ECOTOXICOLOGICAL EFFECTS OF CHROMIUM (VI) ON SEEDLING GROWTH, SOIL NITRIFICATION AND EARTHWORM BEHAVIOR

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ABSTRACT

Soil Cr(VI) contamination is a long existing problem due to large quantities of wastes from various industrial sources being released into the environment. An array of ecotoxicological tests including corn (*Zea mays* L.) and wheat (*Tricitum aestivum* L.) emergence and growth, soil nitrification and earthworm (*Eisenia fetida)* avoidance behavior were used in this study to evaluate the potential effects of Cr(VI) on soil biota. Three South Australian soils specifically Pinnaroo (sandy loam, pH 7.40), Kalangadoo (sandy, pH 7.38) and Mount Compass (sandy, pH 5.94) were used for the plant, soil nitrification and earthworm behavior tests, accordingly. These soils, possessing high sand content, low CEC and low OM, were used to simulate a condition for low Cr(VI) sorption and increase Cr(VI) bioavailability allowing toxic effects to be more apparent for research purposes. Soil samples were spiked with different Cr(VI) concentrations (1-100 mg kg^{-1} for plant tests and 1-500 mg kg^{-1} for nitrification and earthworm tests). In the plant tests (OECD 208), the plant height of corn and wheat were significantly decreased even at low Cr(VI) concentrations of 10 and 20 mg kg^{-1}, respectively. Furthermore, corn and wheat aboveground biomass were significantly decreased at 10 mg Cr(VI) kg^{-1}. Nitrate levels significantly decreased due to increased Cr(VI) concentrations in the soil nitrification test (OECD 216). The avoidance behavior was observed starting at 5 mg Cr(VI) kg^{-1} in the earthworm avoidance assay (ISO 17512-1). The obtained EC$_{50}$ values for Cr(VI) on soil nitrification and earthworm avoidance were 65.2 mg kg^{-1} and 22.4 mg kg^{-1}, respectively. Results showed significant negative effects of Cr(VI) on soil biota as evident on the decreased corn and wheat plant growth, decreased microbially-mediated soil nitrification and increased earthworm avoidance in the contaminated soils. Results of this ecotoxicological study can be used as basis for remediation activities performed on Cr(VI) polluted soil.

Key words: ecotoxicity, seedling growth, nitrification, earthworm avoidance

INTRODUCTION

Chromium(Cr) compounds have various industrial uses. They are used in the production of drilling muds, cleaning agents, electroplating, refractory steel, and catalytic manufacture, in chromic acid production, leather processing and finishing. Hexavalent chromium compounds are used in industry for cooling tower water treatment, hide tanning, metal plating, and wood preservation (Shanker *et al.* 2005). As Cr is very widely used, there are many potential sources of that could result to accumulation of Cr in the soil and natural water system. The amount of Cr in the natural soils ranges between 1 and 2000 mg kg^{-1} depending on the soil type (ATSDR 2012). In the Philippines, several rice growing areas near mining sites are reported to be contaminated with mine tailings. Chromium concentrations ranging from <36.8-1,126.5 mg kg^{-1} have been reported from lowland soil samples all

over the Philippines (Magahud et al. 2014). The highest Cr concentration (1,126.5 mg kg^{-1}) was recorded from soil samples obtained from Sta Cruz, Zambales. This level exceeds the Netherlands intervention values of 180 mg kg^{-1} for Cr (III) and 78 mg kg^{-1} for Cr(VI) (Magahud et al. 2014), and is expected to be toxic to most plants.

Chromium in its anionic form, Cr(VI), is very toxic to living organisms including many plants, animals and microorganisms (Petrilli and De Flora 1977; Shanker et al. 2005; Velma et al. 2009). Although the adverse effects of Cr(VI) have been known for a long time, the exposure to Cr(VI) continues at an increasing rate in some areas. The presence heavy metal contaminants like Cr(VI) affect the soil biota, which are known to play a vital role in maintaining soil health and function, potentially affecting its processes and functions. To site some examples of Cr toxicity to soil biota, Yu and Feng, (2016) reported EC$_{50}$ values of 15.81 mg Cr L^{-1} (based on relative growth rate, %) and 26.80 mg Cr L^{-1} (based on water use efficiency, mg mL^{-1}) for rice seedlings transplanted in solution. Depending on the Cr concentration in the soil, different plant processes including germination, root growth, stem growth, leaf growth and yield are affected and the degree of tolerance differs in each plant (Oliveira 2012; Singh et al. 2013; Ertani et al. 2017). Nitrification in agricultural soil was inhibited by 81% at 269 mg Cr kg^{-1} (Liang and Tabatabai 1978). In the terms of invertebrates, an LC$_{50}$ values for Cr(VI) on earthworm ranges between 222-257 mg kg^{-1} were obtained in 10 different soils (Sivakumar and Subbhuraam 2005).

