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Microcosmic Study of Nickel Stress towards Soil Bacteria and their Biochemical Characterization

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Abstract: Heavy metal contamination of soil decreases microbial diversity and causes bacterial communities to lose part of their degradative capabilities. The present work is mainly related to Nickel (Ni) toxicity. Ni is the twenty fourth most abundant element of the earth's crust and has been detected in the biosphere. High concentration of Nickel frequently inhibits enzymatic activity, DNA replication, transcription and translation by binding to proteins and nucleic acids. We investigated the impact of Ni-stress on the soil bacterial populations in artificial microcosmic conditions as well as compared the artificially created stress with the natural long term heavy metal stress to bacterial populations. Microcosmic conditions were provided by using sterile test tubes for two different intervals (24 and 48 hours) by addition of 20g soil and Ni treatments (500, 1000 and 1500 ppm). Our results showed that Ni stress significantly reduce the bacterial densities as compared to control without Ni contamination. The impact of Ni increased with increase in its concentration. The isolates were picked and characterized using different biochemical tests. The bacterial strains isolated in the study may be useful for bioremediation of heavy metals. Similarly, identifying the enzymes produced in the Ni resistant strains will also be an important future perspective.

Keywords: Bacteria; Bioremediation; Biochemical tests; Microcosms; Ni

Introduction

The heavy metals can cause serious problems related to human health, plants and animals. The environmental change is mainly through human activities i.e. contamination of soil and entry of industrial wastes into soil. The anthropogenic applications including atmospheric deposition, application of agrochemicals and dumping of domestic wastes to the land have aggravated the issue. These contaminants reduced the soil quality of

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agricultural production. Use of pesticides for instance is important contaminant (Sanghi and Sasi, 2001). The heavy metals are toxic to humans, plants and animals because they cannot be naturally degraded like organic pollutants and they accumulate in different parts of the food chain (Smejkalova1 et al., 2003). Nickel is the twenty fourth most abundant element of the earth's crust and has been detected in all parts of the biosphere (Bhadra et al., 2006). Nickel is one of the most toxic heavy metals. Its geochemical properties are similar to cobalt and iron. Due to wide utilization of Nickel in paper, food and chemical industry are the causes of local contamination of environment (Wyszkowska et al., 2007). Nickel inhibits enzymatic activity, DNA replication, transcription and translation by binding to proteins and nucleic acids (Salnikow et al., 1997).

Various concentrations of metals including Ni have already been detected in the polluted soils (Sadiq, 1991). Cd, Ni, Zn, Cu, Pb and Mn were reported to have biological activity and stimulated microbial populations including mycorrhizal fungi (Weissenhorn et al., 1997). However, heavy metal contamination of soil decreased microbial diversity and bacterial communities lost their degradative capabilities (Burkhardt et al., 1993; Giller et al., 1998).

Remediating heavy metal-polluted soils may be based on the removal or conversion of the metals into less bio-available forms. Use of microorganisms in addition to phytoremediation has been found quite useful. For instance, the addition of Sphingomonas macrogoltabidus, Microbacterium liquefaciens, and Microbacterium arabinogalactanolyticum to Alyssum murale were helpful in bioremediation of Ni (Abou-Shanab et al., 2003). Bacteria require nickel as a trace element for enzymes such as urease, CO-dehydrogenase, hydrogenase (Hausinger, 1987). Several nickel resistant bacteria have been isolated from ecosystems polluted by heavy metals (Trajanovska et al., 1997).

The main purpose of our project was to study the impact of Nickel toxicity at various concentrations to bacterial populations. For this purpose artificial stress was created in the microcosms. The temporal effect of the stress was also evaluated over a short period up to two days. In addition to this, a soil from an area with a natural long term heavy metal stress was also considered. The objectives of the present study were to investigate the impact of Ni-stress on the soil bacterial populations in artificial microcosmic conditions; to investigate the temporal metal stress effects on bacterial densities; to compare the artificially created stress with the natural long term heavy metal stress to bacterial populations; and to isolate the resistant bacterial strains and compare their physiology with the strains isolated from the control soils.

Material and Methods

Soil Sampling and Processing

Soil samples from agricultural fields were cleaned by removing stones and debris. The clean soil was then air-dried and stored in paper bags. Sampling was also done from the natural Uranium-stress soil from Karak, Khyber Pakhtunkhwa, Pakistan. Before each experiment the soil was rewetted by addition of (20%) sterile water and was incubated for three days to invoke the microbial functions.

