__Original Research Article__

BIOREMEDIATION OF HEAVY METALS IN THE SOIL BY
PSEUDOMONAS AERUGINOSA **AND** *TRICHODERMA HARZIANUM*
USING *SOLANUM LYCOPERSICUM* **AS TEST PLANT**

ABSTRACT

This study determined the heavy metal concentrations of contaminated stream water and assessed the heavy metal contents of pre- and post-cropped sterilized soil. It also determined the the heavy metal uptake of the *S. lycopersicum* plant. This was with a view to assessing the potential of *Pseudomonas aeruginosa* and *Trichoderma harzianum* for degrading heavy metals in heavy metal contaminated stream water.Experimental pots containing 3000 g of sterilized soil was used for this experiment whereby 60 sample pots were used with various treatments in this study. *Solanum lycopersicum* seeds were raised in the nursery for a period of 3 weeks and treatments applied just before transplanting into the experimental pots. The plants were left for a week so as to be established properly and overcome transplanting shock before watering with the contaminated stream water. Heavy metal analysis using Atomic Absorption Spectroscopy (AAS) method was carried out on the contaminated stream water to determine the amount of heavy metal in the stream water before the commencement of the experiment. The contaminated stream water was applied to the pots in measured quantities; 0, 5 and 0%. Pre and post soil heavy metal analysis were carried out on the soil samples. At harvest, plant tissues were analysed for heavy metals using AAS method. The results showed that heavy metals were present in high concentration in the stream water sample. The values of the heavy metals in the stream water sample used for watering were Iron – 138.15 mg/L, Zinc – 68.4 mg/L, Lead – 7.89 mg/L and Copper – 8.98 mg/L. . Heavy metal analysis of the soil and all the treatments revealed that treatments with *P. aeruginosa* inoculation had the lowest level of Iron, Copper, Zinc and Lead followed by treatments inoculated with *T. harzianum*. The study concluded that the use of contaminated stream water for irrigation could be a potential source of heavy metals in tomato. However, inoculation of microorganisms for the treatment of the heavy metal contaminated sites was effective for increased health, growth and yield of tomato fruits.

KEWORDS: Bioremediation, *Pseudomonas aeruginosa*, T*richoderma harzianum*, *Solanum lycopersicum*

INTRODUCTION

Heavy metals represent a great environmental concern, because of their widespread use and distribution, and particularly their toxicity to human beings and the biosphere. However, they also include some elements that are essential for living organisms at low concentrations (Alloway, 1990). These elements are usually transition metals. They have high densities (>5 g cm-3) when compared with other materials (Baird and Cann, 2005). Human activities such as industrial production, mining, agriculture and transportation lead to release of high amount of heavy metals into the biosphere. The primary sources of metal pollution are the burning of fossil fuels, smelting of metal like ores, municipal wastes, fertilizers, pesticides and sewage (Nriagu, 1979, 1996; Pendias and Pendias, 1989; Rai, 2009). Heavy metal contamination may occur due to factors which could include irrigation with contaminated water, addition of fertilizers and metal based pesticides, industrial emissions, and transportation (Radwan and Salama, 2006; Tuzen and Soylak, 2007; Duran *et al*., 2007). Heavy metal pollution does not only affect the production and quality of crops, it also influences the quality of the atmosphere and water bodies. This threatens the health and life of animals as well as human beings by the way of food chain and most phenomenal is that, this kind of pollution is covert, long term and non-reversible (Zhang, 1999). Heavy metals are also one of the major contaminating agents in our food supply (Zaidi *et al.,* 2005; Khair, 2009). Bioremediation is a process that uses

43 naturally occurring micro-organisms to transform harmful substances to nontoxic compounds, these processes which
44 take advantage of microbial degradation of organic and inorganic substances can be defined as the use of micro-
45 organisms to remove environmental pollutants of soils, water and sediments (Pala *et al*., 2006). Bioremediation
46 involves the use of organisms for the treatment of polluted soils. These organisms which could be micro-organisms
47 or green plants eliminate, attenuate or transform the harmful substances via biological processes to a less harmful
48 substance (Mrayyana and Battikhi, 2005). Micro-organism breaks down organic molecules to carbondioxide,
49 fattyacid and water in order to obtain energy and nutrients. Bioremediation occurs naturally (even though it could be
50 enhanced by a number of processes), thus, it is widely accepted by the general public as a safe way of treating
51 polluted soils. *Trichoderma harzianum* has potential in stimulating phytoremediation directly and indirectly and
52 therefore, inoculation of plants with this fungus could be a feasible approach to enhance the degradation of
53 hydrocarbons in polluted soil. *T. harzianum* also have the ability to solubilize metal ions and produce siderophores
54 to chelate iron, making metal ions required for plant growth more available to the plant (Harman *et al*., 2004). The
55 fungus is thought to colonize roots of annual plants for their entire lifetime by penetrating the outer layers of the
56 roots (Harman *et al*., 2004). This makes the plants release more root exudates to the surrounding soil, thus,
57 stimulating microbial degradation of pollutants. *Trichoderma harzianum* has been shown to induce the production of
58 larger and deeper root systems, and plants inoculated with *Trichoderma harzianum* also produce greater plant
59 biomass. Such plants are more resistant to abiotic stress and take up nutrients more effectively (Harman *et al*.,
60 2004). Edwards *et al*., (2006) noted that various bacteria such as *Pseudomonas aeruginosa* produce surfactants that
61 aid in the biodegradation. A recent study has found a *P. aeruginosa* strain that actually supports plant growth. This
62 characteristic, along with the fact that *P. aeruginosa* can degrade polycyclic aromatic hydrocarbons, suggests the
63 future uses of *P. aeruginosa* for environmental detoxification of synthetic chemicals and pesticides and for industrial
64 purposes (Botzenhardt and Doring, 1993).

