Assessment of *in vitro* effects of chlorpyrifos on biomass and antimicrobial production functionality of *Actinomycetes* isolated from soils in Yola, Nigeria.

Ajunwa, O.M[1]., Audu, J.O[2*]., Adedeji, B.A[1]., and Ja'afaru, M.I[1].

[1.] Department of Microbiology, Modibbo Adama, University of Technology, Yola, Adamawa State, Nigeria.
[2.] Department of Laboratory Technology, Modibbo Adama University of Technology, Yola, Adamawa state, Nigeria
j3suwa@yahoo.com

Abstract: Chlorpyrifos (-0, 0-diethyl 0-(3,5,6- trichloro – 2-pyridyl) phosphorothiote is a broad spectrum organophosphate pesticide with potentials for altering soil microbiota. Actinomycetes were isolated from soils in four sites in Yola, Adamawa State (a hospital waste-dump site, a university campus, a river bank, and an organic farm). The isolates were screened for antibiotic production using perpendicular streaking method against *Staphylococcus aureus* A6 as an indicator organism. Four actinomycete isolates (HS03, RB04, OS02, and FL02) with best inhibition were selected and subjected to *in-vitro* tests with varying concentrations of chlorpyrifos (ranging from 2 to 20g/l) in starch casein medium. Liquid culture-spectrophotometry results (at 600nm) showed growth of all the actinomycete isolates at chlorpyrifos concentrations less than 10g/l but a biomass decline at chlorpyrifos concentrations of $10g/l \leq x \leq 20g/l$. After chlorpyrifos treatment, agar diffusion method with culture filtrate was applied in testing the antimicrobial functionality of actinomycetes exposed to chorpyrifos (20g/L). Actinomycetes OS02 and FL02 (isolated from university campus and organic farm respectively) were heavily affected as they showed reduced efficacy against test organisms *S. aureus* A6 and *Escherichia coli* G3. Actinomycete RB04 and HS03 (isolated from river bank and hospital waste dump site respectively) were however not affected as they produced high zones of inhibition (15mm-26mm) against the test organisms with and without exposure to chlorpyrifos. Biochemical and morphological characteristics identified all actinomycetes as *Streptomyces spp*.
[]Ajunwa, OM, Audu, JO, Adedeji, BA, and Ja'afaru, MI. **Assessment of *in vitro* effects of chlorpyrifos on biomass and antimicrobial production functionality of *Actinomycetes* isolated from soils in Yola, Nigeria.** *N Y Sci J* 2016;9(6):52-57]. ISSN 1554-0200 (print); ISSN 2375-723X (online). http://www.sciencepub.net/newyork. 9. doi:10.7537/marsnys09061609.

Keywords: Chlorpyrifos, actinomycetes, antimicrobials

1. Introduction

Chlorpyrifos is a broad spectrum organophosphate insecticide widely used in agriculture (Mc Connell *et al*., 1997). With a moderate toxicity (Tomlin and Counal, 1994), and a chemical nomenclature of -0, 0-diethyl 0-(3,5,6- trichloro – 2-pyridyl) phosphorothiote, it acts by irreversibly inhibiting acetyl cholinesterase leading to insect deaths (Karalliedde and Senanayahi, 1989). With variability in soil structure in various agricultural climes and environments, chlorpyrifos can last for as long as between 14 days and 150 days or in some cases even up to 1year (Howard, 1991, Lu *et al*., 2013) thus posing large uncertainties both in its degradation dynamics and extrinsic effects to soil microbiota.

Manchus and Montoya (1995), and Singh *et al*. (2003) ascertained that charged molecules derived from hydrolysed clorpyrifos in the environment can lead to contamination of soil and aquatic environments. According to Armbrust (2001), hydrolytic derivatives of chlorpyrifos are among persistent antimicrobial agents as listed by the US Environmental Protection Agency. Feng *et al*. (1997) and Caceres *et al*. (2007) also regard the hydrolytic derivatives as potential chemicals that can detrimentally affect soil microbiota. Actinomycetes are a large group of microorganisms with natural presence as soil microbiota in various environments (Goodfellow and Donnell, 1989). They are Gram positive bacteria with high G + C content (>55%) and possess long branching filaments resembling fungal hyphae with mostly aerobic inclinations and a few anaerobic tolerant strains (Rajesh *et al*., 2013). Actinomycetes are well known for their unique abilities to biosynthesise antibiotics with over 60% of the known antibiotics in the world having their sources as Actinomycetes (Kalyani *et al*., 2012). The effects of chemical additives to soil can be detrimental to the sustainable diversity of industrially important microorganisms like actinomycetes which form our natural bank of bioactive compounds especially antibiotic compounds for pharmaceutical companies.

