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# Use of Microbiological Assay for Study of Bioremediation by *Saccharomyces cerevisiae* and Phytoremediation by *Canna indica* of Heavy Metals (Hg &Cu).

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## Abstract

In this study, techniques of phytoremediation and bioremediation of heavy metals (Hg, Cu) using *Canna indica* and dry yeast granules *(Saccharomyces cerevisiae)* respectively were studied using microbiological assay. Microbiological assay was carried out using *Escherichia coli* as test organism and $CuSO_4$ and $HgCl_2$ solutions in the range of 1mg/L to 10,000mg/L and 0.1mg/L to 1000 mg/L (Serial dilutions) respectively. Zones of inhibition of growth of *E. coli* measured using vemier caliper as well as travelling microscope resulted in the range of diameter, 11.2 mm to 24.6 mm for $HgCl_2$ and 8.3 to 15 mm for CuSO4. A standard graph of log of concentrations of the solutions against the zones of diameters resulted into straight line. When bioremediation of $CuSO_4$ and $HgCl_2$ using 2% and 5% dry yeast granules (enumerated using improved Neubauer's chamber, which was found to be $159x10^6$ yeast cells per granules) each was performed and the solutions were subjected to microbiological assayafter3and4days, the diameter of zone of inhibition after 3 days using $CuSO_4$ was 8.5 mm which on standard graph corresponded to 2mg/L .This was 98% reduction in $CuSO_4$. No zones of inhibition were observed after 4 days. When compared with standard graph, it indicated that the concentration of $CuSO_4$ remaining in the solution was less than 1 mg/L after 4 days. This was equivalent to 99% reduction in the concentration of $CuSO_4$. With $HgCl_2$, no zones of inhibition were seen after 3 and 4 days. The concentration of $HgCl_2$ remaining in the solution after 3 and 4 days, when compared with the standard graph, was less than 0.1mg/L. This was also equivalent to 99% reduction. Same procedure was followed for phytoremediation and it showed the same percent reduction (90% and 99% respectively) as bioremediation but after 4 days. Significance- microbiological assay is inexpensive, feasible as compared to the Atomic Absorption Spectroscopy (AAS) which requires a sophisticated instrument. This technique may be employed at early stage of study for selection of microbial strains and plant species.

**Keywords:** Phytoremediation, Bioremediation, Dry yeast granules, *Canna indica,* Microbiological assay.

## 1. Introduction

Due to explosion of population, there is tremendous increase in anthropogenic activities and massive industrialization which has resulted into unmanaged use of agro-chemicals, uncontrolled burning of fossil fuels and improper dumping of sewage sludge. Such activities have caused soils and waterways to be severely contaminated with heavy metals (Kapachi and Sahaveva, 2019).

Effluents discharged from various industries contain several organic and inorganic pollutants. Among these pollutants, heavy metals are extremely important as they have toxic effects on human beings and other living creatures. They also may prove to be carcinogenic and induce development of various cancers in animals (MacCarty *et al*, 1993; Clement *et al* 1995; Renge *et al*,2012). Unlike organic contaminants, these pollutants are not biodegradable and can be transferred through the food chain via bioaccumulation (Siddique *et al*, 2015). Heavy metals are known to interact with nuclear proteins and DNA and have oxidizing effects responsible for damage of these molecules and associated toxicity (Briffa *et al*, 2020).Some of the heavy metals polluting environment are cadmium (Cd), lead (Pb), aluminum (Al), zinc (Zn), manganese (Mn), chromium (Cr), mercury (Hg) and copper (Cu) which are considered as common toxic heavy metals.

Volesky in 1994 and Domenech in 1998 shortlisted some conventional methods for removing the dissolved heavy metals like chemical precipitation, filtration, ion exchange, oxidation or reduction, reverse osmosis, evaporation, membrane technology, and electrochemical treatment. However, most of these techniques become ineffective when the concentrations of heavy metals are less than 100 mg/L (Ahluwalia & Goyal, 2007). Additionally, use of strong and contaminating reagents for desorption, results into toxic sludge and secondary environmental pollution (Anushree M, 2004).

