Bioremediation of Multi-Heavy Metal Contaminated Lake Sediments Using Indigenous Bacterial Isolates and Consortium

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Abstract
Heavy metal contamination of freshwater sediments poses a serious threat to aquatic ecosystems and public health due to the persistence, toxicity and bioaccumulative nature of metals. The present study investigated the isolation, identification and bioremediation potential of indigenous heavy metal tolerant bacteria from the sediments of Koradi Lake, Maharashtra, India. Sediment samples were serially diluted and cultured using standard microbiological techniques to enumerate and isolate bacterial populations. Pure cultures were characterized based on morphological, biochemical and molecular analyses. Identification was confirmed through 16S rRNA gene sequencing, followed by phylogenetic analysis using the neighbour-joining method. Four dominant bacterial isolates were identified as *Bacillus subtilis*, *Enterobacter aerogenes, Escherichia coli* and *Streptococcus pneumoniae*. The heavy metal tolerance and remediation efficiency of individual isolates and an optimized bacterial consortium were evaluated against selected metals, including Ba, Fe, Cu, Pb, Cd, Cr, and Zn, under controlled experimental conditions. Comparative analyses were conducted using untreated and sterile sediment controls to assess the role of microbial activity in metal reduction.
Results demonstrated minimal heavy metal reduction in sterile sediments (0.5-3%), whereas non-sterile controls showed slightly higher removal (2.9-6.8%), confirming the contribution of indigenous microbial activity. Individual bacterial strains exhibited moderate remediation efficiencies, with metal reduction ranging from 12% to 38%, depending on the metal and bacterial species. In contrast, the bacterial consortium achieved the highest remediation performance, with removal efficiencies ranging from 29% to 59% after four weeks of incubation. Among the tested metals, lead (Pb), copper (Cu), and cadmium (Cd) showed the greatest reduction in the consortium-treated sediments. This study demonstrates that indigenous bacterial consortia represent a cost-effective, eco-friendly, and sustainable strategy for the bioremediation of heavy metal polluted freshwater sediments and offers valuable insights for the development of in situ remediation approaches in contaminated aquatic environments.

Keywords: Heavy metals, bioremediation, lake sediment, bacterial consortium, 16S rRNA sequencing

Introduction

Aquatic ecosystems are among the most vulnerable natural resources due to their direct exposure to anthropogenic activities. Rapid industrialization, urban expansion, agricultural intensification, and population growth have significantly increased the discharge of untreated or partially treated wastes into freshwater bodies. Among various pollutants, heavy metals represent a major class of environmental contaminants because of their non-biodegradable nature, persistence, toxicity and tendency to bioaccumulate and bio magnify through food chains (Alloway, 2013; Tchounwou et al., 2012). Lakes, rivers, and reservoirs often act as sinks for heavy metals, where these pollutants accumulate in sediments and pose long-term ecological and public health risks.
Lake sediments play a crucial role in regulating the fate and transport of heavy metals in aquatic environments. Sediments not only serve as repositories but also act as secondary sources of contamination under changing physicochemical conditions such as pH, redox potential, and microbial activity (Förstner & Wittmann, 2012). Heavy metals such as cadmium (Cd), lead (Pb), chromium (Cr), copper (Cu), zinc (Zn), iron (Fe) and barium (Ba) commonly originate from industrial effluents, mining operations, thermal power plants, urban runoff, and domestic sewage. Once introduced into aquatic systems, these metals persist for decades and exert chronic toxic effects on benthic organisms, fish, plants and microorganisms (Ali et al., 2019).
In India, freshwater lakes located near industrial and urban zones are increasingly threatened by heavy metal pollution. Thermal power plants, metal processing industries and municipal discharges significantly contribute to metal loading in sediments and water columns. Koradi Lake, situated near Nagpur, Maharashtra, is one such water body that receives industrial and anthropogenic inputs. Continuous deposition of contaminated particulates has resulted in elevated concentrations of heavy metals in lake sediments, raising concerns about ecosystem health and potential human exposure through the food web. Understanding the distribution, persistence and biological transformation of heavy metals in such sediments is therefore of paramount importance.
Heavy metals exert toxic effects on living organisms even at low concentrations. Unlike organic pollutants, metals cannot be degraded into harmless end products; instead, they persist and interact with cellular components, disrupting enzymatic activities, damaging DNA, and inducing oxidative stress (Jaishankar et al., 2014). In aquatic environments, heavy metals adversely affect microbial diversity, alter nutrient cycling, reduce primary

productivity, and impair ecosystem functioning. Prolonged exposure can lead to reduced biodiversity and the dominance of metal-tolerant species, thereby altering microbial community structure and ecological balance.

