Inoculation of *Bacillus cereus* enhance phytoremediation efficiency of *Pistia stratiotes* and *Eichhornia crassipes* in removing heavy metal Pb

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**Abstract.** This study investigates the effects of inoculation rhizospheric microbes namely *Bacillus cereus*-NMeHI-Cr2 towards the roots of two locally aquatic macrophytes in removing heavy metals Pb from simulated wastewater. Water lettuce (*Pistia stratiotes*) and water hyacinth (*Eichhornia crassipes*) were selected in this study due to their absorption capability and acts as biological filters by accumulating heavy metals in their tissue’s bodies. The inoculation of *B. cereus*-NMeHI-Cr2 onto the roots of *P. stratiotes* and *E. crassipes* was conducted in non-continuous horizontal flow (NCHF) reactor with addition of 1.5mg/L of Pb comprising of T2: treatment with inoculation of *B. cereus*-NMeHI-Cr2, T4: Non inoculation of *B. cereus*-NMeHI-Cr2 and T6: no plant growth in NCHF reactor (control). After 15 days of cultivation, the treatment integrated with inoculation of *B. cereus*-NMeHI-Cr2 resulted 83.7% removal efficiency of Pb followed by T4 (70.4%) and T6 (2%) respectively. Furthermore, 63.8 ug/g of Pb was observed in T2 treatment with similar distribution pattern as roots > leaves > stalks in *E. crassipes* and roots> leaves in *P. stratiotes*. The physical changes of both plants were also monitored to show any toxicity symptoms experienced as a result of heavy metal Pb. This study validates that the removal of Pb was done via rhizofiltration mechanism as the value of enrichment factor was found below than 1000. The identified plants species with inoculation of *Bacillus cereus*-NMeHI-Cr2 provides a simple and cost-effective method for application of metal polluted water.

1. **Introduction**

Water pollution comes in various forms with its associated effects, depending on the type of pollutant entering the environment [1]. Some heavy metal for example lead is widely used due to its physical and chemical properties such as malleability and ductility. Thus, it can be found in many industries and places. Sources of lead includes leaded gasoline, smelting of lead, lead based paint, as well as ingestion of water obtained from leaded pipes. The emission of lead from these activities can be bioaccumulated in the bones of living things that can subsequently be released into the blood during pregnancies, menopause, or lactation, causing deleterious effects (anaemia, kidney damage, fertility reduction and decrease cognitive function) [2]. Besides that, the excessive uptake of lead in plants will induce stunted growth, the blackening of roots, as well as chlorosis [3]. Phytoremediation can be defined as a low cost, energy-friendly, as well as aesthetic method of using green plants to restore, detoxify, and reclaim places that are polluted with varying levels of environmental pollutants [4]. This
method required simple operation and maintenance, and at the same time is efficient in the removal of various types of pollutants such as organic matter, nutrients, and heavy metals [5]. The combination with rhizoremediation technique is one of the bioremediation processes through which root are inoculated with beneficial microorganisms to increase the efficiency of contaminants in the extraction process [6]. This process works as win-win situation whereby the attachment of microorganisms would increase the availability of the compounds; thus, the roots of plants help in the removal of compound in contaminated water. Previous studies on phytoremediation had shown that the inoculation of beneficial microorganisms towards roots of plants have advantages to host plants against heavy metal stress [7]. Therefore, this study aims to highlight the rhizoremediation effect of both plants with inoculation of B. cereus-NMeHI-Cr2 in removing heavy metal Pb from simulated wastewater. The distribution patterns of lead in both plant tissues will also discussed in this paper.

2. Materials and method

2.1. Aquatic macrophytes sampling
Two locally indigenous aquatic macrophytes Pistia stratiotes (water lettuce) and Eichhornia crassipes (water hyacinth) were selected to access their ability in removing metal Pb from simulated wastewater under laboratory scale. P. stratiotes and E. crassipes (Figure 1) were sampled from unpolluted sites (Kg. Bolong Lama, Tuaran and Kg. Kibabaig, Penampang), Sabah and transported to the laboratory using 20L tank. Both plants were rinsed with tap water and distilled water to remove waste and any undesirable sediment. Cleaned plants were placed into a 40L tank and were acclimatized for 2 weeks in UMS lake water at the laboratory for prior experiments. Light was provided by fluorescent light in the lab and cultivation at room temperature.

![Figure 1](image1.png)

**Figure 1.** Selected plants: (a) *P. stratiotes* and (b) *E. crassipes* growing in natural habitat used as phytoremediation of heavy metal Pb.