The alternative method for detoxification of these metals is bioremediation which refers to growth of certain microorganisms, which have the properties of degrading heavy metals present in the system, according to their capacity and concentration and decrease the level of toxicity of the given system. The main principle is degrading and converting pollutants to less toxic forms. Most of the indigenous microbes have the ability to successfully bring up the environmental restoration via oxidizing, immobilizing, or transforming the contaminants (Vishwakarma, 2020). Number of microbes including aerobic, anaerobic bacteria and fungi are involved in bioremediation process. Bioremediation is highly involved in degradation, eradication, immobilization, or detoxification of diverse chemical wastes and physical hazardous materials from the surrounding through the action of microorganisms. While implementing bioremediation, two main factors are taken into consideration i.e. biotic and abiotic factors. The major biological factors include enzyme activity, interaction (competition, succession, and predation), mutation, horizontal gene transfer, its growth for biomass production, population size and its composition. The abiotic factors involve the interaction of environmental contaminants with metabolic activities, physiochemical properties of targeted microorganism during the process (Indu Sharma, 2020).

Researchers have used several micro-organisms for bioremediation of heavy metals. These mainly include bacteria, molds and yeasts. These microorganisms have evolved various measures to respond to heavy-metal stress via processes such as transport across the cell membrane, biosorption to cell walls and entrapment in extracellular capsules, precipitation, complexation and oxidation-reduction reactions (Veglio & Beolchini, 1997).

One of the most widely available biomass for bioremediation of heavy metals is yeast cells that retain their ability to accumulate a broad range of heavy metals to varying degrees under a wide range of external conditions (Abbas & Badr, 2015). *Saccharomyces cerevisiae* is a yeast that possesses phosphate, amino, carboxyl and hydroxyl groups in its cell wall, which are responsible for the removal of heavy metals (Gohari *et al*, 2013).

Phytoremediation is a technology that exploits the natural capacity of green plants to remove contaminants from the environment. Phytoremediation is preferable because it is safe and has lower cost as compared to physical and chemical remediation. The technologies of metal phytoremediation include phytoextraction, phytostabilization, and phytovolatilization. Among these, phytoextraction is one of the promising techniques being used for reclaiming the metal polluted soils (Hassan, *et al*, 2019).

Advantages of phytoremediation are:

(i) Phytoremediation is an autotrophic system powered by solar energy, hence, easy to manage, and the cost of installation and maintenance is low.
(ii) Environment and eco-friendly—it can reduce exposure of the pollutants to the environment and ecosystem,
(iii) Applicability—it can be applied over a large-scale field and can easily be disposed.
(iv) It prevents erosion and metal leaching through stabilizing heavy metals, reducing the risk of spreading of contaminants.
(v) It can also improve soil fertility by releasing various organic matters to the soil (Wuanna and Okieimen, 2011; Jacob *et al,* 2018).

During the past decades, numerous studies have been conducted to understand the molecular mechanisms underlying heavy metal tolerance and to develop techniques to improve phytoremediation efficiency.

The main disadvantages of phytoremediation are contamination concentration in plant, toxicity, bioavailability and Plant choice and stress tolerance by the plants. Accumulation of pollutant in fruit and other edible parts is not desirable.Due to low biomass production in phytoremediators, several plantings and harvesting is required for decontamination (Pilipović 2008). The process of phytoremediation is slow and season specific (Chintakovid *et al*, 2008).

Reserachers have tried several plants for phytoremediation.Reeves and Baker in 2000 reported total 45 plant families known to contain metal-accumulating species.

Water hyacinth (*Eichhornia spp.)* is a fast growing perennial aquatic macrophyte and has the great reproduction potential. It is resistant to various pollutants and is very commonly used for phytoremediation (Shufield, 2009). Theeta Sricoth *et al* in (2017) studied the ability to remove nutrients, organics, and heavy metals from wastewater by mixture of *Eichhornia crassipes* and *Typha angustifolia.* V. Subhashini and Swamy in 2014 used *Canna indica* for phytoremediation of heavy metals.

Almost all researchers have used atomic absorption spectroscopy (AAS) to measure efficiency of heavy metals removal by bioremediation (Cherlys Infante, 2014; Bhupendra *et al*, 2019) or phytoremediation (V.Subhashini and Swamy, 2014). Though the technique is very sensitive and reliable, the cost of the instrument and availability are major constraints in use of AAS.

