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Bioremediation potential of *Eisenia fetida* and microbes for arsenic contaminated soil and water

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Abstract
Air with hazardous, toxic chemicals, contamination of soils, groundwater are the major problems facing the world today. Contaminated lands and water generally result from past industrial activities when awareness of the health and environmental effects connected with the production, use and disposal of hazardous substances. Heavy metals such as Pb, Cd and As are considered potentially environmental pollutants due to their trends to accumulate on vital organs of humans and animals. Arsenic (As) exists in several forms, which vary in toxicity and occurrence of which arsenate and arsenate. It is used in industry as a wood preservative, paints, dyes, metals, soaps, insecticides and semi-conductors etc. The toxicity and redistribution of arsenic in the environment make it evoking public concern. Earthworms can also tolerate high concentrations of heavy metals in the environment. Several technologies are existing for the remediation of As from contaminated soil and water. The study suggests about the behavior of *Eisenia fetida* and Microbes (strain 23A - unidentified microbes) in arsenic contaminated soil and water. Their bioremediation potential in arsenic rich media was determined with reference to tolerance. Higher tolerance capacity of *Eisenia fetida* and Microbes were observed. At 800 ppb concentration, earthworm shows resistance capacity against arsenic toxicity and then gradually decreases after from 900 ppb to 1400 ppb. After providing different concentration of arsenic to earthworm and microbes, the arsenic in earthworm decreases gradually as the microbes play an important role as arsenic munching.

Keywords: Pollution, Heavy Metal, Arsenic, Bioremediation, *Eisenia fetida* and Microbes

1. Introduction

1.1 Heavy Metal Accumulation in water and soil

Due to rapid industrialization and urbanization pollution of heavy metals becomes a matter of global concern. Cadmium, copper, arsenic, chromium, lead, mercury, nickel and zinc are considered the most hazardous heavy metals. Sources of metals include domestic and industrial effluents, the atmosphere, runoff and lithosphere. The metals can absorb into the soil, runoff into rivers or lakes or leach in the groundwater, an important source of drinking water. Contaminated water, polluted sediments and the accumulation of chemicals in the aquatic food-chain are occurs due to the discharges of inorganic and organic micro-pollutants and radioactivity from various industrial, agricultural and municipal sources. The areas throughout the world that have the worst contamination of groundwater by arsenic are in South Asia, Nepal, India and Bangladesh. Soil pollution has recently been attracting considerable public attention since the magnitude of the problem in our soils calls for immediate action (Garbisu and Alkorta 2003). Among heavy metals Pb, Cd and As are considered potentially important environmental pollutants due to their trends to accumulate on vital organs of humans and animals. The most common metals found at contaminated sites are (U.S. EPA, 1996b), in order: lead (Pb), chromium (Cr), arsenic (As), zinc (Zn), cadmium (Cd), copper (Cu) and mercury (Hg). Heavy metals are among the pollutants that need to be removed from such contaminated sites. It has been stated for example, that Cd has half-life of 10 years once in the human body (Salt *et al.*, 1995) [23]. Additionally, some species of Cd, Cr and Cu have been associated with health effects ranging from dermatitis to various types of cancer (McLaughlin *et al*., 1999) [14]. The presence of these heavy metals in the environment has been a subject of great concern due to their toxicity, non-biodegradable nature and the long biological half-lives for their elimination from biological tissues (Olatunji *et al*., 2009) [21].

1.2 Arsenic Contamination

Arsenic is a member of the nitrogen family with both metallic and nonmetallic properties and is ubiquitous in the environment (soil, water, air and also in living matters) (Tamaki and Frankenberger., 1992). Although the occurrence of arsenic in the environment is mainly from minerals, geogenic sources, human activities such as mining, burning fossil fuels, chemicals in agriculture also cause arsenic distribution in the environment (Bissen and Frimmel, 2003) [2]. A large number of sites worldwide have been contaminated by arsenic from natural and anthropogenic sources (Mandal and Suzuki, 2002) [13]. Many countries, especially Taiwan, Argentina, India, Bangladesh, Mexico, Hungary, and Chile, have reported extensive arsenic contamination of their groundwater supplies (Smedley, 2002; Nikolaidis *et al*., 2004) [27, 19]. It has been used in various fields such as medicine, electronics, agriculture, pesticides, herbicide, insecticides, fertilizer and as wood preservatives etc. Sharma and Sohn, 2009) [25]. Arsenate in reduced state in inorganic is a toxic pollutant in natural environment and is more soluble and mobile than the oxidized state of inorganic arsenic, arsenate (Elangovan and Chalakh, 2006) [6, 30]. The range of arsenic in uncontaminated soil ranges from <1 to 95 mg kg_1. Elevated levels of arsenic in agricultural soil could

