**Toxicity of inorganic mercury to native Australian grass grown in three different soils**

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**Abstract**

In this study, three native Australian grasses namely *Iseilema membranaceum* (Barcoo), *Dichanthium sericeum* (Queensland Blue) and *Sporobolus africanus* (Tussock) were grown in three different soils spiked with different concentrations of inorganic mercury and the root elongation was monitored up to 28 days following the germination. Results showed that mercury at certain concentrations significantly inhibited the root growth of all three tested native grasses grown in three soils, however, the toxicity was less in the soil with high organic carbon content and acidic pH. The calculated EC50 values ranged from 10 – 224 mg/kg total Hg in soil. However, the EC10 values indicated that existing guideline values for mercury may be of protective to the native Australian vegetation. Considering their tolerance to soil mercury, these grass species have the potential for their use in rehabilitation of mercury contaminated sites.

Key words: Inorganic mercury, native Australian grass, Barcoo, Queensland Blue, Tussock.

1. **Introduction**

Plants have been proposed as bio-monitors of soil contamination with heavy metals including mercury (Hg) and root growth as biomarker (indicator of biological response to environmental stress) of the environmental risk of pollutants (Mahbub et al. 2016, Moreno-Jiménez et al. 2006). High levels of Hg in soil lead to accumulation of higher levels in plants which are phytotoxic leading to physiological disorders and cell damage. In plants, intracellular Hg has been shown to bind to –SH groups, phosphate and other active groups in ADP and ATP, alter cell membrane permeability, inhibit mitochondrial activity and photosynthesis reactions (Nagajyoti et al. 2010). Mercury disrupts the antioxidant defense systems of plants by changing the modulations of non – protein thiols, non – enzymatic glutathione, glutathione reductase and superoxide dismutase. Other than these physiological and biochemical alterations, Hg has been shown to be genotoxic as well (Nagajyoti et al. 2010).

Therefore, soil contamination with Hg can have a significant negative effect on growth of plants, especially those of agricultural importance. Unfortunately substantial quantities of mercury are added to agricultural soils with fertilizers, lime, manure and antifungal seed coat dressings (Patra and Sharma 2000) which compete with essential cations for entry into the plant cells. Furthermore, if agricultural crops are planted in soils which have not been properly remediated, and the plants manage to grow, the Hg may be deposited in the plant and harvested in the edible portions. The phyto-toxicity of Hg on crop plants and vegetable plants (Li et al. 2013; Meng et al. 2012; Meng et al. 2014; Moreno-Jiménez et al. 2006; Patra and Sharma 2000; Sahu et al. 2012; Zhang et al. 2010) has been extensively reported.

However, for urban communities, it is critical that after remediation, the contaminated site can support adequate vegetation to stabilize soil particles and re-establish the ecosystem without unnecessary soil erosion and other adverse environmental impacts (Li et al. 2006). Therefore, it is critical to know at what level Hg is inhibitory to plants which may be used for stabilizing rehabilitated sites. Many native Australian plants are well known for their adaptation to infertile soils of low moisture content, and poor soil structure. Therefore, native plants provide unique advantages for the rehabilitation of polluted land. However there is very little knowledge of phytotoxic levels of Hg and other heavy metals to these native plant species. Only a few studies are available which have focused on toxicity of copper, zinc, cadmium and lead on different native Australian tree and grass species (Crawford and Wilkens 1997; Lamb et al. 2010a; Lamb et al. 2010b; Lamb et al. 2012).

Most of the studies on plant toxicity of Hg have been carried out in hydroponic systems, using moistened filter paper or laboratory culture media such as Hoagland nutrient solution or agar spiked with Hg (Cargnelutti 2006, Godbold 1991). The bioavailable Hg concentrations used in these experimental set ups are higher compared to those available in contaminated soils. Therefore, there is a need to conduct experiments in both spiked and properly aged soils and in contaminated soils with appropriate controls. At present, there is no published data on the comparative dose – response behavior of native Australian plants in response to Hg stress in soil.

