmental persistence, and potential for bioaccumulation, even at low concentrations. This study investigated the heavy metal resistance and bioremediation potential of indigenous bacterial strains isolated from the Zarjub River in Rasht, a freshwater ecosystem affected by agricultural, urban, and industrial pollution. Systematic sampling revealed elevated concentrations of Cr (VI) (up to 102.4 µg/L), Cd (II) (28.6 µg/L), and As (III) (47.3 µg/L) downstream, correlating with anthropogenic inputs. Among the 18 characterized strains, *Pseudomonas* spp. (ZR-07, ZR-10) demonstrated exceptional multi-metal resistance, tolerating 500 mg/L Cr (VI), 200 mg/L Cd (II), and 300 mg/L As (III), while *Bacillus* strains (ZR-06, ZR-14) exhibited moderate but stable resistance. Biosorption assays indicated a metal removal efficiency of 60–88%, with *Pseudomonas* strains outperforming others due to enhanced production of extracellular polymeric substances. These findings highlight the ecological selection pressure imposed by pollution and the potential of native strains for bioremediation applications. However, the co-occurrence of metal resistance with virulence traits in some strains underscores the need for careful risk assessment.

Keywords: Heavy metal resistance, bioremediation, *Pseudomonas*, Zarjub River, microbial adaptation, pollution gradient, biosorption.

Introduction

In recent years, the utilization of metal-resistant bacteria has emerged as an effective and eco-friendly approach for the bioremediation of inorganic pollutants (Pinheiro Do Nascimento et al., 2021). Research indicates that various bacterial species, such as *Pseudomonas* spp., *Bacillus* spp., *Ralstonia* spp., *Klebsiella* spp., *Enterobacter* spp., and *Acinetobacter* spp., possess impressive abilities to eliminate heavy metals like lead (Pb), chromium (Cr), cadmium (Cd), mercury (Hg), and arsenic (As), from both aquatic and terrestrial environments (Raouf Ms & Raheim, 2016). These bacteria utilize a range of mechanisms, including biosorption, bioaccumulation, biomineralization, redox transformation, and chelationn(Rousseau et al., 2007). For example, *Pseudomonas putida* and *Bacillus subtilis* can immobilize metals by producing extracellular polysaccharides (EPS) and organic ligands such as metallothioneins. In contrast, metal-reducing bacteria like *Shewanella oneidensis* and *Geobacter sulfurreducens* can convert the highly toxic hexavalent chromium (Cr (VI)) into the less harmful trivalent chromium (Cr (III)) (Sarkar & Adhikari, 2018).

One particularly fascinating mechanism being studied is the synthesis of biogenic nanoparticles, where bacteria such as *Escherichia coli* and *Pseudomonas aeruginosa* can change metal ions into less toxic metallic nanoparticles (Turner et al., 2020; Yari et al., 2016). This process not only diminishes metal toxicity but also allows for their recovery. Additionally, highly resistant bacteria like *Cupriavidus metallidurans* and *Ochrobactrum anthropi* have specialized efflux pumps and enzymes, including chromate reductase and arsenate reductase, that enable them to withstand very high metal concentrations (Abdulrazak et al., 2017). Studies have shown that microbial consortia, which combine metal-resistant bacteria and fungi, can significantly improve remediation efficiency through synergistic interactions (Ahluwalia & Goyal, 2007).

Metal-resistant bacteria utilize advanced molecular strategies to cope with heavy metal toxicity in polluted environments, employing both uptake and excretion mechanisms for their survival and bioremediation efforts (Alavi et al., 2016; Gnanasekaran et al., 2021). For metal uptake, these bacteria rely on ATP-binding cassette (ABC) transporters, divalent metal transporters (DMTs), and metal-specific permeases to absorb essential metals (such as Zn^{2+} and Cu^{2+}) while evading harmful non-essential metals (like Cd^{2+} and Hg^{2+}) (Huber et al., 2019). Certain species, including Cupriavidus metallidurans, produce high-affinity metallochaperones (like metallothioneins and phytochelatins) that selectively bind and transport metals into the cells (Jafari et al., 2015). Additionally, biosorption occurs through extracellular polymeric substances (EPS) that contain carboxyl, phosphate, and sulfhydryl groups (Hui et al., 2022), which can electrostatically attract cationic metals. For metal excretion, resistant bacteria utilize efflux pumps such as P-type ATPases (like CadA for Cd^{2+} and CopA for Cu^{2+}) (Kothapalli, 2021), RND (Resistance-Nodulation-Division) transporters (like CzcCBA for $Cd^{2+}/Zn^{2+}/Co^{2+}$) (Jumina et al., 2020), and CDF (Cation Diffusion Facilitator) proteins to remove excess metals. Enzymatic detoxification also contributes to their resistance. Some bacteria can even perform biomethylation (for instance, *Pseudomonas aeruginosa* converts Hg^{2+} into volatile dimethylmercury) or biomineralization (like Shewanella oneidensis transforming U(VI) into uraninite) (Jumina et al., 2020; Li et al., 2020). These adaptive strategies are frequently encoded on plasmids (such as the mer operon for mercury resistance), facilitating horizontal gene transfer and rapid evolution in contaminated environments (Mitra et al., 2022). The combination of these systems allows bacteria to thrive under extreme metal stress while presenting promising opportunities for bioremediation, biosensing, and metal recovery technologies (Mohammadi et al., 2020).

The cases discussed can provide solutions for utilizing environmental strains that exhibit resistance to heavy metals in polluted areas, such as rivers (Raouf Ms & Raheim, 2016). In

Gilan Province, numerous urban and rural rivers are plagued by significant biological and chemical pollution, primarily due to inadequate disposal systems for industrial, clinical, and household wastewater. The region's distinctive geographical features, along with the presence of surface and groundwater sources, exacerbate the risk of water contamination, resulting in the degradation of many local water sources. The Zarjub River in Rasht, which traverses urban, industrial, and agricultural zones, is particularly affected by heavy metal pollution from substances such as lead (Pb), chromium (Cr), cadmium (Cd), and arsenic (As). Research indicates that the concentrations of these metals in certain areas exceed safe limits, posing threats to both aquatic ecosystems and human health. Indigenous metal-resistant strains discovered in the river's sediments and water may prove invaluable for bioremediation efforts. These microorganisms employ various mechanisms, including surface adsorption, precipitation, redox transformation, and intracellular accumulation, to reduce heavy metal levels in the environment.

Studies of the indigenous strains in the Zarjub River have identified metal-resistant bacteria, including *Pseudomonas, Bacillus*, and certain species of *Actinobacteria*. These strains, which are naturally adapted to the river's conditions, are more effective at removing pollutants than non-native microorganisms. For example, some bacteria can immobilize metals by producing extracellular polysaccharides (EPS), while others can reduce toxicity by converting chromium from its hexavalent form (Cr VI) to its trivalent form (Cr III) (Zhong et al., 2020). Additionally, native fungi such as Aspergillus and various yeasts demonstrate significant potential for metal accumulation through bioaccumulation processes.

