Macedonian Journal of Ecology and Environment
Vol. 26, issue 2
pp. 191 - 199
Skopje (2024)
ISSN 1857 - 8330 (on-line)
ISSN 0354-2491 (print)
Original scientific paper
Available online at www.mjee.org.mk
DOI: https://doi.org/10.59194/MJEE24262191o

Heavy metal stress response in the diazotroph, *Rhizobium petrolearium*

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Abstract

Heavy metals occur at elevated concentrations in the environment mainly due to anthropogenic activities. The response of the hydrocarbon-degrading, diazotroph, *Rhizobium petrolearium*, to varying concentrations of the heavy metals – lead, cadmium and chromium(VI) – was investigated by establishing the effect of loading concentrations of 1 mM to 15 mM on its growth and enzymatic activity with increasing exposure time. The findings revealed a decline in growth and enzymatic activity with increasing heavy metal concentration and increasing exposure period. Mean reductions in abundance of 42.0% – 100.0%, 52.1% – 100.0% and 54.4% – 100.0% for different concentrations of lead, cadmium and chromium(VI) respectively were obtained. For lead and cadmium, no growth (100% decline) was seen by the end of the study at 10mM and 15mM exposure concentrations. There was no growth at the Cr(VI) concentrations of 5mM – 15mM by the end of the study. Statistically significant differences ($p \leq 0.05$) were seen in growth reduction between the different application concentrations. The results inferred the order of toxicity against *R. petrolearium* based on growth response to be chromium(VI) > cadmium > lead. At higher concentrations of 10 mM and 15 mM, all three heavy metals inhibited α-amylase and protease activity but had only minimal impact on oxidase, catalase and cellulase activities. Only Pb and Cr(VI) inhibited protease activity at higher concentrations of 10mM and 15mM. Based on the enzymatic activity inhibition tests, the order of toxicity was Cd > Cr(VI) > Pb. The findings suggest that at high concentrations, the heavy metals, lead, cadmium and chromium(VI) could impact negatively on abundance and interrupt enzymatic activity in soil bacteria like *R. petrolearium*.

Keywords: Bacteria; cadmium; hexavalent chromium; enzyme activity; lead; metallotolerance; toxicity; soil quality

Introduction

Rhizobium is the most well-known genus of the group of microorganisms known as the diazotrophs. These microorganisms are known for their capacity to fix atmospheric nitrogen converting it into plant-available nitrogen in soil and water systems. They often form symbiotic relationships with leguminous plants leading to the formation of root nodules that support nutrient absorption by plants. Diazotrophs contribute to 90% of soil nitrogen, without which, nitrogen, an essential nutrient in any ecosystem, would not be available to other organisms including plants. The bacterium mainly supplies the plant with reduced nitrogen for growth, solubilises phosphates and secretes plant growth hormones like indole acetic acid (Vishnu, 2022; Naik et al. 2023). This process forms an essential part of the food chain in both aquatic and terrestrial systems. *Rhizobium petrolearium* is a lesser known member of the genus. It was first described by Zhang et al. (2012) and has been associated with the biodegradation of hydrocarbon contaminants in the environment (Huang et al. 2016; Osadebe & Chukwu 2023).

Submitted: 18.11.2024;
Accepted: 05.12.2024

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Certain heavy metals are required at low concentrations by organisms for cell metabolism and biochemical reactions, however, when these metals occur at higher than normal concentrations in the environment, they constitute a hazard and have a negative impact on microbial communities both quantitatively and qualitatively. While several heavy metal species may be naturally available in soil and biological systems, their occurrence as contaminants typically stems from various industrial and agricultural processes such as fertiliser and pesticide production and application, sewage disposal, energy and fuel production, mining, leather works, metallurgy and manufacturing amongst numerous anthropogenic activities. When both essential and non-essential heavy metals from these anthropogenic sources exceed threshold levels in the environment, they form highly toxic unspecific complexes in microbial cells. These complexes hamper physiological function within the cell (Henao & Ghneim-Herrerra 2021; Syed et al. 2021). The most common heavy metals associated with anthropogenic activities are arsenic, cadmium, lead, chromium, nickel, copper and zinc. Lead, chromium, particularly chromium(VI) and cadmium are heavy metals of public health importance and have been known to deactivate enzyme activity in microorganisms (Balali-Mood et al. 2021; Kumar et al. 2022; Li et al. 2022).