pose a serious threat to plants and human health and the environment through the food chain pathways (Bruce *et al.*, 2003; Duxbury *et al*., 2003) [3, 5] (Fig 1). Arsenic toxicity depends on its speciation, and generally inorganic arsenic species are more toxic than those of organic species (Meharg and Hartley-Whitaker, 2002) [15]. Therefore, removal of metals such as arsenic from contaminated sites is foremost importance. The need to remediate these sites has led to the development of new technologies that emphasize the destruction of the pollutants rather than the conventional approach of disposal.

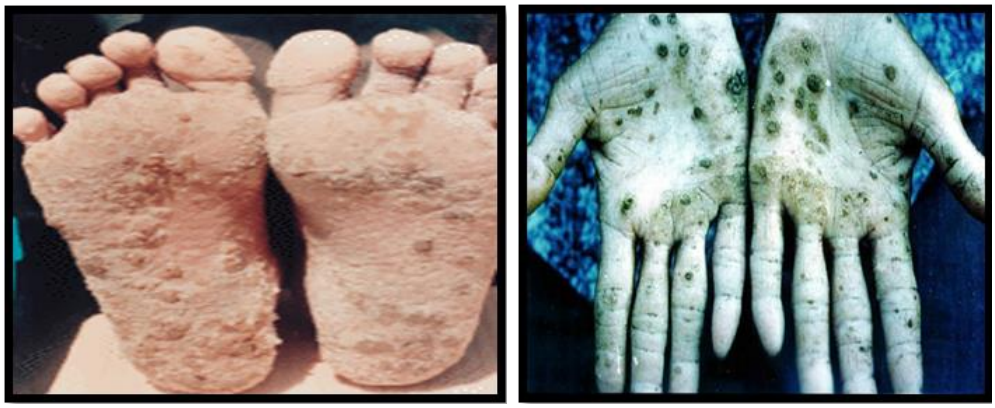

Fig 1: Lesions of Arsenic

1.3 General Remediation Approach

Various methods for the remediation of heavy metals from contaminated soil and water are consist of general approaches to remediate isolation, immobilization, toxicity, reduction, physical separation and extraction. (La Grega *et al.*, 1994). The techniques for arsenic removal from contaminated water, development of new techniques along with enhancement and cost reduction are essential for common people (Mondal *et al.*, 2006) [17].

1.4 Bioremediation

Bioremediation is the technology that uses microorganism metabolism to remove pollutants it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. It is the addition of matched microbe strains to the medium to enhance the resident microbe population's ability to break down contaminants. This technology includes biostimulatio, bioaccumulation, biosorption, phytoremediation and rhizoremediation etc. Rapid advances in the last few years have helped us in the understanding of process of bioremediation.

1.4 Earthworms the environmental managers on earth

The earthworms have over 600 million years of history in waste and environmental management. No wonder then, Charles Darwin called them as the ‘unheralded soldiers of mankind,’ and the Greek philosopher Aristotle called them as the ‘intestine of earth,’ meaning digesting a wide variety of organic materials including the waste organics, from earth (Darwin and Seward 1903; Martin, 1976) [4]. Earthworms are long, narrow, cylindrical, bilaterally symmetrical, segmented animals without bones. Earthworms harbor millions of ‘nitrogen-fixing’ and ‘decomposer microbes’ in their gut (Fig 2; 3). The distribution of earthworms in soil depends on factors like soil moisture, availability of organic matter and pH of the soil. They occur in diverse habitats specially those which are dark and moist. Earthworms are generally absent or rare in soil with a very coarse texture and high clay content or soil with pH 4 (Gunathilagraj, 1996) [8]. In a study made by (Kerr and Stewart, 2006) [11] that E. fetida can tolerate soils nearly half as salty as seawater. Earthworms can also tolerate toxic chemicals in environment. Earthworms which ingested TCDD contaminated soils were shown to bioaccumulate dioxin in their tissues and concentrate it on average 14.5 fold (Satchell, 1983) [24]. E. fetida also survived 1.5% crude oil containing several toxic organic pollutants (OECD, 2000; Safwat *et al.*, 2002) [20]. Some species have been found to bioaccumulate up to 7600 mg of lead (Pb) /gm of the dry weight of their tissues (Ireland, 1983) [10]. They can tolerate a temperature range between 5 and 29 C. A temperature of 20–25_C and moisture of 60–75 % are optimum for good worm function (Hand, 1988) [9].

