Advanced Journal of Microbiology Research Vol. 2017
Available online at http://internationalscholarsjournals.org/journal/ajmr

Full Length Research Paper

In vitro effects of four heavy metals on glyphosate utilization by some bacteria isolated from rice fields

Okpala N. Gloria* and A. N. Moneke

Department of Microbiology, University of Nigeria, Nsukka, Enugu State, Nigeria.

Accepted 4 August, 2017

The effect of heavy metals (zinc, cadmium, chromium and lead) at concentrations 50, 100 and 500 g/ml of the heavy metal salts on glyphosate utilization by some bacterial species isolated from rice fields were studied, the addition of lead (Pb), cadmium (Cd) and zinc (Zn) to the glyphosate mineral salt medium used in growing the *Acetobacter* sp. significantly (P < 0.05) increased the rate of glyphosate utilization as indicated by the increase in the growth of the organism and could be attributed to easy uptake of the metal-glyphosate complex by the organism. The growth of *Pseudomonas fluorescens* was enhanced in the presence of zinc in the glyphosate medium, when compared to its growth in the medium without the heavy metals. This was followed by lead, cadmium and chromium, respectively.

Key words: Glyphosate herbicide, utilization, metal-glyphosate complex.

INTRODUCTION

Most agricultural lands are faced with contamination not just with herbicides and other xenobiotics but also with toxic metals. These metals find their way into the soil via runoffs, as constituents of chemicals (e.g. fertilizers) added to enhance soil productivity and through treated sewage sludge applied to the soil for soil enrichment and reinforcement.

Glyphosate [N-(phosphonomethyl) glycine] is a broad-spectrum, non-selective post emergence herbicide used widely as its isopropylamine salt (Roundup®) by rice and oil palm plantation farmers in Nigeria to get rid of weeds and also to optimize agricultural resources. Although this herbicide is not applied directly to the soil, a significant amount may reach the soil during early-season or pre-plant applications (Haney et al., 2000). The local farmers make use of the hand operated knapsack sprayers to distribute the herbicide at a field application rate of 2% v/v of the commercial formulation.

Glyphosate in the soil has been found to have profound effects on soil microorganisms (Krzysko-Lupicka and Sudol, 2008; Carlisle and Trevors, 1988). This is because it inhibits the enzyme 5-enolpyruvylshikimmic acid-3-phosphate synthase required for the synthesis of three essential aromatic amino acids (tryptophan, phenylalanine and tyrosine).

Roundup® contains polyoxyethylene amine (POEA) surfactant that enhances its herbicidal efficacy and is thought to contribute significantly to its toxicity (Tsui et al., 2005). In the soil, glyphosate binds to organic matter, broken bonds of clay minerals and oxides of iron and aluminium (Morillo et al., 2000). The binding of glyphosate is said to be strongly influenced by cations associated with the soil (Carlisle and Trevors, 1988).

Glyphosate is a metal-chelating herbicide (Subramaniam and Hoggard, 1988) which strongly binds to cations, most especially transition metals due to its three functional groups (the carboxylate, phosphonate and amine) to form metal - glyphosate complexes (Tsui et al., 2005). The formation of these complexes leads to reduction in the herbicidal activity of glyphosate as well as affecting its bioavailability to microorganisms (Hall et al*.,* 2000; Tsui et al*.,* 2005).

Heavy metals are a dangerous group of soil pollutants that cannot be naturally degraded and as such accumulate in different parts of the food chain (Šmejkalová et al*.,* 2003). Although some are essential for growth, others can be harmful to living organisms by forming complexes with protein molecules thereby rendering them inactive (Kim, 1985; Azza et al., 2009). Heavy metals have been shown to have detrimental effects on microorganisms even at low concentrations (Kim, 1985) by affecting microbial growth, morphology, biochemical activities and enzymatic processes (Nweke et al., 2007). However,

*Corresponding author. E-mail: glocos2006@yahoo.com. Tel: +2348060215515.

some microorganisms are able be tolerate these metals at varying concentrations. The ability of an organism to survive in an environment with high metal concentration or its capacity to accumulate high concentration of heavy metal without dying reflects its capacity to tolerate metals (Azza et al., 2009). Heavy metals have been reported to be powerful inhibitors of biodegradation activities (El. Deeb and Altalhi, 2009). Hence, their presence may hinder or affect the rate of glyphosate degradation in soil and water.

The present work was aimed at evaluating the effects of different concentrations of zinc, lead, cadmium and chromium on some soil bacterial isolates previously known to utilize glyphosate even at high concentrations (Moneke et al., 2010).

MATERIALS AND METHODS

Chemical

The isopropylamine salt of glyphosate as Roundup® (containing 360 g/l a.e of glyphosate; Monsanto) was purchased from a local dealer's store in Nsukka, Enugu state, Nigeria. The heavy metal ions, Pb^{2+}, Cd^{2+}, Zn^{2+} and Cr^{2+} were used as $PbCl_2$, $CdCl_2$, $ZnSO_4$ and $CrCl_2$, respectively. All other chemicals were of the highest purity commercially available.

Microorganisms

The bacterial isolates (*Pseudomonas fluorescens* and *Acetobacter* sp.) used for this study were previously isolated from glyphosate contaminated rice fields and their ability to utilize glyphosate at different concentrations evaluated in that study (Moneke et al., 2010).

Medium

A modified mineral salts medium (MSM) of Dworkin and Foster (1958) was used and consisted of (g/l): glucose (1.0), $(NH_4)_2SO_4$, (0.375); $MgSO_4$, (0.075); $CaC0_3$, (0.03); $FeSO_4.7H_2O$, (0.001); H_3BO_3, (0.000001), $MnSO_4$, (0.000001), and Tris buffer (6.05). The pH of the medium was adjusted to 7.0.