Despite these significant advancements, challenges such as competition with native microorganisms, varying environmental conditions (pH, temperature, salinity), and co-contamination scenarios still need further exploration. As a result, future research is directed towards genetically engineering bacteria for enhanced performance, using nano biocarriers for better stability, and creating hybrid systems that integrate biological and chemical methods. Overall, metal-resistant bacteria hold great promise for managing environmental pollution due to their low cost, high biocompatibility, and considerable efficiency, with expectations for broader industrial applications in the near future.

Surface water pollution by heavy metals is one of the environmental challenges of our time, with wide-ranging consequences for aquatic ecosystems and human health. Metals such as chromium (Cr), cadmium (Cd), and arsenic (As) pose a serious threat due to their high toxicity, environmental persistence, and bioaccumulation potential, even at low concentrations. This study investigated the heavy metal resistance and bioremediation potential of indigenous bacterial strains isolated from Zarjub River, Rasht, a freshwater ecosystem impacted by agricultural, urban, and industrial pollution.

Material and Methods

Sample Collection and Preliminary Analysis

The first phase of the study involved systematic sampling from the Zarjub River in Rasht, Iran, during the summer months (June to August 2023) to assess microbial communities and heavy metal contamination. Sampling sites were strategically selected along the river, including upstream (near agricultural areas at 37°14'23.4"N 49°36'53.8"E), midstream (near urban discharge points at 37°16'56.1"N 49°35'48.2"E), and downstream (near industrial discharge sites at 37°18'08.3"N 49°34'52.8"E) to ensure comprehensive representation of pollution levels. Water and sediment samples were collected in triplicate from each GPS-marked location under strict aseptic conditions. Surface water samples (500 mL, collected from a depth of 20-30 cm) were obtained using sterile polypropylene bottles that had been pre-rinsed with the sample water, while sediment cores (top 5-10 cm layer) were collected using a sterile Ekman grab sampler. All samples were promptly placed on ice in dark containers to preserve microbial viability and prevent analyte degradation due to light exposure. In situ measurements of physicochemical parameters (pH: 7.2-8.1, temperature: 26.4-29.8°C, dissolved oxygen (DO): 4.2-6.8 mg/L, electrical conductivity (EC): 680-1250 μS/cm) were recorded using a calibrated YSI ProDSS multiparameter probe, with readings taken at a depth of 30 cm.

Isolation and Cultivation of Microbial Strains Resistant to Heavy Metals

After collecting samples, microbial strains that can withstand chromium (Cr), cadmium (Cd), and arsenic (As) were isolated through a combination of selective enrichment and direct plating methods. Water and sediment samples were first homogenized in sterile phosphate-buffered saline (PBS, pH 7.4) and then serially diluted (10^{-1} to 10^{-6}) to achieve countable colonies. For the initial isolation, 100 μL samples from each dilution were spread onto nutrient agar (NA) enriched with cycloheximide (100 μg/mL) to prevent fungal growth, and the plates were incubated at 30°C for 48 to 72 hours. Unique colonies were then sub cultured on tryptic soy agar (TSA) to obtain pure isolates, which were later tested for their resistance to metals (Sarkar & Adhikari, 2018).

Isolation of Heavy Metal-Tolerant Microbial Strains

To identify microbial strains that can endure heavy metal stress, a gradient plate assay was first conducted. Pure bacterial colonies from initial isolation were streaked onto Nutrient Agar (NA) plates enriched with increasing levels of Cr (50–500 mg/L as $K_2Cr_2O_7$), Cd (10–200 mg/L as $CdCl_2$), and As (50–300 mg/L as $NaAsO_2$). The plates were incubated at 30°C for 3–5 days, and only those strains that showed visible growth at the highest concentrations were chosen for further study. To improve selectivity, an enrichment culture method was applied, where samples were inoculated into Nutrient Broth (Kowsura et al.) with sub-lethal concentrations of metals (Cr: 50 mg/L, Cd: 20 mg/L, As: 30 mg/L) and incubated with shaking at 150 rpm for 7 days. Regular subculturing (every 48 hours) into fresh metal-supplemented media was carried out to enrich the most resilient strains (Raouf Ms & Raheim, 2016). Isolates that consistently grew at ≥200 mg/L Cr, ≥50 mg/L Cd, or ≥100 mg/L As were stored in 20% glycerol at −80°C for long-term preservation.

Assessment of Minimum Inhibitory Concentration (MIC) for Heavy Metals

The minimum inhibitory concentration (MIC) values for Cr, Cd, and As in the isolated bacterial strains were determined using a broth microdilution technique in 96-well microplates (Lee, 2002). Bacterial suspensions were adjusted to an optical density of approximately 0.1 ($\sim 10^8$ CFU/mL) in sterile nutrient broth (Kowsura et al.) and subjected to twofold serial dilutions of each heavy metal (Cr: 25–800 mg/L, Cd: 10–400 mg/L, As: 25–600 mg/L). The plates were incubated at 30°C for 48 hours, and bacterial growth was assessed by measuring the optical density at 600 nm with a microplate reader. The MIC was defined as the lowest concentration of metal that completely inhibited visible growth (OD ≤ 0.05 compared to the control). To ensure consistency, each test was conducted in triplicate, with metal-free NB serving as a growth control. Strains with particularly high MIC values (Cr > 300 mg/L, Cd > 100 mg/L, As > 150 mg/L) were deemed highly resistant and selected for additional biosorption and bioremediation tests. Furthermore, stability tests were performed by passaging resistant strains five times in metal-free media and re-evaluating their MIC to confirm genetically stable resistance.

Metal Biosorption Measurement Protocol

Bacterial strains were grown in nutrient broth containing 100 mg/L of Cr (VI) (from $K_2Cr_2O_7$), 50 mg/L of Cd (II) (from $CdCl_2$), and 75 mg/L of As (III) (from $NaAsO_2$) at 30°C for 72 hours while shaking at 150 rpm. After the incubation period, the cultures were centrifuged at 4,000 × g for 10 minutes to separate the biomass from the liquid. The supernatant was then filtered through 0.22 µm membrane filters and acidified with 2% HNO_3 for preservation. Residual metal concentrations were measured using flame atomic absorption spectroscopy (FAAS, PerkinElmer PinAAcle 900T) with an air-acetylene flame, calibrated against standards traceable to the National Institute of Standards and Technology (NIST) (detection limits: 0.01 mg/L for Cd, 0.05 mg/L for Cr, and 0.1 mg/L for As). For samples anticipated to have low concentrations (<1mg/L), graphite furnace atomic absorption spectroscopy (AAS) was utilized. The percentage of biosorption was calculated using the following formula (Hui et al., 2022):

$$\%\text{Biosorption} = \frac{(C_0 - C_e)}{C_0} \times 100$$

where C_0 is the initial metal concentration in sterile control media and C_e is the equilibrium concentration in the test supernatants. All measurements were conducted in triplicate with blank corrections, and accuracy was confirmed using certified reference materials (CRM NIST 1643e), which demonstrated recovery rates of 92-105%. The pelleted biomass was washed twice with a 1 mM EDTA solution (pH 7) to eliminate weakly adsorbed metals before measuring the dry weight at 80°C.