Ecological toxicity tests, involving the use plants, invertebrates, and microorganisms as the test targets, are important methods to diagnose toxicity and adverse effects due to soil contaminants (Yang et al. 2001; Wang and Zhou 2006). Higher plants are an important component of the soil ecosystem, the degree of soil pollution can be assessed through their growth and development (Gong et al. 2001). Microbially-catalyzed transformation processes including soil nitrification (conversion of ammonium-N to plant available nitrate-N) provides an important insight on the soil microbial communities involved in N-cycling within the soil. Earthworms are important decomposers and are crucial to soil fertility in many terrestrial ecosystems. The avoidance behavior of soil invertebrates, including earthworms, enchytraeids and collembolans, is based on the fact that soil organisms is capable of avoiding unfavorable conditions indicating the presence of soil contaminants. In this study, effects of Cr(VI) contamination on soil biota were investigated using ecotoxicological tests including seedling emergence and growth, soil nitrification and earthworm avoidance behavior. These tests are reflective of plants, microbial functions, and invertebrate activities.

MATERIALS AND METHODS

Seeds and test organism. Corn (*Zea mays* L.) and wheat (*Triticum aestivum* L.) seeds were used in the seedling emergence and seedling growth experiment. The test organism used for the earthworm avoidance experiment was adult *Eisenia fetida* obtained from a local earthworm breeder at Fullarton, South Australia. The adult worms with visible clitellum (250- 600 mg each) were acclimatized in uncontaminated test soil for 24 h prior to the avoidance test.

Test soils. Three uncontaminated South Australian soils (Pinnaroo, Kalangadoo and Mount Compass) were used. Pinnaroo soil (sandy loam, pH 7.40) was used in the seedling emergence and growth test due to difficulties growing crops in a sandy soil. The Kalangadoo soil (sandy, pH 7.38) was used in the soil nitrification test, while Mount Compass soil (sandy, pH 5.94) was used in the earthworm avoidance test. Different soil types were used due to limited quantity of the soil samples. The selected soils possess low cation exchange capacity (CEC) as well as low organic matter (OM) content, which create conditions for low Cr(VI) sorption and increase its bioavailability simulating a 'worse-case' scenario allowing for the toxic effects of the Cr(VI) to be more apparent for investigative purposes. The soils were air-dried and sieved (<2 mm). Selected physio-chemical characteristics of the three soils are detailed in Table 1.

Table 1. Selected physio-chemical properties of Kalangadoo and Mount Compass soil.

Soil Characteristics	Sandy loam (Pinnaroo)	Sandy (Kalangadoo)	Sandy (Mount Compass)
Particle size distribution	77% sand, 4% silt, 19% clay	94% sand, 2.4% silt, 2.3% clay	96.1% sand, 1.7% silt, 1.6% clay
pH (1:5 soil:water)	7.40	7.38	5.94
Electrical conductivity (1:5 soil:water)	0.01 dS m^{-1}	24.1 µS m^{-1}	0.08 dS m^{-1}
Total carbon (C) %	1.00%	0.48%	0.50%
Cation exchange capacity	13.6 cmol$_{(c)}$ kg^{-1}	3.7 cmol$_{(c)}$ kg^{-1}	3.6 cmol$_{(c)}$ kg^{-1}
Exchangeable cations, Ca^{2+}	4.16 cmol$_{(c)}$ kg^{-1}	3.5 cmol$_{(c)}$ kg^{-1}	1.5 cmol$_{(c)}$ kg^{-1}
Exchangeable cations, Mg^{2+}	7.52 cmol$_{(c)}$ kg^{-1}	0.2 cmol$_{(c)}$ kg^{-1}	0.31 cmol$_{(c)}$ kg^{-1}
Exchangeable cations, Na$^+$	0.58 cmol$_{(c)}$ kg^{-1}	0.1 cmol$_{(c)}$ kg^{-1}	<0.1 cmol$_{(c)}$ kg^{-1}
Exchangeable cations, K$^+$	6.67 cmol$_{(c)}$ kg^{-1}	0.1 cmol$_{(c)}$ kg^{-1}	0.08 cmol$_{(c)}$ kg^{-1}
Hexavalent chromium, Cr^{6+}	<1 mg kg^{-1}	<1 mg kg^{-1}	<1 mg kg^{-1}