Inoculum preparation and standardization

Inocula used for the study were prepared by inoculating isolates into nutrient broth and incubated at 30°C for 24 h using sterile normal saline, the cells from the above cultures were resuspended to a 0.5 McFarland nephelometer standard (Optical density of 0.17 at 660 nm).

Growth conditions

Prior to media preparation, all glassware were washed with 1 N HCl and thoroughly rinsed with deionized water to remove contaminating phosphate ions. Experiments were performed using 300 ml Erlenmeyer flasks containing 150 ml of medium, which received 1 ml inocula containing (0.5 Macfarlane). All cultures were incubated at 30°C on a rotary shaker (Gallenkamp, England) at 120 rpm. Culture turbidity measurements were made by withdrawing Samples (5 ml) aseptically at intervals with a spectrophotometer (Spectronic 20, USA) at 660 nm.

Comparative effects of heavy metals on *Pseudomonas fluorescens* and *Acetobacter* sp.

A concentration 25 g/ml of each of the salts of the selected heavy metals ions (Pb^{2+}, Cd^{2+}, Zn^{2+} and Cr^{2+}) were added to 150 ml of the mineral salts medium. The medium was autoclaved prior to the addition of 1 ml of the filter-sterilized Roundup® (7.2 mg/ml of glyphosate). This concentration was used because it is equivalent to the field application rate. 1 ml aliquot of each isolate was used in inoculating the cultures and incubation was carried out for 5 days.

Effect of different concentrations of heavy metals

The medium for the study was prepared as described earlier and was autoclaved prior to the addition of the filter -sterilized Roundup®. The medium was supplemented with the following heavy metal ions Pb^{2+}, Cd^{2+}, Zn^{2+} and Cr^{2+} at concentrations of 50, 100 and 500 (g/ml of their salts). Filter-sterilized glyphosate (7.2 mg/ml) was added to each replicate flask and also, freshly prepared 1 ml inoculum of the isolates was used in inoculating the flasks and growth monitored for 120 h. Inoculated flasks were incubated at 30°C on a rotary shaker (Gallenkamp, England) at 120 rpm. Samples (5 ml) were aseptically withdrawn at 12 h intervals and used to assay for growth by measuring the optical density with a spectrophotometer (Spectronic 20, USA) at 660 nm.

Treatment effects on the growth of the bacterial isolates at the different time periods were analysed using 2 way ANOVA. Number of replicates for each treatment was 3 and the level of significance was concluded using least significant difference at 5% probability level ($P < 0.05$). The statistical package used was GENSTAT.

RESULTS

Comparative effects of heavy metals on *Acetobacter* sp. and *Pseudomonas fluorescens*

Figure 1 depicts the effects of the different heavy metals on the growth of *Acetobacter* sp. and *P. fluorescens* for 5 days. For *Acetobacter* sp. maximum growth was observed in the medium containing lead (mean OD = 0.1434). This was highly significant ($P < 0.05$) when compared to the growth of *Acetobacter* sp. in thepresence of the other heavy metals. The growth of *Acetobacter* sp. in the medium containing zinc was also significantly ($P < 0.05$) higher when compared with its growth in cadmium (0.1143) and chromium (0.0783), which gave the least growth. The growth of *P. fluorescens* in the medium containing zinc gave the most significant ($P < 0.05$) growth (mean OD = 0.1338). This was closely followed by lead, cadmium and chromium (mean OD = 0.1184, 0.1004 and 0.09927), respectively.

Effects of different concentrations of cadmium on the growth of *Acetobacter* sp.

As shown in Figure 2, the growth of *Acetobacter* sp in the medium containing 500 g/ml was significantly ($P < 0.05$)

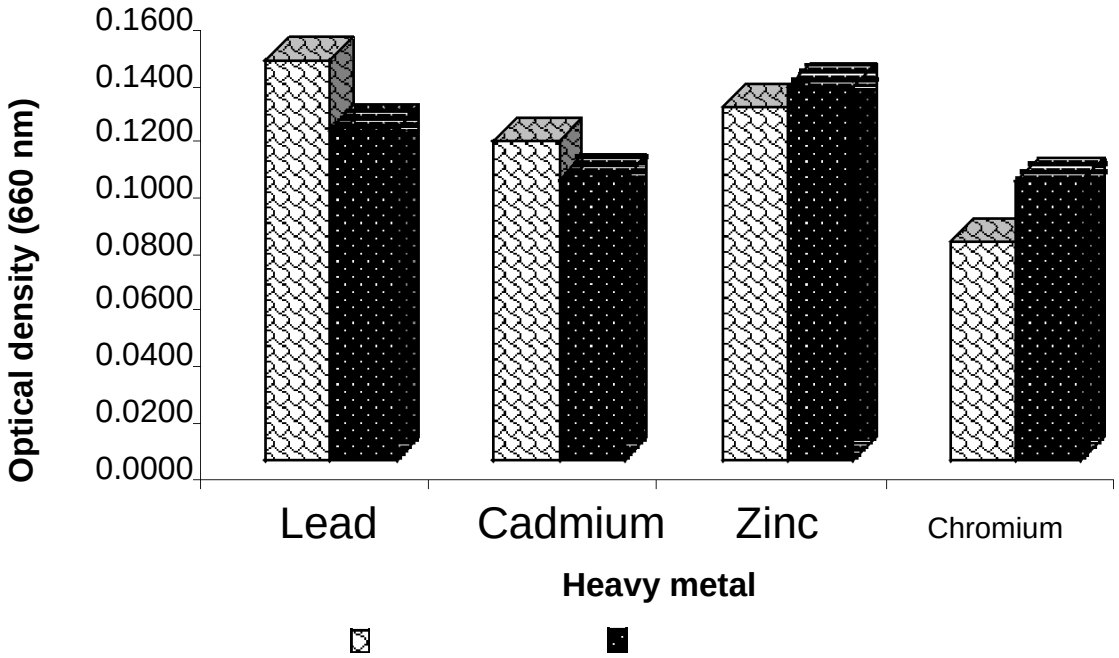

Figure 1. Comparative effects of heavy metals on *Acetobacter* sp. and *P. Fluorescens.*