Fig 2: *Eisenia fetida*

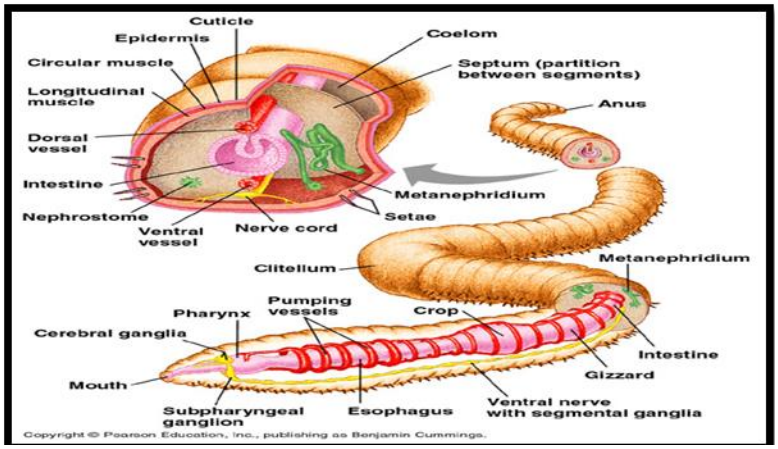

Fig 3: Biology of earthworm

1.6 Vermifiltration technology

Vermifiltration of wastewater using waste eater earthworms is a newly conceived novel innovative technology developed by us at Griffith University, Australia. Earthworms body work as a 'biofilter' and they have been found to remove the 5 days BOD (BOD) by over 90 %, COD by 80-90 %, total dissolved solids (TDS) by 90-92 % and the total suspended solids (TSS) by 90-95 % from wastewater by the general mechanism of 'ingestion' and biodegradation of organic wastes, heavy metals and solids from wastewater and also by their 'absorption' through body walls. Most successful species are the Tiger Worms (*Eisenia fetida*). Vermi-filtration system is low energy dependent and has distinct advantage over all the conventional biological wastewater treatment systems- the 'activated slude process', 'trickling filters' & 'rotating biological contractor' which are highly energy intensive, costly to install and operate and do not generate any income. This is also an odor free process. The most significant advantage is that there is 'no sludge formation' in the process as the earthworms eat the solids simultaneously and excrete them as vermicast. This plagues most municipal council in world as the sludge being a biohazards requires additional expenditure on safe disposal in secured landfills. In the vermifilter process there is 100 % capture of organic & inorganic materials and any pathogen and the capital and operating costs are much less. Earthworm's bioaccumulate all toxic chemicals including the 'endocrine disrupting chemicals' (EDCs) from sewage which cannot be removed by the conventional systems. A pilot study on vermifiltration of sewage was made by (Xing *et al*., 2005) [33] at Shanghai Quyang Wastewater Treatment Facility in China. (Taylor, 2003) [31] studied the treatment of domestic wastewater using vermifilter beds and concluded that worms can reduce BOD and COD loads as well as the TDSS (total dissolved and suspended solids) significantly by more than 70–80%. (Hartenstein and Bisesi, 1989) studied the use of earthworms for the management of effluents from intensively housed livestock which contain very heavy loads of BOD, TDSS and nutrients nitrogen (N) and phosphorus (P). The worms produced clean effluents and also nutrient-rich vermicompost. (Bajsa *et al.,* 2003) [1] also studied the vermifiltration of domestic wastewater using vermicomposting worms with significant results.

1.7 Factors Affecting Vermifiltration of Wastewater

1). Worm Population & Density (Biomass) in Vermifilter Bed (Soil)

As the earthworms play the critical role in wastewater purification, their number and population density (biomass) in soil, maturity and health are important factors. This may range from several hundred to several thousands. There are reports about 8-10,000 numbers of worms per square meter of the worm bed and in quantity (biomass) as 10 kg per cubic meter (cum) of soil for optimal function (Sinha *et al*., 2008) [28].