65

66 ## MATERIALS AND METHOD
67 ### Collection of Contaminated water, seeds and microorganisms
68 Heavy metals contaminated stream water was obtained from a flowing stream. It is situated at 7°30' Northern
69 latitude and 4°28' Eastern longitude. The sampling point was located at the back of the Ife Iron and Steel Nigeria
70 Limited along Ife-Ibadan expressway. Surface water samples was collected at downstream into clean plastic kegs.
71 The water samples were collected during the month of April, 2015. Seeds of *Solanum lycopersicum* cultivar (ROMA
72 VF) were obtained from Institute of Agricultural Research and Training, Moor Plantation, Ibadan.
73 ### Culturing of Organisms
74 A culture of *Pseudomonas aeruginosa* was obtained from the Department of Microbiology, Obafemi Awolowo
75 University (OAU), Ile-Ife. A culture of *Trichoderma harzianum* was also obtained from the Mycology unit of the
76 Department of Crop Production and Protection, OAU, Ile-Ife. A single colony of *P. aeruginosa* was subcultured by
77 using nutrient agar in petri dishes and kept in the incubator for 48 hours at 37°C to a medium after which it was
78 harvested by flooding with sterile distilled water. The bacterium inoculum was prepared by streaking a single colony
79 of *P. aeruginosa* earlier isolated on plated nutrient agar plate and incubated at 37°C for 48 hours. Cells of *P.*
80 *aeruginosa* were harvested from agar plates by flooding with sterile distilled water and standardized using a
81 colorimeter to 10^8 CFU/ml. Spores of *Trichoderma harzianum* was subcultured by using potato dextrose agar in
82 petri dishes and kept in the incubator for 7 days at 37°C to a medium after which it was harvested by flooding with
83 sterile distilled water. The fungal spore solution was prepared by picking spores of *T. harzianum* earlier isolated on
84 plated potato dextrose agar plate and incubated at 37°C for 7 days. Spores of *T. harzianum* were harvested from agar
85 plates by flooding with sterile distilled water and standardized using a colorimeter to 10^7 spores/ml.
86 ### Planting of seeds and contamination of experimental pots
87 Seedlings of *S. lycopersicum* were raised on nursery beds for a period of three weeks. Sixty pots, each containing
88 three kilograms of soil from sterilized soil was used for this study. *Pseudomonas aeruginosa* inoculum solution (30
89 ml) was poured into a hole that was made in the middle of a set of 15 experimental pots containing sterized soil
90 before *S. lycopersicum* seedlings are transplanted to it. *Trichoderma harzianum* spore solution (30 ml) was also

91 poured into a hole that was made in the middle of another set of 15 experimental pots before *S. lycopersicum*
92 seedlings are transplanted to them. The third set of 15 pots received dual inoculation of *Trichoderma harzianum*
93 spore solution (15 ml) and *P. aeruginosa* innoculum before *S. lycopersicum* seedlings were transplanted into it; with
94 the final set of 15 pots acting as control at various levels. Thereafter, pot preparation was arranged in a completely
95 randomized design in the screenhouse.
96 Seedlings were left for a week to establish and overcome transplanting shock before wetting with the contaminated
97 stream water at various concentrations of 0%, 5% and 10% v/v. Contaminated stream water was quantified using the
98 formula: percentage soil contamination = (Volume of polluted stream water applied / Volume of soil) x 100. Each
99 treatment of the experiment was replicated three times. Twenty four pots were watered with the contaminated stream
100 water once during the experiment and another 24 pots watered daily with the contaminated stream water. The
101 remaining 12 pots which served as the control experiment were watered daily with distilled water. Pots containing *S.*
102 *lycopersicum* was watered regularly to ensure adequate moisture. Heavy metal analysis on the contaminated stream
103 water was carried out using AAS (Atomic Absorption Spectrophotometer) for Iron, Copper, Lead, and Zinc pre
104 experiment. Plant samples were also subjected to heavy metal analysis using AAS (Atomic Absorption
105 Spectrophotometer) for Iron, Copper, Lead, and Zinc post experiment. Pre and post – soil tests were carried out to
106 determine soil nutrients. Soil samples were also subjected to heavy metal analysis using AAS (Atomic Absorption
107 Spectrophotometer) for Iron, Copper, Lead, and Zinc pre and post – soil tests. Data obtained was subjected to
108 statistical analysis using descriptive and inferential methods.