Conventional methods for heavy metal remediation include physical and chemical techniques such as dredging, excavation, chemical precipitation, ion exchange, membrane filtration, and solidification/stabilization. While these methods can be effective, they are often expensive, energy-intensive, environmentally disruptive, and generate secondary pollution in the form of toxic sludge (Fu & Wang, 2011). Additionally, such approaches are not always feasible for large-scale or in situ remediation of contaminated sediments. These limitations have driven increasing interest in bioremediation, an eco-friendly and cost-effective alternative for the management of heavy metal pollution.

Bioremediation exploits the natural metabolic capabilities of microorganisms and plants to detoxify, transform, immobilize, or remove pollutants from contaminated environments. Among biological agents, microorganisms particularly bacteria are widely recognized as effective tools for heavy metal remediation due to their rapid growth, metabolic versatility, adaptability to extreme conditions, and diverse resistance mechanisms (Gadd, 2010). Bacteria can interact with metals through various processes such as biosorption, bioaccumulation, biotransformation, precipitation, and redox reactions, thereby reducing metal bioavailability and toxicity.

Sediment-associated bacteria are especially important in bioremediation because they are naturally adapted to polluted environments and often develop resistance to high concentrations of heavy metals. Long-term exposure to metals exerts selective pressure, leading to the enrichment of metal-tolerant and metal-resistant microbial populations (Nies, 2003). These bacteria possess genetic and physiological mechanisms such as efflux pumps, metal-binding proteins, enzymatic detoxification systems, and extracellular polymeric substances (EPS) that enable them to survive and function in contaminated habitats.

Several bacterial genera, including Bacillus, Enterobacter, Escherichia, and Streptococcus, have been reported to exhibit significant tolerance and remediation potential for various heavy metals (Volesky, 2007; Wang & Chen, 2009). Members of the genus *Bacillus* are particularly attractive for bioremediation due to their ability to form endospores, produce metal-binding cell wall components, and survive under harsh environmental conditions. Similarly, *Enterobacter* and *Escherichia* species are known for their efficient metal uptake, reduction, and intracellular sequestration mechanisms. Although *Streptococcus* species are less frequently studied in environmental remediation, emerging evidence suggests their potential role in metal tolerance and transformation.

Microbial remediation efficiency can be influenced by environmental factors such as pH, temperature, nutrient availability, metal concentration, and the presence of competing ions. Moreover, individual bacterial strains may show selective affinity toward specific metals, limiting their overall remediation efficiency. To overcome this limitation, bacterial consortia or blends are increasingly employed. Mixed microbial communities often demonstrate synergistic interactions, broader metabolic capabilities, and enhanced pollutant removal efficiency compared to single strains (Singh & Ward, 2004). Such consortia better mimic natural ecosystems and offer improved stability and resilience under fluctuating environmental conditions.

Accurate identification and characterization of metal-tolerant bacteria are essential for understanding their remediation potential. Traditional phenotypic methods, including colony morphology, Gram staining, and biochemical tests, provide preliminary identification; however, these approaches may lack precision. Molecular techniques, particularly 16S rRNA gene sequencing, have become the gold standard for bacterial identification due to their high specificity, reliability, and phylogenetic relevance (Woese et al., 1990). Combining biochemical and molecular approaches enables robust identification and functional assessment of environmental isolates.

In sediment ecosystems, microbial processes play a central role in controlling metal mobility and bioavailability. Microorganisms can mediate metal speciation through oxidation–reduction reactions, thereby influencing metal solubility and toxicity. For instance, bacterial reduction of hexavalent chromium [Cr(VI)] to trivalent chromium [Cr(III)] significantly reduces toxicity and mobility. Similarly, microbial interactions with iron and manganese oxides can indirectly affect the behavior of other associated heavy metals (Lovley, 1991). Despite growing recognition of microbial bioremediation, site-specific studies are essential because microbial diversity and remediation potential vary depending on local environmental conditions and pollution history. Indigenous microorganisms isolated from contaminated sites are often more effective than introduced strains because they are already adapted to prevailing conditions (Vidali, 2001). Therefore, investigating native bacterial communities in contaminated lake sediments is a critical step toward developing effective and sustainable remediation strategies.

