BIOREMEDIATION OF HEAVY METALS IN AGRICULTURAL SOILS IRRIGATED WITH TREATED WASTEWATER

PHADU, M. L. – KGOPA, P. M.*

School of Agricultural and Environmental Sciences, University of Limpopo, Sovenga 0727, South Africa
(phone: +27-15-268-2377)

**Corresponding author*
e-mail: moedishamaphuti@gmail.com; phone: +27-72-882-9989

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Abstract. The current study investigated the bioremediation abilities of bacteria on non-essential heavy metals in treated wastewater irrigated fields. Two fields, namely, cultivated field and fallowed field, each being 4 ha, were divided into 40 equal grids, for soil sample collection. Samples were analysed for five non-essential heavy metals namely arsenic, aluminium, cadmium, chromium, and lead. The isolated bacteria were identified as *Providencia rettgeri*, *Enterobacter cloacae*, *Bacillus cereus* and *Arthrobacter aurescens.* The bacteria were cultured and inoculated into heavy metal-contaminated soils and incubated for 12 weeks. Results showed that gram positive bacteria reduced concentrations of non-essential heavy metals separately and combined, especially in fallowed field. Cadmium and lead were significantly reduced by the combination of gram-positive bacteria by 95% and 83% respectively. Among the selected non-essential heavy metals chromium was the one which was most efficiently bioremediated with a 100% removal by *Providencia rettgeri* in cultivated field. No reduction was observed for cadmium by *Arthrobacter aurescens* in fallowed field. This study proved that bioremediation coupled with fallowing could be considered a solution in ameliorating heavy metal toxicity while naturally improving the quality of the soil.
Keywords: *water re-use, heavy metals toxicity, soil regeneration, soil health*

Introduction

Industries are rapidly expanding and improving and while that happens, great amounts of toxic wastes such as heavy metals get released and spread into the environment and water sources (USDA, 2016). These heavy metals are then transferred into wastewater through runoffs, whereby the wastewater is collected, purified and used for irrigation in agricultural fields. The treated wastewater containing large amounts of heavy metals results in soil heavy metal pollution (Hussain et al., 2019). There are several techniques that have been used to remove these heavy metals from water and soil and which includes chemical precipitation, oxidation or reduction, filtration, ion-exchange, reverse osmosis, membrane technology, evaporation and electrochemical inoculum (Selvi et al., 2019). Most of these techniques become unsuccessful when the concentrations of heavy metals are less than 100 mg/l (Masindi and Muedi, 2018). These cleaning methods are expensive, time consuming and still release some other toxic wastes after removal of heavy metals (Kanamarlapudi et al., 2018). Most heavy metal salts are water-soluble and get dissolved in wastewater, which means they cannot be separated by physical separation methods (Khulbe and Matsuura, 2018).

The use of microorganisms such as bacteria and fungi for remediation purposes is thus a more sustainable solution for heavy metal pollution since it includes maintainable remediation technologies to rectify and restore the natural condition of soil without giving off any more toxic substances (Dixit et al., 2015). The use of microorganisms

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will not only remove the toxic heavy metals in the soil but will lead to improved soil health; for example, more bacterial species in the soil will encourage relations with other organisms such as fungi. More fungi in the soil will help build stable aggregates with their hyphae or with the use of glomalin which is a carbon rich compound that they release (Wu et al., 2014). The build-up of carbons and presence of more stable aggregates in the soil will improve soil structure and improve water holding capacity of the soil.

Bioremediation refers to the use of microorganisms such as bacteria and fungi to detoxify heavy metals by either absorbing them or converting them into carbon oxide, energy or methane (Garima and Singh, 2014). Bioremediation relies on microbes that live naturally in soil and groundwater, and these microbes pose no threat to people at the site or in the community (EPA, 2016). This is further assured using indigenous microbes, which guarantees that the organisms can tolerate and exist in that particular ecosystem. Microbes that participate in bioremediation are referred to as bioremediators, and common examples are bacteria (*Bacillus subtilis, Pseudomonus putida, Enterobacter cloacae*) and fungi (*Penicillium, Aspergillus, Rhizopus);* they are potential microbial agents for the removal of heavy metals from aqueous solutions or in contaminated soils (Bahafi et al., 2013). Studies further reported that fungi were more tolerant to heavy metals as a group than bacteria (Igiri et al., 2018) However, diversity of bioremediators in agricultural soils remain necessary. Therefore, the objective of this study was to investigate the bioremediation abilities of indigenous gram-positive and gram-negative bacteria on non-essential heavy metals in treated wastewater irrigated agricultural fields. The research aimed at identifying specific microbes that were indigenous to the soils cultivated and fallowed soils contaminated with heavy metals following irrigation with treated wastewater, then used as inoculants for bioremediation.