Microcomic Preparations

Microcosms were prepared in sterile test tubes taking 20 g soil treating with Nickel (500, 1000 and 1500 ppm) and incubating for two different intervals (24 or 48 hour) at 37 °C. The 10000 ppm mother

solution was prepared by taking 2.213 gm of Nickel Chloride in 100 mL of distilled water. After incubation the tubes were stored at 4 °C until further analyzed.

Bacterial Densities

Bacterial densities were assessed by colony forming unit (cfu). Colony forming unit was performed by adding 5 g of soil into 45 ml of distilled water. After mixing in a mechanical shaker for 20 min, the 10 times serial dilutions were prepared from the soil suspension.

Pour plate method was performed for microbial counting. In this method the nutrient agar was added to the Petri plate containing 100 µL of soil suspension of the different dilutions. Each experiment was performed in triplicates. Colonies were counted every 24 hours up to 3 days. Control soil was not treated with Nickel. In addition to this, Uranium rich soil from Karak was also evaluated for cfu. The data obtained were analyzed by analysis of variance (ANOVA).

Isolation of Bacterial Strains

Isolation of pure culture was performed from cfu plates. Single colonies were picked and cultured on nutrient agar plates. Each isolate was then purified by sub-culturing.

Table 1: Name and Origin of Isolates.

S.No	Strains	Origin
1	Mlk-1	**Malakand**
2	Mlk-2	**Malakand**
3	Krk-1	**Karak**
4	Krk-2	**Karak**
5	Krk-3	**Karak**
6	Ni-1	**Malakand**
7	Ni-2	**Malakand**
8	Ni-3	**Malakand**
9	**Ni-4**	**Malakand**

Biochemical Tests

Indole Test

Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and de-aminating tryptophan with the production of indole, pyruvic acid and ammonia. Indole reacts with the Kovac's reagent to form rosindole dye which is red in color (indole +ve). The different isolates were cultured in peptone water medium containing tryptophan in a screw capped tube, and incubated for 24 hours at 37°C. Kovac's reagent (0.5ml) was added into 24 hours broth culture.

Motility Test

This test is used to detect the motility of the test organism. If an organism is motile than the growth will radiate form the stab mark and make the entire tube appear turbid. Semi solid nutrient agar was prepared and then deeps of the agar were made in test tubes. Colony was picked form 18-24 hours fresh culture of organism and then inoculated into deeps of nutrient agar with the help of a straight wire loop and incubated at 37°C for 18-24 hours. After incubation result was noted.

Citrate Utilization Test

Simmons Citrate agar slant was used for this test. The slant was prepared such that citrate as the only carbon source, thus forcing the organisms to use it as a nutrient. The medium also contains a pH indicator called Bromthymol blue. This characteristic differentiates between members of family Enterobacteriaceae and other gram-negative rods. The test organism was inoculated into sterile Simon's citrate medium with the help of sterile straight wire and incubated at 37°C for 24-48 hours.

Catalase Test

Fresh 1% solution of hydrogen peroxide was prepared and 3ml of solution was distributed in each test tube. And with help of glass rod, colony was picked from culture plate and was dipped in hydrogen peroxide solution. And result was noted.

Triple Sugar Irons (TSI) Agar Test

The test organism was inoculated on TSI agar slant and butt with the help of platinum wire. Wire loop was used for inoculating on slants and straight wire was used to inoculate the butt.

Gram Staining

Using sterile techniques, a smear of each isolate was prepared, dried and heat fixed on slides. The smear was flooded with crystal violet and allowed to stand for one minute. It was then wash with distilled water and flooded with Gram's Iodine. That was allowed to stand for one minute and then it was washed with distilled water; it was decolorized with 95% ethyl alcohol and again washed with distilled water. After that, it was counter stained with Safranin for 45 seconds and washed with distilled water. The slide was wash and examined under the oil emulsion.

Results

Effect of Nickel on soil Bacteria

The Nickel contamination reduced bacterial densities significanlty. The Nickel contamination of 500, 1000 and 1500 ppm significantly reduced the bacterial densities as compared to soil without treatments. As compared to 500 ppm Nickel concentration, the bacterial densities were significantly reduced in 1000 and 1500 ppm, while there was no differences between bacterial densities at 1000 and 1500 ppm concetrations (Fig. 1A). Similar results were observed after 48 hours of microcosmic incubation (Fig. 1B).