Microbiological assay is a simple, economical method which can be used for study of efficiency of bioremediation as well as phytoremediation. Though this method is not as sensitive as AAS, it can be used in early stages of study to compare efficiency of various microorganisms for bioremediation and plants for phytoremediation. After selection of particular micro-organism or plant, in the last stage of study, AAS may be employed to confirm that the levels of toxic heavy metals have been reduced to permissible level which are 1.3mg/L for Cu and 0.001mg/L for Hg (As per Maharashtra Pollution Control Board) . Hence, in the current study microbiological assay has been used as a tool to measure the efficiency of heavy metal(Cu and Hg) removal by bioremediation and phytoremediation.

## 2. Materials and Methods

All experiments were carried out in triplicate and mean values were considered for calculations.

### 2. 1 Bioremediation of Cu and Hg using dry yeast granules (*Saccharomyces cerevisiae*)

#### 2.1.1 Standard graph preparation for microbiological assay of $CuSO_4$ and $HgCl_2$ using *Escherichia coli*.

For estimation of $CuSO_4$ and $HgCl_2$, microbiological assay was used. It was carried out by agar well diffusion method (Bauer *et al,*1966) Standard graph for this assay was prepared by spreading 0.1ml of 24 hours old culture of *Escherichia coli* (grown in sterile MacConkey's broth)on sterile MacConkey's agar( Selective medium for *Escherichia coli*). Using alcohol sterilized cork borer (8mm diameter) wells were made in the medium. Different dilutions of $CuSO_4$ in the range of 1mg/L to 10,000 mg/L and of $HgCl_2$ in the range of 0.1mg/L to 1000mg/L were filled in these wells(Dilutions were prepared using serial dilution method). All dilutions were prepared using sterile distilled water. One well was filled with sterile distilled water and was used as 'control'. All plates were incubated at $37^0$C for 24 hours. After incubation, zones of inhibition of growth of *Escherichia coli* were observed and measured using Vernier caliper and

travelling microscope (**Figures 1 and 2**). A *standard* graph was prepared by plotting log of concentration versus diameter of zone of inhibition (Cooper, K.E. edited by Kavanagh, F., 1963).


**Figure 1**: Measurement of diameter of inhibition zone by Vernier caliper

**Figure 2**: Measurement of diameter of inhibition zone by travelling microscope

### 2.1.2 Enumeration of yeast cells per granule using improved Neubauer's chamber

Dry yeast granules (*Saccharomyces cerevisiae*) of same size were selected from the commercially available packet. Average number of yeast cells per granule was determined by enumerating them using improved Neubauer's chamber. For counting 4 corner squares (1mm x1mm) were used.

### 2.1.3 Inoculation of yeast cells in $CuSO_4$ and $HgCl_2$ solutions for bioremediation

Two flasks of 500ml of $CuSO_4$ solution with 100mg/L concentration, pH 7 were prepared using sterile distilled water. One flask was inoculated with 2% yeast granules (Total 10) and the other one was inoculated with 5% yeast granules (Total 25).Using the same method two flasks of $HgCl_2$ solution with 10mg/L concentration, pH7 were prepared. One flask was inoculated with 2% yeast granules and the other with 5% yeast granules (**Figures 3& 4** ).

All flasks were kept at room temperature and estimation of $CuSO_4$ and $HgCl_2$from these flasks was carried out after 1,3 and 4 days using the same method for microbiological assay, i.e solution was filled in a well, made on sterile MacConkey's agar spread with 24 hours old culture of *Escherichia coli*, incubated for 24 hours, diameter of zone of inhibition of growth was measured. The value was plotted on the standard graph and extrapolated on X-axis to determine the concentration of both the solutions.