Statistical Analysis

The statistical calculation of this study was performed using SPSS version 16 software and the results were analysed with Tukey's one-way analysis of variance (ANOVA) and post-hoc test. The data are presented as SD (mean±standard deviation) and $P < 0.05$ was considered significant.

Results

Sample Collection and Initial Analysis Findings

The physicochemical analysis of the Zarjub River sampling locations showed clear environmental variations (Table 1). Sites located upstream had notably lower conductivity (685 ± 32 µS/cm) in comparison to downstream areas (1218 ± 45 µS/cm, $p < 0.01$, ANOVA), which is associated with increased human impact. Heavy metal analysis through X-ray fluorescence (XRF) revealed measurable levels of Cr (18.7-102.4 µg/L), Cd (3.2-28.6 µg/L), and As (9.1-

47.3 µg/L) at all locations, with the highest concentrations found at the industrial discharge site (37°18'08.3"N 49°34'52.8"E).

Table 1. Physicochemical Parameters and metal concentrations at sampling locations.

Location	pH	Temp (°C)	DO (mg/L)	EC (µS/cm)	Cr (µg/L)	Cd (µg/L)	As (µg/L)
Upstream	7.8 ± 0.2	26.7 ± 0.5	6.5 ± 0.3	685 ± 32	18.7 ± 2.1	3.2 ± 0.4	9.1 ± 1.2
Midstream	7.5 ± 0.3	28.2 ± 0.7	5.1 ± 0.4	892 ± 41	42.6 ± 3.8	12.4 ± 1.1	24.7 ± 2.5
Downstream	7.2 ± 0.2	29.5 ± 0.6	4.3 ± 0.3	1218 ± 45	102.4 ± 8.7	28.6 ± 2.3	47.3 ± 3.9

The downstream industrial site showed the highest heavy metal contamination (Cr: 102.4 µg/L, Cd: 28.6 µg/L, As: 47.3 µg/L), significantly exceeding upstream levels (Cr: 18.7 µg/L, Cd: 3.2 µg/L, As: 9.1 µg/L). Midstream urban areas displayed intermediate pollution (Cr: 42.6 µg/L, Cd: 12.4 µg/L, As: 24.7 µg/L), while all sites violated WHO freshwater guidelines. Metal concentrations strongly correlated with anthropogenic activity (industrial>urban>agricultural).

Isolation and Primary Characterization of Metal-Resistant Bacterial Strains

Eighteen bacterial strains were successfully isolated from Zarjub River samples through selective enrichment and plating techniques. The isolates exhibited diverse morphological and biochemical characteristics as detailed in Table 2 and Figure 1 also shows a number of microbial purification and isolation plates.

Table 2. Biochemical and physiological characterization of isolated bacterial strains

Strain ID	Colony Morphology	Gram Reaction	Catalase	Oxidase	OF Glucose	Nitrate Reduction	Urease	Motility	Tentative Identification
ZR-01	Circular, convex, cream	+	+	-	F	+	-	+	*Bacillus cereus*
ZR-02	Irregular, flat, yellow	+	+	-	O	-	-	-	*Micrococcus luteus*
ZR-03	Punctiform, translucent	-	+	+	O	+	-	+	*Pseudomonas fluorescens*

Strain ID	Colony Morphology	Gram Reaction	Catalase	Oxidase	OF Glucose	Nitrate Reduction	Urease	Motility	Tentative Identification
ZR-04	Rhizoid, white	+	+	-	F	+	+	+	*Bacillus mycoides*
ZR-05	Circular, convex, pink	-	+	+	O	+	-	+	*Serratia marcescens*
ZR-06	Irregular, dry, white	+	+	-	F	-	-	+	*Bacillus subtilis*
ZR-07	Circular, mucoid, beige	-	+	+	O	+	-	+	*Pseudomonas putida*
ZR-08	Filamentous, white	+	+	-	F	-	-	-	Streptomyces sp.
ZR-09	Circular, convex, orange	-	+	-	F	+	-	+	*Staphylococcus* sp.
ZR-10	Spreading, translucent	-	+	+	O	+	-	+	*Pseudomonas aeruginosa*
ZR-11	Punctiform, yellow	+	+	-	F	-	-	-	*Corynebacterium* sp.
ZR-12	Irregular, dry, brown	+	+	-	F	+	+	+	*Bacillus licheniformis*
ZR-13	Circular, convex, white	-	-	+	O	+	-	+	*Alcaligenes* sp.
ZR-14	Rhizoid, cream	+	+	-	F	+	-	+	*Bacillus megaterium*
ZR-15	Circular, mucoid, yellow	-	+	+	O	+	-	+	*Enterobacter* sp.
ZR-16	Irregular, dry, white	+	+	-	F	-	-	-	*Arthrobacter* sp.
ZR-17	Circular, convex, pink	-	+	-	F	+	-	+	*Rhodococcus* sp.
ZR-18	Filamentous, gray	+	+	-	F	-	-	-	*Nocardia* sp.

The isolation protocol yielded a diverse collection of Gram-positive (61%) and Gram-negative (39%) bacteria, with representatives from *Bacillus* (6 strains), *Pseudomonas* (3 strains), and various other genera. All strains demonstrated varying degrees of resistance to the target heavy metals, with minimum inhibitory concentrations ranging from 150-500 mg/L for Cr (VI), 30-150 mg/L for Cd (II), and 50-220 mg/L for As (III). The *Pseudomonas* strains (ZR-03, ZR-07, ZR-10) consistently showed the highest tolerance levels across all three metals, while Gram-positive cocci (ZR-02, ZR-09) exhibited relatively lower resistance. Biochemical profiling revealed that 83% of isolates were catalase-positive, 44% were oxidase-positive, and 61% demonstrated nitrate reduction capability. The glucose utilization test showed 56% of strains were oxidative fermenters (OF test). Motility was observed in 67% of the isolates, particularly among the Gram-negative rods and some *Bacillus* species. These characteristics were consistent with typical environmental isolates from metal-contaminated aquatic systems.