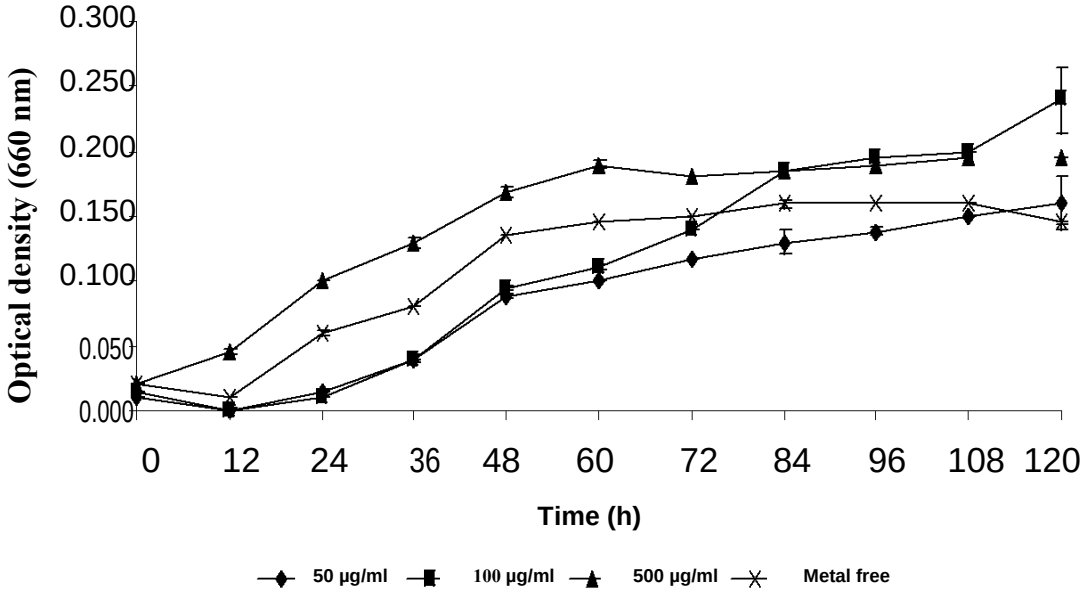

Figure 2. Growth of *Acetobacter* sp. on glyphosate mineral salts medium containing different concentrations of cadmium.

higher when compared with its growth at the other concentrations used. A 12 h lag period was observed at concentrations of 50 and 100 g/ml before any appreciable growth was observed after the 36 h incubation. The highest (peak) growth was seen in the medium containing 100 g/ml after 120 h. This increase was significant ($P < 0.05$) when compared with others after 120 h incubation.

Effects of different concentration of chromium on the growth of *Acetobacter* sp.

The growth of the isolate at the different concentration of

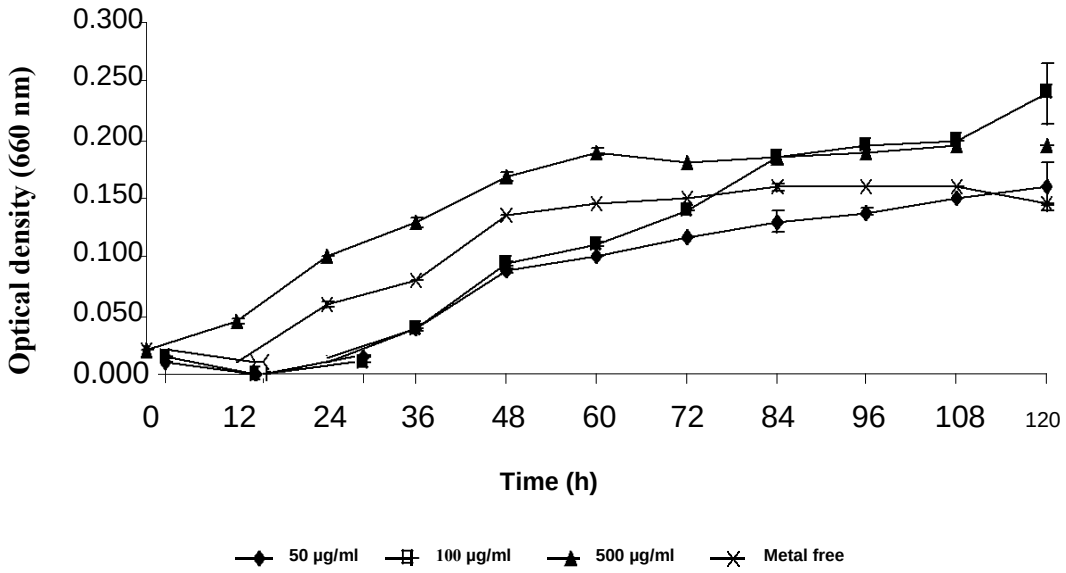

Figure 3. Growth of *Acetobacter* sp on glyphosate mineral salts medium containing different concentrations of chromium.