Soil spiking and incubation. For the seedling emergence and seedling growth experiment, 1,500 g air-dried soil were weighed into large heavy duty bags. Each soil sample was spiked with different Cr(VI) concentrations (10, 20, 40, 60, 80 and 100 mg kg^{-1} soil). For the soil nitrification test and earthworm avoidance assay, 1,000 g air-dried soil samples were spiked to achieve concentrations equivalent to the effective concentration of Cr(VI) that caused 50% reduction in nitrification or earthworm avoidance compared to the uncontaminated (control) soil (EC$_{50}$). To determine the EC$_{50}$ values, the soils were spiked with varying Cr(VI) concentrations. The range of Cr(VI) concentrations used for the nitrification test were 0.1, 1, 5, 10, 50, 100, 200, and 500 mg kg^{-1} while 1, 5, 10, 15, 25, 30, 35, 40, 50, 100, 150, 300, 400 and 500 mg kg^{-1} were used for the earthworm avoidance assay. Uncontaminated soil samples were used as control in each test. Cr(VI) solutions were made by dissolving K$_2$Cr$_2$O$_7$ in Milli-Q water equivalent to 10% MWHC of the soil. Soil samples were thoroughly mixed by tightly sealing up the bags with plastic cable clips and mixing the soil to evenly distribute the spike solutions. The samples were incubated for 3 days to equilibrate before conducting the ecotoxicological tests. Cr exists predominately as Cr(VI) under the aerobic test conditions. In addition, the low OM content of the test soils prevents the reduction of Cr(VI) to Cr(III).

Seedling emergence and seedling growth test. Seedling emergence and seedling growth test (OECD 208) assesses the effects of contaminants on seedling emergence and early growth of higher plants following exposure to the test substance in the soil (or other suitable soil matrix). Endpoints measured in the test are visual assessment of seedling emergence, dry shoot weight (alternatively fresh shoot weight) and in certain cases shoot height (OECD, 2006). The effects of Cr(VI) on corn and wheat emergence and growth were assessed according to OECD 208. From spiked bags, 300 g (dry weight basis) subsamples were transferred into pre-weighed black circular polypropylene pots (Genfac 440 mL 117.5 mm x 60 mm). Pots without holes were used to avoid Cr(VI) leaching. Moisture content of the soil was adjusted with deionized water to 60% MWHC. Five wheat seeds and four corn seeds were placed into individual pots following the plant density guide of OECD 208. Plants were grown in a controlled environment chamber at 20°C with a 12:12 light: dark cycle and a light intensity of >75 W m^{-2} (from 400 to 700 nm). Thinning was performed 7 days after germination only for the corn experiment to conform with OECD 208. Only two corn seedlings per pot were retained. Ruakura nutrient solution (5 mL) was applied twice weekly to overcome any macronutrient/micronutrient deficiency. Cr(VI) effects were assessed following 21 d after 50% of the seedlings in control samples had emerged from the soil. Care and maintenance of the plant samples were done promptly and

properly. At the end of the growing period, individual plant height was measured and the seedlings were cut just above the surface of the soil for the aboveground fresh weight. Four pots (replicates) were prepared for each Cr(VI) concentration.

Nitrogen transformation test. Nitrogen transformation test (OECD 216) is designed to detect long-term adverse effects of a substance on the process of nitrogen transformation in aerobic surface soils. Nitrate levels formed in treated and control samples are measured after 28 d of incubation and EC_{50} value is calculated (OECD, 2000). The effect of Cr(VI) on soil nitrogen transformation, as soil nitrification, was investigated following OECD 216. Cr(VI)-spiked soils (25 g) were first pre-incubated in 50 mL polypropylene tubes at 60% MWHC. Centrifuge tubes were left open to prevent development of anaerobic condition. Powdered lucerne meal (C:N molar ratio of 13.6:1) was added to the samples at a rate of 5 mg g^{-1} soil (dry weight). Moisture levels for each treatment were maintained during the 28-day incubation period (60% MWHC) using Milli-Q water. To homogenize, the samples were mixed daily using a vortex mixer. Subsamples (4 g) from each treatment were transferred to a 50 mL polypropelyne tubes after 28 d and extracted with 2M KCl (1:5 soil:solution ratio) by mixing in an end-over-end shaker (2 h). Afterwards, the samples were then centrifuged (4000 rpm, 20 min). The supernatants were collected and freeze-dried for the analysis of nitrate content. Four replicates were prepared for each Cr(VI) concentration. The amount of soil nitrate-N in the KCl extracts were measured colorimetrically based on the reduction of nitrate by vanadium(III) combined with detection by the acidic Griess reaction (Miranda et al.2001). Concisely, samples were reacted with a two color reagents containing vanadium in dilute acid solution and Griess reagent (sulfanilamide and N-(1-naphthyl)-ethylenediamine), producing a pink-colored dye. The absorbance was measured in 96-well plates at 540 nm using a spectrophotometer (MultiskanTM GO Microplate, Thermo Scientific). The nitrate concentrations in the extracts were calculated from a calibration curve of absorbance plotted against concentrations of standard nitrate solutions.