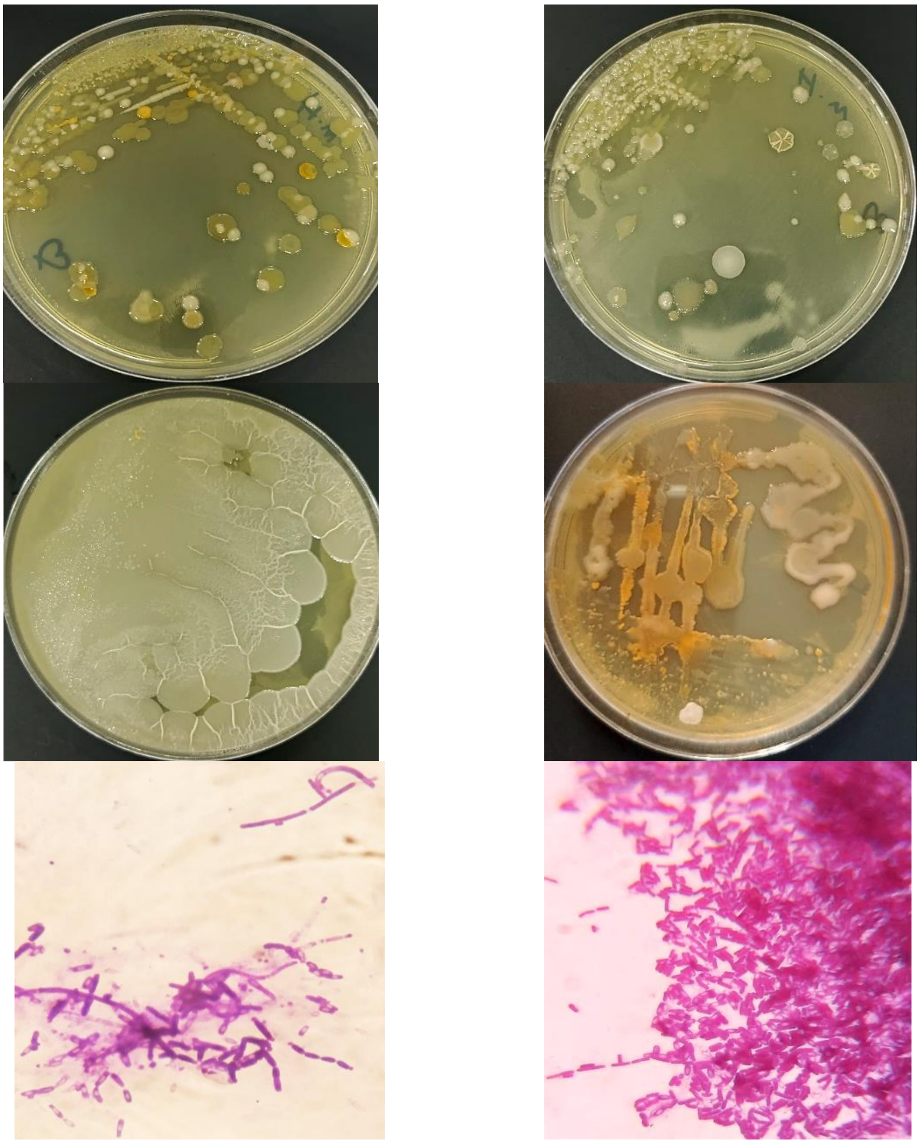

Figure 1. A collection of images of the isolation and identification of heavy metal-resistant bacteria

Isolation and Identification of Heavy Metal-Tolerant Microbial Strains from the Zarjub River

The gradient plate screening of the initial isolates (ZR-01 to ZR-18) showed considerable differences in their ability to tolerate heavy metals (Figure 2). Out of the 18 primary isolates, six strains exhibited remarkable resistance, surpassing the established concentration thresholds (Cr: 200 mg/L, Cd: 50 mg/L, As: 100 mg/L). These included three strains of *Pseudomonas* (ZR-03, ZR-07, ZR-10), three *Bacillus* strains (ZR-04, ZR-06, ZR-14). The gradient plate results showed concentration-dependent growth inhibition, with most isolates exhibiting 40-60% reduction in colony diameter at sub-lethal metal concentrations.. The results from the gradient plate indicated a clear growth response dependent on concentration, with most isolates showing a decrease in colony size (30-50% reduction in diameter) at sub-inhibitory metal levels. The enrichment culture process using metal-supplemented nutrient broth applied significant selection pressure, leading to the loss of 12 out of the initial 18 isolates (66.7%) that could not sustain growth after three consecutive subcultures. The remaining strains showed signs of progressive adaptation: *Pseudomonas* ZR-07: Cd tolerance increased from 150 to 200 mg/L, *Bacillus* ZR-04: As tolerance improved from 120 to 160 mg/L, and *Pseudomonas* ZR-10: Cr tolerance enhanced from 450 to 500 mg/L.