In this study we investigated the relative toxicity of inorganic Hg to three native Australian grass species grown in three different soils using root growth as an endpoint. Dose – response analyses were performed to obtain effective concentrations of total and water soluble Hg in soil that caused 50%, 20% and 10% decrease in root growth.

1. **Materials and methods**

Top soils (0 – 15 cm) were collected from three different sites in South Australia, Australia where soils varied mainly in their pHs. Soils were sieved through 2 mm sieve. Soil texture was determined by the micro pipette method (Miller and Miller 1987). Soil pH was determined electrometrically on a 1:5 dry soil: water suspension after 2 h stirring using a glass membrane electrode at 25 °C. Electrical conductivity (EC) was determined with an EC probe in the aqueous extract of a 1:5 soil-water suspension and recorded in deciSiemens/m at 25 °C. Maximum water holding capacities of three soils were determined by gravimetry with the oven-dry method (Gardner and Klute 1986). Total organic carbon content and total nitrogen were determined by dry combustion at 1250 °C using a Tru Mac (LECO, Japan) CNS elemental analyzer.

Collected soils (3 kg of each sample) were spiked with 9 different concentrations of inorganic mercury using a stock solution of 5000 mg/L Hg, prepared by dissolving HgCl2 in sterile de-ionized water, kept in polyethylene containers by maintaining 70% of the soil’s maximum water holding capacity. The spiked and control soils (not spiked with Hg) were mixed thoroughly in an end-over-end shaker, stored in covered polyethylene containers and aged for 90 d at 25 °C to provide a minimum time for Hg to be complexed with soil particles. Immediate analysis after spiking might have resulted in overestimation of toxic doses. The final Hg concentrations in soils were intended to be 0, 5, 10, 50, 100, 150, 200, 250, 300, 400 and 500 mg/kg.These concentrations were chosen to obtain proper dose - response curves to estimate effective concentration (EC) values and these are the concentrations that are commonly found in different Hg contaminated sites.

A microwave digestion system (Model: MARS 5, CEM) was employed for the digestion of soil samples with aqua regia after 90 d of aging. The USEPA method 3051 was employed for sample digestion (Melgar et al. 2009). For water soluble Hg analysis, 5 g soil was taken following 90 d of aging process and mixed with 50 ml de-ionized water, and then shaken overnight in an end-over-end shaker. The soil extract was collected by centrifugation at 2000 g for 20 min followed by filtration with 0.45 µm filters (Millipore, Billerica, Massachusetts, USA). The water extract and microwave digested soil samples were examined for Hg using Inductively Coupled Plasma Triple Quad Mass Spectrometry (ICP-QQQ-MS, Agilent Technologies 8800, Santa Clara, California, USA) following 100X dilution with 1% HCl. The detection limit of Hg for this instrument was 0.5 µg/L.

Three native Australian plant species were chosen for the present study. All three plants were grass species namely *Iseilema membranaceum* (Barcoo), *Dichanthium sericeum* (Queensland Blue) and *Sporobolus africanus* (Tussock). The plant species were selected because they are widespread and are known to be used for revegetation purposes. Plant seeds were purchased from Native Seeds Pty Ltd, Victoria, Australia. Plant growth experiment was carried out according to a published method (Kader et al. 2016). Triplicate plastic pots (120×125×120 mm) with 300 g of soil from each concentration was prepared. Pots were kept moistened throughout the study with distilled water to 70% of respective soil’s water holding capacity. Twenty seeds of individual plant were placed on top of soil to ensure germination. After 1 week of emergence, each pot was thinned to 6 plants and grown for 28 d under green house condition (20 – 25 ºC). After 28 d, plants were harvested and roots were carefully separated from soil, washed with distilled water.

Root lengths of plants grown in control and spiked soils were measured in millimeter and averaged from triplicate pots. Relative growth (%RG) was calculated by comparing the root length in treated soils with that in respective control soils. Dose – response curve was obtained by plotting soil total Hg concentrations and %RG and soil water soluble Hg concentration and %RG. Dose – response relation was analyzed by non – linear regression using four parametric logistic model to obtain 50% (EC50), 20% (EC20) and 10% (EC10) effects employing IBP SPSS 17 software. Where regression relation was not significant at 95% level, root elongation data were subjected to one – way analysis of variance (ANOVA) followed by Tukey’s post hoc analysis to observe any significant difference from control.