Materials and methods

Description of the study site

The experiment was carried out in the Soil Science laboratory of University of Limpopo (28° 0’ 59.76” E; 25° 36’ 54” S), South Africa. Soil samples for the experiment were collected from a cultivated field (CF) in its third of onion cultivation and irrigated with treated water; and fallowed field (FF) which has been fallowed for 5 years following 3 years of cultivation and irrigation with treated wastewater. The 2 fields were adjacent to each other at University of Limpopo Experimental Farm (UL Farm) (-23°50’42.86” E; 29°42’44” S) (*Fig. 1*).

Research design

The experiment was a 2 × 7 factorial study in completely randomised design. The first factor was the 2 fields which were CF and FF and the second factor was the microorganism inoculants. The second factor was made up of a control with no inoculant (this sample was sterilised of microorganisms) (0I); two Gram-negative microorganisms (*Providencia rettgeri* (A) and *Enterobacter cloacae* (B)); the combination of the 2 Gram-negative microorganisms (AB); 2 Gram-positive microorganisms (*Bacillus cereus* (C) and *Arthrobacter aurescens* (D)) and the combination of two Gram-positive microorganisms (CD). Samples were collected at a depth of 0-20 cm and mixed to get a composite sample.

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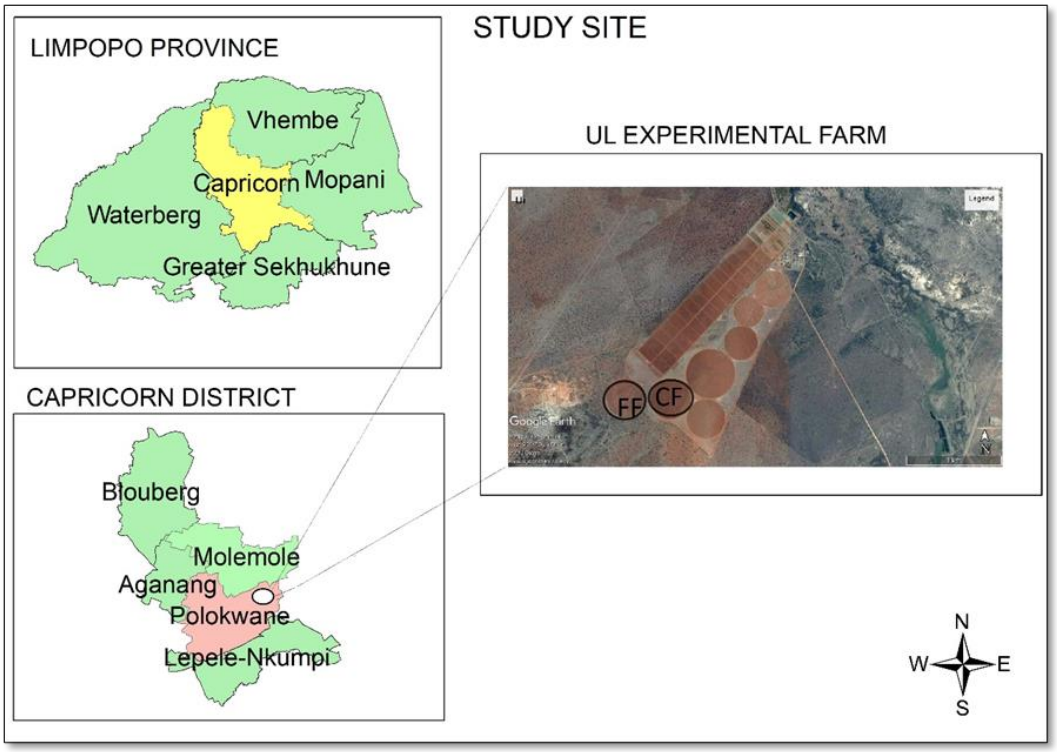

Figure 1. *Study area*

Data collection

Soil pH and heavy metal analysis

Soil pH (H_2O) and pH (KCl) were analysed using an electrode soil pH meter Mettler Toledo before commencing with the experiment (Reeuwijk, 2002). Non-essential heavy metals (As, Al, Cd, Cr and Pb) were extracted from the soil samples through the use of the nitric acid digestion method (Lu et al., 2017), whereby 1 g of soil was mixed together with 5 ml of 65% concentrated nitric acid in a centrifuge tube, heated in water bath at 95 °C temperature, cooled and then filtered and analysed through an ICP-OES Spectrophotometer machine from Ultima Expert (Habte et al., 2016).