The present study focuses on the isolation, identification, and characterization of heavy metal-tolerant bacteria from Koradi Lake sediments and evaluates their potential for bioremediation. By employing standard microbiological techniques, biochemical characterization, and 16S rRNA gene sequencing, dominant bacterial isolates were identified. The ability of individual bacterial strains and a bacterial consortium to remediate heavy metals such as Ba, Fe, Cu, Pb, Cd, Cr, and Zn was systematically assessed under controlled conditions. The study also compares remediation efficiency between sterile and non-sterile sediments, highlighting the crucial role of microbial activity in metal removal.

Materials And Methods

Sampling methods for Microbial analysis

The samples for microbiological examination were collected first to avoid the danger of contamination of the sampling point. The changes that occur in the bacterial content of water on storage were reduced to a minimum by ensuring that samples were not exposed to light. They were kept cool, preferable between 4°C and 10°C but not frozen. After sampling, analysis was taken up as soon as possible.

Isolation of Bacteria
From the collected sediment sample, 1 mL was taken and it is serially diluted with 100ml sterile distilled water. The serial dilution was done up to$10^{-9.}$ From each dilution, 100 µL of sample was spread on Plate count agar. The plates were then incubated at 37ºC for 24 to 48 hours for the bacterial growth. All the procedures were carried out in sterile conditions. Colonies developed on the plate were counted and expressed as cfu/g. The isolates were purified and stored on the agar slants.

Determination of the Coliform Bacteria
Total coliform bacteria were determined by the standard multiple tube fermentation technique. Coliforms were detected by inoculation of samples into tubes of lauryl tryptose broth. The three-tube procedure using this broth was used to detect the coliform and determine the most probable number (MPN) of coliform bacilli. 10 ml, 1 ml and 0.1 ml of lake sediment samples were inoculated into tubes with 10 ml of lauryl tryptose broth and incubated at 35 ± 0.5°C. The productions of gas formation after 24 hours (a bubble filling the concavity of Durham's tube) were considered presumptive positive growths of coliforms. Cultures showing no production of gas in 48 hours were considered negative. Confirmed test done by transferring a loopful of culture from a positive tube from the presumptive test into a tube of brilliant green lactose bile broth with Durham's tubes. The tubes were incubated at 37°C for 24-48 hours for total coliforms observed for gas production. Completed test was carried out by streaking a loopful of broth from a positive tube into eosin methylene blue (EMB) agar plate for pure colonies. The plates were incubated at 37°C for 24-48 hours. The most probable number (MPN) per 100 ml of sample was determined using the completed test. The MPN table according to the quantity of positive fermentation tubes and calculate the most probable number of the quantity of the total coliforms in a given water sample.

Bacterial identification
Biochemical analysis of bacteria
The morphological characteristics of the isolates were identified by gram stain and biochemical reactions. The biochemical reactions include glucose fermentation, Indole, Methyl Red (MR), Voges-Proskauer (VP), Nitrate, Citrate, Urease, Triple sugar Iron (TSI), Catalase, Glucose, Fructose, Maltose, Gelatin and Starch were performed. Bacteria were isolated, identified and named based on morphological, physiological and biochemical characteristics presented in Bergey's Manual of Determinative Bacteriology and the APi Kit profiles.

DNA isolation from bacteria
2 ml overnight isolated culture is taken and the cells are harvested by centrifugation for 10 minutes. Remove the supernatant and 100 µl of TE buffer is added to the cell pellet and the cells are resuspended in the buffer by gentle mixing. 100 µl of 10% SDS and 5 µl of Proteinase K are added to the cells. The above mixture is mixed well and incubated at 37ºC for an hour in an incubator. 1 ml of phenol-chloroform mixture is added to the contents, mixed well by inverting and incubated at room temperature for 5 minutes. The contents are centrifuged at 10,000 rpm for 10 minutes at 4ºC. The highly viscous jelly like supernatant is collected using cut tips and is transferred to a fresh tube. The process is repeated once again with phenol-chloroform mixture and the supernatant is collected in a fresh tube. 100 µl of 5 M sodium acetate is added to the contents and is mixed gently. 2 ml of isopropanol is added and mixed gently by inversion till white strands of DNA precipitates out. The contents are centrifuged at 5,000 rpm for 10 minutes. The supernatant is removed and 1 ml 70% ethanol is added. The above contents are centrifuged at 5,000 rpm for 10 minutes. After air drying for 5 minutes 200 µl of TE buffer or distilled water is added. 10 µl of DNA sample is taken and is diluted to 1 or 2 ml with distilled water. The concentration of DNA is determined using a spectrophotometer at 260/280 nm. The remaining samples are stored for further experiments.