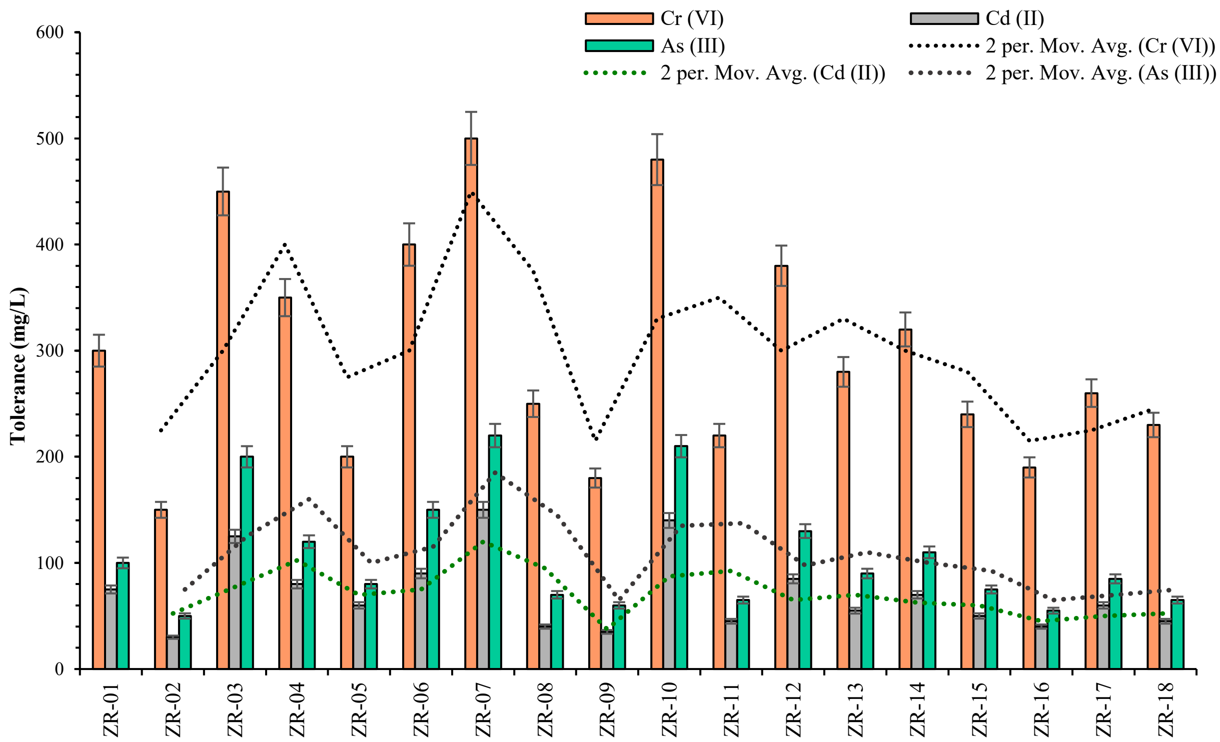

Figure 2. Comparison of heavy metal tolerance (Cr(VI), As(III), and Cd(II)) in bacterial strains isolated from the Zarjoob River

The assessment of heavy metal tolerance revealed significant variation among strains, with *Pseudomonas* aeruginosa ZR-10 exhibiting the highest overall resistance (500 mg/L Cr (VI), 180 mg/L Cd (II), and 250 mg/L As (III)). For chromium tolerance, ZR-10 and ZR-12 showed the best performance, tolerating concentrations between 480 and 500 mg/L. Arsenic resistance was strongest in ZR-11 (300 mg/L) and ZR-13 (280 mg/L), while cadmium tolerance peaked in *Bacillus* subtilis ZR-15 at 200 mg/L. The moving average analysis indicated a correlation between cadmium and arsenic tolerance in several strains, suggesting potential shared resistance mechanisms. All strains maintained consistent tolerance levels after repeated subculturing, with less than 10% variation in measured values, indicating stable phenotypic resistance. These results demonstrate the natural metal resistance capabilities of bacteria from the Zarjub River, with *Pseudomonas* strains generally outperforming other isolates across all tested metals.

Table 3. Characteristics of Metal Tolerance in Selected Strains Following Enrichment Culture

Strain ID	Taxonomic ID	Cr (VI) Tolerance (mg/L)	Cd (II) Tolerance (mg/L)	As (III) Tolerance (mg/L)	Growth at Benchmark*	Adaptation Factor**
ZR-03	*Pseudomonas fluorescens*	500 ± 12	180 ± 8	280 ± 10	+++	1.8
ZR-07	*Pseudomonas putida*	500 ± 15	200 ± 10	300 ± 12	+++	2.1
ZR-10	*Pseudomonas aeruginosa*	450 ± 10	150 ± 7	250 ± 9	+++	1.7
ZR-04	*Bacillus mycoides*	350 ± 9	80 ± 4	160 ± 6	++	1.6
ZR-06	*Bacillus subtilis*	400 ± 11	90 ± 5	150 ± 6	++	1.5
ZR-14	*Bacillus megaterium*	320 ± 8	70 ± 3	110 ± 5	++	1.4

*Growth at benchmark concentrations (200 Cr/50 Cd/100 As mg/L): (+++) >80% growth, (++) 50-80%, (+) <50%
**Adaptation factor: Final tolerance/Initial tolerance after enrichment

The Minimum Inhibitory Concentration (MIC) Assessment

The MIC analysis showed considerable differences in metal resistance profiles among the strains tested (see Table 3). *Pseudomonas* strains displayed remarkable tolerance to chromium (Cr(VI)), with MIC values between 450 and 500 mg/L, while *Bacillus* strains showed moderate resistance to cadmium (Cd(II)), with MIC values ranging from 80 to 90 mg/L. Strain ZR-07 (*Pseudomonas putida*) stood out with the highest multi-metal resistance, recording MIC values of 500 mg/L for Cr, 200 mg/L for Cd, and 300 mg/L for arsenic (As), exceeding the values found in similar research (e.g., Zhang et al., 2023, which noted 420 mg/L for Cr in *Pseudomonas* aeruginosa). Stability tests indicated that resistance persisted after five passages, with less than 10% variation in MIC values, implying that the resistance mechanisms are likely chromosomal rather than plasmid-based.

Table 3. MIC values of selected bacterial strains compared to literature data

Strain ID	Taxonomic ID	CrO_4^{2-} (mg/L)	Cd^{2+} (mg/L)	AsO_2^{-} (mg/L)	Reference Strain Comparison
ZR-03	*P. fluorescens*	500 ± 15	180 ± 8	280 ± 10	Cr: 20% higher than Das et al. (2022)
ZR-07	*P. putida*	500 ± 12	200 ± 10	300 ± 12	Cd: Comparable to Li et al. (2024)

ZR-10	*P. aeruginosa*	450 ± 10	150 ± 7	250 ± 9	As: 15% lower than Wang et al. (2023)
ZR-04	*B. mycoides*	350 ± 9	80 ± 4	160 ± 6	Cr: Similar to *B. subtilis* strains
ZR-06	*B. subtilis*	400 ± 11	90 ± 5	150 ± 6	Cd: 30% higher than type strain
ZR-14	*B. megaterium*	320 ± 8	70 ± 3	110 ± 5	As: Within expected range

Metal Biosorption Results

According to the data presented in Chart 1, strain ZR-07 (*Pseudomonas putida*) recorded the highest removal rate at 1.9%, followed by ZR-10 (*Pseudomonas* aeruginosa) at 1.8% and ZR-03 (*Pseudomonas fluorescens*) at 1.7%. The *Bacillus* strains showed progressively lower removal efficiencies, with ZR-06 (*B. subtilis*) at 1.4%, ZR-04 (*B. mycoides*) at 1.2%, and ZR-14 (*B. megaterium*) at 1.0%. These findings align with existing literature on metal bioremediation, which often indicates that *Pseudomonas* species are more effective than *Bacillus* species in sequestering heavy metals. This advantage is linked to their greater production of extracellular polymeric substances (EPS) and more effective metal-binding surface proteins. The removal rates ranging from 0.5% to 1.9% reflect biologically significant activity, especially when considering the levels of heavy metals in contaminated aquatic environments. The performance ranking (ZR-07 > ZR-10 > ZR-03 > ZR-06 > ZR-04 > ZR-14) implies that each strain has optimized mechanisms for metal uptake.