2). Hydraulic Retention Time (HRT)

Hydraulic retention time is the time taken by the wastewater to flow through the soil profile (vermifilter bed) in which earthworms inhabits. It is very essential for the wastewater to remain in the vermifiltration system and be in contact with the worms for certain period of time. HRT depends on the flow rate of wastewater to the vermifiltration unit, volume of soil profile and quality of soil used. HRT is very critical, because this is the actual time spent by earthworms with wastewater to retrieve organic matter from it as food.

3.) Objectives were to determine the bioremediation potential of the *Eisenia fetida* and Microbes (strain 23A - unidentified microbes) in arsenic contaminated soil and water. Their tolerance potential and remediation efficacy was also checked in an arsenic rich media.

2. Microbe Culture Process

2.1 Preparation of smear

It is thick, dense smear, which concentrate a large no. of cells on the slides. Such this type of preparation diminished the amount of light that can pass through and make it difficult to visualize the morphology of single cells. Smears require only a small amount of the bacterial culture. A good smear is when it is dried, appears as a thin whitish layer or film. Those made for broth cultures or cultures from a solid medium require variations in technique.

2.2 Broth culture

Resuspend the culture by tapping the tube with finger. Depending on the size of the loop, one or two loopfuls applied to the centre of the slide with a sterile inoculating

loop and spread evenly over an area about the size of a dime. Set the smear on the lab and allowed it to dry.

2.3 Culture from solid medium

Organisms cultured in a solid medium produce thick, dense surface growth are not amenable to direct transfer to the glass slide. These cultures must be diluted by placing one or two loopfuls of water on the centre of the slides in which the cells are emulsified. Transfer of the cell requires the use of a sterile inoculating loop or a needle is preferred. Only the tip of the needle should touch the culture to prevent the transfer of too many cells. Suspension is accomplished by spreading the cells in a circulating motion in the drop of water with the loop or needle. The finished smear should occupy an area about the size of nuclei and should appear as a translucent or semitransparent confluent whitish film.

2.4 Microbe culture procedure

2.4.1 Reagent preparation

About 1.25g of beef extract, 0.75 g peptone, 1.25g Nacl and 4 g agar agar was dissolved in 250 ml distilled water.

2.4.2 Procedure of staining:

A smear formation was done by putting 1 drop of distilled water on slide with the help of loop by taking microbes from slant. In staining process crystal violet of about 2-4 drops was putted on slide after heat fixation. Kept for 1 min then washed it. Air dried the slide for 5 min then focused at microscope.

3. Experimental Design

3.1 Vermifiltration bed setup

The Vermifiltration bed was setup contained gravels with a layer of garden soil on top. It was to collect the filtered water at the bottom in a chamber which opens out through a pipe fitted with tap. The chamber lays the net of wire mesh to allowed only water to trickle down while holding the gravels above. The bottommost layer was made of gravel aggregates of size 7.5 cm and it fills up to the depth of 5 cm. Above this the aggregates of 3.5–4.5 cm sizes filling up to another. On the top of this is the 5-cm layer of aggregates 10–12 mm sizes mixed with sand and 2 cm wood sand. The topmost layer of about 10 cm consists of pure soil in which the earthworms were released. The worms were given around one week settling time in the soil bed to acclimatize in the new environment. Here 24 hrs retention periods was provided to earthworm to perform its action (Fig 4).

3.2 Preparation of Synthetic Wastewater

Synthetic wastewater was prepared in the laboratory daily for vermifilter technology and after every 3 days for constructed wetland to avoid organic matter degradation. As arsenic stock solution was used as source. Stock solution was prepared by dissolving 0.416 g of sodium arsenate in 1000 ml of distilled water. Synthetic wastewater for constructed wetland was prepared for 500 ppb dissolving 10 ml of stock solution in 20 liters of tap water. Similarly 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400 (ppb) respectively dissolved in 12, 14, 16, 18, 20, 22, 24, 26, 28 ml in 20 liters of tap water. Synthetic wastewater for vermifilter bed was prepared 500, 600, 700, 800, 900, 1000, 1100, 1200, 1300, 1400 ppb respectively and dissolved in 2.5, 3.0, 3.5, 4.0. 4.5, 5.0, 5.5, 6.0, 6.5, 7.0 ml in 5 liters.