This present research study was designed to determine the direct effects of chlorpyrifos pesticide on the growth and biofunctionality of antibiotic producing actinomycetes species isolates from soils in Yola, north-eastern Nigeria.

The study was carried out in the month of March/April when the average atmospheric

temperature of Yola was 39/40^{o}C; which we suggest can allow a preponderance of actinomycetes life forms.

2. Materials and methods

Collection of soil samples

Soil samples were collected from four (4) sites in Yola, Adamawa state of Nigeria; (i) a hospital waste dump site, (ii) the bank of River Benue, (iii) an open space within a public university in Yola, (iv) an organic farm land site. Approximately 100g of soil were collected from each of the sites using a clean spatula and clean and dry polyethylene bags. The samples were then transported to the laboratory and subsequently oven dried for 45 minutes at 45^{o}C. The samples were then allowed to cool to room temperature before isolation of actinomycetes.

Isolation of actinomycetes from soil samples

Actinomycetes' isolation was carried out according to the modified methods of Kalyani *et al.* (2012) and Thawai *et al*. (2005). One (1) gram of each soil samples was introduced into 9 ml of sterile distilled water supplemented with 0.05% sodium lauryl suphate. The diluted samples were shaken thoroughly and 10 fold serial dilutions were carried out to obtain dilution 10^{-1} to 10^{-10}. The samples were ten plated on Actinomycetes isolation agar medium which was supplemented with 50mg/L nystatin and inoculated for 4 days at 30^{o}C. at the end of the incubation period, white to brown colonies with smell akin to soil and hardened surfaces was observed. The isolates were sub-cultured unto freshly prepared Actinomycetes agar slants and further subjected to antimicrobial production assay and identification

Antimicrobial assay

Isolates were screened for antimicrobial production assay against *Staphylococcus aureus* A6 (obtained from FIIRO, Oshodi Lagos) as an indicator strain, using the perpendicular streaking method on nutrient agar according to the procedure of Gulve and Deshmukh (2012). A single streak of each isolate was done in a straight line on the nutrient agar surface and incubated for 3 days after which the indicator strain was streaked at right angle perpendicular to the line of streak of the test isolates and plates were incubated at 30^{o}C for 24 h. The plates were observed afterwards, and a decrease in length of the indicator organism less than the line of streak especially at the point of perpendicular contact with the test isolate, indicated an antimicrobial production. Test isolates with highest reduction (in millimeters) of streak line of indicator organism were selected for further studies.

Identification and characterization of isolates

Isolates were identified based on their macroscopic and microscopic morphology, physiological properties, biochemical and cell wall based chemotaxonomic features**.** Gram staining, cultural observations, spore staining and acid fast staining techniques were carried out on the selected isolates to determine their macroscopic and microscopic features. Physiological characterization of the isolates based on sodium chloride (NaCl) concentration tolerance was done by growing the isolates in starch casein broth supplemented with 2, 5 and 10% w/v NaCl concentrations and spectrophotometrically monitored at 600nm. Determinations of temperature and pH tolerance were also spectrophotometrically observed with variations of 5^{o}C, 15^{o}C, 35^{o}C, 55^{o}C and 5, 7, 9 for temperature and pH respectively.

Biochemical tests carried out on the isolates included catalase, oxidase, casein hydrolysis, gelatin liquefaction, starch hydrolysis, methyl red, Voges Proskaeur, nitrate reduction, indole production, H_2S production and sugar fermentation tests using glucose, sucrose, arabinose fructose, innositol, xylose, galactose. The presence of saccharides and diaminopimelic acid (DAP) with the cell wall of isolates was used to determine the cell wall-chemotaxonomy and identity of the actinomycete isolates using thin layer chromatography according to the procedure of Boone and Pine (1968)

Effects of chorpyrifos on biomass of test actinomycetes

In vitro soil based liquid culture spectrophotometric determinations (at 600nm) using 10ml of starch casein broth with the addition of 2, 5, 10, 15 and 20 g/litre chlorpyrifos was carried out. The set ups were incubated for 5 days, and at the end of the 5th day, increase or decrease in biomass was monitored by absorbance values in optical density.