Identification of isolated pure cultures microorganisms

Microorganisms were identified using a Maldi Biotyper through formic acid extraction method (Singhal, 2015), at the Biotechnology Unit, University of Limpopo. 14 Eppendorf tubes were sterilised and labelled according to the inoculums. 300 µl of deionized water was pipetted and transferred into each of the Eppendorf tubes. A quantity of pure culture biological material grown on agar plates (*Fig. 2*) (between one colony and 5-10 mg) was transferred into the tubes in accordance with the labels and the respective samples and mixed thoroughly by vortexing. 900 µl of alcohol was added into the tubes and mixed thoroughly. The samples were then centrifuged at maximum speed (15 000 rpm) for 2 min. The supernatant was decanted, and the samples were centrifuged again until the remaining alcohol was removed without disturbing the pellet. The alcohol-pellets were allowed to dry at room temperature for 2-3 min. 10 ml of 70% formic acid was then added to the pellets and then mixed thoroughly by vortexing.

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10 ml of acetonitrile was then added to the samples, and the samples were mixed thoroughly by vortexing. Samples were then centrifuged at 15 000 rpm for 2 min. 1 µl of the supernatant was transferred onto a Maldi target plate and allowed to dry at room temperature. The samples on the Maldi targets were then overlayed with 1 µl of α-Cyano-4-hydroxycinnamic acid (HCCA) solution within 1 h and allowed to dry at room temperature before being placed into the Maldi-tof for identification (Singhal, 2015).

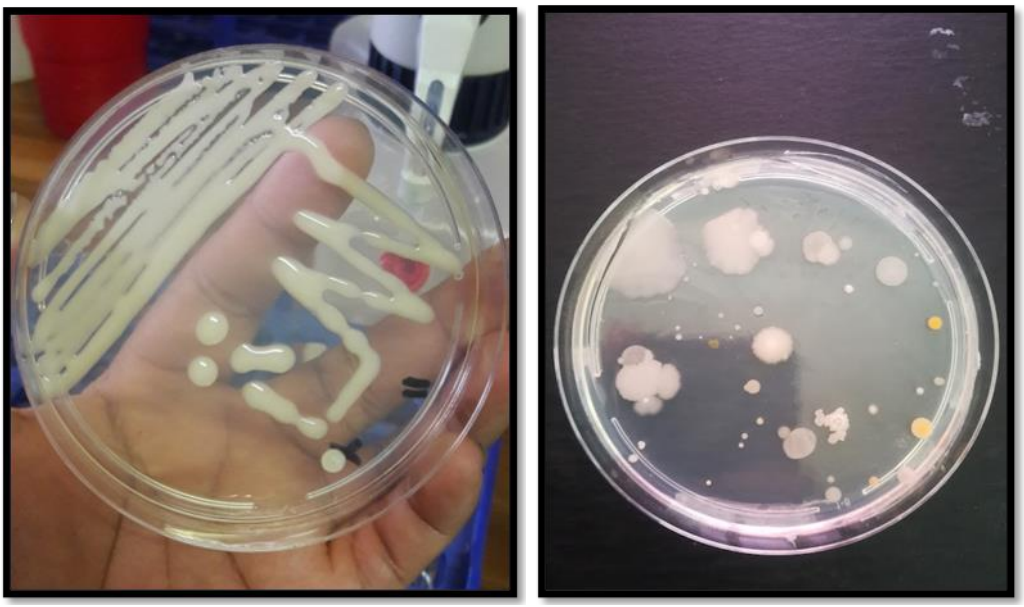

Figure 2. *Bacteria isolates sample used for bioremediation*

Morphological, microscopical and gram staining characteristics of the microorganisms used for the process of bioremediation

The morphological, microscopical and gram staining characteristics of the microorganisms used for the process of bioremediation are presented in *Table 1*. The first bacterium was identified as *P. rettgeri* which developed as a cream colony with medium rods, and it tested negative on gram stain. The second Gram-negative bacterium was identified as *E. cloacae* which grew as cream white colony with medium sized rods. The 2 Gram-positive bacteria were *B. cereus* which grew as small rods, light brown colony and the other one was *A. aurescens* grew as pink colony with big rounds (*Table 1*).