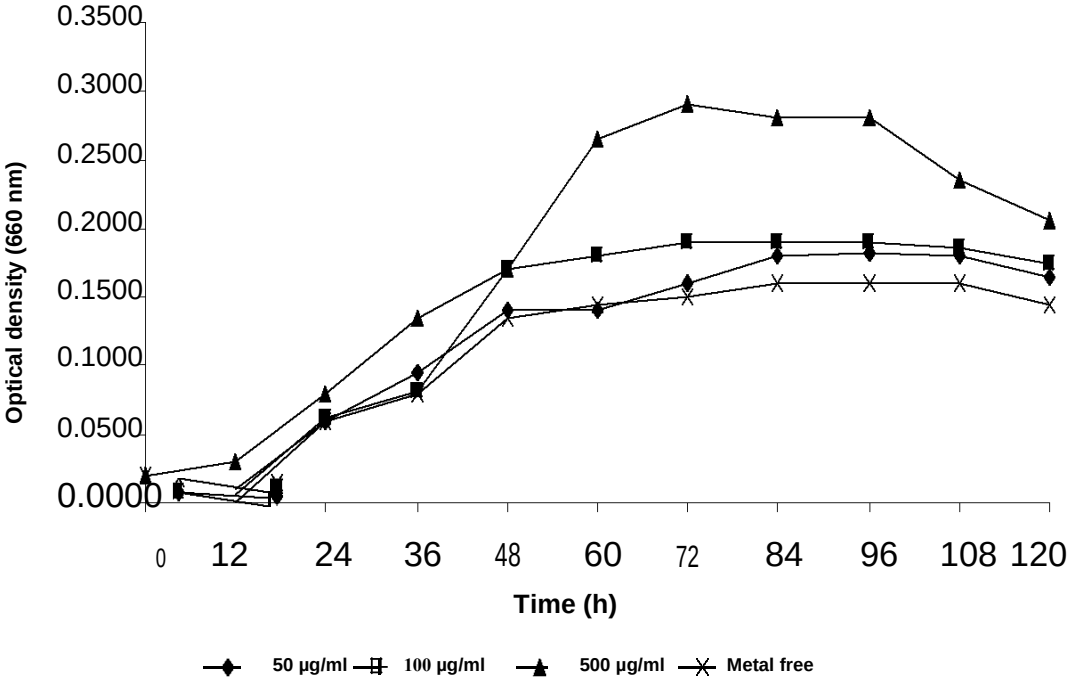

Figure 4. Growth of *Acetobacter* sp. on glyphosate mineral salts medium containing different concentrations of lead.

chromium followed a similar pattern as shown in Figure 3. A 12 h time lag was observed in the medium containing 50 and 100 g/ml of chromium. Maximum growth was observed in the medium containing 50 g/ml of chromium after 96 h of incubation (mean OD = 0.170) and it was significant when compared to others.

Effects of different concentrations of lead on the growth of *Acetobacter* sp.

The growth of *Acetobacter* sp. increased progressively after a 12 h lag period at the different concentrations (Figure 4). The growth at a concentration of 500 g/ml of

lead gave the most significant ($P < 0.05$) yield, with maximum growth observed after the 72 h (mean OD = 0.2900).

Effects of different concentrations of zinc on the growth of *Acetobacter* sp.

The addition of zinc at a concentration of 500 g/ml gave the most significant ($P < 0.05$) yield (Figure 5). Maximum growth was observed after 96 h with a mean OD of 0.3250. After 12 h of incubation, a lag period was observed with the three different concentrations before any appreciable increase in growth.

Effects of different concentrations of cadmium on the growth of *Pseudomonas fluorescens*

Appreciable growth of *P. fluorescens* in the medium containing cadmium (at different concentrations) was only observed after 24 h (50 g/ml) and 36 h (100 and 500 g/ml) of incubation. Figure 6 shows a progressive increase in the growth of *P. fluorescens* at the different concentrations of cadmium. Its growth in the medium containing 500 g/ml concentration was significantly higher ($P < 0.05$) when compared with others. Peak growth at this concentration was seen after 96 h incubation (mean OD = 0.2350).

Effects of different concentrations of chromium on the growth of *P. fluorescens*

In Figure 7, a consistent increase in growth of *P. fluorescens* was observed in the media containing 50 and 100 g/ml concentrations of chromium all through 120 h of monitoring. Maximum growth was recorded in the medium containing 100 g/ml of chromium after 96 h incubation (mean OD = 0.180) and was significant ($P < 0.05$) when compared with other concentrations at that time. The growth of the isolate in the medium containing 500 g/ml had its peak growth after 36 h (mean OD of 0.130) and thereafter, there was a steady decline.

Effects of different concentrations of lead on the growth of *Pseudomonas fluorescens*

The growth pattern of *P. fluorescens* in the medium containing lead at different concentrations was similar (Figure 8).

However, after 72 h incubation, a significant ($P < 0.05$) increase in growth was observed in the medium containing 500 g/ml ((mean OD = 0.210). Also, after 108 h incubation another growth peak was observed in the medium containing 50 g/ml (mean OD = 0.2130).

Effects of different concentrations of zinc on the growth of *P. fluorescens*

The addition of 500 g/ml of zinc to the glyphosate mineral salt medium yielded the most significant ($P < 0.05$) growth when compared with its growth at 50 and 100 g/ml concentrations. A growth peak was recorded after 96 h incubation (mean OD = 0.335) in this medium (Figure 9).

DISCUSSION

Gram negative bacteria show higher tolerance to heavy metals than their gram positive counterparts due to their higher level of intrinsic metal resistance (Ahmad et al., 2005). This difference is based on the chemical composition of their cell wall. The two bacterial species used are gram negative organisms thus supporting their ability to tolerate high concentrations of the heavy metals. Noghabi et al. (2007) reported the high capability of heavy metals bioaccumulation by gram negative bacteria. Many bacterial-resistance systems for toxic metals are plasmid- encoded (Silver, 1999; Jankowska et al., 2006). However, some are chromosomal (Gupta et al., 1999).