Microorganisms are fundamental to a thriving, healthy ecosystem, thus, any factor that influences their community structure and activity, adversely or otherwise, is of ecological significance. Microbial diversity, abundance and metabolic activities all serve as a suitable and immediate measure of ecosystem health and potential pollutant toxicity. Changes in the metabolic activities of microorganisms within an ecosystem are considered an accurate indication of the toxic effects of an environmental stressor on indigenous species (Hellal et al. 2023). The diazotrophs are particularly significant because of their role in driving nutrient cycling within the ecosystem. The aim of this preliminary study was to establish the effect of varying concentrations of lead, chromium(VI) and cadmium on the growth and enzymatic activity of the soil bacterium, *Rhizobium petrolearium*.

Materials and methods

Isolation and characterisation of Rhizobium petrolearium

Soil was obtained from hydrocarbon-contaminated agricultural soil in Bomu in Gokana Local Government Area of Rivers State, Nigeria. The top 20 cm of soil was collected at different points and transferred to the laboratory in polyethylene bags. The soil samples were homogenised then sieved, using a 2 mm sieve, to remove debris, stones and large particles. About 1 g of the sieved soil was serially diluted and selected dilutions plated out individually on sterile yeast extract mannitol agar (YEMA) and nutrient agar (NA) in triplicates. The plates were incubated at 30 ºC ± 2 ºC for 48 h. The streaking method was used to obtain pure isolates from discrete colonies. The pure isolates of *R. petrolearium* were stored on YEMA slants until required for further studies (Holt *et al*., 1994; Cheesbrough, 2006). The isolate was identified by sequencing the 16S region of the bacterial gene as described in Osadebe and Chukwu (2023) and registered with the NCBI® GenBank under accession number, MF547450.

Heavy metal toxicity assay

The heavy metals tested were cadmium, lead and chromium(VI). Testing was done in batch systems. The nitrate salts of lead and cadmium and potassium dichromate were employed in the study. Testing was carried out at four concentrations of the heavy metals – 1 mM, 5 mM, 10 mM and 15 mM. The media containing the heavy metal salts were sterilised in a closed system using a 0.22 µm membrane filter. About 1 mL of a 24 h culture of the isolate (*R. petrolearium*) was added into 100 mL yeast extract mannitol broth in 250 mL Erlenmeyer flasks amended with different concentrations of the heavy metals. Incubation was at 30 ºC ± 2 ºC for 7 days. The abundance and enzymatic activity of the isolate were monitored at 24 h intervals. All set-ups were done with replicates. The control studies consisted of inoculated media without the addition of heavy metals.

Growth response of R. petrolearium to heavy metals

Enumeration of the isolate was done on YEMA via standard plate count technique. Precisely 0.1 mL aliquots were extracted from the tainted broth cultures at 24 h intervals and inoculated onto fresh YEMA plates via the spread plate method. Following 48 h incubation at 30 ºC ± 2 ºC, the number of discrete visible colony forming units (CFU) was determined using an automated digital colony counter (Balance Instrument co., China). Plates having CFU counts in excess of 300 were excluded.

Enzyme Activity Assays

Alpha-amylase production (starch hydrolysis)

The isolate was streaked in parallel lines on 1 % starch agar plates and incubated at 37 ºC for 48 h. Following incubation, the plates were covered with 1 % iodine solution and observed for the appearance of a clear zone around the isolate. The clear zone is indicative of starch hydrolysis due to α-amylase activity.

Protease production

Extracellular protease production and proteolytic activity in *R. petrolearium* was assessed by determining casein utilisation in skim milk agar (SMA) consisting of 1 % skimmed milk. The isolate was streaked on the agar in parallel lines and incubated at 37 °C for 24 h. A clear zone around the bacterial colony confirmed protease activity (Purwaningsih *et al.*, 2021).

Cellulase production

Cellulolytic activity was assessed by streaking the isolates onto cellulose agar containing 2 % cellulose and Congo red at a concentration of 0.2 g/L as described in Gupta et al. (2012). Incubation was at 37 °C for 48 h after which the plates were observed for the appearance of a clear zone around the colonies. The clear zone is considered indicative of cellulase production.