Earthworm avoidance test. Earthworm avoidance behavior assay (ISO 17512-1:2008) is a rapid screening method for evaluating the influence of contaminants and chemicals on earthworm behavior. It is a rapid method that reflects the bioavailability of contaminants in natural soils to *Eisenia fetida* (ISO, 2008). The two-section avoidance test, was performed to determine the Cr(VI) effect on earthworm activity. The test containers were made of a 750 mL black rectangular polypropylene box (Castaway 120 mm x 175 mm x 55 mm) and a removable cardboard divider wall dividing the container into two equal sections. One of the sections was filled with 250 g of uncontaminated soil (control section) and the other with the Cr(VI)-spiked soil (test section). Five containers (replicates) were used for each Cr(VI) concentration. The test also included boric acid treatment (750 mg kg^{-1}) as a reference material. In each container, ten adult *Eisenia fetida* (250– 600 mg) previously washed and wiped dry, were placed on the middle line of the soil surface after removing the cardboard divider. Containers were covered with transparent lids with holes. To prevent the earthworms from escaping from the containers, holes were covered with gauze permeable to air and light. The containers were incubated at 20 ± 2 °C at a day/night rhythm of 16/8 h for a time period of 48 h. The cardboard dividers were inserted back at the end of the test period. Each section was independently searched for earthworms. The avoidance endpoint was expressed as the percentage of earthworms that avoided the treated soil in the test container from the total number of earthworms in the container. The Avoidance Rate (AR) was calculated as: AR = (CS-TS)/N * 100; where, C: number of earthworms in the control section, T: number of earthworms in the test section, N: total number of earthworms/replicate.

Evaluation and statistical analysis. One-way analysis of variance (ANOVA) (Gomez and Gomez, 1984) was performed to assess the effects of Cr(VI) on different soil ecotoxicity endpoints. Tukey's HSD test (at $p < 0.05$) was used to determine whether means differed significantly. A dose-response curve was made by plotting the Cr(VI) concentrations against nitrate content/earthworm avoidance of the soil. The EC_{50} was calculated using a spreadsheet tool (Barnes et al. 2003).