Heavy metals have been reported to stimulate microbial growth (Gikas, 2007; Gikas et al., 2009) . Kools et al. (2005) reported that the presence of heavy metals increased the rate of glyphosate utilization. They proposed that this could be because metal-glyphosate complexes are transported more efficiently across microbial cell walls than the sole compound. This supports the findings of this study which showed that the addition of lead (Pb), cadmium (Cd), and zinc (Zn) to the glyphosate medium used in growing the *Acetobacter* sp. increased the rate of glyphosate utilization as indicated by the increase in the growth of the organism. None of the metals used completely inhibited the growth of the organism. The organisms grew very well in the presence of lead at all the concentrations (50, 100, 500 µg/ml) used.

This was closely followed by zinc, cadmium and chromium. The highest growth of the organism was observed at the highest concentration of the heavy metal (500 g/ml) with the exception of chromium. Although chromium serves as an essential trace metal over-exposure or very high concentrations of it has cytotoxic and genotoxic effects (cell death, cell transformation and mutation) (Carmago et al., 2005).

The growth of *P. fluorescens* was more enhanced in the presence of zinc (Zinc is an essential element for the normal activity of DNA polymerase and protein synthesis) in the glyphosate medium. This was followed by lead, cadmium and chromium respectively. Unlike the response of *Acetobacter* sp. to 500 µg/ml of chromium, *P. fluorescens* showed more tolerance to chromium at that concentration. Studies by Bopp et al. (1983) and Viti et al. (2006) showed that *P. fluorescens* has the capacity to

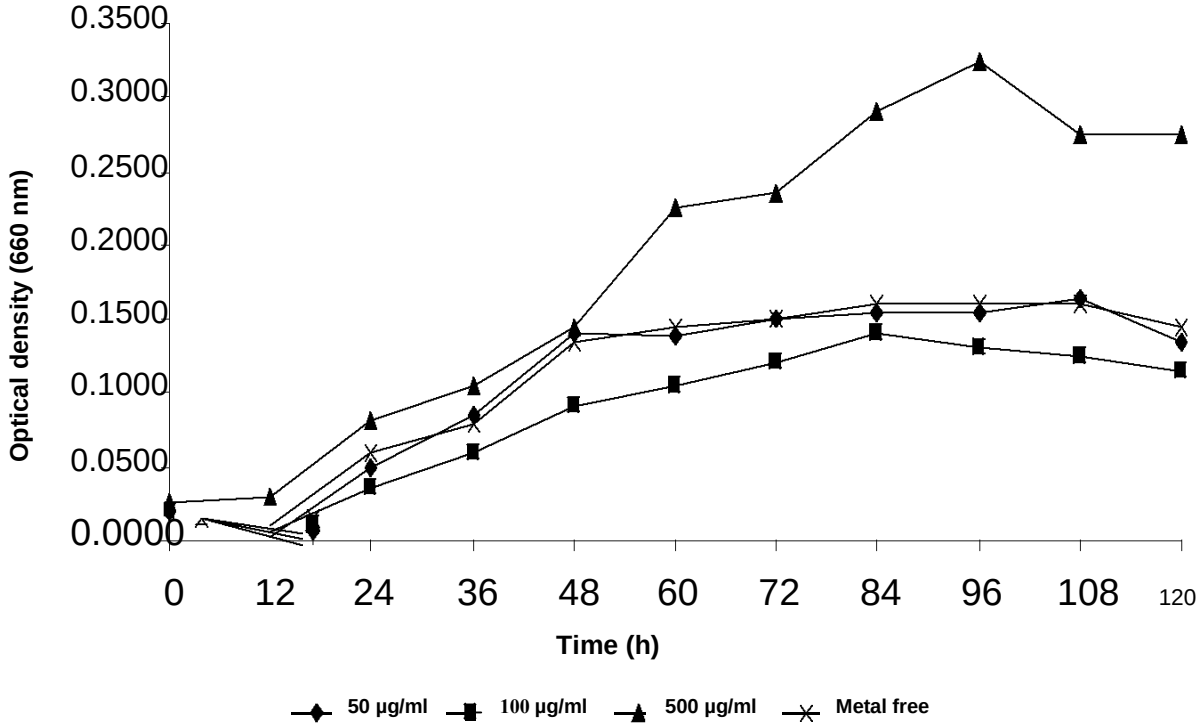

Figure 5. Growth of *Acetobacter* sp. on glyphosate mineral salts medium containing different concentrations of zinc.