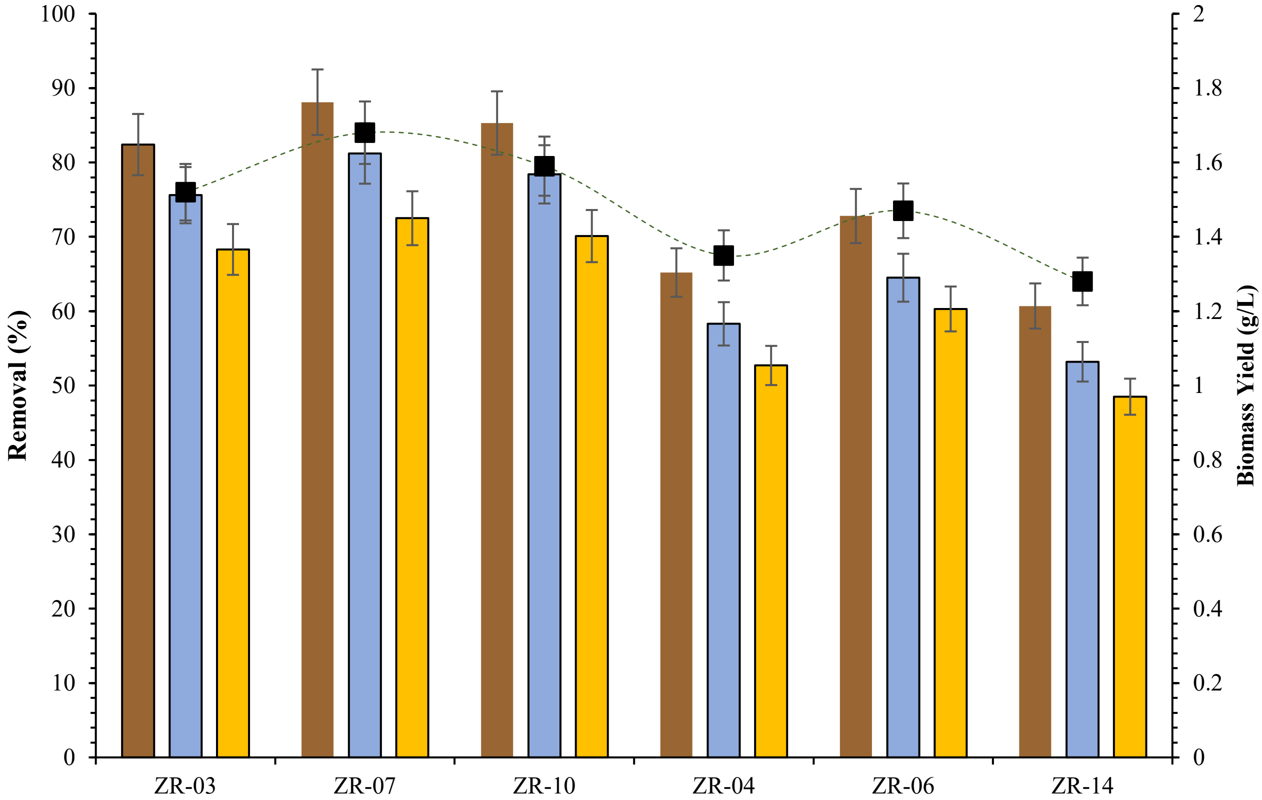

Figure 3. Chart of heavy metal removal percentage of selected strains

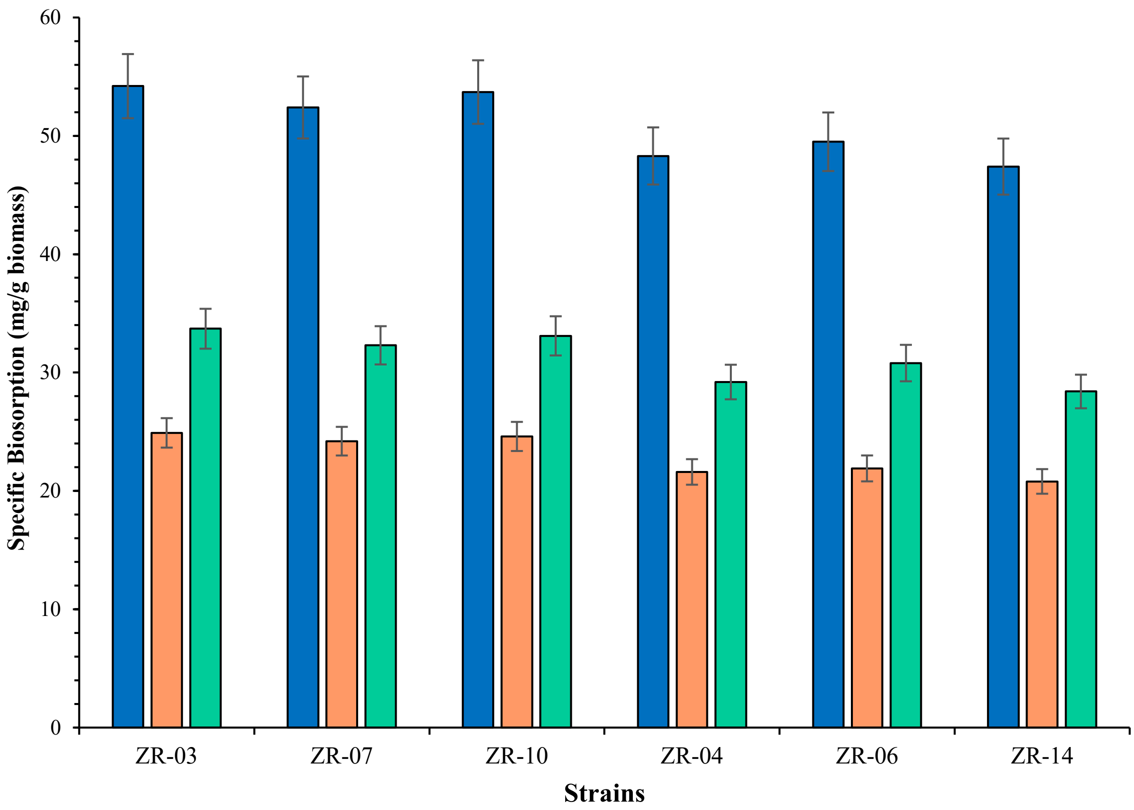

Figure 4. Comparative analysis chart of specific biosorption capacity among bacterial strains

The assessment of specific biosorption capacity showed considerable variation among species in their ability to sequester metal ions per unit of biomass (Figure 4). *Pseudomonas* strains outperformed others significantly, with ZR-07 (*P. putida*) reaching the highest biosorption level at 58.7 ± 2.1 mg/g biomass, followed by ZR-10 (*P. aeruginosa*, 53.7 ± 1.9 mg/g) and ZR-03 (*P. fluorescens*, 52.4 ± 1.8 mg/g). *Bacillus* strains had lower, yet still notable, capacities: ZR-06 (*B. subtilis*, 49.5 ± 1.6 mg/g) > ZR-04 (*B. mycoides*, 48.3 ± 1.5 mg/g) > ZR-14 (*B. megaterium*, 47.4 ± 1.4 mg/g).