Comparison of Natural Uranium Stress with the Artificial Nickel Stress

The effect of metal stress was also checked against the naturally long term U-rich soil from Karak. The Karak soil was directly processed and was not exposed to the temporal metal effect. The results obtained showed that the bacterial densities in the U-rich soil were significantly reduced in comparison to soil with no stress (Control). However bacterial densities were significantly higher in the U-rich soil as compared to different nickel treatments (Fig. 2).

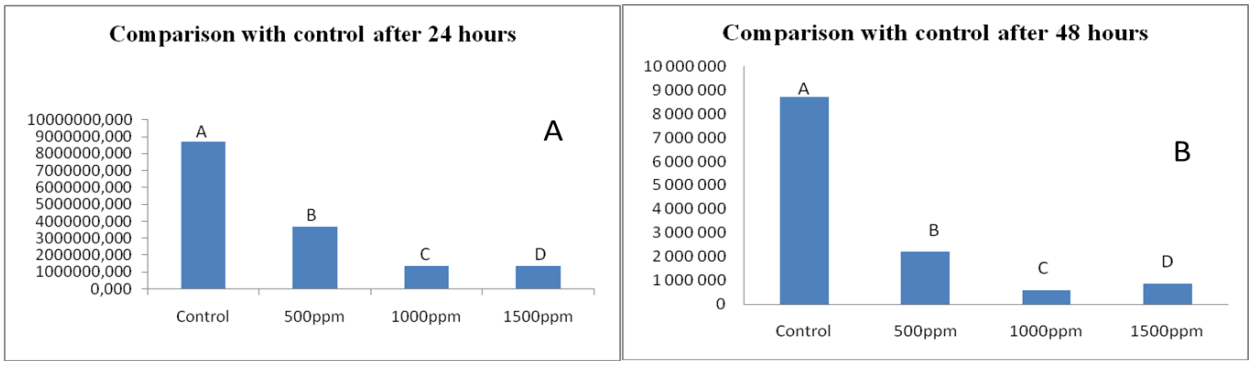


Fig. 1. Bacterial Densities (cfu/gm of dry soil) contaminated with different concentrations of Ni (500, 1000 & 1500 ppm) after 24 (A) and 48 (B) hours. Each bar indicates the mean of three independents replicates. Different small letters indicate the significant differences tested through ANOVA and Fisher LSD test.

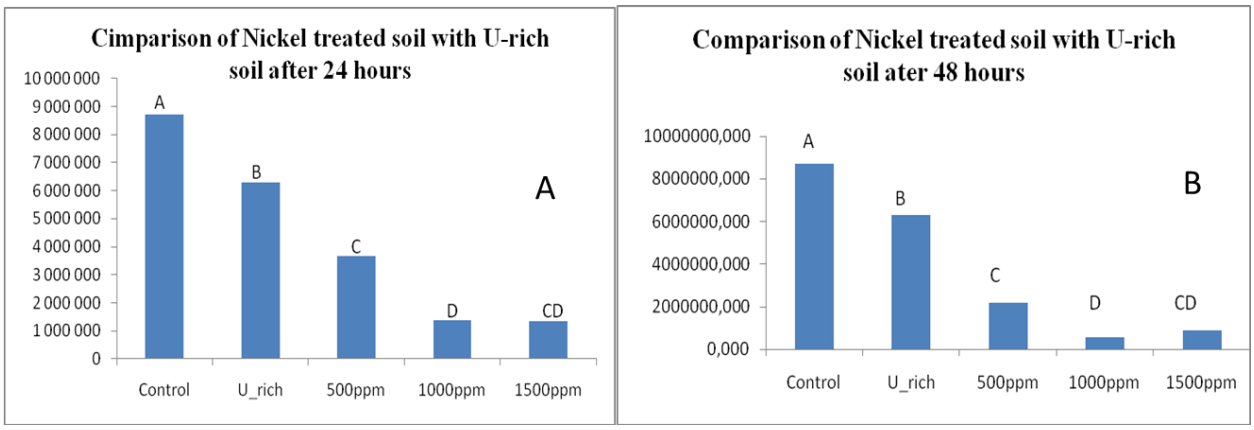


Fig. 2. Bacterial Densities (cfu/gm of dry soil) present in U-rich soil in comparison to Control and Nickel treatments after 24 (A) and 48 (B) hours. Each bar indicates the mean of three independents replicates. Different small letters indicate the significant differences tested through ANOVA and Fisher LSD test.