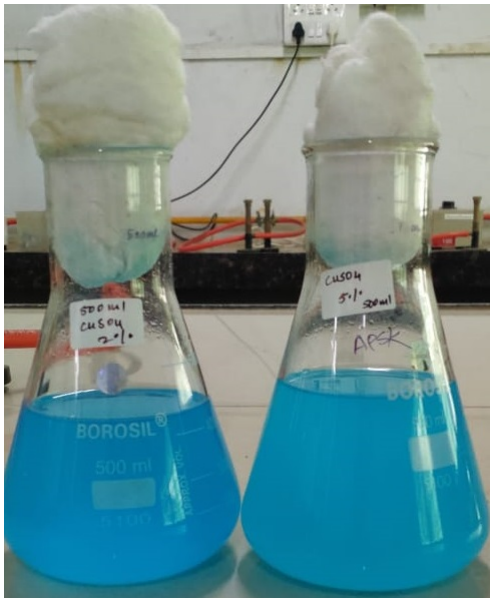


**Figure 3**: $CuSO_4$ solution (100mg/L) inoculated with 2% and 5% yeast granules


**Figure 4**: $HgCl_2$ solution (10 mg/L) inoculated with 2% and 5% yeast granules

## 2.2 Phytoremediation of Cu and Hg using *Canna indica*.

### 2.2.1 Standard graph preparation for microbiological assay.

Using the same technique as for bioremediation, a standard graph was prepared and used for estimating efficacy of phytoremediation by *Canna indica*.

### 2.2.2 Placing *Canna indica* saplings in $CuSO_4$ and $HgCl_2$ solutions for phytoremediation

Two saplings of *Canna indica* of equal size and with intact roots were selected. They were immersed in distilled water for 24 hours and then thoroughly washed several times with distilled water to remove all adhering soil particles. Finally they were placed in bottles filled with 500 ml of 100mg/L $CuSO_4$solution and 10mg/L $HgCl_2$ solution (**Figures 5 & 6**). Estimation of concentration of $CuSO_4$ and $HgCl_2$ was done by microbiological assay after 1, 3 and 4 days.

**Figure 5**: *Canna indica* placed in $CuSO_4$ solution

**Figure 6**: *Canna indica* placed in $HgCl_2$ solution

## 3. Results and Discussion

### 3.1 Bioremediation of Cu an Hg using dry yeast granules (*Saccharomyces cerevisiae*)

#### 3.1.1 Standard graph preparation for microbiological assay of CuSO4 and $HgCl_2$ using *E. coli*.

Clear zones of inhibition (**Figure 9**) were observed around almost all wells. They were measured in mm (**Tables 1&2**). Graphs were plotted taking log of concentration Vs. diameter of zone of inhibition.A straight line graph was obtained for both$CuSO_4$ and $HgCl_2$ (**Figures 7 &8**).

The results obtained in the current study could not be compared with other researchers as almost all have used directly atomic absorption spectroscopy for determination of efficacy of bioremediation.

**Table 1:** Results of microbiological assay for $HgCl_2$

| Sr. NO. | Concentration of $HgCl_2$ Mg/L | Diameter of zone of inhibition (mm) |
|---|---|---|
| 1 | Control | 0 |
| 2 | 0.1 | 11.2 |
| 3 | 1 | 12.4 |
| 4 | 10 | 16.4 |
| 5 | 100 | 21.7 |
| 6 | 1000 | 24.6 |