Oxidase activity

The oxidase test was conducted using tetramethyl-p-phenylenediamine dihydrochloride (TMPD) as an electron acceptor. Colonies of an 18 h old culture of R. petrolearium were transferred onto sterile filter paper (Whatmann's no. 1) using a sterile wooden pick. A drop of TMPD reagent was added and the set-up observed for immediate colour change to bluish-purple indicating a positive reaction for oxidase.

Catalase activity

Catalase activity was ascertained by dropping 10 % hydrogen peroxide onto a small portion of the bacterial colony placed on a clean glass slide. The evolution of oxygen gas as indicated by effervescence represented a positive result for catalase production. Catalase enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas.

Results and discussion

The study's findings indicated a decline in bacterial growth and enzymatic activity with increasing heavy metal concentration and exposure duration. The growth profile of the test isolate, shown in Figure 1, revealed the greatest decline in total viable bacterial abundance with cadmium and chromium(VI). Exposure to lead showed decreases in abundance of 41.99 %

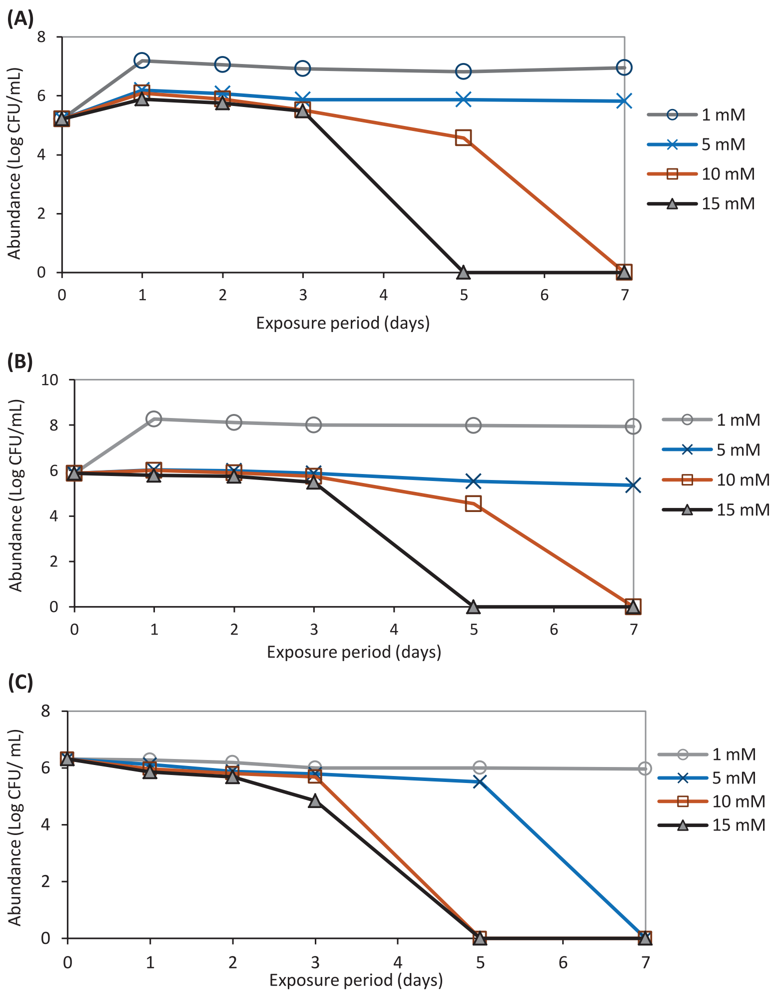

Figure 1. Growth response of *R. petrolearium* to varying concentrations of (A) lead, (B) cadmium and (C) chromium(VI)

and 58.32% on day 7 at concentrations of 1 mM and 5 mM respectively while cadmium exposure resulted in declines in abundance of about 51.14 % and 78.96 % for 1 mM and 5 mM concentrations respectively at the end of the study (day 7).