Microbial culturing of the identified microorganisms

Prior to inoculation, 4 microorganisms were cultured (Kim et al., 2021). Briefly, 4 Erlenmeyer flasks (500 ml) were sterilized and labelled as per organism. Cultures of the microorganisms were transferred into the flasks filled with 250 ml nutrient broth. The flasks were incubated on a shaker at 150 rpm at 25^0C for 72 h. After 72 h the samples were transferred into sterile centrifuge tubes and centrifuged at maximum speed in order to obtain single pellets. The supernatant was then discarded without disturbing the pellets. In reference to a paper by Kim et al. (2021), 5 ml of deionized water was added into the centrifuge tubes and mixed thoroughly. The samples were then ready to be used as inoculants (*Fig. 3a*) (Kim et al., 2021).

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Table 1. *Identified microorganisms that were used for bioremediation non-essential heavy metals on cultivated and fallowed fields*

Sample names	Morphological characteristics	Shapes under microscope	Gram staining results
Providencia rettgeri	Cream colony	Medium rods	Negative
Enterobacter cloacae	Cream-white colony	Medium rods	Negative
Bacillus cereus	Light-brown colony	Small rods	Positive
Arthrobacter aurescens	Pink colony	Big rounds	Positive

a **b**

Figure 3. *Inoculated samples (a) ready for incubation, and (b) incubated at 37 °C*

Bioremediation process

Composite samples were made from all the samples collected from 0-20 cm with respect to each field (CF and FF). Eight 50 g of soil were weighed from composite samples of CF and FF and transferred into 16 (100 ml) sterile glass beakers. Each of the glass beakers were replicated 3 times and labelled according to the inoculums of the microorganisms. Each glass beaker was inoculated with the prepared samples in the centrifuge tubes except for the control. The samples were incubated at 37 °C for 12 weeks as it was tested and proven to be a duration that will yield the most effective results (Fawole, 2017) and to maintain adequate moisture, the samples were irrigated two times a week with 20 ml of distilled water. After 12 weeks the soils were analysed again for bioavailable heavy metals (*Fig. 3b*) (Fawole, 2017). The incubators were well aerated and monitored for excess moisture.

Data analysis

All data were subjected to analysis of variance (ANOVA) through Statistix 10.0 version. Mean separation was done for significant means using Tukey's multiple range test at $P \leq 0.05$. Heavy metal reductions and additions are presented as relative impact (RI%), which was computed as follows:

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$$R.I(\%) = [(Inoculum\ treatment/Before\ Inoculum) - 1] \times 100. \quad \text{(Eq.1)}$$

A negative RI% indicates a reduction in non-essential heavy metals, while a positive RI% indicates addition. Before inoculum samples were used as a reference point.

Results

Description summary of soil pH for cultivated and fallowed field

Soil pH (H_20) and pH (KCl) values of CF ranged between minimum of 4.27 and 4.10 with maximum values of 8.94 and 6.605, respectively. Soil pH (H_2O) and pH (KCl) had average values of 7.67 ± 0.6381 and 5.54 ± 0.43 (*Table 2*).

Table 2. *Descriptive summary of soil pH for cultivated (CP) and fallowed (FF) fields*

Soil properties	Cultivated field (CF)				Fallowed field (FF)			
	Min	Max	Mean	St Dev	Min	Max	Mean	St Dev
pH (H_20)	4.27	8.94	7.67	0.63	5.58	7.58	5.35	0.46
pH (KCl)	4.10	6.60	5.54	0.49	4.55	6.58	5.35	0.44

Non-essential heavy metal distribution following bioremediation

The field × inoculation effects were significant on Cd, Cr and Pb but were not significant on Al and As. Factor A (field) was not significant for all the selected non-essential heavy metals. Factor B (Inoculum) was highly significant on Al, As, Cd, Cr and Pb. Since the field × inoculation and Factor A (field) effects were not significant on Al and As, RI% was only computed to report on effects of Factor B (Inoculum) for the two metals.

Distribution of cadmium

Relative to the reference sample in CF, OI reduced Cd by 67% whereas, AB, CD, A, B, C, and D reduced Cd concentration by 77%, 95%, 73%, 80%, 58% and 90%, respectively. Relative to the reference sample in FF, OI reduced Cd by 54% whereas, AB, CD, B, C, and D reduced Cd concentration by 3%, 26%, 60%, 14% and 0%, respectively. A increased Cd concentration by 8%, respectively (*Fig. 4*).