RESULTS AND DISCUSSION

Effects of Cr(VI) on seed emergence and seedling growth. Cr(VI) effects on corn and wheat emergence and seedling growth are shown in Figures 1-3. Increasing Cr(VI) concentrations (10-100 mg kg^{-1}) had no significant effect on corn and wheat germinations (Fig. 3a). This suggests that seeds utilize their own nutrient reserves during germination and are therefore less vulnerable to metal interference. Similar results have been reported with mung bean, pakchoi cabbage and rice (Hu et al. 2016). Furthermore, the toxic effects of Cr(VI) was probably mitigated by the soil itself as it can reduce Cr(VI) to Cr(III) and bind Cr(VI) making it less bioavailable for the seeds.
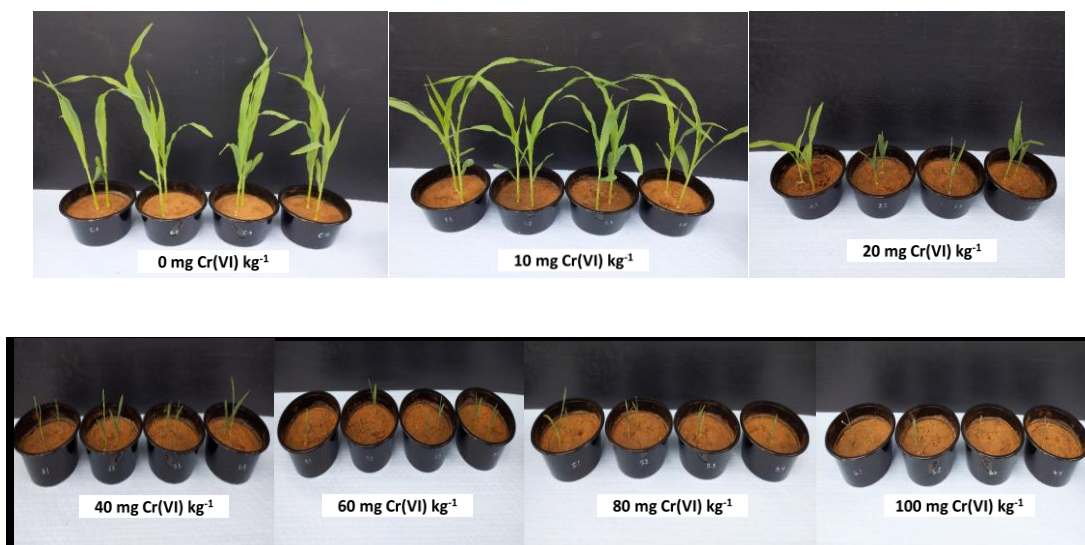
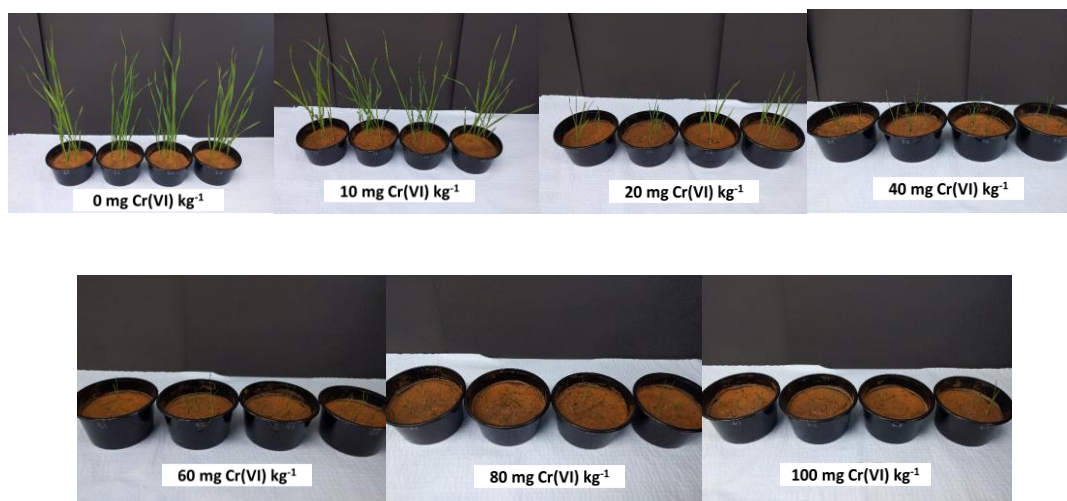**Fig. 1.** Corn seedlings under different Cr(VI) concentrations (0-100 mg kg^{-1}).