2.2. Experimental set-up
Non-continuous horizontal flow (NCHF) reactor was designed with 1 glass tanks being set up horizontally. The measurements glass of the tank is (30 x 60 x 30 cm) as illustrated in Figure 2. A bacterial suspension with $10^8$ cell mL$^{-1}$ was prepared and submerged in the roots of plants for 10 minutes as described by [8]. The details of experiments conducted were presented in Table 1.
Figure 2. Experimental set-up on the removal of Pb using Non-Continuous Horizontal Flow (NCHF) reactor

| Treatment (T) | Experiment |
|--------------|------------|
| T2 | 50g of *P. stratiotes* and *E. crassipes* were inoculated with rhizospheric bacteria *B. cereus*-NMeHI-Cr2. Approximately 1.5 mg/L of Pb was added at 1.5 mg/L concentration in 40L Lake UMS water. |
| T4 | 50g of *P. stratiotes* and *E. crassipes* were cultivated in 40L Lake UMS water with 1.5 mg/L concentration of Pb was added in the NCHF reactor. |
| T6 (Control) | There are no plants cultivated in 1.5 mg/L concentration of Pb in the NCHF reactor. |

2.3. Sample analysis
The simulated wastewater was sampled at 15 days intervals throughout the experiment. The concentration of Pb in the samples were collected in duplicate each day with the use of clean 50 mL polyethylene bottles. Prior to sampling, the simulated wastewater was gently stirred to ensure the collection of a homogenous sample. For heavy metal analysis in the plant samples, the roots, leaves, and stalks were collected at Day 0 and day 15 in duplicate. The samples obtained were placed into a clean aluminium container and dried in an oven at 75°C for 24 hours. The samples were digested using Nitric acid as according to USEPA method 3050b. The final solution was poured into an acid-washed polyethylene bottle and placed in refrigeration at below 4°C prior to heavy metal analysis using Inductively Coupled Plasma Spectroscopy (ICP – OES). Standard calibration curve for metal Pb was established to determine their concentrations.

2.3.1. Removal efficiency and distribution of metals Pb by both aquatic plants
Heavy metal removal efficiency was used to determine how well both plants to remove Pb from simulated wastewater [9]. The formula used to calculate heavy metal removal efficiency is shown below:

\[ Removal (\%) = \frac{T_i - T_f}{T_i} \times 100 \]  

where:
\[ T_i = \text{Total Pb initial (mg/L)} \]
$T_f = \text{Total Pb final (mg/L)}$

\[ Uptake \ of \ Pb \ in \ plant \ parts \ (\mu g/g) = \frac{C_f - C_i}{D_w} \times T \] (2)

where:
- $C_f$ = Concentration final in plant parts (ug/mL)
- $C_i$ = Concentration initial in plant parts (ug/mL)
- $T_v$ = Total volume (mL)
- $D_w$ = Weight of biomass (g)

2.3.2. *Phytoremediation mechanism uptakes of P.stratiotes and E. crassipes by determining the Enrichment Factors (EF) and Translocation Factors (TF).*

The enrichment factors and translocation factors of heavy metal Pb in simulated wastewater and plant parts were calculated. The enrichment factors (EF) measuring the degree of enrichment and transfer of metals into both plant samples from simulated wastewater was evaluated by the ratio of the metal in plants (ug/g) to the metal in the simulated wastewater (mg/L). The translocation factors (TF) which refers to the transfer of metals (ug/g) to the shoot from the roots (ug/g). The EF \cite{10} and TF \cite{11} formulae was calculated as below:

\[ EF = \frac{C_{plants}}{C_{water}} \] (3)

where:
- EF = Enrichment factors
- $C_{plants}$ = concentration in plants
- $C_{water}$ = concentration in water

\[ TF = \frac{C_{shoot}}{C_{root}} \] (4)

where:
- TF = Translocation factors
- $C_{shoot}$ = concentration in shoots
- $C_{root}$ = concentration in roots

3. Results and discussion

3.1. *Effect of inoculation on removal efficiency of Pb by both aquatic plants in NCHF reactor*