109 **RESULTS**
110 The heavy metals analysis of the stream water showed that heavy metals (Iron, Zinc, Copper and Lead) were present
111 in high concentration in the water. Iron (Fe) had the highest concentration of 138.15 mg/L followed by zinc (Zn)
112 which had a concentration of 68.4 mg/L . The order of concentration was Fe>Zn>Cu>Pb.
113 After the soils were subjected to heavy metal analysis, it was observed that iron concentration of the soil increased
114 as the contaminated stream water concentration increased in all the treatments without any inoculation of
115 microorganism (Fig. 1). Treatments 3 and 3d inoculated with *P. aeruginosa* were lower in concentration of iron
116 compared to treatments 2 and 2d which were inoculated with *T. harzianum*. Treatment 1d without any inoculation of
117 microorganisms had highest iron concentration followed by treatment 1 also without any inoculation of
118 microorganisms at 5% and 10% contaminated stream water concentration. The order of iron concentration across the
119 treatments with 5% and 10% contaminated stream water concentration was 1d>1>4d>4>2d>2>3d>3 and
120 1d>1>4d>4>2d>2>3d>3 respectively.
121 Treatment 1d had the highest zinc concentration at 10% contaminated stream concentration followed by treatment 1
122 at the same 10% concentration. Treatment 3 at 0% concentration had the lowest iron concentration. Soil samples
123 treated with single or both micro-organisms had the lowest value in zinc compared to soil polluted with
124 contaminated stream water without any treatment with microorganisms (Fig 2). Treatment 3 had the lowest copper
125 level of 2.46 part per million (ppm) at 5% contaminated stream water concentration while treatment 1d had the
126 highest level of copper with 3.86 ppm at the same concentration (Fig 3). The order of copper concentration in 0%
127 and 10% was treatment4>2>2>1>3and 1d>1>4d>4>2d>2>3d>3 respectively. Lead analyses in the soil indicated
128 that the order of the concentration in 5% and 10% was 1d>1>4d>4>>2d>2>3d>3 and 1d>1>4d>4>>2d>2>3d>3
129 respectively, treatment 1d had the highest level of lead concentration followed by treatment 1 both at 10%
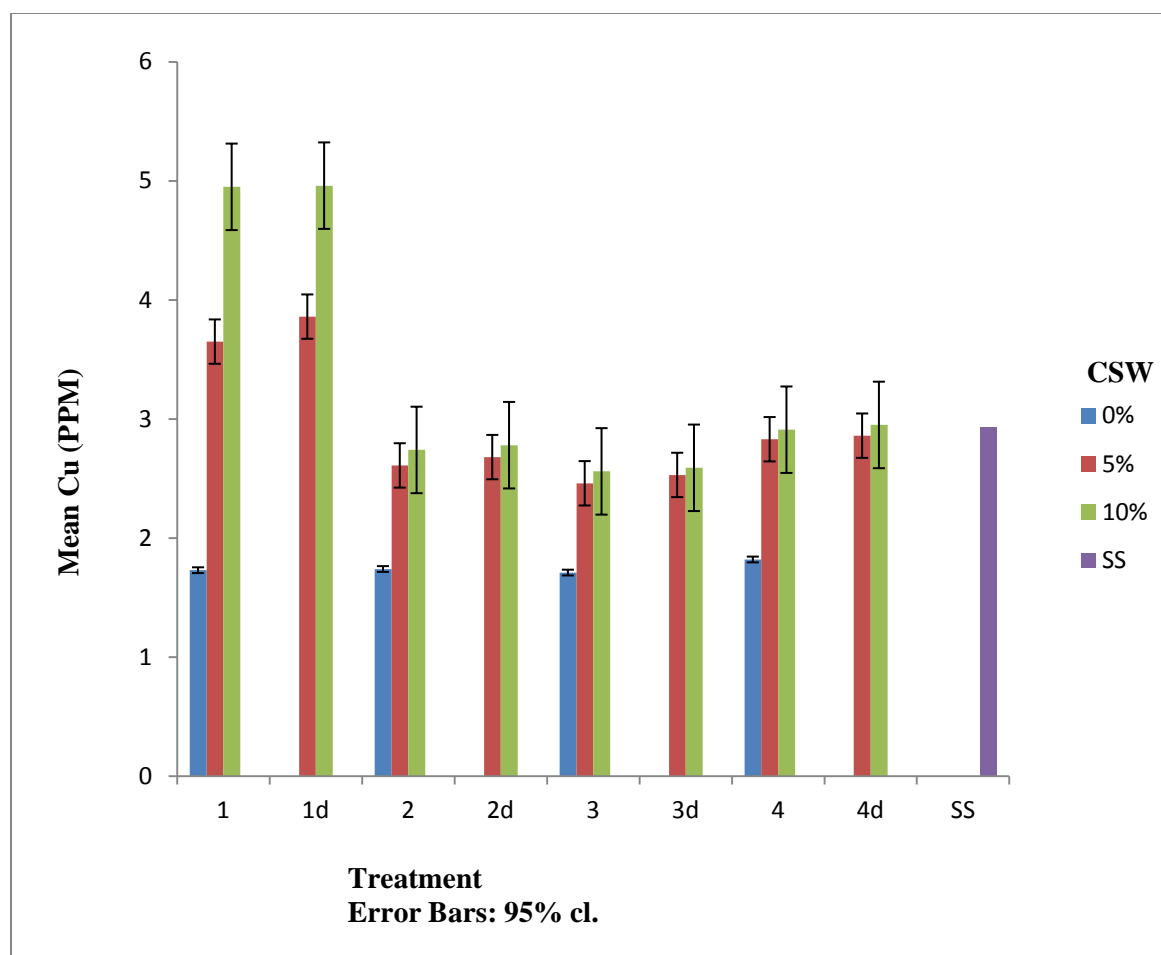130 contaminated stream water concentration while treatment 2 had the lowest at 0% (Fig. 4).
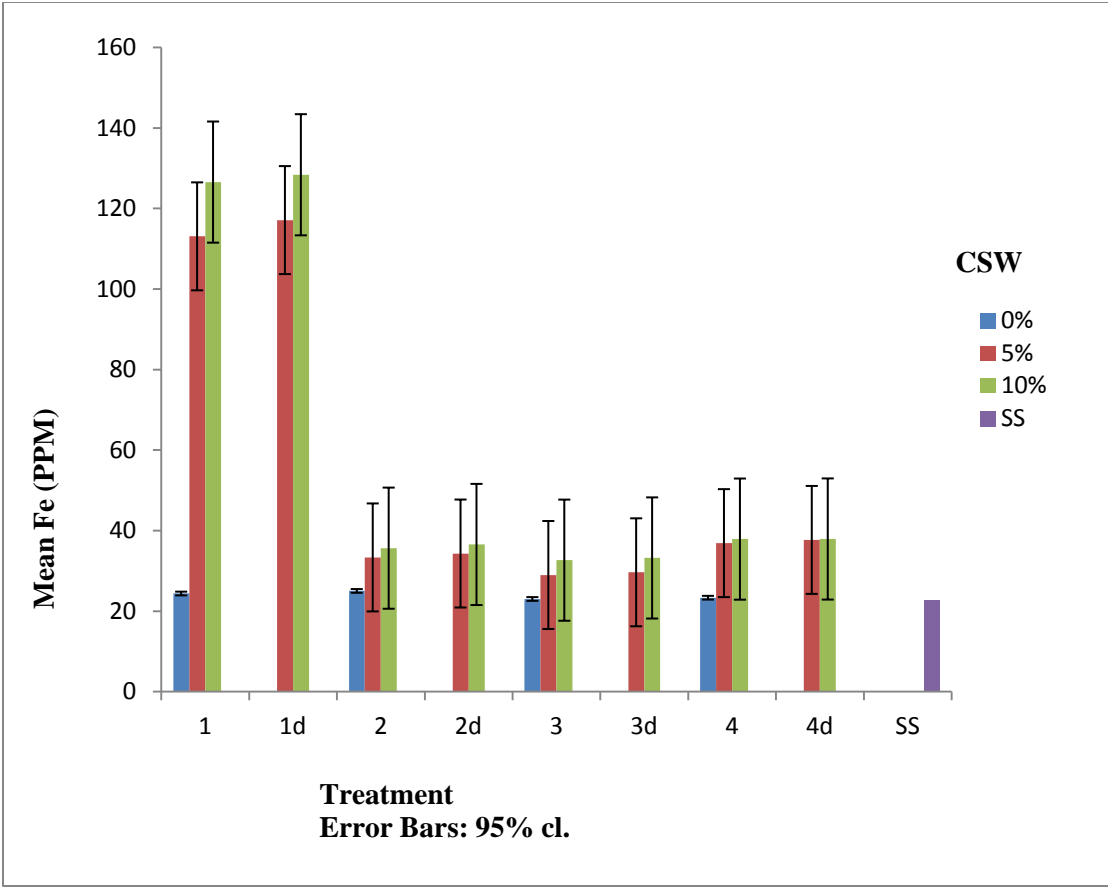
Figure 1: Iron (PPM) content of Pre and Post Planting Soil Samples