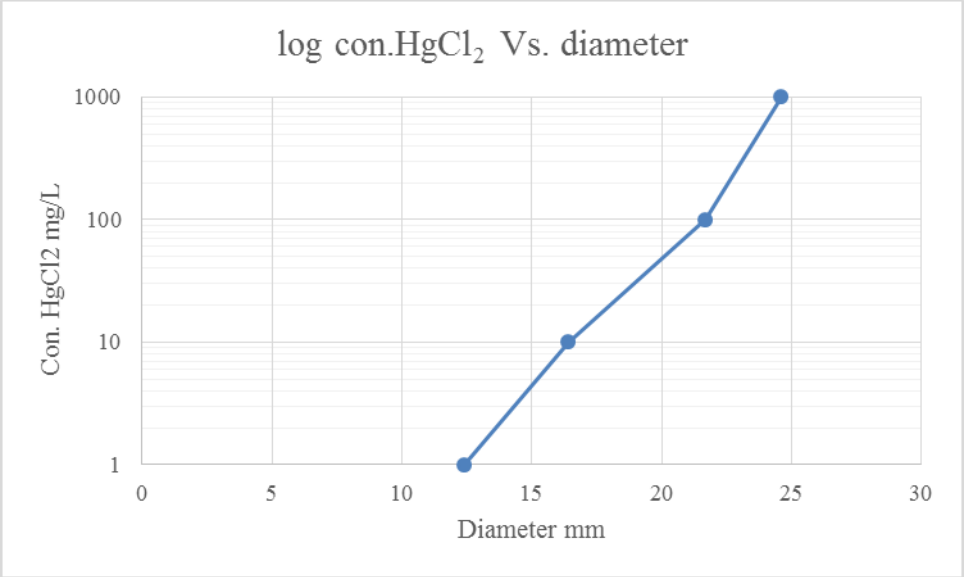


**Figure 7:** Standard graph of assay for $HgCl_2$

**Table2:** Results of microbiological assay for $CuSO_4$

| Sr. NO. | Concentration of $CuSO_4$ mg/L | Diameter of zone of inhibition (mm) |
|---|---|---|
| 1 | Control | 0 |
| 2 | 1 | 8.3 |
| 3 | 10 | 10.2 |
| 4 | 100 | 11.2 |
| 5 | 1000 | 13.4 |
| 6 | 10000 | 15 |

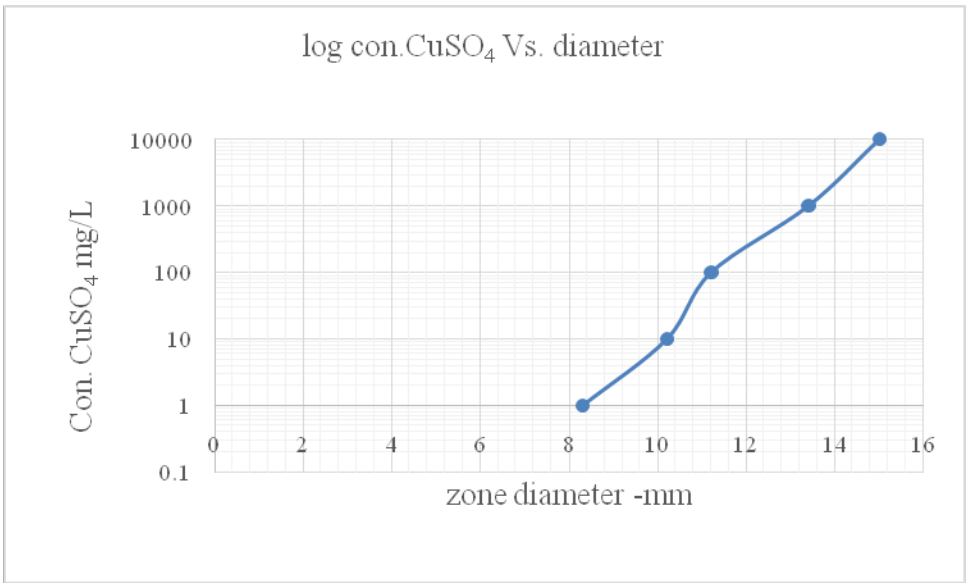


**Figure 8:** Standard graph of assay for $CuSO_4$

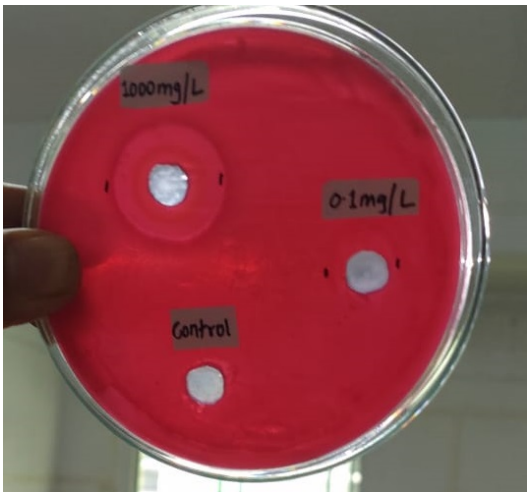


**Figure 9:** Zones of inhibition around wells filled with $HgCl_2$ solution.

### 3.1.2 Enumeration of yeast cells per granule using improved Neubauer's chamber

When yeast granules were subjected to enumeration by improved Neubauer's chamber the average count of yeast cells per granule was 159 x $10^6$. In order to provide sufficient number of yeast cells, these granules were used at 2% and 5% inoculation.