3.3 Sample collection

Daily outflow and inflow samples were collected for earthworm and earthworm with microbes and then preserved. For plant inflow and outflow, samples were collected after 72 hr retention period and preserved with 50 % HCL.

3.4 Experimental procedure

Vermifilter bed was made in 3 carets – in first control used only earthworm was used with simple tap water, in second unit earthworm was used with synthetic water of different concentration and third unit was prepared with earthworm and microbe's then different concentration of synthetic water was used. Influent of different concentration was poured and after 1 day retention period effluent was collected and preserved with 50 % HCL.

3.5 Chemical analysis

3.5.1Testing of arsenic in the water and soil sample

There are number of methods available to identify and determine total arsenic and arsenate. Unpolluted freshwater normally does not contain organic compounds, but may contain inorganic arsenic compounds. SDDC method was used with UV- spectrophotometer for the testing of arsenic contaminated sample in which arsine gas was generated in acidic solution, to the determination of total inorganic arsenic when interferences was absent and when the sample contained no methyl arsenic compounds.

3.5.2 Principal

Arsenic, in presence of Zn in acid medium gets reduced to arsine gas, AsH_3. Then it was passed through a scrubber tube containing glass wool soaked with lead acetate and later was absorbed in silver diethyl-dithicarbomate dissolved in pyridine. Arsenic reacted with the silver salt to form a red complex which can be determined calorimetrically. Many other metals such as Cr, Co, Cu, Hg, Mo, Ni, Pt, Sb and Ag interfere in the detection of As, but the concentration of these metals normally encountered in the waters were often less to produce any significant interference.

3.5.3 Procedure

About 35.0 ml samples was took into the arsenic generator and 5ml conc. HCl, 2ml KI solution, and 0.4ml (8drops) $SnCl_2$ reagent was added and mixed the sample after each addition. Kept for about 15 minutes. Then the glass wool was soaked in scrubber with lead acetate solution. Then 5ml of SDDC reagent was taken in the absorber tube and 3 gm Zn was added in the generator. Kept for about 30 minutes for the generation of arsine with slight heating of the generator. The gas absorbed in the SDDC reagent. The solution was removed from the absorber and the intensity was measured at the 535 nm using the reagent blank as a reference. Standard curve was prepared in the range of 0.0 to 10.0 µg. As by taking suitable volume of the standard solution and flowing the same procedure as for the sample.

Fig 4: Vermifiltration setups Earthworm species Tiger Worm (*Eisenia fetida*) was used.

4. Results and Discussion

Upon being providing Arsenic concentration from 500 ppb to 700 ppb the Arsenic concentration in effluent increases and at 800 ppb, the concentration of arsenic in effluent was maximum. At this point earthworm shows resistance capacity against arsenic toxicity and then after from 900 ppb to 1400 ppb the arsenic concentration in effluent was decreases gradually (Table 1). The result shows that after providing different concentration of Arsenic to earthworm and microbes the Arsenic in earthworm decreases gradually that means microbes play an important role as Arsenic munching (Table 2). This result indicates that the arsenic accumulation by earthworm is less as compared to earthworm with microbes (Table 3). It shows that microbes support earthworm or itself accumulate arsenic. Higher tolerance capacity of *Eisenia fetida* and Microbes were observed (Fig 5; 6; 7). All the results are shown in the form of table and the results are discussed below the tables

4.1 Bioremediation potential of *Eisenia fetida* and Microbes

Table 1: Result of sample water of Earthworm

Provided conc.	control	Effluent(Earthworm)
500	6	17
600	8	20
700	10	22
800	10	79
900	8	63
1000	10	59
1100	10	47
1200	8	38
1300	8	27
1400	10	21