Gram's Reaction

Gram Positive:

Under Oil immersion lens (100X) we observed blue color cells having rod shaped appearance. Some of them were in clustered form while some were in chain form. The strains C-1, C-2, K-2, K-3, Ni-2 and Ni-3 were Gram's positive (Table 2).

Gram Negative:

Under Oil immersion lens (100X) we observe red to pinkish color cells having rod shaped appearance. They were in chain form. The strains K-1, Ni-1 and Ni-4 were Gram negative respectively (Table 2).

Biochemical Characteristics

Indole Test

The indole tests were performed for all isolates and the results were noted after 10 to 15 min. The results obtained were negative for all isolates. No ring was formed which indicated that they are indole negative. Indole positive bacteria form ring at the top.

Catalase Test

The Malakand soil sample isolates were tested for catalase test. The gas in the form of bubbles was seen if the tests were positive while no bubbles formation occur if the test were negative. Catalase tests were positive for C-1, C-2, K-2, K-3 and Ni-4 while it was negative for K-1 and Ni-1,2,3 respectively (Table 2).

Motility Test

All soil samples isolates were motile (Fig. 5).

TSI Test

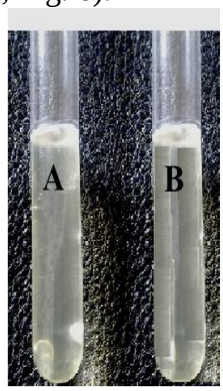
The growth on TSI is shown (Table 1). Slop was red in color and butt was yellow. Blackening in the middle indicates formation of H_2S gas. Other gases are also formed in bottom. The results indicate that a single bacterium (C-2) utilized the three main carbohydrate sources present in the media i.e. Sucrose, Fructose and Lactose.

Table 1: TSI Test for soil microbial strains isolated form stress and non-stressed soil.

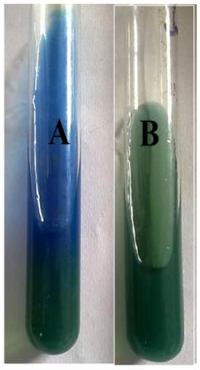
	Triple Sugar Iron (TSI)			
Strains	**Slant**	**Butt**	**H_2S**	Other gases
C-1	Red	Yellow	Negative	Negative
C-2	Yellow	Yellow	Negative	Negative
K-1	Red	Yellow	Negative	Negative
K-2	Red	Yellow	Negative	Negative
K-3	Red	Yellow	Negative	Negative
Ni-1	Red	Yellow	Negative	Negative
Ni-2	Red	Yellow	Negative	Negative
Ni-3	Red	Yellow	Negative	Negative
Ni-4	Red	Yellow	Negative	Negative

Citrate Utilization Test

This characteristic differentiates between members of family Enterobacteriaceae and other gram-negative rods. The medium also contains a pH indicator called Bromthymol blue. The test isolates of bacteria were positive for strains, C-1, K-2, K-3, and Ni-2 while negative for C-2, K-1, Ni-1, Ni-3 and Ni-4 respectively (Table 2; Fig. 6).



A (Positive) **B** (Negative)
Fig. 5. Motility test.



A (Positive) **B** (Negative)
Fig. 6. Citrate Utilization Test.

Table 2: Catalase, Citrate and Gram's Staining of the different isolates from soil.

Strains	Catalase Test	Citrate Test	Gram's Stain
C-1	Positive	Positive	Positive
C-2	Positive	Negative	Positive
K-1	Negative	Negative	Negative
K-2	Positive	Positive	Positive
K-3	Positive	Positive	Positive
Ni-1	Negative	Negative	Negative
Ni-2	Negative	Positive	Positive
Ni-3	Negative	Negative	Positive
Ni-4	Positive	Negative	Negative

DISCUSSION

Heavy metals present inside the soil is because of human activities, industrial wastes or sewage sludge and agricultural management. These heavy metals may cause serious problems related to human's health, plants or animals because most of heavy metals cannot be naturally degraded (Smejkalova1 et al., 2003). Besides effect on humans, plants and animals, these heavy metals also reduced the soil quality, fertility and texture. The main purpose of the present study was to study the impact of nickel on soil bacterial populations in microcosmic conditions and their comparison with naturally present Uranium rich soil as an external control.