Distribution of chromium

Relative to the reference sample in CF, OI reduced Cr by 89% whereas, AB, CD, A, B, C, and D reduced Cr concentration by 84%, 93%, 100%, 76%, 98% and 63%. Relative to the reference sample in FF, OI reduced Cr by 64%, whereas AB, CD, B, C, *D* and A reduced Cd concentration by 64%, 98%, 46%, 65%, 73% and 83%, respectively (*Fig. 5*).

Distribution of lead

Relative to the reference sample in CF, OI reduced Pb by 80% whereas, AB, CD, A, B, C, and D reduced Pb concentration by 77%, 80%, 80%, 83%, 83% and 82%, respectively. Relative to the reference sample in FF, OI reduced Cr by 79% whereas, AB, CD, B, C, D and A reduced Pb concentration by 79%, 80%, 83%, 83%, 83% and 82%, respectively (*Fig. 6*).

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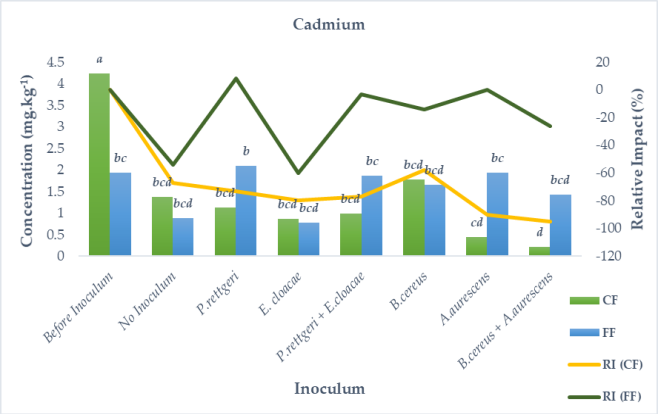

Figure 4. *Response of cadmium (Cd) in cultivated field (CF) and fallowed field (FF) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level p < 0.05)*

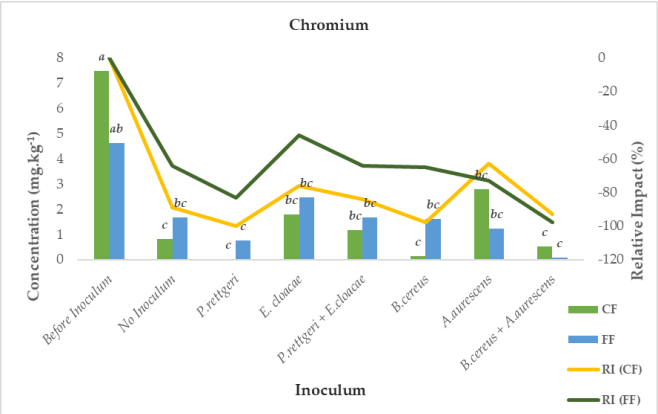

Figure 5. *Response of chromium (Cr) in cultivated field (CF) and fallowed field (FF) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level p < 0.05)*

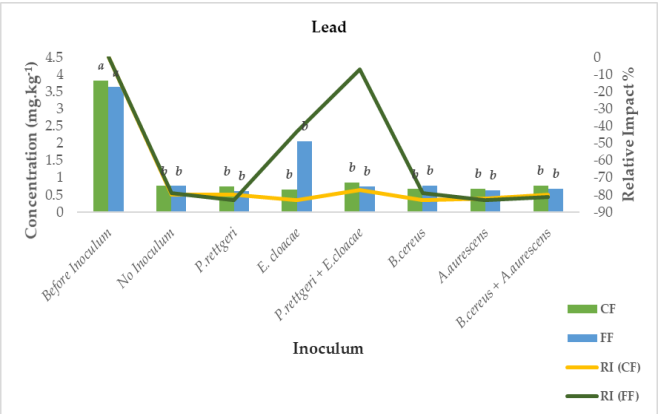

Figure 6. *Response of lead (Pb) in cultivated field (CF) and fallowed field following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level p < 0.05)*

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Distribution of aluminium

Relative to the reference sample, OI reduced Al by 61% whereas, AB, CD, A, B, C, and D reduced Al concentration by 46%, 62%, 63%, 60%, 61% and 59%, respectively (*Fig. 7*).