Polymerase chain reaction (PCR)
PCR is a sensitive technique which is used for amplification of desire gene sequence. 16S rDNA sequence are conserve region in bacteria from evolution so it is used for identification of bacterial strains. The isolated genomic DNA was amplified with 16S rDNA primer. The master mix was prepared by mixing template DNA (50 nanograms) with the polymerase reaction buffer, dNTPs (100 µM), Reverse and Forward primers (100 ng each), 1.0 U TaqDNA polymerase and DW.

Agarose gel electrophoresis
1% (w/v) agarose powder in tris-acetate EDTA buffer (1X TAE) and melt on hot plate to dissolve the agarose. Cool the solution to 45ºC-50ºC and 1% EtBr (0.5 ug / ml) was added. Mixed the solution properly. Before pouring the solution into gel casting tray washed the tray and comb with DW. Comb was set in the casting tray and poured the agarose gel and leaved for solidify. Casting apparatus was dipped into 1X TAE buffer and carefully removed the comb. Connected the electrode with power pack and current was checked. The DNA sample was mixed with bromophenol blue dye and carefully loaded the sample into the wells. A ladder DNA of standard molecular weight was loaded in a separate well along with the samples. It will help in assessing the size of the PCR product. The DNA bands were observed under the UV transilluminator.

Sequencing
The purified PCR products were subjected to paired end sequencing with universal primers 785F and 907R using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems).

Result And Discussion

Microorganisms and plants have the potential to remove heavy metals from wastewater and contaminated soils. Microorganisms' removal of heavy metals from soil and sediment has proven effective in bioremediation studies. Bioremediation is based on the natural ability of certain microorganisms, such as bacteria, to degrade pollutants. Microorganisms must receive the right amounts of essential nutrients and chemicals for them to be able to detoxify the contamination. Microbial enzymes can utilize pollutants as substrates, making them suitable agent for this process. Bacteria are among the most widely utilized microorganisms in bioremediation processes.

In the present study, the abundance and distribution of bacteria in Koradi lake sediment was investigated. The bacteria were isolated from all the lakes sediment sample by serial diluting the sample and plating them on the Nutrient Agar plate. The plates were then incubated it at 37°C for 24-48 hrs. The colony appeared on the plate was closely monitored for recording their morphological diversity such as size, shape, form, edge, optical characteristic, colour and texture. The distinct colonies from the plate were picked up and plated on a separate plate for further purification. All the purified bacterial cultures were maintained on Nutrient Agar slant for further study. Pure cultures were identified based on microscopic observation, the colour of cell suspension, pigments present, colony morphology, and 16S rRNA gene sequence analysis. From this stock, only 4 isolates showed viability after storage and these 4 isolates were identified up to generic level.