Effects of chlorpyrifos on antimicrobial production functionality of actinomycetes

About 1ml of each of the broths of the highest concentration of chlorpyrifos (20g/L) set-ups with test isolates was obtained and transferred into 20ml of freshly prepared Actinomycetes isolation media. The isolates were incubated for 7 days after which it was centrifuged at 5000 rpm for 15 mins, and the culture supernatant was obtained. With sterile cork borers, agar wells were made on the freshly prepared agar plates carrying the seeded organisms – *S. aureus* A6 and *Escherichia coli* G3 (both obtained from FIIRO, Lagos) with 0.1ml of the supernatant transferred into the wells. The experiments were carried out with the actinomycetes isolates untreated with chlorpyrifos in comparison. All the plates were incubated for 24 hours and observed afterwards.

3. Results

A total of 19 isolates were obtained from culturing on Actinomycetes Isolation media. The

isolates were labeled based on their source as HS- hospital (Specialist Hospital Yola, Nigeria) waste dump site, RB- bank of River Benue, OS- open space in the campus of Modibbo Adama University of Technology, Yola, FL- organic farm land (with a history of non-chemical practices). Seven (7) of the isolates were from hospital waste dump site; 6 isolates were from the bank of River Benue; 3 isolates were from the open space soil while the remaining 3 were from the organic farm land.

Primary screening results (Figure 1) showed that all isolates had some degree of inhibition against *Staphylococcus* aureus A6. Actinomycetes isolates HS02 and OS02 however had the highest values of 13mm zones of inhibition while isolate HS05 had the least zone of inhibition (3mm). From the results of screening, the four best isolates were HS03 (13mm), OS02 (13mm), RB04 (12mm), and FL02 (11mm). From further identification and characterization of the isolates (table 1) HS03 and RB04were macroscopically observed as white hardened colonies with smell like soil, while OS02 and FL02had brown to white colonies. Isolates HS03 and RB04 had a physiological growth capability between of up to 5% NaCl concentration, while isolates OS02 and FL02 were only able to tolerate 2% NaCl concentration. None of the isolates however were able to tolerate up to 10% NaCl concentration. All isolates had a pH tolerance at pH values 5 and 7, while none tolerated up to pH 9. Temperature tolerances ranged from 15-55^{O}C with all the isolates. All isolates were spore and acid fast negative, and had varied biochemical properties and abilities to ferment different saccharides. The isolates however lacked cell wall saccharides, but possessed cell enveloped diaminopimelic acids. All isolates were identified as *Streptomyces* species.

Treatments with chorpyrifos concentrations of 2 and 5g/L had no effect on the growth of the test isolates. However, increased values of 10, 15 and 20g/L affected the growth of test isolates as it was inimical to their biomass increase shown by decline in the optical density readings of each isolate.

Inhibitory biofunctionality of the actinomycetes isolates against *S. aureus* A6 when treated with 20g/L chlorpyrifos concentrations showed that, isolate HS03 and isolate RB04 were unaffected by the treatment as they produced antimicrobial compounds regardless of the treatment with substantial zones (12mm) of inhibition similar in diameter with the untreated controls. Isolates OS02 and FL02 had a reduced biofunctionality (3 and 4mm respectively) as they produced less anitimicrobial compounds when compared with their untreated controls. Against *E. coli* G3, a similar trend was observed with the antimicrobial biofunctionalities of the treated isolates as HS03 and RB04 still produced significant zones of inhibition (17 and 19mm respectively). There was a little reduction in the zones of diameter compared with their controls however (with 20 and 26mm for respective controls). There was however a significant decrease in the zones of inhibition of chlorpyrifos treated OS02 (from 20mm to 2mm), and FL02 (from 19mm to 0mm).