Fig. 2. Wheat seedlings under different Cr(VI) concentrations (0-100 mg kg^{-1}).
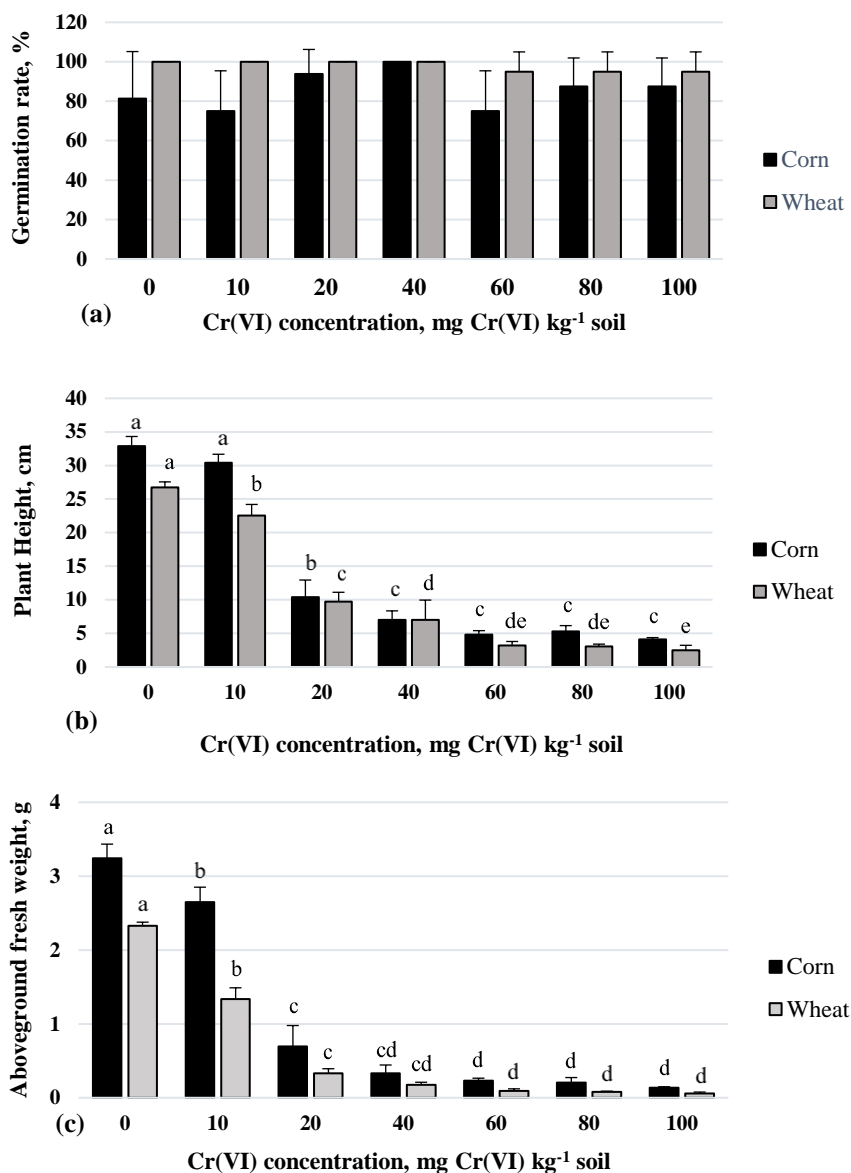
Fig. 3. Inhibition of (a) seed germination, (b) plant height, and (c) aboveground fresh weight following the exposure of corn and wheat to different Cr(VI) concentrations. Error bars represent the standard deviation (n=4). Different letters indicate significant difference among means (P < 0.05) according to HSD test.

The plant height of corn and wheat seedlings were severely affected with increasing Cr(VI) concentrations (Fig. 3b). The lowest Cr(VI) concentration (10 mg kg^{-1}), resulted to the inhibition of corn and wheat plant height by 7.42 and 14.22%, respectively. At the highest Cr(VI) concentration (100 mg kg^{-1}), the corn and wheat plant height were severely reduced by 87.52% and 90.53%. The plant height reduction due to Cr is a direct consequence of the destruction of root cells causing a decrease in nutrient and water mobility from root to shoot (Saddiqe et al. 2015). Other researchers also reported reduced plant height using different test plants due to Cr(VI). Plant height was reduced in cucumber, lettuce and proso millet (Joseph et al. 1995) and wheat sown in sand in a glasshouse trial (Sharma and

Sharma 1993). A significant reduction in plant height in white mustard was likewise observed when Cr was given along with N, P, K and S fertilizers (Hanus and Thomas 1993).

Significant negative effects were observed with respect to the aboveground biomass of corn and wheat as it decreased with increasing Cr(VI) concentrations (Fig. 3c). At the lowest Cr(VI) concentration (10 mg kg^{-1}), corn and wheat aboveground biomass were reduced by 18.31% and 42.62%, respectively. The highest Cr(VI) concentration (100 mg kg^{-1}) reduced the corn and wheat aboveground biomass by 95.85% and 97.45%, respectively. Other researchers observed that higher Cr(VI) concentrations inhibit various plant activities (Dube et al. 2003). Depending on Cr(VI) concentration, toxicity symptoms include inhibition of germination, seedling growth and development, biomass production besides physiological and biochemical alterations (Shanker et al. 2009).