Table 1 Isolation of bacteria from Koradi lake sediment

Dilution	CFU Value (cfu/ml)
10-3	TNTC
10-5	TNTC
10-7	237 × 108
10-9	54 × 1010

*TNTC- Too Numerous to Count

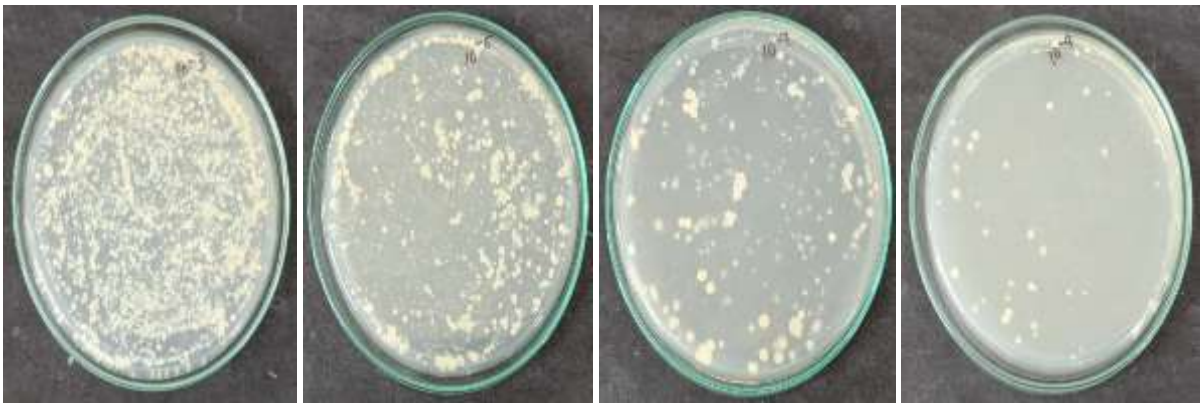

Figure I Cultured serial dilution with spread plate technique of the test sample

Table 2 Biochemical characteristics of bacterial isolates

Biochemical Test		BAC1 (*Enterobacter*)	BAC 2 (*Streptococcus)*	BAC3 *Escherichia*	BAC4 *Bacillus*
Indole		–	–	+	–
Methyl Red		-	+	+	+
VP		+	–	–	+
Nitrate		+	–	+	+
Citrate		–	–	–	V
Urease		–	–	–	–
TSI		V	–	–	–
Carbohydrate Fermentation	**Glucose**	+	+	+	+
	Sucrose	+	+	–	+
	Maltose	+	+	+	+

Figure II Biochemical characteristics of bacterial isolates from Bac1

Figure III Biochemical characteristics of bacterial isolates from Bac 2

Figure IV Biochemical characteristics of bacterial isolates from Bac 3

Figure V Biochemical characteristics of bacterial isolates from Bac 4

The biochemical characteristics of these 4 bacterial isolates were used to tentatively classify the isolates. for Indole, Methyl red, Voges Proskauer's, nitrate, Citrate Utilization, urease, TSI, carbohydrates (Glucose, Sucrose and maltose). The isolated bacterial strains were screened for 10 biochemical test and the results were positive and negative summarized in (Table 1,2,3). In carbohydrate utilization test all the isolates were able to utilize Glucose, maltose, and sucrose while one of the isolates was able to utilize sucrose. Based on biochemical characteristic, isolated bacteria from koradi lake consisted of members of the family as *Bacillus*, *Enterobacter*, *Escherichia* and *Streptococcus*.

The degradation of heavy metals in non-sterile soil ranged from 2.9% to 6.8%**,** indicating the natural activity of indigenous soil microorganisms. In contrast, sterile soil showed a much lower reduction rate of only 0.5% to 3%**,** confirming that microbial activity plays a major role in heavy metal removal. When evaluated individually, each bacterial strain demonstrated a higher capacity for heavy metal reduction compared to the controls. *Bacillus subtilis* achieved a removal rate of 20% to 35%, while *Enterobacter aerogenes*. showed a reduction of 20% to 38%**.** *Escherichia coli* exhibited a degradation range of 20% to 36%**,** and *Streptococcus pneumoniae* showed reductions between 12% and 38%**.** Whereas the optimized bacterial blend produced the highest removal efficiency, with heavy metal reductions ranging from 29% to 59%**.** Overall, he results indicated that the bacterial consortium was significantly more effective than individual isolates or the controls. The blend facilitated significant decrease by bacterial blend were observed in the soil sediment and degrade the heavy metal Ba, Fe, Cu, Pb, Cd, Cr, and Zn after 4weeks of experimental trials.

Table 3 Percentage Reduction of heavy metals

Metal	**Untreated control**	**Sterile control**	***Bacillus subtilis***	***Enterobacter aerogenes***	***Escherichia coli***	***Streptococcus pneumoniae***	**bacterial blend**
Ba	5.7	2	28	22	20	32	50
Fe	2.9	0.5	25	22	30	33	32
Cu	6.8	3	20	24	32	38	52
Pb	5.6	3	30	28	35	33	59
Cd	5.9	3	35	38	36	32	48
Cr	4.5	2	25	33	22	25	38
Zn	3.9	1	23	20	18	12	29