1. **Results and discussion**

The physico-chemical properties of the experimental soils are presented in Table 1. S-1 and S-2 soils had neutral and alkaline pH. S-3 soil was different from the other two soils as it was much lower in pH (4.2) and higher in organic carbon content (4%). S-3 soil had the highest percentage of sand and lowest clay content compared to the other two soils.

Table 1: Soil characteristics

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| **Soils** | **pH** | **EC (µs/cm)** | **%**  **WHC** | **%**  **TOC** | **%**  **N** | **% Sand** | **% Silt** | **% Clay** |
| S-1 | 7.6 | 323 | 42 | 2.07 | 0.23 | 51 | 36 | 13 |
| S-2 | 8.5 | 232 | 39 | 2.2 | 0.22 | 42 | 44 | 14 |
| S-3 | 4.2 | 52 | 28 | 4.0 | 0.24 | 89 | 9 | 2 |

Values are represented as mean (n=4), standard deviations were <10% of the mean value; EC – electrical conductivity, WHC- water holding capacity, TOC- total organic carbon, N- nitrogen

Total Hg content after 90 d of aging in spiked soils varied from 0.2 to 644 mg/kg (80 to 120% recovery, Table 2). The concentrations of total Hg in three control soils with no Hg spiking were ~0.5 mg/kg which is similar to background concentration of Hg in soil. Recovery of total Hg in all three spiked soils was almost 90 - 100%. The water soluble fraction of Hg in experimental soils varied from 0.05% to 1.5% of the total spiked amount (Table 2). In the present investigation, the water soluble Hg portion in S-3 soils was found approximately 2 to 3 times lower than the water soluble Hg fraction in other soils spiked with 50 mg kg-1 to 500 mg/kg. Hg has high affinity to soil organic matter which results in decreased solubility of Hg in soil, especially in acidic soil (Busto et al. 2012; Neculita et al. 2005; Reis et al. 2014). In this study, the lower water extractable fraction of Hg in acidic higher organic carbon containing S-3 soil is consistent with other researcher’s findings (Gu et al. 2011; Skyllberg et al. 2006).

Table 2: Recovery of total and water extractable mercury from mercury spiked soil samples

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Spiked Hg (mg/L)** | **Soil 1 (S-1)** | | **Soil 2 (S-2)** | | **Soil 3 (S-3)** | |
| **Total (mg/kg)** | **H2O extract**  **(mg/kg)** | **Total (mg/kg)** | **H2O extract**  **(mg/kg)** | **Total (mg/kg)** | **H2O extract**  **(mg/kg)** |
| 0 | 0.6 | 0.025 | 0.2 | 0.002 | 0.28 | 0.018 |
| 5 | 5.9 | 0.082 | 4.4 | 0.074 | 5.3 | 0.073 |
| 10 | 9.9 | 0.089 | 9.7 | 0.156 | 10.4 | 0.158 |
| 50 | 54.3 | 0.55 | 53.9 | 0.675 | 52.6 | 0.285 |
| 100 | 98.1 | 0.641 | 113.9 | 0.728 | 111.6 | 0.26 |
| 150 | 124.1 | 0.773 | 130.1 | 0.873 | 135.8 | 0.317 |
| 200 | 172.7 | 1.101 | 175.9 | 0.904 | 170.5 | 0.540 |
| 250 | 225.8 | 1.382 | 218.5 | 0.941 | 228.1 | 0.640 |
| 300 | 268.6 | 1.711 | 261.2 | 1.031 | 265.2 | 0.805 |
| 500 | 464 | 2.1 | 452 | 1.89 | 454 | 1.18 |

Values are represented as mean (n=4), standard deviations were <10% of the mean value

Root elongation is considered a sensitive indicator of heavy metal toxicity (Lamb et al. 2010b). Root elongation data of three native Australian grass and their dose – response curves are depicted in Figures 1 and 2. Dose – response curves were prepared for both total recovered Hg (Figure 1) and water extractable Hg (Figure 2) concentrations in three experimental soils to estimate EC50, EC20 and EC10 values. It was observed that Hg significantly inhibited root growth (expressed as % RG) of all experimental grass grown in all the three soils. The estimated EC values for total and water soluble Hg are presented in Table 3.