Effects of Cr(VI) on soil nitrification. The effect of Cr(VI) on microbially-mediated N transformation activity (nitrification) in the soil was performed in a 28 d incubation experiment. The dose-dependent effect of Cr(VI) on soil nitrification is presented in Table 2. The nitrate content of uncontaminated soil is 28.57 mg kg^{-1}. The nitrate levels of soil with very low Cr(VI) concentrations (0.1- 1 mg kg^{-1}) were not significantly different from uncontaminated soil. Significant decrease in nitrate level (25.47 mg kg^{-1}) was first observed when the amount of Cr(VI) in the soil reached 5 mg kg^{-1}. Increase in Cr(VI) concentrations resulted in further significant decrease in soil nitrate levels. At the highest Cr(VI) concentration (500 mg kg^{-1}), the nitrate level (1.62 mg kg^{-1}) was extremely low. Considerable reduction in nitrate levels due to Cr(VI) contamination is detrimental to plant growth as nitrate is the major source of nitrogen assimilated by higher plants. Using the nitrate concentrations derived from Cr(VI)-spiked soils, the EC$_{50}$ value on nitrification is 65.2 mg Cr(VI) kg^{-1} (Fig. 4). At this concentration, 50% of the soil nitrification activity was inhibited.

Table 2. Effect of different Cr(VI) concentrations on soil nitrification (presented as nitrate-N, mg kg^{-1} soil) in Kalangadoo soil (sandy, pH 7.38). Data are expressed as Mean±SD.

Cr(VI) concentrations (mg kg^{-1})	Nitrate-N (mg kg^{-1})
0	28.57 ± 1.13[a]
0.1	30.05 ± 1.16[a]
1	28.73 ± 2.27[ab]
5	25.47 ± 1.63[bc]
10	22.4 ± 3.38[cd]
50	19.47 ± 0.38[d]
75	9.54 ± 0.25[e]
100	5.81 ± 1[f]
250	1.73 ± 0.27[g]
500	1.62 ± 0.21[g]

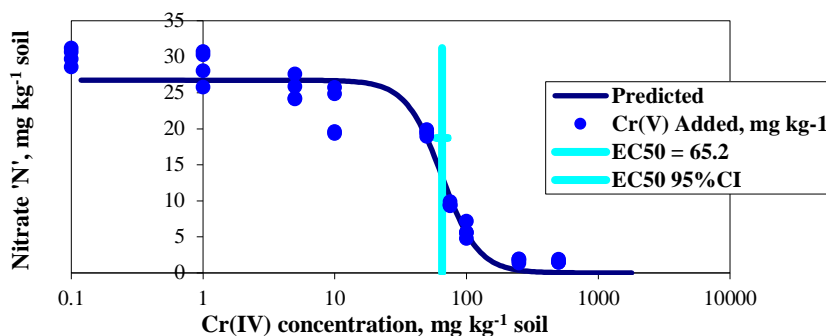Different letters indicate significant difference among means (P < 0.05) according to HSD test.

Fig. 4. Dose-response curve for soil microbial nitrification in Cr(VI)-spiked soil at 0.1- 500 mg Cr(VI) kg^{-1} soil. Blue vertical line shows the 50% effect concentration (EC$_{50}$). Blue horizontal line shows the 95% confidence interval of EC$_{50}$.

The negative effects of Cr(VI) on soil microbial transformation particularly on nitrification were also observed by other researchers using different methods. Kapoor et al. (2016) provided genetic results of Cr(III) and Cr(VI) inhibiting nitrification by decreasing Nitrosomonas activity in wastewater nitrifying enrichments. A similar result was obtained by Zheng et al. (2017), where Cr(VI) had a significant inhibitory effect (maximal inhibition rate of 139.19%) on autotrophic nitrification.

Effects of Cr(VI) on earthworm avoidance. The two-section earthworm avoidance test was performed to determine the avoidance of *Eisenia fetida* to increasing Cr(VI) contamination in the soil. The avoidance behavior was recorded after the 48 h incubation period. Results showed that Cr(VI) concentrations 5 mg kg^{-1} and above induced avoidance response in *Eisenia fetida* (Fig. 5). No avoidance was observed under the uncontaminated soil. Negative responses like the one observed in the lowest Cr(VI) concentration (1 mg kg^{-1}) are treated as no avoidance. The average earthworm avoidance in the boric acid reference treatment was 68%. Earthworms are considered bioindicators of soil quality. They influence OM decomposition, C/N cycling in soil, soil porosity, aeration, and water infiltration, and soil structure. The abundance and behavior (avoidance) of earthworms on Cr(VI) contaminated soil is indicative of the lower soil quality of the soil due to Cr(VI) contamination.
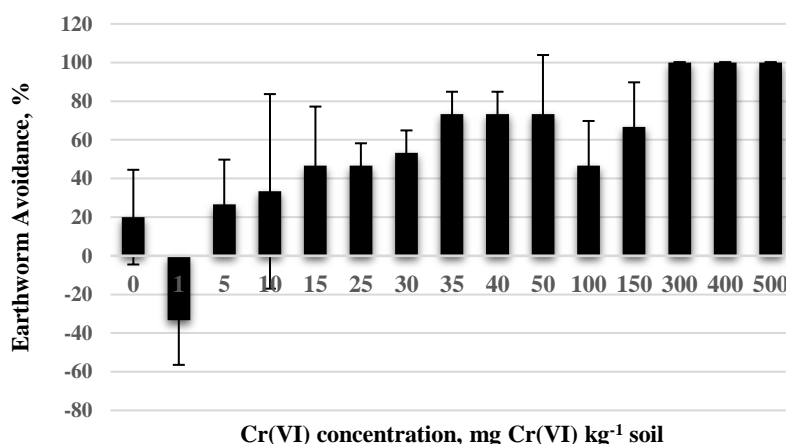