Legend

1-SS + TP

1d-SS + TP

2-SS + TH+ TP

2d-SS + TH + TP

3-SS + PA + TP

3d-SS + PA + TP

4-SS + TH + PA + TP

4d-SS + TH +PA +TP

SS - Sterilized soil before planting

d – Daily wetting of plants with contaminated stream water

Cl- Confidence level

TH – *T. harzianum*

PA - *P. aeruginosa*

TP - Test Plant

CSW- Contaminated Stream Water
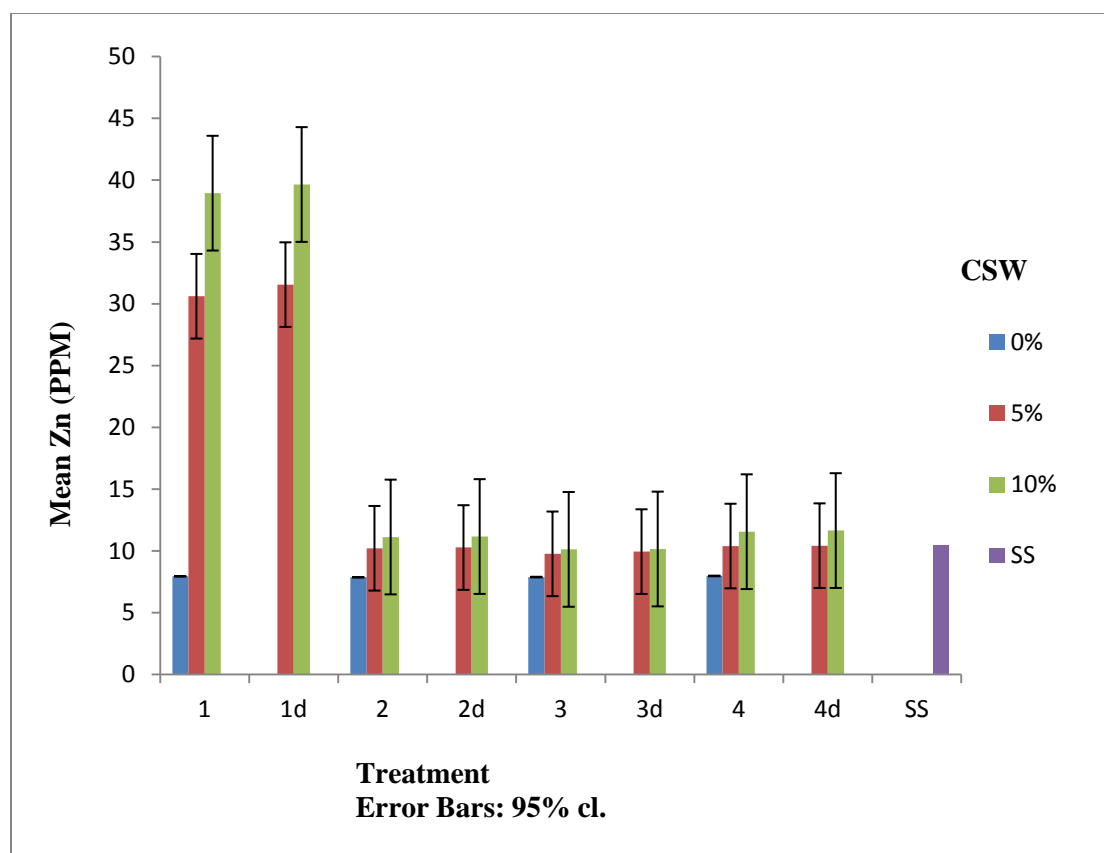
**Figure 2: Zinc (PPM) content of Pre and Post Planting Soil Samples**