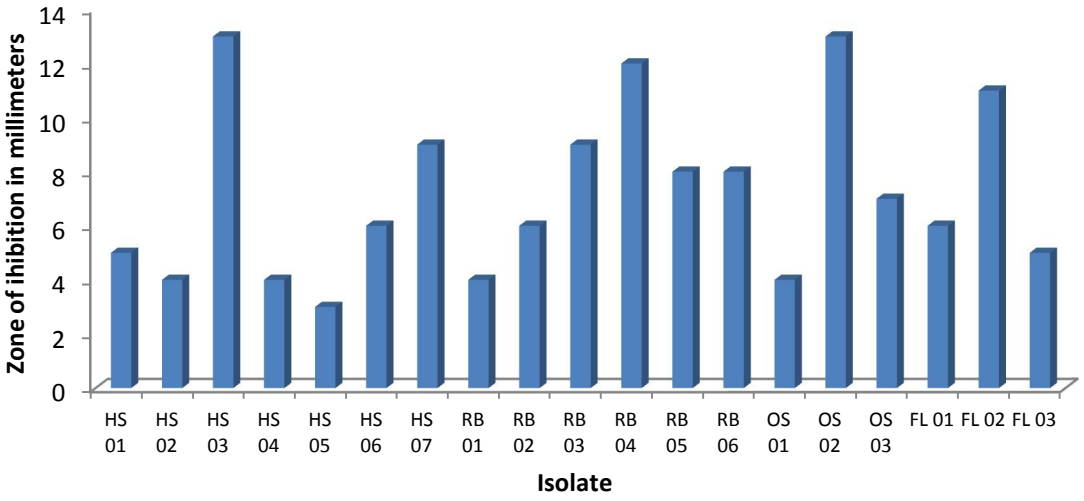

Figure 1: Primary screening of Actinomycetes isolates for antimicrobial production against *Staphylococcus aureus* A6

Table 1: Morphological, physiological and biochemical properties of most efficient Actinomycetes isolates.

Test	**Isolates properties**			
Macroscopy and microscopy	**HS 03**	**RB 04**	**OS 02**	**FL 02**
Gram staining	+	+	+	+
Cultural observations	White hardened colonies with smell like soil	White hardened collies with smell like soil	Brown to white colonies	Brown to white colonies
Spore staining	-	-	-	-
Acid fast staining	-	-	-	-
Physiology				
NaCl (% w/v) tolerance				
2	+	+	+	+
5	+	+	-	-
10	-	-	-	-
pH tolerance				
5	+	+	+	+
7	+	+	+	+
9	-	-	-	-
Temperature tolerance (^{o}C)				
5	-	-	-	-
15	-	+	-	+
35	+	+	+	+
55	+	+	+	+
Biochemical properties				
Catalase	+	+	+	+
Oxidase	-	-	-	-
Casein hydrolysis	+	+	+	+
Gelatin liquefaction	+	+	+	+
Starch hydrolysis	+	+	+	+
Methyl red	-	-	+	+
Voges Proskaeur	+	+	+	+
Nitrate reduction	+	+	+	+
Indole production	-	-	-	-
H_2S production	-	-	-	-
Chemotaxonomic property				
Diaminopimelic acid	+	+	+	+
Cell wall saccharides	-	-	-	-
Sugar utilization properties				
Glucose	+	+	+	+
Sucrose	+	+	+	+
Arabinose	-	-	-	-
Fructose	+	+	-	-
Innositol	-	-	-	-
Xylose	-	+	-	-
Galactose	-	-	-	-
PROBABLE IDENTITY	*Streptomyces* sp	*Streptomyces* sp	*Streptomyces* sp	*Streptomyces* sp

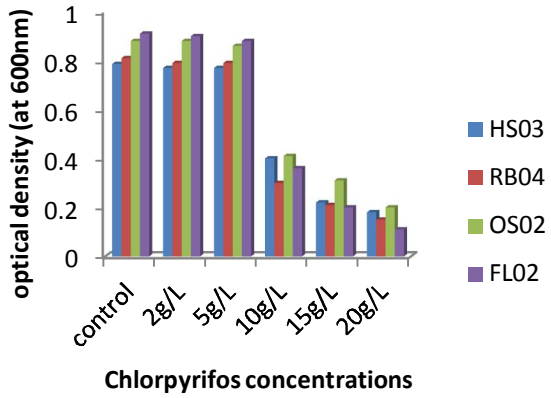

Figure 2: Effect of different chlorpyrifos concentrations on the biomass yield of Actinomycetes isolates