Conclusion

The present study provides a comprehensive assessment of the role of indigenous sediment bacteria in the bioremediation of heavy metal contaminated freshwater environments, with specific reference to Koradi Lake sediments. The findings clearly demonstrate that lake sediments harbor diverse and metabolically active bacterial communities capable of tolerating and remediating multiple heavy metals. Through systematic isolation, biochemical characterization, and molecular identification using 16S rRNA gene sequencing, four dominant heavy metal–tolerant bacterial strains Bacillus subtilis, Enterobacter aerogenes, Escherichia coli, and Streptococcus pneumoniae were successfully identified and evaluated for their remediation potential. Comparative analysis between sterile and non-sterile sediment controls confirmed that microbial activity plays a pivotal role in heavy metal removal. Sterile sediments exhibited only negligible reductions (0.5–3%), whereas untreated non-sterile sediments showed slightly higher reductions (2.9–6.8%), reflecting the natural background activity of indigenous microorganisms. These results underscore the importance of biological processes in regulating metal mobility and persistence in sediment ecosystems. When evaluated individually, each bacterial isolate demonstrated a measurable capacity to reduce heavy metal concentrations, with removal efficiencies ranging from 12% to 38%, depending on the metal species and bacterial strain. Among the isolates, *Bacillus subtilis* and *Enterobacter aerogenes* showed comparatively higher remediation efficiencies, which can be attributed to their robust metabolic activity, adaptive resistance mechanisms, and ability to interact with metals through biosorption and bioaccumulation processes. However, the performance of individual strains remained limited by metal specificity and physiological constraints. In contrast, the bacterial consortium exhibited a significantly enhanced bioremediation efficiency, achieving heavy metal reductions ranging from 29% to 59% within four weeks of treatment. The superior performance of the consortium highlights the importance of synergistic interactions among bacterial species, where complementary metabolic pathways and cooperative metal-binding mechanisms collectively improved remediation outcomes. Notably, substantial reductions were observed for highly toxic metals such as lead, cadmium, and copper, indicating the practical relevance of consortium-based approaches for mitigating environmental and health risks associated with heavy metal pollution. The outcomes of this research contribute valuable insights into sediment microbial ecology and environmental biotechnology and provide a scientific basis for developing in situ bioremediation strategies for contaminated freshwater ecosystems. Future studies should focus on optimizing environmental parameters, scaling up field

applications, and elucidating the molecular mechanisms underlying metal–microbe interactions to further enhance remediation efficiency and long-term sustainability.

Acknowledgements
The authors acknowledge the support provided by RTM University for laboratory facilities and research assistance.

DECLARATIONS

Funding: No funds, grants, or other support was received.

Conflict of Interest: The authors declare no conflict of interest.

Ethical Approval: Not applicable.

Consent to Participate: Not applicable.

Consent for Publication: All authors have read and approved the final manuscript for publication.

Availability of Data and Material: All data generated or analyzed during this study are included in this published article.

Code Availability: Not applicable.

References

1. Ali, H., Khan, E., & Ilahi, I. (2019). Environmental chemistry and ecotoxicology of hazardous heavy metals: Environmental persistence, toxicity, and bioaccumulation. *Journal of Chemistry*, 2019, 1–14.
2. Alloway, B. J. (2013). *Heavy metals in soils: Trace metals and metalloids in soils and their bioavailability* (3rd ed.). Springer.
3. Förstner, U., & Wittmann, G. T. W. (2012). *Metal pollution in the aquatic environment*. Springer.
4. Fu, F., & Wang, Q. (2011). Removal of heavy metal ions from wastewaters: A review. *Journal of Environmental Management*, 92(3), 407–418.
5. Gadd, G. M. (2010). Metals, minerals and microbes: Geomicrobiology and bioremediation. *Microbiology*, 156(3), 609–643.
6. Jaishankar, M., Tseten, T., Anbalagan, N., Mathew, B. B., & Beeregowda, K. N. (2014). Toxicity, mechanism and health effects of some heavy metals. *Interdisciplinary Toxicology*, 7(2), 60–72.
7. Lovley, D. R. (1991). Dissimilatory Fe(III) and Mn(IV) reduction. *Microbiological Reviews*, 55(2), 259–287.
8. Nies, D. H. (2003). Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiology Reviews*, 27(2–3), 313–339.
9. Singh, A., & Ward, O. P. (2004). *Applied bioremediation and phytoremediation*. Springer.
10. Tchounwou, P. B., Yedjou, C. G., Patlolla, A. K., & Sutton, D. J. (2012). Heavy metal toxicity and the environment. *EXS*, 101, 133–164.
11. Vidali, M. (2001). Bioremediation: An overview. *Pure and Applied Chemistry*, 73(7), 1163–1172.
12. Volesky, B. (2007). Biosorption and me. *Water Research*, 41(18), 4017–4029.
13. Wang, J., & Chen, C. (2009). Biosorbents for heavy metals removal and their future. *Biotechnology Advances*, 27(2), 195–226.
14. Woese, C. R., Kandler, O., & Wheelis, M. L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences*, 87(12), 4576–4579.