**Figure 3: Copper (PPM) content of Pre and Post Planting Soil Samples**
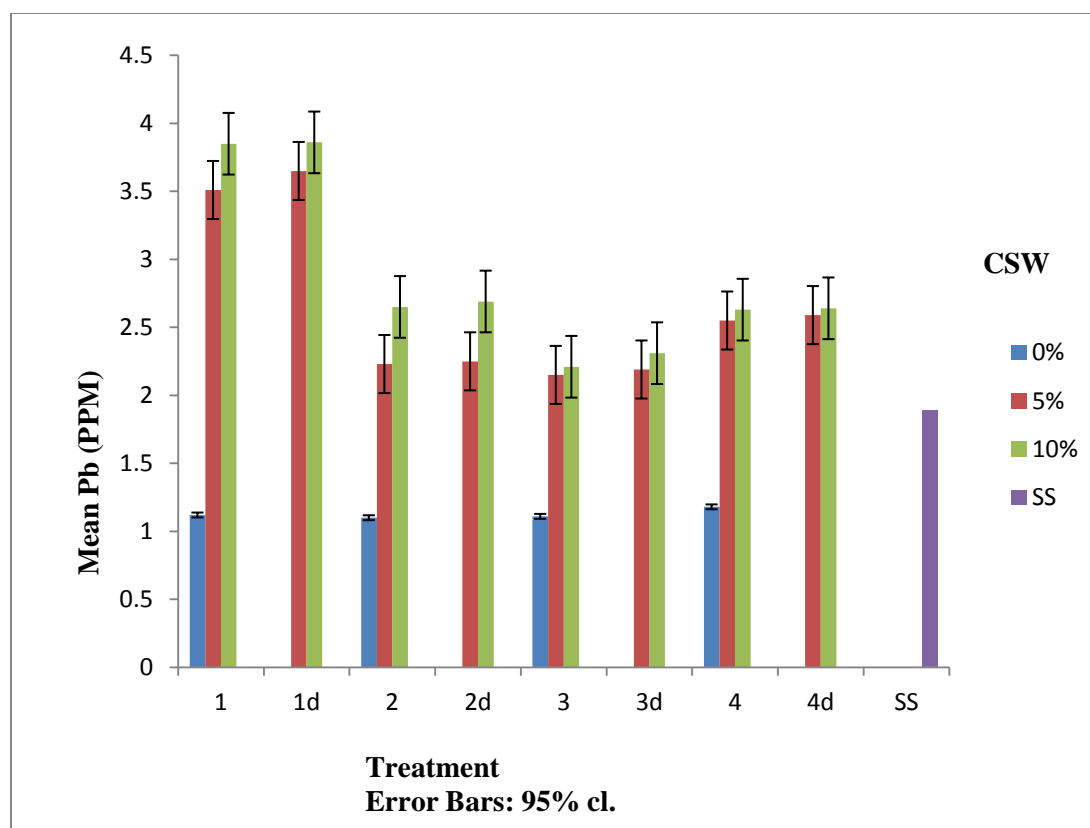
Figure 4: Lead (PPM) content of Pre and Post Planting Soil Samples

Heavy metal analysis carried out on plant samples showed that plants from soil samples without inoculation of micro-organisms had the highest heavy metal uptake as the concentration of contaminated stream water increased. For 5% contaminated stream water concentration, treatment 1 had the highest level of iron at 77.78 ppm followed by treatment1d with 77.71 ppm while treatment 3 had the lowest concentration of iron with 13.91 ppm (Fig 5).

The order of concentration in iron at 10% was sample 1>1d>4d>4>2d>2>3d>3. Zinc at 10% contaminated stream water concentration had the highest concentration in treatment 1d and the lowest at treatment 3 at same 10%. The order of zinc concentration at 5% was 1d>1>4d>4>2d>2>3d>3 while 10% was 1d>1>4d>2>2d>4>3d>3 (Fig. 6).

Copper in treatment 1d without any inoculation had the highest concentration at 5% and 10% followed by treatment 1 at same concentrations with treatment 3 inoculated with *P. aeruginosa* having the lowest value (Fig.7).