![Figure 3. Pb removal efficiency by P. stratiotes and E. crassipes in NCHF reactors at different treatments: T2: inoculation of B. cereus-NMeHI-Cr2; T4: Non inoculation of B. cereus-NMeHI-Cr2 and T6: Non cultivation of plants in NCHF reactor (Control)](image-url)
Figure 3 shows the residual concentration of Pb at different times upon different exposure experiments in NCHF reactors. On the contrary, treatment T6 (without plants) in control reactors do not evidence significant changes. The data shows that experiment containing P. stratiotes and E. crassipes with inoculation of B. cereus-NMeHI-Cr2 (T2) has a sudden drop on the first days of the experiment and the removal efficiency was continuing to decrease until day 15. On the other hand, T4 with non-inoculation of rhizospheric microbes shows that the removal of Pb was slightly decrease. Overall, after 15 days of the experiment the removal of Pb was highest in treatment T2 with 84% followed by T4 (70%) and control; T6 (2%) respectively. The results indicates that the effects of rhizospheric microbes towards both plants had increased the removal efficiency of Pb. This finding agrees with [12] stated that inoculation of beneficial microorganisms in the roots had aid in Pb removal process.

The characteristics of B. cereus -NMeHI-Cr2 which is Gram positive bacteria that contains of teichoic acids and acids associated to the cell wall, plays an important role in the uptake of Pb. These results are in line with [13], whereby the treatment of contaminated soil with heavy metal by Trofoliumrefens had shown that removal efficiency of metals is highly increased with inoculation of B. cereus as compared to control treatment. He found that the associated acids Indole acetic acid (IAA) was produce by B. cereus during the treatment process thus increase the bioaccumulation ability exhibited by the bacterial cells. The data proved that the inoculation of B. cereus -NMeHI-Cr2 towards roots of P. stratiotes and E. crassipes had increased the amount of Pb removal by both plants. Along the experiment, the physical changes of both plants were observed indicates that toxicity symptoms might occur due to toxic effect of Pb. Based on the observation, both plants in treatment T2 were recorded healthy within the exposure times of experiments. However, a physical change on P. stratiotes was observed in T4 whereby the leaves of this plant are wilting and yellowing after day 10 of exposure. This is due to the toxic effect of Pb whereby the capability to tolerate to this high concentration of Pb are decreased as exposure time is increased. Table 2 shows the summary observation that have been recorded during 15 days of the experiment.

Table 2. Pb removal efficiency by P. stratiotes and E. crassipes using NCHF reactors and toxicity symptoms observed during 15 days of treatment

| Treatments                        | Removal (%) | Toxicity symptoms | Roots | Leaves | Stalks |
|----------------------------------|-------------|-------------------|-------|--------|--------|
|                                  |             |                   | Fragile | Decay | Yellowing | Wilting | Wilting |
| T2 - Inoculation of B. cereus-NMeHI-Cr2 | 83.7        | -                 | -      | -      | √       | -       | -       |
| T4 - Non inoculation of B. cereus-NMeHI-Cr2 | 70.4        | -                 | -      | -      | √       | -       | -       |
| T6 - Control (No cultivation of plants) | 2.0         | -                 | -      | -      | √       | -       | -       |

*P = P. stratiotes, *E = E. crassipes * √ = positive physical changes observed

3.2. Uptake and distribution of Pb in plant tissues of P. stratiotes and E. crassipes
Figure 4 showed the accumulation of Pb in plant tissues of P. stratiotes and E. crassipes after 15 days of cultivation. The results showed that the highest uptake of Pb was observed in roots of E. crassipes inoculated with B. cereus-NMeHI-Cr2 with 25.6 µg/g whereas the stalks in T4 reactor showed the
lowest uptake with 6 ug/g. It can be note that a similar pattern of distribution in both plants’ parts can be found higher in roots as compared to leaves and stalks. This finding is similar to previous studies done by [14] mentioned that the amounts of metals are mainly deposited in roots rather than leaves and stalks. [14] stated that the roots is the major storage and collection of metals in plants followed by vacuoles of epidermis and bundle sheath of leaves respectively.

![Image of distribution of Pb](image)

**Figure 4.** Distribution of 1.5 mg/L of Pb in roots and leaves (*P. stratiotes*) and roots, leaves and stalks (*E. crassipes*)