Fig. 5. The earthworm avoidance in the presence of Cr(VI) at 1-500 mg kg^{-1} soil.

Using the dose-response curve generated, the EC$_{50}$ on earthworm avoidance is 22.4 mg Cr(VI) kg^{-1} (Fig. 6). The low EC$_{50}$ value can be attributed to the soil properties of the test soil. A sandy soil

with low OM and CEC was used. Hence, the amount of Cr(VI) bioavailable to earthworm was high. Similar EC_{50} value was observed by Yang et al. (2018). In an artificial soil, they studied the combined effects of four pesticides and chromium(VI) on the earthworm avoidance behavior obtaining an EC_{50} value of 23.80 mg Cr(VI) kg^{-1} soil. The obtained EC_{50} from the avoidance test is relatively lower compared to results obtained from earthworm acute toxicity tests. LC_{50} values of 225-257 mg Cr(VI) kg^{-1} soil were reported on ten different soils in acute toxicity tests (Sivakumar and Subbhuraam 2005). This is due to the earthworms' ability to avoid unfavorable conditions; thus avoidance tests are more sensitive compared to acute or reproduction assays. The results obtained in this avoidance test allowed the early detection of the effects of increasing Cr(VI) concentrations than in earthworm acute tests. However, researcher observed higher coefficient of variation in these avoidance tests compared to acute tests (Hund-Rinke et al. 2003; Judy et al. 2019).
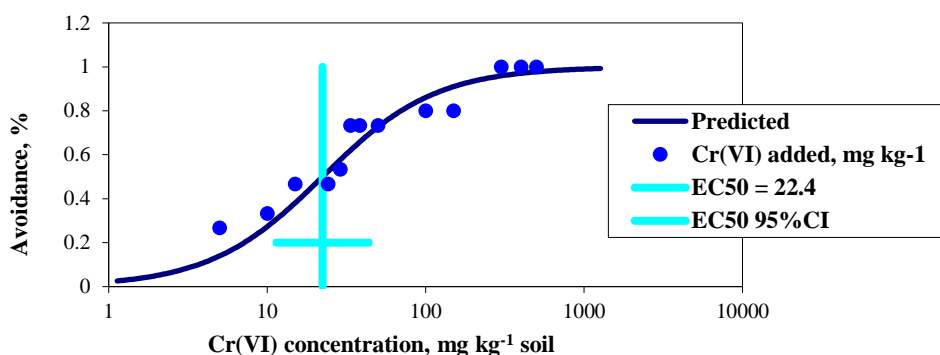

Fig. 6. Dose-response curve for earthworm avoidance in the presence of Cr(VI) at 1- 500 mg kg^{-1} soil. Blue vertical line shows the 50% effect concentration (EC_{50}). Blue horizontal line shows the 95% confidence interval of EC_{50}.

CONCLUSIONS

Multiple ecotoxicological tests involving plant growth, microbial functions and earthworm activities showed significant effects of soil Cr(VI) contamination towards soil biota. A direct correlation was observed between Cr(VI) concentrations in the soil and the adverse effects on corn and wheat growth, soil nitrification and earthworm avoidance. This has important environmental implications. Decreased nitrate levels reduce plant available N and decrease the N-use efficiency. The reduced earthworm abundance in Cr(VI) contaminated soil is an indication of poor soil quality. A variety of remediation technologies can be developed using the results of this study. Further ecotoxicological studies can be performed to provide additional information on the effects of Cr(VI) contamination under different soil environmental conditions. This study could also lead to other studies using a variety of ecotoxicological endpoints in investigating the effects of other contaminants in the soil.

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