For lead and cadmium, no growth (100% decline) was seen by the end of the study period at 10 mM and 15 mM exposure concentrations. The mean percentage reduction in abundance of the test isolate after exposure to chromium(VI) for 7 days was 54.39 % at 1 mM concentration; at the higher concentrations of 5 mM – 15 mM, no growth was seen demonstrating a 100 % drop in abundance. This 100 % decline in abundance was obtained on day 5 at concentrations of 10 mM and 15 mM. These results would infer the order of toxicity based on abundance levels to be chromium(VI) > cadmium > lead. Overall, it was found that the reductions in growth obtained differed significantly ($p \leq 0.05$) between groups (among the different heavy metals studied) and within groups (from one heavy metal concentration to the other).

The overall effect of the different heavy metal concentrations on enzymatic activity in *R. petrolearium* is shown in Tables 2 – 5. The heavy metals had the strongest adverse influence on starch hydrolysis indicating the possible inhibition or impairment of α-amylase enzyme activity (Table 3). There was no impact on oxidase, catalase and cellulase activity and only minimal impact on protease activity. Only Pb and Cr(VI) inhibited protease activity at higher concentrations of 10 mM and 15 mM. Alpha-amylase activity was inhibited with increasing concentration and progressing contact time. Based on the enzymatic activity inhibition tests, the order of toxicity was Pb < Cr(VI) < Cd.

Angon *et al.* (2024) agree that heavy metals in soil decrease overall microbial biomass. The decrease in abundance with increasing concentration and increasing contact time seen in the current study correlates with the report of Ngwewa *et al.* (2022) who also found a decrease in microbial load with an increase in the concentration of selected heavy metals. Their study on heavy metal toxicity documented that the growth of *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* exhibited an inversely proportional relationship with the concentrations of cadmium and lead alongside copper, zinc and cobalt at concentrations up to 1000 ppm. Similarly, Yuan et al. (2015) suggested that long-term exposure to heavy metals like lead reduced microbial abundance, biomass and diversity. This is also the finding of Nwuche and Ugoji (2008), who studied the impact of heavy metal pollution on microbial activity in soil. They reported a negative correlation between microbial respiration, abundance and heavy metal concentration. Several other studies have reported decreases in soil enzymatic activity and microbial abundance due to heavy metal contaminants, consistent with the findings of the present study (Li et al. 2020; Cheng et al. 2022; Nurzhan et al. 2022). Li et al. (2020) stated that lead, chromium, zinc, copper, nickel and manganese diminished microbial bioactivity, species richness and microbial diversity by mean values of 55.1% – 87.7% while another study verified that lead-related stress modified the microbial community structure at levels of 2.5% and 5.0% lead in contaminated soil. The reduction levels in microbial abundance in response to lead obtained in those studies are akin to the 41.99 % – 100 % reduction in the counts of *Rhizobium petrolearium* reported in the current study. Heavy metal levels over specific threshold concentrations are known to exert inhibitory effects on microorganisms by modifying key molecules or by transforming the active conformations of biological molecules (Syed *et al.*, 2021).

The negative impact of heavy metals on soil microorganisms has been linked to their effect on soil pH levels. One study revealed that lead contamination

Table 1. Effect of varying concentrations of heavy metals on oxidase activity in *R. petrolearium*

Heavy Metal	Concentration	EXPOSURE PERIOD					
		Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
Lead	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	n/a
	10 mM	+	+	+	+	+	n/a
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+
Cadmium	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	+
	10 mM	+	+	+	+	+	n/a
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+
Chromium(VI)	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	n/a
	10 mM	+	+	+	+	+	n/a
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+

+ positive oxidase activity; – Negative for oxidase activity; n/a – not applicable as no growth observed

Table 2. Effect of varying concentrations of heavy metals on catalase activity in *R. petrolearium*

Heavy Metal	Concentration	EXPOSURE PERIOD Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
Lead	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	n/a
	10 mM	+	+	+	+	+	n/a
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+
Cadmium	1 mM	+	+	+	+	+	n/a
	5 mM	+	+	+	+	+	n/a
	10 mM	+	+	+	+	+	n/a
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+
Chromium(VI)	1 mM	+	+	+	+	+	n/a
	5 mM	+	+	+	+	+	n/a
	10 mM	+	+	+	+	+	n/a
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+

+ Positive result;– Negative result; n/a – not applicable as no growth observed

Table 3. Effect of varying concentrations of heavy metals on α-amylase activity (starch hydrolysis) in *R. petrolearium*