Response of *Iseilema* *membranaceum* grass to Hg stress was different in three different soils (Figure 2a). In neutral (S-1) and acidic (S-3) soils, an initial tolerance was observed up to >54 mg/kg and 124 mg/kg Hg respectively and root growth was inhibited beyond these concentrations. Whereas in alkaline S-2 soil, the tolerance was observed up to 6 mg/kg total Hg concentration and 50% inhibition in root growth was observed at 10 mg/kg. The estimated EC values confirms that *Iseilema membranaceum* grass was more sensitive to Hg in S-2 soil (EC50 10) followed by S-1 (EC50 200) and S-3 (EC50 224). Acidic soil with more organic carbon contents was proved to be more protective for *Iseilema membranaceum* grass from Hg stress. This can be related to less recovery of water soluble Hg in that soil (S-3) compared to other two soils (S-1 and S-2).

*Dichanthium sericeum* grass showed no significant inhibition in root growth up to >54 mg/kg, >124 mg/kg and <6 mg/kg total Hg concentration in S-1, S-2 and S-3 soils, respectively (Figure 2b). In acidic organic content rich S-3 soil, the highest experimental concentration of Hg 454 mg/kg caused only ~50% reduction in root growth; therefore the regression analysis and estimated EC values were not significant (P>0.05) (Table 3). However, the calculated EC values show that Hg exerted almost similar toxicity to root growth of *Dichanthium sericeum* grass in S-1 soil (EC50 126), and S-2 soil (EC50 132). Acidic soil with more organic carbon content was proved to be more protective for *Dichanthium sericeum* grass as well which might be due to chelation of available Hg with organic matter.

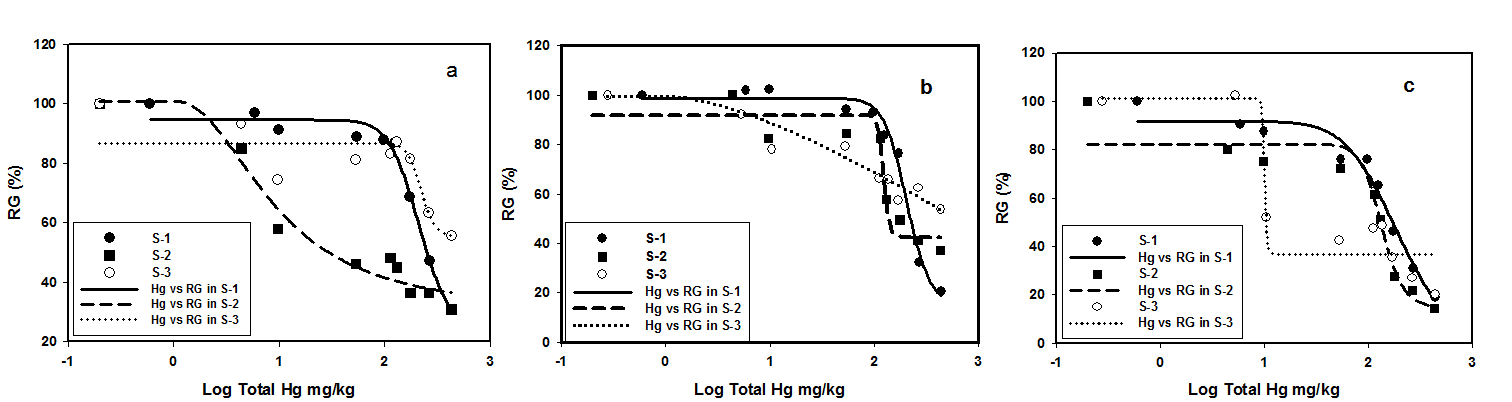


Figure 1: Non - linear regression of plant’s relative root growth and log transformed total Hg concentrations in 3 soils - a) *Iseilema* *membranaceum*, b) *Dichanthium* *sericeum*, c) *Sporobolus* *africanus*. Straight lines, broken lines and dotted lines represent fitting in S-1, S-2 and S-3 respectively.