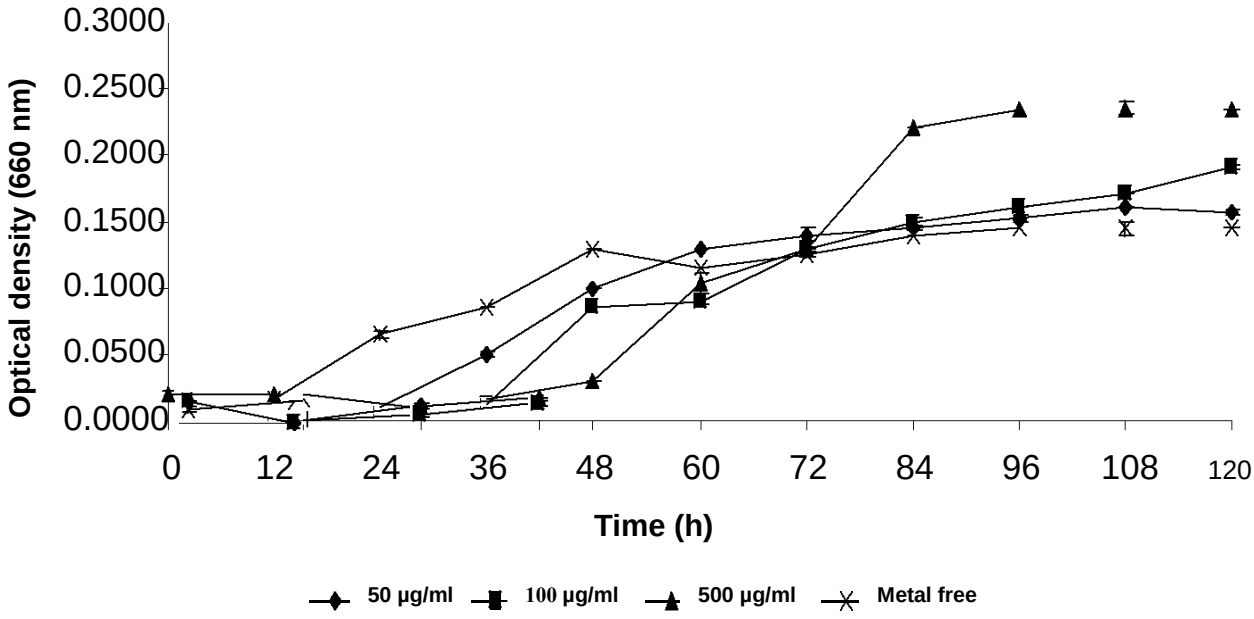

Figure 6. Growth of kinetics of *Pseudomonas fluorescens* on glyphosate mineral salts medium containing different concentrations of cadmium.

resist chromate and grow. This report is consistent with our findings.

In this study, it was observed that 500 ug/ml of chromium in the glyphosate medium caused a reduction in the growth of *P. fluorescens*. Chromate resistance in *Pseudomonas* sp. has been shown to be plasmid-

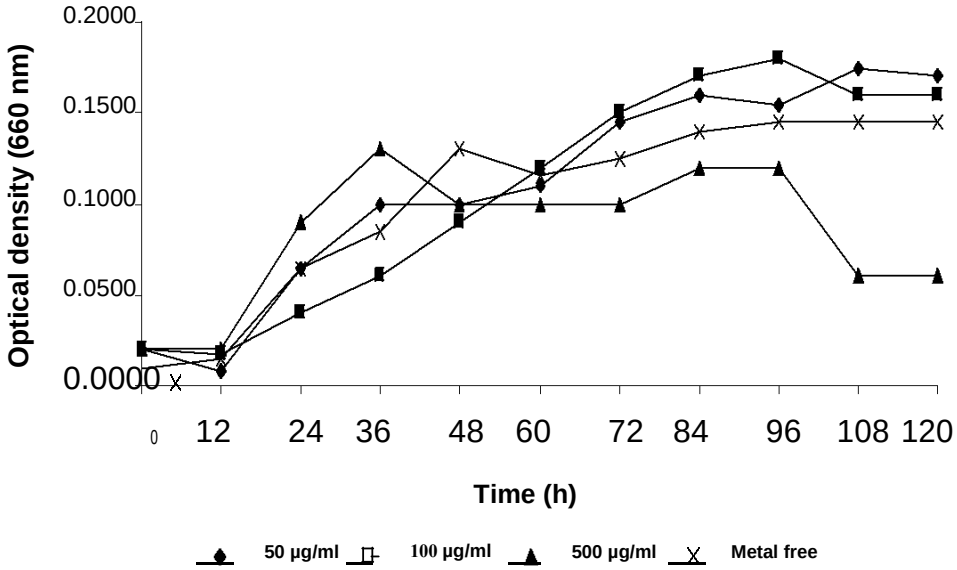

Figure 7. Growth of kinetics of *P. fluorescens* on glyphosate mineral salts medium containing different concentrations of chromium.