The summary distribution patterns of these metals in plant tissues of *P. stratiotes* are in descending order from roots to leaves whereas for *E. crassipes* the pattern is as roots> leaves> stalks. It is important to note that the correlation of the amount of metal removed from simulated wastewater with the amount of metal uptake by both plants was significant. This is proved by the metal’s uptake by both aquatic plants in NCHF reactor with the decreasing metals concentration after 15 days of the cultivation. It is important to note that the effects of rhizospheric microbes and roots of both plants had great influence in uptake Pb from simulated wastewater. In this study, both plants and *B. cereus-NMeHI-Cr2* had shown synergistic effects whereby the plant provides bacteria with a specific carbon source, thus this induces bacteria to reduce the phytotoxicity of contaminated water. It is interesting to know that roots of *P. stratiotes* and *E. crassipes* form an association with *B. cereus-NMeHI-Cr2* which increase the remediation activity thus stimulate the microbial community to boost metabolic activity in degrading Pb [15]. The present findings suggested that the effects of rhizospheric microbes (*B. cereus-NMeHI-Cr2*) increase both plant biomass and thereby stabilize the biochemical process [16]. Besides that, *B. cereus-NMeHI-Cr2* have shown to produce auxin which in turn eliciting the plant growth, increase the metal uptake and enhance systematic resistance. This suggest that that both plants can be used as phytoremediation plants for removal of Pb in industrial wastewater.

### 3.3. Enrichment factor of Pb in *P. stratiotes* and *E. crassipes*

Figure 5 showed the enrichment factors of Pb in roots and shoots of *P. stratiotes* and *E. crassipes* cultivated in NCHF reactors. The EF of Pb in both plants are observed in range of 12.3–28.3. The highest EF can be found in *E. crassipes* in T2 with (28.3) whereas the lowest can be observed in *P. stratiotes* in T4 with (12.3). This finding shows that the roots of both plants play as a main role in close with simulated wastewater. As roots is the main source of nutrients for these plants, the inoculation of *B. cereus-NMeHI-Cr2* in T2 had showed that both plant roots growth healthy with hairy
branching roots thus makes it available in absorbed metals into roots as compared to shoots. This finding is in line with [17], whereby the effects of rhizospheric microbes towards both roots system had resulted in high EF of roots as compared to shoots in both plants studied.

According to [16] some bacteria had shown internal absorption of metal in certain part of bacteria cells or dispersed throughout the cell in nanoparticle and had a mechanism in protecting itself from acute toxicity effect by the shortening and forming wooly coat around the cells in order to decrease surface area from further contact with metal ions. However, both plants in this study are not considered as hyperaccumulator of Pb as the EF obtained in roots and shoots are below than 1000 [10]. Since the EF of Pb in both plants are in the range of 12.3 – 28.3, they are only best classified as rhizofiltration plants as suggested by [10].

![Graph 1](image1.png)

**Figure 5.** Enrichment factors of Pb in roots and shoots of *P. stratiotes* and *E. crassipes* at different treatments studied

3.4. **Translocation factor of Pb in *P. stratiotes* and *E. crassipes***

![Graph 2](image2.png)

**Figure 6.** Translocation factors of roots and shoots in *P. stratiotes* and *E. crassipes* cultivated in NCHF reactors

Translocation factor is an important indicator to mark the accumulation and storage of heavy metals from roots to aerial part. Figure 6 showed the translocation factors of Pb in roots and shoots of *P. stratiotes* and *E. crassipes*. Data on TF showed that the average value for by both plants studied are
below than 1 which indicates that these plants have limited translocation of Pb to the aerial part. The highest TF value was found in *P. stratiotes* (T4) with 0.76 while the lowest was observed in *E. crassipes* (T4) with 0.58. [18] reported that the TF value which is higher than 1 indicates that the plants is accumulator species whereby this plant have an efficient metal transporter system to transport metal from roots to leaves [19]. Otherwise for TF value lower than 1, this plant generally known as metal excluder species. It is worth to know that the effects of rhizospheric microbes *B. cereus*-NMeHI-Cr2 towards roots of *P. stratiotes* and *E. crassipes* had displayed a low significance in translocating Pb into the aerial part. However, *P. stratiotes* and *E. crassipes* had perform as good rhizofiltration mechanism plants and can be used to remove heavy metal especially Pb from industrial wastewater.

4. Conclusion

The effects of rhizospheric microbes *B. cereus* -NMeHI-Cr2 onto the roots of *P. stratiotes* and *E. crassipes* had displayed higher removal efficiency of Pb with 83.7%. On the other hand, experiments with non-inoculation showed a lowest removal percentage with 13.3% differences. The data suggested that the inoculation of *B. cereus* -NMeHI-Cr2 had evidently form a synergistic effect for removal of Pb. The finding also reported that both plants have similar accumulation patterns whereby the distribution of Pb was higher in roots followed by leaves and stalks. Therefore, this study concludes that *P. stratiotes* and *E. crassipes* have a huge potential to remove metal polluted water. It is important to note that both plants must be harvested regularly to avoid the metals release back in to the environment.

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