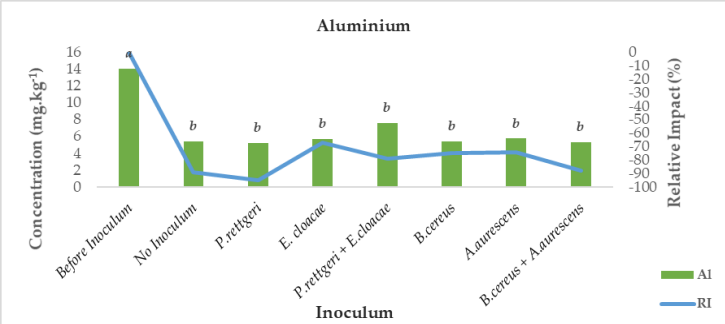

Figure 7. *Response of aluminium (Al) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level p < 0.05)*

Distribution of arsenic

Relative to the reference sample, OI reduced As by 89% whereas, AB, CD, A, B, C, and D reduced As concentration by %, 80%, 95%, 67%, 75% and 74%, respectively (*Fig. 8*).

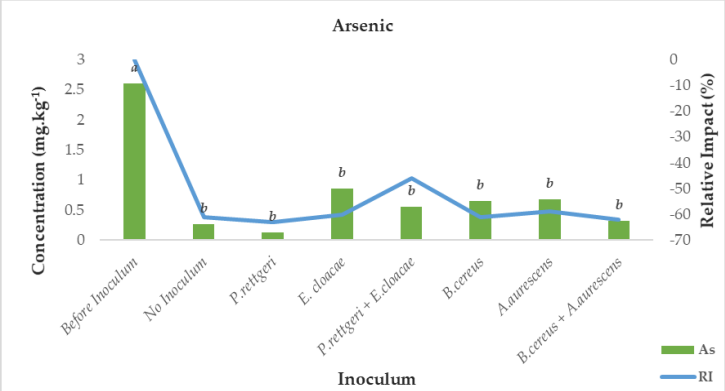

Figure 8. *Response of arsenic (As) following bioremediation with selected bacteria. (Different letters means statistical difference in the mean values according to the Turkey's multiple range test at the probability level p < 0.05)*

Discussion

Soil pH is a very crucial chemical indicator of organism habitat, which to an extent determines soil microbial diversities. Different organisms grow differently in varying pH ranges, with reports indicating that most bacteria prefer neutral pH, as acidity may impact microbial growth and functions (Sullivan et al., 2017). Soil pH means for the current study was found to be neutral in both CF and slightly acidic in FF, respectively. This observation could have influenced the performance of the isolated bacteria,

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however, this study was designed to show potential of indigenous bacteria as bioremediators, where there was contamination, which promotes resilience even when other conditions are not conducive.

Inoculum A (Providencia rettgeri)

Providencia rettgeri is a Gram-negative bacterium that is commonly found in both water and land environments (Triverdi et al., 2015). A strain of *P. rettgeri* was isolated from wastewater and solid water compost in Tunisia, and it showed tolerance to chromium, copper and other heavy metals (Das and Osborne, 2018). Likewise, it could be obtained in polluted effluents (Foti et al., 2009). Since it is an ubiquitous microorganism, it could have been in the soil naturally, brought by run off or even brought by the treated wastewater during irrigation at the study site. The trend of heavy metals was Cr > As > Pb > Cd > Al at CF and As > Pb > Cr > Al > Cd in order of the most reduced heavy metals to the least reduced by *P. rettgeri*. It was able to reduce 83% of Cd at its highest bioremediation compared to the other microorganisms. *Providencia rettgeri* could grow and reduce chromate to 100% at a concentration ranging from 100–300 mg/l and 99.31% at a concentration of 400 mg/l, pH 7 and temperature 37 °C (Thacker et al., 2006). The latter finding was better than the current research results. This could be due to the fact that the pH at both CF and FF was not kept constant at 7 like that of the latter report (Thacker et al., 2006). A different study reported that among other microorganisms, *P. rettgeri* was highly resistant to high concentrations of cadmium, copper and cobalt in polluted activated sludge (Bestawy et al., 2013).