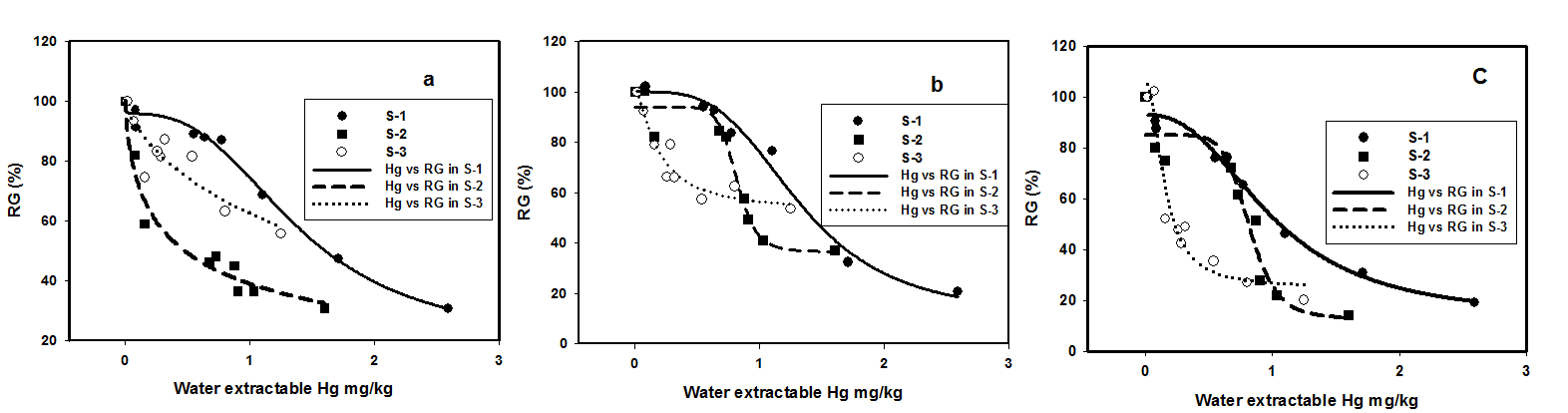


Figure 2: Non - linear regression of plant’s relative root growth and water extractable Hg concentrations in 3 soils - a) *Iseilema membranaceum*, b) *Dichanthium sericeum*, c) *Sporobolus africanus*. Straight lines, broken lines and dotted lines represent fitting in S-1, S-2 and S-3 respectively.

Table 3: Estimated effective concentrations of Hg obtained from non-linear regression analysis of mercury gradients in three soils vs plants’ relative root growth

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| ***Iseilema membranaceum*** | | | | | | | | |
| **Soil** | **Total Hg (mg/kg)** | | | | **Water soluble Hg (mg/kg)** | | | |
| **EC50** | **EC20** | **EC10** | **R2** | **EC50** | **EC20** | **EC10** | **R2** |
| S-1 | 200 | 163 | 145 | 0.98 | 1.4 | 1.3 | 1.2 | 0.98 |
| S-2 | 10 | 2.2 | 0.95 | 0.96 | 0.41 | <0.002 | <0.002 | ns |
| S-3 | 224 | 200 | 190 | 0.73 | 1.9 | <0.018 | <0.018 | ns |
| ***Dichanthium sericeum*** | | | | | | | | |
| **Soil** | **Total Hg (mg/kg)** | | | | **Water soluble Hg (mg/kg)** | | | |
|  | **EC50** | **EC20** | **EC10** | **R2** | **EC50** | **EC20** | **EC10** | **R2** |
| S-1 | 126 | 111 | 103 | 0.93 | 1.32 | 1.19 | 1.1 | 0.99 |
| S-2 | 123 | 118 | 115 | 0.93 | 0.82 | 0.78 | 0.75 | 0.95 |
| S-3 | >109 | >109 | >109 | ns | 0.19 | 0.16 | 0.14 | ns |
| ***Sporobolus africanus*** | | | | | | | | |
| **Soil** | **Total Hg (mg/kg)** | | | | **Water soluble Hg (mg/kg)** | | | |
|  | **EC50** | **EC20** | **EC10** | **R2** | **EC50** | **EC20** | **EC10** | **R2** |
| S-1 | 209 | 139 | 111 | 0.97 | 1 | 0.88 | 0.81 | 0.98 |
| S-2 | 132 | 108 | 98 | 0.93 | 0.82 | 0.71 | 0.65 | 0.92 |
| S-3 | nd | nd | nd | ns | 0.15 | 0.1 | 0.06 | 0.94 |