### 3.1.3 Inoculation of yeast cells in $CuSO_4$ and $HgCl_2$ solutions for bioremediation

When 100mg/L solution of $CuSO_4$ and 10mg/L solution of $HgCl_2$ were inoculated with 2% and 5% yeast granules for bioremediation and subjected to microbiological assay after 3 and 4 days, the diameter of zone of inhibition after 3 days using $CuSO_4$ was 8.5 mm which on standard graph corresponded to 2mg/L .This was 98% reduction in $CuSO_4$. There were no zones of inhibition after 4 days. When compared with standard graph, it indicated that the concentration of $CuSO_4$ remaining in the solution was less than 1mg/L after 4 days. This was equivalent to 99% reduction in the concentration of $CuSO_4$.

With $HgCl_2$, Zones of inhibition obtained after 3 and 4 days were very minute (**Figure 10**) (almost unmeasurable). The concentration of $HgCl_2$ remaining in the solution after 3 and 4 days, when compared with the standard graph, was less than 0.1mg/L. This was also equivalent to 99% reduction.

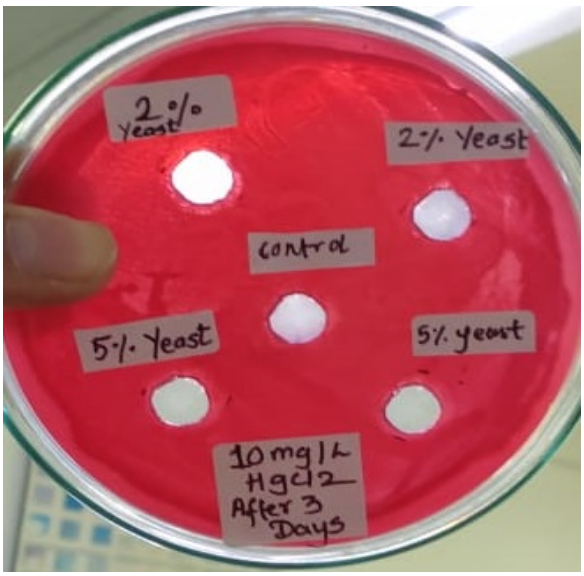


**Figure 10:** Results of bioremediation after 3 days

Infante *et al* in 2014 had also used *Saccharomyces cerevisiae* for removal of lead, mercury and nickel from aqueous solution. They have reported 69.7% removal of mercury. In current study, higher % reduction in concentration of both the heavy metals solutions was noted.

### 3.2 Phytoremediation of Cu and Hg using *Canna indica.*

#### 3.2.1 Standard graph preparation for microbiological assay

The same standard graphs for microbiological assays prepared using concentration of $CuSO_4$ and $HgCl_2$ versus diameter of inhibition zones in mm were used for interpretation of results of phytoremediation.

#### 3.2.2 Placing *Canna indica* saplings in $CuSO_4$ and $HgCl_2$ solutions for phytoremediation

When saplings of *Canna indica* were placed in $CuSO_4$ and $HgCl_2$ solutions and microbiological assays were carried out for both the solutions after 4 days, no zones of inhibition were observed (**Figure 11**).No adverse effect was observed on plants upto 3 days. On 4$^{th}$ day slight wilting of leaves of plant immersed in $HgCl_2$ solution was observed.


**Figure 11:** Results of phytoremediation after 4 days

These results indicated efficiency of phytoremediation equivalent to 90% for $CuSO_4$ and 99% for $HgCl_2$. V., Subhashini and Swamy A.V.V.S. in 2014 had experimented phytoremediation of Metals (Pb, Ni, Zn, Cd and Cr) Contaminated Soils Using *Canna indica*. The results obtained in the current study are comparable with them.

## 4. Conclusions

Microbiological assay proved to be an useful method to study bioremediation and phytoremediation of heavy metals at larger concentrations. Though it cannot be used to detect permissible concentrations of these heavy metals in water which are very low and beyond the sensitivity of this technique. At such low concentrations, they can be estimated only by sophisticated instrument like atomic absorption spectroscopy. However, initial study for selection of efficient microbial strain or a suitable plant, this method can be definitely employed. Advantages of this method are, it does not require any sophisticated instrument which is very expensive, simple to perform and results can be easily observed and interpreted. In the current study efficiencies of bioremediation of Cu and Hg by yeast cells and phytoremediation of these heavy metals by plant *Canna indica* was excellent(90 to 99%) and comparable with other researchers. In the last step use of AAS may help to ensure reduction in these heavy metals up to permissible levels.

## Statement of conflict of interest

The authors declare that there is no conflict of interest.

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