Inoculum B (Enterobacter cloacae)

Enterobacter cloacae is a rod-shaped Gram-negative bacterium that live in mesophilic environments with an optimal temperature of 37 °C (Devin-Regli, 2015). It is aerobic and facultative anaerobic. *Enterobacter cloacae* is a human pathogen that can cause infections but can also act as a bioremediator. Under anaerobic conditions, it is able to convert toxic selenite in water sources that come from fossil fuel combustion to elemental, insoluble and non-toxic selenium (Devin-Regli, 2015). The trend of the bioremediated heavy metals was Pb > Cd > Cr > Al > As in CF and in FF was As > Al > Cd > Cr > Pb in order of the most reduced heavy metal to the least reduced heavy metal. Its lowest reduction in FF was Pb but it was its highest remediated heavy metal in CF. On average it performed best in CF. In a different study, maximum resistance was tested against *E. cloacae* with increasing concentrations of silver (Ag), Pb, and Cd. The maximum biosorption capacities of *E. cloacae* to the heavy metals were reported to be 65% at 200 mg/kg, 54.28% at 100 mg/kg and 74.46% at 300 mg/kg (Banerjee et al., 2009). In a polluted soil bioremediation study conducted in 2015 in India, *E. cloacae* bioaccumulated 95.25% of Pb, followed by 64.17% of Cd then by 36.77% Ni after 72 h of inoculation (Banerjee et al., 2009). The results of this research were more comparable to the latter, and this proves the ability and resilience of the organism.

Inoculum C (Bacillus cereus)

Bacillus cereus is said to be aerobic and facultatively anaerobic. This means that it makes adenosine try-phosphate (ATP) by aerobic respiration if oxygen is present but can switch to fermentation or anaerobic if oxygen is absent (Rohini and Jayalakshmi, 2015). It is also motile and commonly found in soil and food (Olaniran et al., 2013).

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Bacillus cereus is widely reported as a soil bacterium and occurs in the rhizosphere of some plants (Xiao et al., 2017), and some strains of *B. cereus* produce antibiotics able to suppress fungal diseases of the rhizosphere (Thacker et al., 2006). *Bacillus cereus* and *Bacillus thuringiensis* have been reported to increase extraction of Cd and Zn from soil and soil polluted with effluent from metal industry (Chibuike and Obiora, 2014).

A 70% decrease of chromium from the soil by two strains of *B. cereus* was reported (Ghalib et al., 2009). From the results of this study, it was observed that *B. cereus* was able to remediate Cr > Pb > As > Al > Cd in CF and in FF the trend was Pb > As > Cr > Al > Cd in order of the most reduced heavy metal to the least reduced. Based on the results obtained from this research, *B. cereus* was able to reduce 100% of the bioavailable Cr in CF, which was a huge improvement from the previous study. *Bacillus cereus* bioremediates heavy metals by bio-absorbing them from the soil solution, assisted by its cell wall characteristic (Thacker et al., 2006). This is due to affinity of hydroxylated and carboxylic functional group molecules on bacterial surfaces for heavy metals leading to their effective adsorption and precipitation and hence it shows resilience in bioremediating rather the most toxic heavy metals such as Pb (Thacker et al., 2006). Its least performance was on Cd, whereby, it increased it by 8% in FF. One study indicated that *B. cereus* was tolerant to a minimum level of 100 ppm to the metals, Cd and Co (Garima and Singh, 2014; Selvi et al., 2019) and this was in contrast with the current study as it could not bioremediate Cd efficiently.

Inoculum D (Arthrobacter aurescens)

Arthrobacter aurescens are basic soil bacteria that can fix nitrogen in the soil (Mongodin et al., 2006; Zhang et al., 2011) and perform several important functions of removal of toxic chemicals (Singhal et al., 2015; Wu et al., 2014). It has been reported that *A. aurescens* can reduce hexavalent chromium, which can cause severe irritations to humans, and they are also known to degrade agricultural pesticides in the soil (Fu et al., 2014). Hexavalent chromium is 100 times more toxic than trivalent chromium because of its oxidation state, and is also much more soluble in water, allowing it to seep into groundwater very easily (Fu et al., 2014). This research revealed that *A. aurescens* was able to reduce heavy metals in CF at a trend of Cd > Pb > As > Cr > Al in CF and in FF, it was Pb > As > Cr > Al > Cd in order of the most reduced heavy metal to the least reduced. Which is a very interesting finding because it was able to reduce more of Cd in CF but the lowest in FF.