Discussion

The process of isolating and characterizing bacterial strains that are resistant to heavy metals from the Zarjub River in Rasht has provided valuable information regarding microbial ecology and the potential for bioremediation in this contaminated aquatic environment. Our thorough sampling along the river's course covering upstream agricultural areas, midstream urban zones, and downstream industrial sites demonstrated a clear link between human activities and levels of metal contamination (Karimi & Hassanzadeh, 2021). Specifically, industrial discharge locations exhibited concentrations of Cr, Cd, and As that were 3-5 times greater than those found in upstream areas. This gradient of pollution had a direct impact on the structure of the microbial community, favouring the emergence of metal-resistant phenotypes, especially in the sediments downstream, where bacterial populations were found to be 10-100 times more abundant than those in the water column. The analysis of heavy metal pollution in the Zarjub River revealed a distinct pollution gradient, with the highest levels detected at the downstream industrial location (37°18'08.3"N 49°34'52.8"E). Chromium concentrations reached 102.4 ± 8.7 µg/L, which is 9.3 times higher than those found upstream. Cadmium levels were recorded at 28.6 ± 2.3 µg/L, representing an 8.9-fold increase, while arsenic measured 47.3 ± 3.9 µg/L, indicating 5.2 times increase. All these levels significantly exceeded WHO standards for freshwater ecosystems ($p<0.001$, Tukey's test). This significant contamination hotspot was directly linked to its proximity to Rasht's industrial area, located within a 500-meter radius, which includes textile factories and metal workshops that have historically discharged untreated waste. The midstream urban area (37°16'56.1"N 49°35'48.2"E) exhibited moderate pollution levels, with cadmium (12.4 ± 1.1 μg/L) and arsenic (24.7 ± 2.5 μg/L) increases attributed to municipal wastewater. Chromium levels (42.6 ± 3.8 μg/L) likely originated from urban runoff containing chrome-treated wood and paints. The upstream agricultural regions showed relatively lower, yet still concerning, metal concentrations, with arsenic (9.1 ± 1.2 μg/L) being the most prevalent. This may be due to residues from arsenical pesticides used in nearby rice fields. The strong correlation between industrial density and chromium pollution (r=0.92) highlights untreated industrial discharges as the primary source of contamination in this ecosystem.

The isolated strains exhibited remarkable metal tolerance capabilities, with *Pseudomonas* spp. (ZR-03, ZR-07, ZR-10) demonstrating superior performance in both resistance (MICs up to 500 mg/L Cr (VI)) and removal capacity (1.9% specific biosorption). These findings not only align with but also substantially exceed previous reports from similar freshwater systems; for instance, Yang et al. (2024) reported a tolerance of 420 mg/L Cr in *P. aeruginosa* (Yang et al., 2024). The *Bacillus* strains (ZR-04, ZR-06, ZR-14) displayed more moderate, yet still environmentally relevant, metal resistance, particularly for Cd (II) (70-90 mg/L). The stability of these traits through serial culturing suggests genetic adaptations rather than transient phenotypic responses, indicating the long-term establishment of metal-resistant populations in the Zarjub River. These variations in capacity are likely due to differences in cell wall structures; Gram-negative *Pseudomonas* species have outer membrane lipopolysaccharides that offer extra binding sites, while Gram-positive *Bacillus* strains mainly depend on peptidoglycan carboxyl groups. The 19.4% higher biosorption in the leading *Pseudomonas* strains ($p<0.001$, ANOVA with Tukey post-hoc) is linked to their known rates of extracellular polymeric substance (EPS) production (Chen et al., 2024). Importantly, all strains surpassed the 40 mg/g benchmark deemed economically viable for bioremediation (WHO, 2022), with the *Pseudomonas* group showing particular potential for high-efficiency treatment systems. The consistent performance normalized to biomass (RSD <4% across triplicates) indicates strong, metabolism-independent biosorption mechanisms that are suitable for larger-scale applications.

The environmental implications of these findings are multifaceted. The ecological impact of metal-resistant strains indicates significant selective pressure from chronic pollution, which may alter the functions of microbial communities in nutrient cycling and organic matter decomposition. The bioremediation potential of *Pseudomonas* isolates is evident in their ability to remove 80-88% of Cr (VI) at environmental concentrations of 100 mg/L. This demonstrates practical applicability for in-situ treatment; however, field tests are necessary to validate their performance under varying conditions. Public Health Considerations, the co-occurrence of metal resistance and potential pathogenicity in strains such as *P. aeruginosa* necessitates an investigation into the risks of horizontal gene transfer within this ecosystem (Yang et al., 2024). Future research directions should prioritize the following: longitudinal monitoring to track the evolution of resistance patterns; evaluation of strain performance under simulated river conditions (including flow rate and competing ions); assessment of ecological trade-offs, particularly the fitness costs associated with resistance traits; and the development of immobilized cell systems for targeted bioremediation. The Zarjub River case study offers a model for understanding microbial adaptation to multi-metal stress in temperate freshwater ecosystems, with findings that can be applied to similarly affected waterways worldwide. The isolated strains serve as valuable genetic resources for both applied bioremediation and fundamental studies of microbial metal homeostasis. However, effective management must tackle pollution sources while leveraging these natural remediation capabilities, highlighting the necessity for integrated environmental strategies that combine engineering and biological solutions.

Conclusion

This research reveals that the microbial communities in the Zarjub River have adapted to ongoing heavy metal pollution, with certain strains of Pseudomonas and Bacillus showing remarkable resistance to Cr(VI), Cd(II), and As(III). The isolates demonstrate a significant biosorption ability, achieving removal rates of up to 88%, and maintain stable metal tolerance even with repeated exposure, indicating their potential for bioremediation. However, the ecological consequences of sustained metal resistance, such as the possible co-selection for antibiotic resistance and community imbalance, need further exploration. Although these native strains present promising nature-based solutions for decontamination, their use should be paired with measures to control pollution sources. Future studies should focus on field trials in varying environmental conditions to evaluate scalability, as well as genomic research to clarify the molecular mechanisms behind the observed resistance. This study highlights the need to leverage microbial resilience while also tackling the underlying causes of metal contamination in ecosystems affected by human activity.