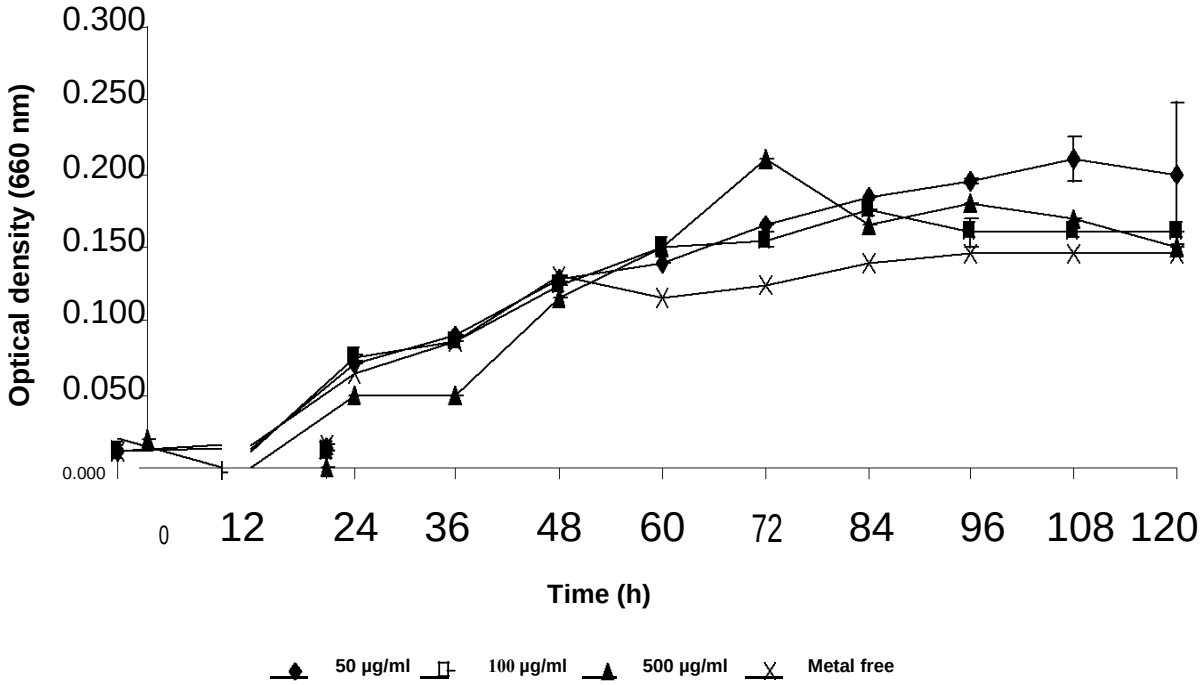

Figure 8. Growth of kinetics of *P. fluorescens* on glyphosate mineral salts medium containing different concentrations of lead.

associated and transmissible to a restricted host range (Nriagu and Nieboer, 2000; El.Deeb and Altahli, 2009). Chromium at a concentration of 500 ug/ml caused a significant reduction in the growth of *Acetobacter sp*. with an eventual inhibition of the isolate in the medium. In Figure 3, two peaks were observed for the growth of the organism on chromium, Viti et al. (2006) suggests a possible explanation for this; the sudden drop could be due to toxic shock that chromium had on the isolate causing cell lysis. Thereafter, leaking nutrients from the lysed cells aided the growth of the more resistant cells. The overall reduction in the growth of the isolates by

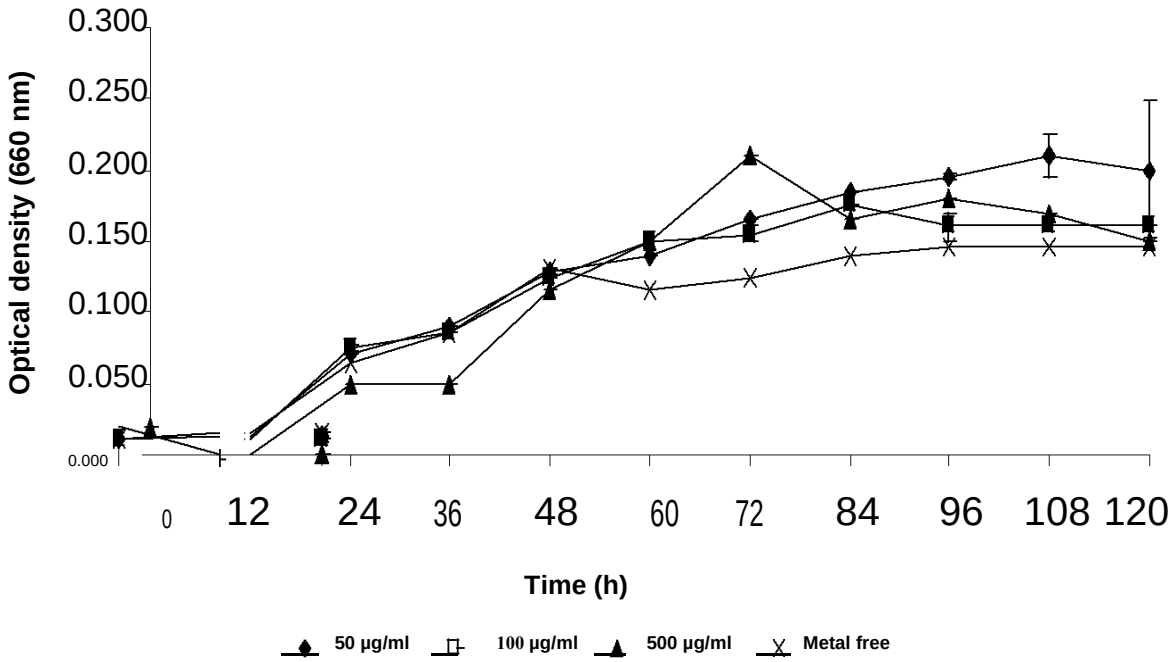

Figure 9. Growth of kinetics of *Pseudomonas fluorescens* on glyphosate mineral salts medium containing different concentrations of zinc.

chromate might be explained by the higher requirements for maintenance energy in the presence of the metal (Giller et al., 1998) or due to cell lysing as an effect of the exposure to heavy metal, as growth was measured by the optical density. In the first case, the bacteria expend energy to repair the cell damages caused by the metal toxicity, or because they have to use alternative enzymatic pathways to adapt to the new environmental conditions (Gikas et al., 2009).

The effect of increasing concentrations (50, 100, 500 ug/ml) of Zn was examined. It can be concluded that low concentrations of Zn (50 and 100 ug/ml) did not exhibit any inhibitory effect on the isolates. However, at low concentration of this metal, the growth of the isolates was similar as in the control (glyphosate alone) . A ten-fold increase in the concentration of the Zn caused a significant increase in the growth of the isolates. A probable explanation for this is that Zn is an essential trace element that is important in forming complexes (such as zinc fingers in DNA) and as a component in cellular enzymes (Spain and Alm, 2003). Zinc is usually accumulated by an unspecific uptake mechanism that is generally coupled to magnesium.

In conclusion, the results of this study showed increased uptake of glyphosate in the presence of the metals. When the metals were tested individually at the concentrations used, they did not show any detrimental effect on the growth of isolates. Further studies are ongoing in evaluating the effects these metals would have on the isolates when combined at different concentrations. Since in the field these metals (Zn, Pb, Cd, Cr) and others co-exist in the soil and affect the rate at which this organisms degrade glyphosate.