“ns“ - the estimated regression relation was not significant (P>0.05); although the treatments were different from controls with background concentration of Hg (P<0.05). “ns” – not significant, “nd” – not detected.

The dose response curve reveals no significant inhibition in root growth of *Sporobolus africanus* from 10 – 54 mg/kg total Hg concentration in S-1 and S-2 (Figure 2c), whereas in S-3 this was up to 5 mg/kg total Hg. Dose – response regression was significant in S-1 and S-2, but the regression analysis was not significant in case of S-3 and therefore EC values could not be estimated in S-3 soil using the four parametric logistic model (Table 3). The EC values elucidate that Hg was more toxic to *Sporobolus africanus* grass in alkaline S-2 soil (EC50 132) followed by S-1 soil (EC50 209).

Hg has been shown to be highly toxic to plant’s root elongation when nutrient solutions were used as media. For example, approximately 1 – 3 mg/L of inorganic Hg in a chemically defined nutrient solution completely inhibited root growth of *Picea* *abies* (Godbold 1991). In another study, 50 – 100 mg/L Hg reduced 96 – 98% root length of *Cucumis sativus* while grown in Hg supplemented agar media (Cargnelutti et al. 2006). On the other hand, a study done by Moreno-Jiménez et al. (2006) found no inhibition in root and shoot growth of *Rumex induratus* and *Marrubium vulgare* when the seedlings were grown in contaminated soils with 122 and 550 mg/kg Hg. In the present investigation, it was also observed that the grass species were tolerant to Hg up to 124 mg/kg (Figure 2). Hence, the Hg toxicity varies depending on the media used. Therefore, toxicity studies using nutrient solution or agar media may not reflect the real toxicity in contaminated soils.

When the regression analysis was carried out by plotting water soluble fraction of Hg in soils, it was clear that although soluble fraction of Hg in soil is very low, it can still exert toxicity on plant. The estimated EC10 values indicate that very small amount of water soluble Hg can cause significant inhibition on root growth of the tested three native Australian grasses (Table 3). EC10 values are considered as more reliable indicator than No Observed Effect Concentration (NOEC) (Warne et al. 2014). The EC10 values obtained in this study indicate that soil inorganic Hg may not be harmful to native Australian plants if the pollution is controlled according to the existing safe limits (ranging from 1 – 170 mg/kg Hg) set by different industrial countries (CCME 1997; EA 2009; GON 2000; NEPM 2013; USEPA 2013). Since all of tested grass species can withstand considerable amount of Hg in soil (reflected by EC20 values at Table 3), these native grasses can be used for revegetation in mercury contaminated sites.

1. **Conclusion**

In this study, toxicity of inorganic Hg to three native Australian grass species was determined by growing them in three different natural soils spiked with a range of Hg concentrations. Mercury was observed to inhibit the plant’s root growth at certain concentration in a sigmoidal pattern in varying degrees. Effective toxic Hg concentrations were estimated from dose – response relationships which showed that high doses of Hg (98 – 115 mg/kg total Hg) were required in all soils to inhibit 10% root growth of all tested plants (except *Iseilema membranaceum* grownin alkaline soil). Massive root growth inhibition (50% inhibition) of three plants in three soils was observed at 10, 123, 126, 132, 200, 209, and 224 mg/kg total Hg concentrations. Further study is required to investigate the accumulation of Hg in native Australian grasses and its effect on their biochemistry and genetics.

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