Lead content in the plant samples was highest in treatment 1d, followed by those from treatment 1 but lowest in treatment 3. Order of increase of lead is treatment 1d>1>4d>4>2d>2>3d>3 (Fig 8). Treatments 2 and 2d inoculated with *T. harzianum* had more of the heavy metal in plant tissue compared to treatments 3 and 3d treated with *P. aeruginosa*.
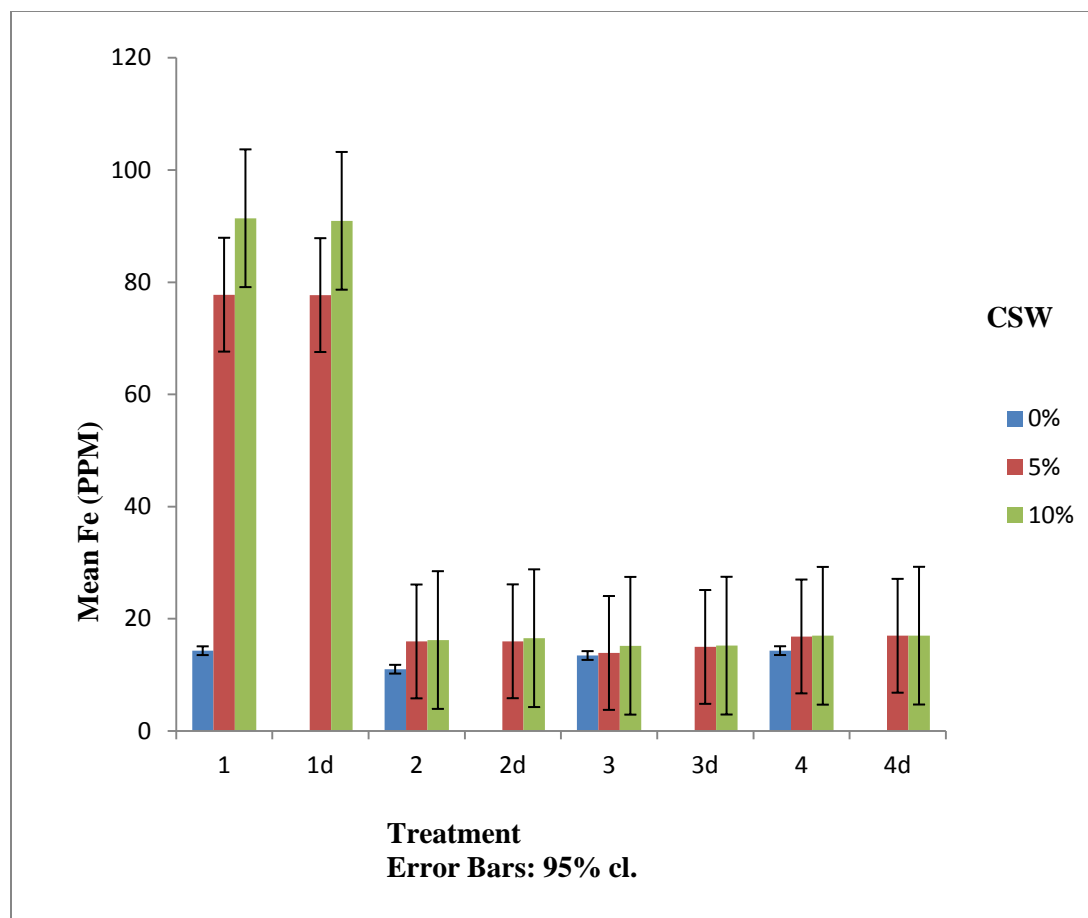
Figure 5: Iron (PPM) content of *S. lycopersicum* across all the treatments
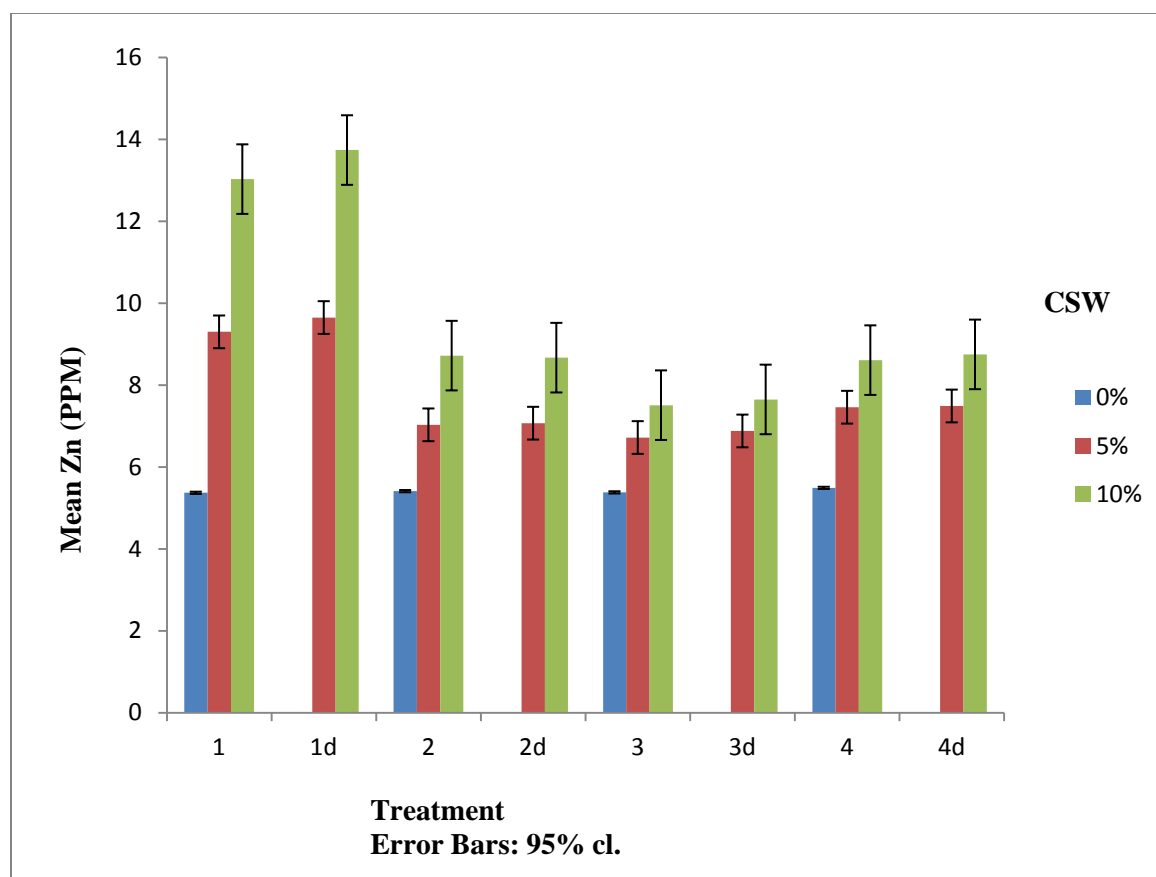
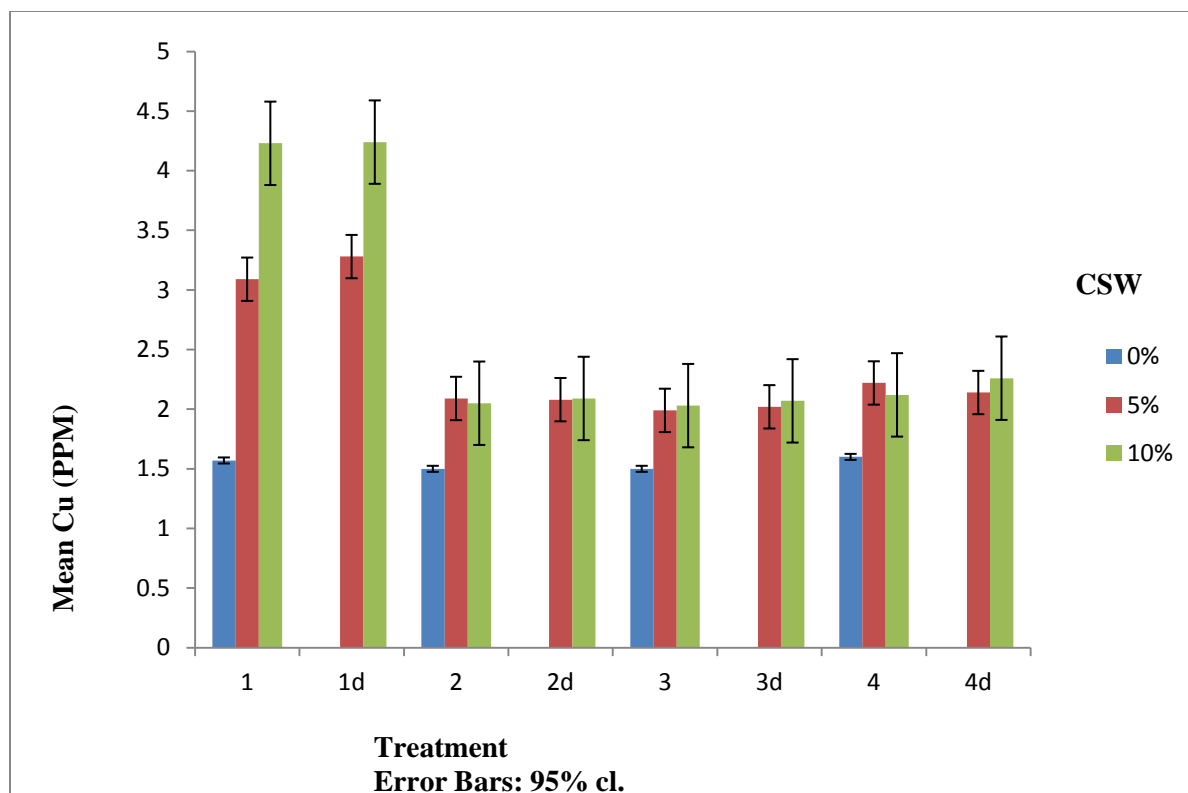**Figure 6: Zinc (PPM) content of *S. lycopersicum* across all the treatments**