The artificially created nickel-stress in the present study affected the bacterial densities and the density decreased by increasing their concentrations of the metal. The impact of nickel in 500 ppm artificial stress was significantly lesser showing higher number of bacterial densities as compared to 1000 and 1500 ppm. The effect of nickel increased with the increase in concentration till 1000 ppm. Hence 1000 ppm concentration may be the threshold for the nickel stress. Moreover, the U-rich soil also showed a suppressive effect on the microflora. Ansari and Malik (2009) also showed that the microbial count in soil as

well as in wastewater decreased as the metal concentration increased. Smejkalova1 et al. (2003) studied that heavy metals decreased the soil bacterial population by inhibiting various metabolic and molecular aspects. On the other hand only 24 hour exposure to Nickel stress reduced the densities of bacteria more than the long term U-exposed soil. This may be due to the temporal adaptation of the bacterial communities towards the stress that, of course, needs time for the evolution.

The different colonies were also picked and purified in the present study. The isolated strains were characterized through the biochemical tests although the results similar to Ansari and Malik (2009) were also obtained but it did not confirm the identification. Biochemical characteristics mainly involve the conversion of biomolecules, enzymes and utilization of specific substrate which change the color of medium. The present study may be a landmark for obtaining such bacterial strains that may be important for bioremediation of heavy metals especially Nickel related compounds. Moreover, the genetic and molecular level studies for the identification of resistant gene may also be an important perspective.

References

Abou-Shanab, R. A., Angle, J. S.,Delorme, T.A.,Chaney, R. L.,Berkum, P. V.,Moawad, H., Ghanem, K. and Ghozlan,H. A. 2003. Rhizobacterial effects on nickel extraction from soil and uptake by *Alyssum murale*. New Phytol. 158:219-224.

Bhadra, B., Nanda, A. K. and Chakraborty, R. 2006. Inducible nickel resistance in a river isolate of India phylogenetically ascertained as a novel strain of *Acinetobacterjunii*. World J. Microbiol. Biotechnol. 22:225–232.

Burkhardt, C., Insam, H., Hutchinson,T. C. and Reber,H. H. 1993. Impact of heavy metals on the degradative capabilities of soil bacterial communities: Biol. Fertil. Soils, 16:154-156.

Giller, K. E., Witter,E.and McGrath, S. P. 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils. Soil Biol. Biochem. 30:1389-1414.

Hausinger, R.P. 1987. Nickel utilization by microorganism. Microbiol. Rev. 51:22–42.

Ansari, M. I. and Malik, A. 2009. Seasonal variation of different microorganisms with nickel and cadmium in the industrial wastewater and agricultural soils. Environ. Monit. Assess. 167:151–163.

Smejkalova1, M., Mikanova, O. and Boruvka, L. 2003. Effects of heavy metal concentrations on biological activity of soil micro-organisms. Plant Soil Environ. 49:321–326.

Sadiq, M. 1991. Impact of Kuwait oil fires and other war activities on inorganic atmospheric pollution in Saudi Arabia, The Kuwaiti Oil Fires, Presentation Abstract, American Academy of Arts and Sciences, Cambridge.

Salnikow, K., Cosentino, S., Klein, C. and Costa, M. 1997. The loss of thrombospondin transcription activity in nickel-transformed cells. Mole. Cell Biol. 14:851–858.

Sanghi, R. and Sasi, K. S. 2001. Pesticides and heavy metals in agricultural soils of Kanpur, India. Bull. Environ. Contaminat. Toxicol. 67:446–454.

Trajanovska, S., Brits, M. L. and Bhave, M. 1997. Detection of heavy metal ion resistance genes in gram-positive and gram-negative bacteria isolated from lead-contaminated site. Biodegradation, 8:113–124.

Weissenhorn, I., Mench, M. and Leyval,C. 1997. Bioavailability of heavy metals and arbuscularmycorrhiza in a sewage sludge-amended sandy soil. Soil Biol. Biochem. 27:287–296.

Wyszkowska, J., Boros, E.and Kucharski, J. 2007. Effect of interactions between nickeland other heavy metals on the soil microbiological properties. PlantSoil Environ. 53:544–552.