Combination of Gram-positive and Gram-negative bacteria

From the results obtained, the combination of the Gram-positive bacteria (*B. cereus* + *A. aurescens*) always had the highest reduction of heavy metals than the combination of Gram-negative bacteria (*P. rettgeri* + *E. cloacae*). To add, the reduction was always highest at FF than at CF, meaning that bioremediation in this case was highly favourable at conditions of FF than that of CF. Although *B. cereus* + *A. aurescens* generally performed the best in comparison with *P. rettgeri* + *E. cloacae*, it was observed to have the lowest reduction of Cd in FF. Even when the individual microbes were used, they still had a poor performance. This could be because the Gram-positive bacteria used are not resistant to high levels of Cd. The trend of the non-essential heavy metals in CF was Cr > As > Cd > Pb > Al for *P. rettgeri* + *E. cloacae* and Cd > Cr > As > Pb > Al in for *B. cereus* + *A. aurescens* in the order of the most reduced

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heavy metal to the least. In FF the trends were As > Pb > Cr > Al > Cd for *P. rettgeri + E. cloacae* and Cr > As > Pb > Al > Cd for *B. cereus + A. aurescens* in the order of the most reduced to the least reduced heavy metal.

Both Gram-negative and Gram-positive bacteria have their cell wall charged with a negative charge. This is due to carboxyl, hydroxyl and phosphyl groups, thus in the presence of positive heavy metal cations, these groups are very important in cation sorption (Bahafi et al., 2013). Metals and metalloids get attached to these ligands on cell surfaces, which displace essential metals from their normal binding sites (Ayangbenro and Babalola, 2017). Once the metal and metalloid are bound, microbial cells can transform them from one oxidation state to another, thus reducing their toxicity (Gupta et al., 2015; Lesmana et al., 2009). By so saying, this defines the act of bioremediation observed in the study whereby the concentrations of non-essential heavy metals reduced in the soil.

Conclusion

Gram-positive bacteria performed the best individually and as a combination in bioremediation of the bioavailable non-essential heavy metals. Generally, this was mostly observed at FF than at CF. This means that fallowing of soils helps in bioremediation process, and this could be because the soil conditions are not constantly changed through the irrigation with treated wastewater. All the identified microbes were able to reduce the heavy metal concentration in the soil at different conditions of CF and FF, but worrying observations were seen with low reduction of concentrations of Cd at FF such that bacteria *A* increased it by 8% and D could not even reduce it at all. Another observation was that there wasn't any significant difference between the effectiveness of the inoculums of Pb, Al and As, further investigation needs to be done to see what the cause of this is. For further studies, these microorganisms must be screened for Cd resistance in soils of FF to understand the negative performance observed. More research must also be done on bioremediation of these non-essential heavy metals on both treated wastewater and polluted soils, especially with varying bacteria strains. Furthermore, a follow-up study that will investigate the mechanism with which this remediation occurs must be conducted, as well as a study that will implement the recommended findings in agricultural fields. It can be concluded that there are other soil factors that contributes to the effectiveness of this process and also that must be investigated. In conclusion, bioremediation using bacteria coupled with fallowing has shown to have great potential in the removal of non-essential heavy metals in soils. Therefore, it could be recommended for adoption by farmers who experience heavy metal pollution in their fields and as well as those who rely on treated wastewater for irrigation purposes.

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APPENDIX

Table A1. *Analysis of variance for Cd at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	0.41	0.21		
Field	1	0.46	0.46	1.61	0.21
Trt	7	22.31	3.19	11.18	0.00
Field #Trt	7	15.98	2.28	8.01	0.00
Error	30	8.55	0.29		
Total	47	47.71			

Table A2. *Analysis of variance for Cr at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	4.86	2.43		
Field	1	0.07	0.07	0.05	0.82
Trt	7	142.60	20.37	14.98	0.00
Field #Trt	7	22.32	3.19	2.34	0.04
Error	30	40.81	1.36		
Total	47	210.65			

Table A3. *Analysis of variance for Al at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	10.08	5.04		
Field	1	0.12	0.12	0.02	0.90
Trt	7	385.49	55.07	7.82	0.00
Field #Trt	7	34.88	4.98	0.71	0.67
Error	30	211.17	7.04		
Total	47	641.74			

Table A4. *Analysis of variance for As at cultivated and fallowed fields following bioremediation with seven (7) Gram-negative and positive bacterial species*

Source	DF	SS	MS	F	P
Rep	2	1.89	0.94		
Field	1	1.20	1.20	1.69	0.20
Trt	7	25.75	3.68	5.17	0.00
Field #Trt	7	1.51	0.22	0.30	0.95
Error	30	21.37	0.71		
Total	47	51.71			

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