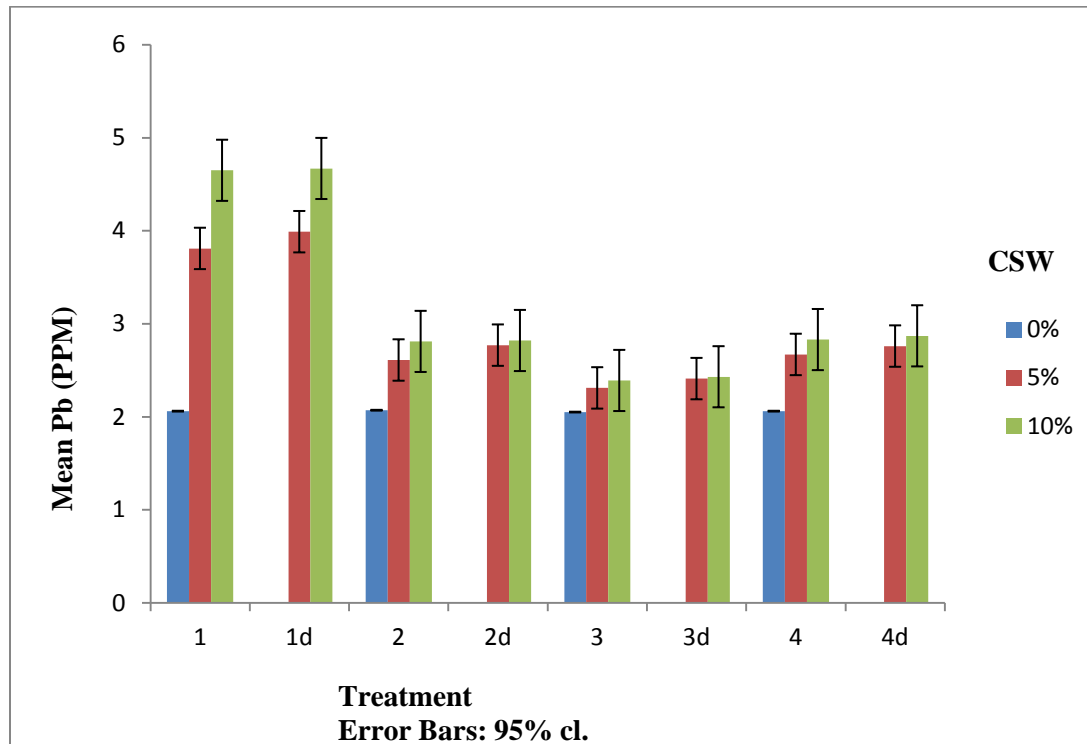**Figure 7: Copper (PPM) content of *S. lycopersicum* across all the treatments**

Figure 8: Lead (PPM) content of *S. lycopersicum* across all the treatments

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193 **DISCUSSION**

194 Heavy metals are elements that exhibit metallic properties such as ductility, malleability, conductivity, cation
195 stability, and ligand specificity (Opaoluwa, 2010). They are characterized by relatively high density and high
196 relative atomic weight with an atomic number greater than 20. Industrial effluents are usually considered as
197 undesirable for arable soil, plants, animals and human health. This is due to the contained heavy and trace metals
198 like Cr, Mn, Fe, Cu, Co, Zn, Ni, As, Cd and Pb that are discharged continuously into water source (streams/ nullahs,
199 canals and rivers). These are allowed to spread on agricultural lands. The unplanned disposal of these effluents has
200 increased the threat of environmental pollution (Gulfraz *et al.,* 2003). Soils, whether in urban or agricultural areas
201 represent a major sink for metals released into the environment from a wide variety of anthropogenic sources
202 (Niragu, 1991).