Heavy Metal	Concentration	EXPOSURE PERIOD Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
Lead	1 mM	+	+	+	+	–	–
	5 mM	+	+	+	–	–	n/a
	10 mM	+	+	+	–	–	n/a
	15 mM	+	+	–	–	n/a	n/a
	Control	+	+	+	+	+	+
Cadmium	1 mM	+	+	+	–	–	–
	5 mM	+	+	+	–	–	n/a
	10 mM	+	+	+	–	–	n/a
	15 mM	+	+	–	–	–	n/a
	Control	+	+	+	+	+	+
Chromium(VI)	1 mM	+	+	+	–	–	n/a
	5 mM	+	+	+	–	–	n/a
	10 mM	+	+	–	–	n/a	n/a
	15 mM	+	–	–	–	n/a	n/a
	Control	+	+	+	+	+	+

+ Positive result;– Negative result; n/a – not applicable as no growth observed

Table 4. Effect of varying concentrations of heavy metals on protease activity in *R. petrolearium*

Heavy Metal	Concentration	EXPOSURE PERIOD Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
Lead	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	+
	10 mM	+	+	+	+	–	–
	15 mM	+	+	+	–	–	n/a
	Control	+	+	+	+	+	+
Cadmium	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	+
	10 mM	+	+	+	+	+	+
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+
Chromium(VI)	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	+
	10 mM	+	+	+	+	+	–
	15 mM	+	+	+	+	–	n/a
	Control	+	+	+	+	+	+

+ Positive result;– Negative result; n/a – not applicable as no growth observed often precipitated soil acidification which could, in turn, limit the growth and activity of soil microorganisms (Collin et al. 2022). Cadmium has also been linked to reductions in soil pH, resulting in similar impacts on the soil microbial community and their enzymatic activities (Ukalska-Jaruga *et al.* 2022). Even though Ali et al. (2023) identified that chromium greatly inhibited soil microbial abundance and enzymatic activity, the heavy metal did not have any significant impact on soil pH levels in that study. The findings in the current study and comparable studies conflict with those of Meng et al. (2023) who noted that exposure to lead triggered increases in the abundance of microbial species related to the Actinobacteria, Bacteroidata and Myxococcota phyla. Bandara *et al.* (2022) also found that prolonged exposure to the heavy metal, cadmium, resulted in marked increases in the viable counts of members of the Bacteroidata and proteobacteria while concurrently decreasing the abundance of acidobacteria, firmicutes, chloroflexi, myxococcota and Gemmatimonadota phyla. *Rhizobium* spp. belong to the proteobacteria phylum taxonomic rank which would indicate that the findings of the present study oppose of Bandara *et al.* (2022).

In contrast, *Brandt et al.* (2010) determined that soil bacterial communities displayed structural and metabolic resilience against cadmium at exposure concentrations between 0 and 500 ppm. This was not the case in the current study where cadmium exposure inhibited the counts of *Rhizobium petrolearium* by 51.14 % – 100 % at concentrations of 1 mM to 15 mM. Agricultural soils in China containing a combination of copper (43.6 – 249.9 mg/kg), lead (43.8 – 144.5 mg/kg), Zinc (70.3 – 293.1 mg/kg) and cadmium (0.3 – 2.9 mg/kg) due to acid mine drainage was predominated by species of Acidobacteria phylum. The control studies consisting of uncontaminated soil were dominated by species of the phylum Chloroflexi and Proteobacteria, to which *Rhizobium* spp. belong (Wang et al. 2018). These finding are in agreement with those of the current study.