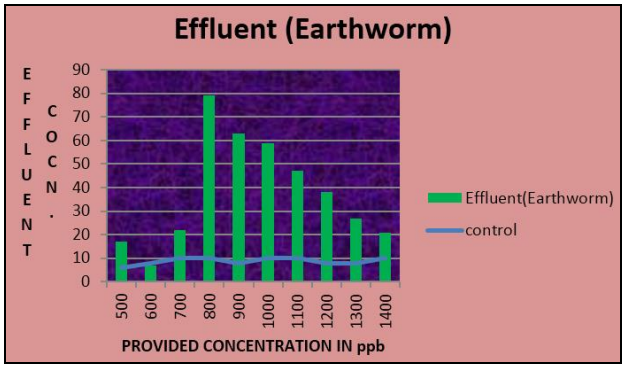

Fig 5: Effluent concentration (Earthworm)

Table 2: Result of sample water of Earthworm with Microbes

Provided conc.	Control	Effluent (earthworm +microbes)
500	6	10
600	8	14
700	10	7
800	10	5
900	8	5
1000	10	8
1100	10	7
1200	8	5
1300	8	9
1400	10	3

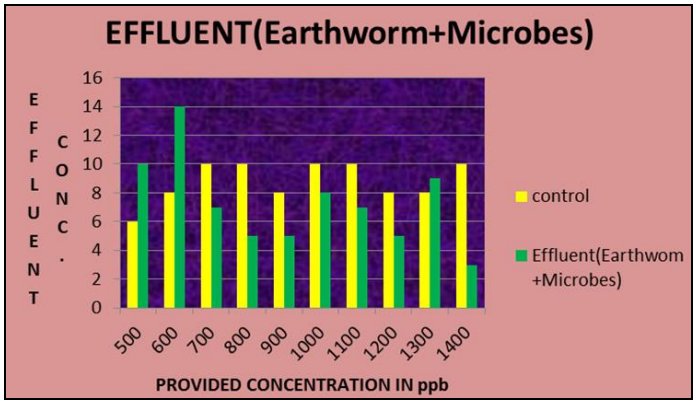

Fig 6: Effluent concentration (Earthworm + Microbes)

Table 3: Result of water sample Earthworm and Earthworm + Microbes

Provided conc.	control	Effluent (Earthworm)	Effluent (Earthworm with Microbes)
500	6	17	10
600	8	20	14
700	10	22	7
800	10	79	5
900	8	63	5
1000	10	59	8
1100	10	47	7
1200	8	38	5
1300	8	27	9
1400	10	21	3

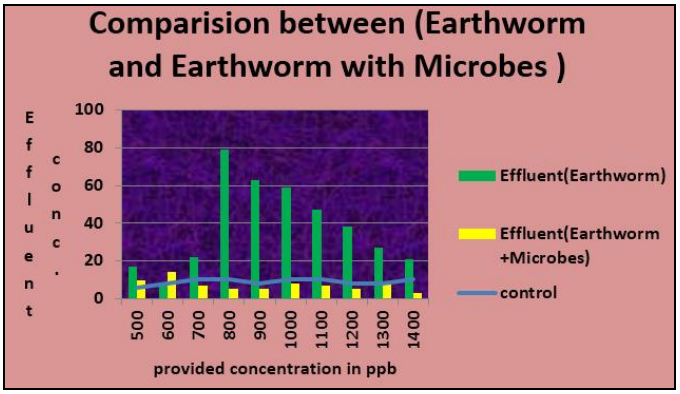

Fig 7: Effluent of Earthworm and Earthworm with Microbes

5. Conclusion

Exposure to the heavy metals through ingestion or uptake of drinking water particularly where water is reused and foods can lead to accumulation in animals, plants and humans. Several heavy metals such as Cd, as and Cr are considered hazardous waste metals that can accumulate in the human body with a relatively large half-life. Arsenic contamination in ground water in many areas of downstream Himalayas is mainly due to fluvial deposits, mainly of holocene age and later due to extraction of ground water. Arsenic is present in the environment both naturally and due to certain human activities. Earthworms are tolerant to moderate salt salinity in soil, but some species like the tiger worms (*Eisenia fetida*) has been found to be highly salt tolerant. The study suggests that the behavior of *Esenia fetida* alone with reference to tolerance of arsenic containing solution is not uniform. This indicates that microbes resist the transfer of arsenic to the test plant. It also indicates that microbes having the munching capacity of the arsenic. It was found that earthworm has capacity to remediate arsenic more as compared to plant. And in the next setup earthworm with microbes were used due to which arsenic remediating capacity increased. In this experiment the best bioremediation potential was earthworm with microbes interaction for arsenic contaminated water.

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