203 Su *et al.* (2014) reported that low concentration of heavy metals could stimulate microbial growth and increase
204 microbial biomass, while high concentration could decrease soil microbial biomass significantly. The
205 microorganisms used in this study (*T. harzianum* and *P. aeruginosa*) were highly effective in biodegrading heavy
206 metals. The bio-sorption potential of the organisms used in this study showed that *T. harzianum* and *P. aeruginosa*
207 posses effective heavy metal absorption capacity. It was discovered in this study that at higher concentrations of
208 these metals, there were reductions in plant growth. This may be due to the decrease in growth parameters of *S.*
209 *lycopersicum* as the contaminated stream water concentration increased in this study. Heavy metals of soil in all the
210 soil samples showed an increase as the contaminated stream water increased in concentration. Treatments inoculated
211 with *P. aeruginosa* were found to have lower concentration of heavy metals (Fe, Zn, Cu and Pb) followed by
212 treatments inoculated with *T. harzianum*. Due to a change in their oxidation state, heavy metals can be transformed
213 to become either less toxic, easily volatilized, more water soluble (and thus can be removed through leaching), less
214 water soluble (which allows them to precipitate and become easily removed from the environment) or less
215 bioavailable (Marques *et. al*., 2009).

216 The biodegrading ability of *P. aeruginosa* which showed the most efficient heavy metal uptake from the soil is in
217 agreement with report of Lewis *et al.* (2002) and Odeyemi *et al.* (2011) which stated that *Psedomonas* spp have a
218 high biodegrading ability. Report from Jankiewicz *et al.* (2000) also support the findings from this study which
219 noted that *P. aeruginosa* cells grown in biofilms accumulate higher amounts of heavy metals. Also, many species of
220 soil fungi including *Trichoderma* are able to dissolve through the release of chelating compounds of organic acids.
221 The fungus releasing organic acids causes acidification of the environment, which helps increase the mobility of
222 heavy metals (Barea *et al.,* 2005; Ledin, 2000; Wang and Chen, 2009). This study confirms this reports. Treatments
223 inoculated with dual inoculation of *T. harzianum* and *P. aeruginosa* were found to have slightly higher
224 concentration of heavy metals than treatments inoculated with *P. aeruginosa* or *T. harzianum*. However treatments
225 with no inoculation of one or two microorganisms showed very high concentration of heavy metals in the soil in
226 comparison with treatments with dual microorganisms. This confirms that the microorganisms used in this study
227 biodegraded the heavy metals in the soil. This also revealed that there is positive and productive interaction between
228 *T. harzianum* and *P. aeruginosa* in bioremediation of heavy metals heavy metals polluted soil.

229 Many species of plants have been successful in absorbing contaminants such as lead, cadmium, chromium, arsenic,
230 and various radionuclides from soils. Some metals with unknown biological function (Cd, Fe, Zn, Cu, Cr, Pb, Co,
231 Ag, Se, Hg) can also be accumulated (Cho-Ruk *et al.,* 2006). Contaminant uptake by plants and its mechanisms have
232 been being explored by several researchers. It could be used to optimize the factors to improve the performance of
233 plant uptake. According to Sinha *et al.* (2008), the plants act both as "accumulators" and "excluders". Accumulators
234 survive despite concentrating contaminants in their aerial tissues. They biodegrade or biotransform the contaminants
235 into inert forms in their tissues. The excluders restrict contaminant uptake into their biomass. Plant has a lot of
236 consequences from heavy metal pollution in soil (Liao 1993, Su *et al.,* 2014, Wu *et al.,* 1998), plants were also seen
237 to be polluted by heavy metals (Yin *et al*., 1999), which consequently threatens the health of animals and human
238 beings via the food chain (Wang *et al*., 2001).

239 Heavy metals such as cadmium and lead are non-essential elements for plants. Microbial populations are generally
240 higher in the rhizosphere than in the root-free soil. This is due to a symbiotic relationship between soil
241 microorganisms and plants. This symbiotic relationship can enhance some bioremediation processes. Plant roots also
242 may provide surfaces for sorption or precipitation of metal contaminants (Sas-Nowosielska *et al.,* 2008) .This study
243 were found to show reduction in growth parameters as heavy metals increased which is brought by increase in
244 contaminated stream water concentration. Iron, Zinc, Copper and Lead level were higher in plant tissues from soil
245 samples containing no inoculation of microorganisms at 5% and 10% contaminated stream water concentration. This
246 was discovered to affect the growth of the plants. Su *et al.,* (2014) reported that dicots, leafy vegetable crops are
247 sensitive to Zn toxicity, especially spinach and beet; because of their inherent high Zn uptake capacity. However soil
248 samples containing *P. aeruginosa* was generally the lowest in plant heavy metal uptake of iron, zinc, copper and
249 lead followed by samples containing *T. harzianum*. This may be an indication that the heavy metals in the soil had
250 been degraded by the microorganisms used which also showed there is low amount of heavy metals in soil left for
251 the plant to absorb. This result was found to be consistent with the work of Soumitra *et al.* (2014) which
252 demonstrated that *P. aeruginosa* reduced heavy metal uptake in *Oryza sativa* L. and increase its growth. Also
253 *Trichoderma* spp. produces organic acids such as gluconic acid, fumaric acid, and citric acid, which can decrease the
254 pH of the soil and allow for the dissolution of phosphate, as well as macro- and micronutrients such as iron,
255 manganese, and magnesium, which are necessary for plant metabolism (Ociepa, 2011; Cao *et al.,* 2008) Treatments
256 inoculated with a combination of *T. harzianum and P. aeruginosa* in this study had lower concentration of heavy
257 metals in their plant tissue compared to treatments without inoculation of microorganisms. This may insinuate that
258 there is positive and effective interaction between *T. harzianum* and *P. aeruginosa* in the reduction of heavy metals
259 build up in plant cultivated on heavy metals polluted soil. concentrations of metals were attributed to the
260 contaminated stream water irrigation. The results from this study indicates that there is a serious potential health risk
261 associated with heavy metals in tomato by using contaminated water for irrigation by farmers for tomato production.
262

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