It has been highlighted that the toxic effects of heavy metal exposure are normally enhanced at higher concentrations of heavy metals. One such toxic effect is decreased soil enzymatic activity. This decline in soil enzyme activity has been linked to the associated alteration in the soil microbial community and the decrease in both microbial abundance and diversity which diminishes the available species for enzyme synthesis (Singh and Kalamdhad, 2016; Bakshi *et al.*, 2018). The continued catalase and oxidase activity seen in the current study seemed to demonstrate that the enzymes retain their functional properties in the presence of heavy metal environmental stressors. This resilience is fundamental to the survival of *R. petrolearium*. This is significant because in the absence of the catalase enzyme, for example, hydrogen peroxide accumulates in the cell and may become toxic to the bacterium (Heck et al. 2015). Cytochrome c oxidase in aerobic bacteria supports the final step in the respiratory chain – reduction of oxygen into water – and drives ATP synthesis and other cellular processes that require energy. Inactivation of this enzyme would eventually result in cellular death in selected bacteria (Hederstedt, 2022). Unlike catalase and cytochrome c oxidase, the activity of α-amylase responsible for starch hydrolysis was impacted by extended exposure to the heavy metals. Loss of this function would inhibit the ability of the *R. petrolearium* to breakdown more complex energy sources and may limit its survival in the environment.

Lead and cadmium have been highlighted for their capacity to interrupt microbial processes including nutrient cycling capabilities, metabolic processes and enzymatic activity (Abedi et al. 2022; Rehman et al. 2023). Consistent with the findings of the current study, other studies also indicated that the production and activities of catalase, urease and proteases were severely lim-

Table 5. Effect of varying concentrations of heavy metals on cellulase activity in *R. petrolearium*

Heavy Metal	Concentration	EXPOSURE PERIOD					
		Day 0	Day 1	Day 2	Day 3	Day 5	Day 7
Lead	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	+
	10 mM	+	+	+	+	+	+
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+
Cadmium	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	+
	10 mM	+	+	+	+	+	+
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+
Chromium(VI)	1 mM	+	+	+	+	+	+
	5 mM	+	+	+	+	+	+
	10 mM	+	+	+	+	+	n/a
	15 mM	+	+	+	+	+	n/a
	Control	+	+	+	+	+	+

+ Positive result;– Negative result; n/a – not applicable as no growth observed ited in the presence of lead and cadmium (Nyiramigsha, 2021; Xiao et al. 2022). In contrast to the results obtained in the current study, *Rhizobium* was seen to reduce copper-induced heavy metal stress in alfalfa and support microbial community structure in rhizosphere soil (Duan et al. 2022). Akin to the findings of the current study, exposure to cadmium was confirmed to result in a decline in catalase activity, alongside urease and dehydrogenase activities with increasing contact time and increasing concentrations from 0 mg/L – 10 mg/L in a 45-day study by Yeboah et al. (2021). Catalase activity was greatest at the lowest cadmium concentration of 0.1 mg/L. The drop in catalase activity continues gradually till day 10 when it seems to show slight increases.

R. petrolearium, in the present study, did not exhibit the anti-toxic responses that have been associated with other members of the genera. Species of *Rhizobium* have been associated with intracellular sequestration of heavy metals as a mechanism to reduce toxic stress. They typically do this via the formation of complexes using low molecular proteins within the cell. *Rhizobium leguminosarum* has been identified by Nonnoi *et al*. (2012) for high tolerance of mercury and other heavy metals in soil due to its distinct capacity for heavy metal sequestration. One study found that the bacterium employed cellular gluthathione to build up and sequester the heavy metal, cadmium, within its cell (Lima *et al*., 2006). Other bacterial genera like *Bacillus*, *Acinetobacter* and *Micrococcus* are also able to moderate the toxicity of heavy metals like chromium(VI) via oxidation, reduction, biological chelation and metabolic transformation in addition to biofilm formation (Adetunji *et al*. 2023).

Conclusion

Heavy metal contamination is currently an area of global interest owing to their association with environmental persistence and toxicity. These elements are known to compromise balance within the ecosystem and impact on human health. In the current study, the heavy metals – lead, cadmium and chromium(VI) – proved toxic to *R. petrolearium* at elevated concentrations and with prolonged exposure period. High concentrations of 10 mM and over limited growth and enzymatic activity in the bacterium. Based on the results, it can be concluded that that increased concentrations of heavy metals in soil could lead to diminished *R. petrolearium* populations which could, in turn, have negative knock-on effects on nitrogen cycling efficiency in the impacted ecosystem. An impaired nitrogen cycle and subsequent decrease in nitrogen availability could result in nutrient imbalances within the ecosystem, deterioration in plant health and an overall decline in biodiversity.

Abbreviations: NCBI, National Centre for Biotechnology Information; CFU, Colony forming Units

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