REFERENCES

Ahmad I, Hayat S, Ahmad A, Inam A, Samiullah (2005). Effect of heavy metal on survival of certain groups of indegenious soil microbial popuplation. J. Appl. Sci. Environ. Manage., 9(1): 115-121.

Azza A, Abou Z,Wesam AH, Hedayat MS, Ghada A, Fahd A (2009). Biosorption of some heavy metal ions using bacterial species isolated from agriculture waste water drains in Egypt. J. Appl. Sci. Res., 5(4): 372-383.

Bopp LH, Chakrabarty AM, Ehrlich HL (1983). Chromate resistance plasmid in *Pseudomonas fluorescens*. J. Bacteriol., 155: 1105-1109.

Carmago FAO, Okeke BC, Bento FM, Frankenberger WT (2005). Diversity of chromium-resistant bacteria isolated from soils contaminated with dichromate. Appl. Soil Ecol. 29: 193–202.

Carlisle SM, Trevors JT (1988). Glyphosate in the environment. Water, Air Soil Pollut., 39: 409-420.

Dworkin M, Foster JW (1958). Experiments with some microorganisms which utilized ethane and hydrogen. J. Bacteriol. 75: 592-603.

El.Deeb B, Altalhi D (2009). Degradative plasmid and heavy metal resistance plasmid naturally coexist in phenol and cyanide assimilating bacteria. Am. J. of Biochem. Biotech., 5(2): 84-93.

Gikas P (2008).Single and combined effects of nickel (Ni(II)) and cobalt (Co(II)) ions on activated sludge and on other aerobic microorganisms: A review. J. Hazardous Mater., 159: 187-203.

Gikas P (2007). Kinetic responses of activated sludge to individual and joint nickel (Ni(II)) and cobalt (Co(II)) - an isobolographic approach. J. Hazardous Mater., 143: 246-256.

Gikas P, Sengor SS, Ginn T, Moberly J, Peyton B (2009). The effects of heavy metals and temperature on microbial growth and lag. Global Nest J., (in press).

Giller KE, Witter E, McGrath SP (1998). Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. Soil Biol. Biochem., 30: 1389-1414.

Gupta A, Phung L, Chakravarty L Silver S (1999). Mercury resistance in

Bacillus cereus RC607: transcriptional organization and two new open reading frames. J. Bacteriol. 181: 7080-7086.
Hall GJ, Hart CA, Jones CA (2000). Plants as sources of cations antagonistic to glyphosate activity. Pest Manage. Sci. 56: 351-358.
Haney RL, Senseman SA, Hons FM, Zuberer DA (2000). Effect of glyphosate on soil microbial activity and biomass. Weed Sci., 48: 89-93.
Jankowska K, Ola czuk-Neyman K, Kulbat E (2006). The sensitivity of bacteria to heavy metals in the presence of mineral ship motor oil in coastal marine sediments and waters**.** Polish J. Environ. Stud., 15(6): 935-941.
Kim S (1985). Effect of heavy metals on natural populations of bacteria from surface microlayers and subsurface water. Marine Ecology – Progress Series, 26: 203-206.
Kools SAE, van Roovert M, van Gestel CAM, van Straalen NM (2005). Glyphosate degradation as a soil health indicator for heavy metal polluted soils. Soil Biol. Biochem., 37: 130-1307.
Krzysko-Lupicka T, Sudol T (2008). Interactions between glyphosate and autochthonous soil fungi surviving in aqueous solution of glyphosate. Chemosphere, 71: 1386-1391.
Moneke AN, Okpala GN, Anyanwu CU (2010). Biodegradation of glyphosate herbicide *in vitro* using bacterial isolates form four rice fields. Afr. J. Biotech., 9(26): 4067-4074.
Morillo E, Undabeytia T, Maqueda C, Ramos A (2000). Glyphosate adsorption on soils of different characteristics: Influence of copper addition. Chemosphere, 40: 103-107.
Noghabi KA, Zahiri HS, Yoon SC (2007). The production of a cold-induced extracellular biopolymer by *Pseudomonas fluorescens* bm07 under various growth conditions and its role in heavy metals absorption. Process Biochem., 42(5): 847-855.
Nriagu JO, Nieboer E (2007). Chromium in the natural and human environments. Advances in Environ. Sci. Tech., 20: 339-340.
Nweke CO, Ntinugwa C, Obah IF, Ike SC, Eme GE, Opara EC, Okolo JC, Nwanyanwu CE (2007). *In vitro* effects of metals and pesticides on dehydrogenase activity in microbial community of cowpea (*Vigna unguiculata*) rhizoplane. Afr. J. Biotech*.,* 6(3): 290-295.
Silver S (1996). Bacterial resistances to toxic metal ions – A review. Gene, 179(1): 9.
Šmejkalová M, Mikanová O, Bor ka L (2003). Effects of heavy metal concentrations on biological activity of soil microorganisms. Plant Soil Environ., 49(7): 321-326.
Spain A, Alm E (2003). Implications of microbial heavy metal tolerance in the environment. Rev. Undergraduate Res., 2: 1-6.
Subbramanian V, Hoggard PE (1988). Metal complexes of glyphosate. J. Agric. Food Chem., 36: 1326-1329.
Tsui M, Chu LM (2003). Aquatic toxicity of glyphosate-based formulation: comparison between different organisms and the effects of environmental factors. Chemosphere, 52: 1189-1197.
Tsui MTK, Wang W, Chu LM (2005). Influence of glyphosate and its formulation (roundup) on the toxicity and bioavailability of metals to *Ceriodaphnia dubia.* Environ. Pollut., 138: 59-68.
Viti C, Mini A, Ranalli G, Lustrato G, Giovannetti L (2006). Response of microbial communities to different doses of chromate in soil microcosms. Appl. Soil Ecol., 34: 125–139.