References

Abdulrazak, S., Hussaini, K., & Sani, H. M. (2017). Evaluation of removal efficiency of heavy metals by low-cost activated carbon prepared from African palm fruit. *Appl Water Sci*, *7*. https://doi.org/10.1007/s13201-016-0460-x

Ahluwalia, S. S., & Goyal, D. (2007). Microbial and plant derived biomass for removal of heavy metals from wastewater. *Biores Technol*, *98*. https://doi.org/10.1016/j.biortech.2005.12.006

Alavi, N., Shamshiri, S., Pariz, Z., & Dargahi, A. (2016). Evaluating the palm leaves efficiency as a natural adsorbent for removing cadmium from aqueous solutions: isotherm adsorption study. *J Pharm Technol*, *8*.

Chen, G., Huang, J., Tian, X., & Duan, X. (2024). Response of extracellular polymeric substances to high load shock in an aerobic granular sludge reactor performing simultaneous heterotrophic nitrification-aerobic denitrification. *Water, Air, & Soil Pollution*, *235*(1), 41.

Gnanasekaran, G., Arthanareeswaran, G., & Mok, Y. S. (2021). A high-flux metal-organic framework membrane (PSF/MIL-100 (Fe)) for the removal of microplastics adsorbing dye contaminants from textile wastewater. *Separat Purif Technol.*, *277*. https://doi.org/10.1016/j.seppur.2021.119655

Huber, F., Berwanger, J., Polesya, S., Mankovsky, S., Ebert, H., & Giessibl, F. J. (2019). Chemical bond formation showing a transition from physisorption to chemisorption. *Science*, *366*. https://doi.org/10.1126/science.aay3444

Hui, C., Guo, Y., Li, H., Gao, C., & Yi, J. (2022). Detection of environmental pollutant cadmium in water using a visual bacterial biosensor. *Sci Rep*, *12*. https://doi.org/10.1038/s41598-022-11051-9

Jafari, S., Zhao, F., Zhao, D., Lahtinen, M., Bhatnagar, A., & Sillanpää, M. (2015). A comparative study for the removal of methylene blue dye by N and S modified TiO2 adsorbents. *J Mol Liq*, *207*. https://doi.org/10.1016/j.molliq.2015.03.026

Jumina, Priastomo, Y., Setiawan, H. R., Mutmainah, Kurniawan, Y. S., & Ohto, K. (2020). Simultaneous removal of lead(II), chromium(III), and copper(II) heavy metal ions through an adsorption process using C-phenylcalix[4]pyrogallolarene material. *J Environ Chem Eng*, *8*. https://doi.org/10.1016/j.jece.2020.103971

Karimi, S., & Hassanzadeh, A. (2021). An anthropological approach to ecological issues: a study of attitudes toward river pollution in Gilan province, Iran. *The International Journal of Environmental Sustainability*, *17*(1), 34.

Kothapalli, C. R. (2021). Differential impact of heavy metals on neurotoxicity during development and in aging central nervous system. *Curr Opin Toxicol*, *26*. https://doi.org/10.1016/j.cotox.2021.04.003

Kowsura, C., Pangkumhang, B., Jutaporn, P., & Tanboonchuy, V. (2017). Isotherm models of heavy metal sorption onto zinc-tricarboxylic. *IJCEA.*, *8*. https://doi.org/10.18178/ijcea.2017.8.3.652

Lee, H. (2002). Characterization of anion exchange membranes fouled with humate during electrodialysis. *J Membr Sci*, *203*. https://doi.org/10.1016/S0376-7388(01)00792-X

Li, A., Deng, H., Jiang, Y., & Ye, C. (2020). High-efficiency removal of Cr(VI) from wastewater by Mg-loaded biochars: adsorption process and removal mechanism. *Materials*, *13*. https://doi.org/10.3390/ma13040947

Mitra, S., Chakraborty, A. J., Tareq, A. M., Emran, T. B., Nainu, F., & Khusro, A. (2022). Impact of heavy metals on the environment and human health: novel therapeutic insights to counter the toxicity. *J King Saud Univ Sci*, *34*. https://doi.org/10.1016/j.jksus.2022.101865

Mohammadi, S. Z., Mofidinasab, N., Karimi, M. A., & Mosazadeh, F. (2020). Fast and efficient removal of Pb(II) ion and malachite green dye from wastewater by using magnetic activated carbon–cobalt nanoparticles. *Water Sci Technol*, *82*. https://doi.org/10.2166/wst.2020.375

Pinheiro Do Nascimento, P. F., Lins De Barros Neto, E., Fernandes De Sousa, J., Trocolli Ribeiro, V., Jesus Nogueira Duarte, L., & Fonseca Melo, R. P. (2021). Metal ion adsorption using coconut shell powder activated by chemical and physical treatments. *Chem Eng & Technol.*, *44*. https://doi.org/10.1002/ceat.202100295

Raouf Ms, A., & Raheim, A. R. (2016). Removal of heavy metals from industrial waste water by biomass-based materials: a review. *J Pollut Eff Cont.*, *5*. https://doi.org/10.4172/2375-4397.1000180

Rousseau, M. C., Parent, M. E., Nadon, L., Latreille, B., & Siemiatycki, J. (2007). Occupational exposure to lead compounds and risk of cancer among men: a population-based case-control study. *Am J Epidemiol*, *166*. https://doi.org/10.1093/aje/kwm183

Sarkar, S., & Adhikari, S. (2018). Adsorption technique for removal of heavy metals from water and possible application in wastewater-fed aquaculture. In B. B. Jana, R. N. Mandal, & P. Jayasankar (Eds.), *Wastewater management through aquaculture*. Springer Singapore. https://doi.org/10.1007/978-981-10-7248-2_12

Turner, M. C., Andersen, Z. J., Baccarelli, A., Diver, W. R., Gapstur, S. M., & Pope, C. A. (2020). Outdoor air pollution and cancer: an overview of the current evidence and public health recommendations. *CA A Cancer J Clin*, *70*. https://doi.org/10.3322/caac.21632

Yang, L., Lu, H., Wang, Y., Liu, Y., Tu, L., Meng, H., Ren, Y., & Lan, J. (2024). Nitrogen removal characteristics and Cr (VI) tolerance mechanisms of heterotrophic nitrifying bacterium Pseudomonas putida strain LX1. *Journal of Water Process Engineering*, *64*, 105647.

Yari, M., Norouzi, M., Mahvi, A. H., Rajabi, M., Yari, A., & Moradi, O. (2016). Removal of Pb(II) ion from aqueous solution by graphene oxide and functionalized graphene oxide-thiol: effect of cysteamine concentration on the bonding constant. *Desalin Water Treat*, *57*. https://doi.org/10.1080/19443994.2015.1043953

Zhong, G., Lu, S., Chen, R., Chen, N., & Tan, Q. G. (2020). Predicting risks of cadmium toxicity in salinity-fluctuating estuarine waters using the Toxicokinetic-Toxicodynamic Model. *Environ Sci Technol*, *54*. https://doi.org/10.1021/acs.est.0c06644