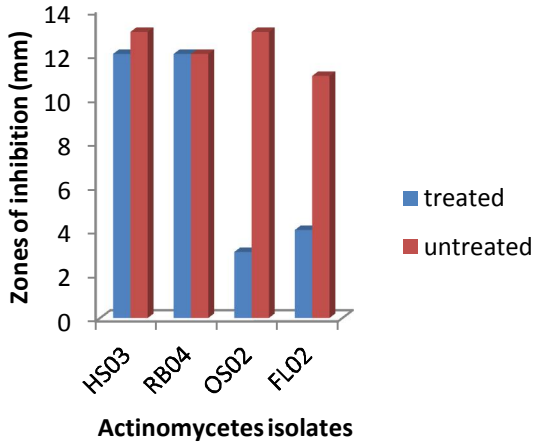

Figure 3: Effects of 20g/l chlorpyrifos treatment on the antimicrobial biofunctionality of Actinomycetes isolates against *Staphylococcus aureus* A6

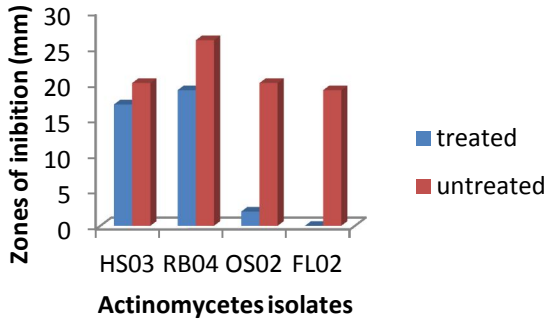

Figure 3: Effects of 20g/l chlorpyrifos treatment on the antimicrobial biofunctionality of Actinomycetes isolates against *Escherichia coli* G3

4. Discussions

From the results, soil samples from different environments harboured actinomycetes with varying degrees of antimicrobial production capabilities. Their abundance in nature (especially soil environments) and their antimicrobial potentials are a major reason for their high economic importance. However, the type of soil and the anthropogenic activities occurring within the soil area, can affect the natural presence of the organisms (Dhananjeyan *et al*., 2010). This was observed in the varying number of actinomycetes isolates obtained from the different soils sites sampled. Their varying inhibitory properties were also as a result of their diverse sources and their specie based properties. High inhibitory strains (>10mm) from the primary screening were however found within all soil environments sampled.

The most inhibitory isolates were identified as *Streptomyces* species. This is in line with reports by Rajesh *et al.* (2013) which stated that *Streptomyces* species are responsible for the production of over two-thirds of the medically important antibiotics of natural origin. There are very important organisms as they serve as a repository of highly effective bioactive compounds, and their presence in nature is of high industrial interests. The effects of chlorpyrifos on the biomass of *Streptomyces* species was concentration dependent as lower concentrations had no effect on biomass yield, while increased concentrations negatively affected their biomass. This is as a result of the inimical effects of high concentrations of chlorpyrifos on microorganisms. In corroboration, this is in line with the antimicrobial potentials of chlorpyrifos and chlorpyrifos-like chemicals as stated by Armbrust (2001), which regarded the hydrolytic derivatives of chlorpyrifos as persistent antimicrobial agents as listed by the US Environmental Protection Agency.

From the results in this work, the effects of high concentrations of chlorpyrifos has varying levels of effects on the antimicrobial properties of the actinomycetes as the different samples showed different responses in antimicrobial functionality of the isolates after exposure to high doses of chlorpyrifos. This mechanism of loss of antimicrobial biofunctionality in *Streptomyces* species exposed to chlorpyrifos was not investigated in this work. However, the environments of isolation could play a role in the inductive nature of exposure to antimicrobial agents as the isolates. The two isolates with proven unaffected/mildly affected biofuntionalities were isolated from a hospital waste dump site and river bank, which are potential sites for exposure to antimicrobial agents from the anthropogenic activities they are exposed to (chemical from hospital drugs and effluent discharge into river

body). The other two isolates that had their biofunctionalities affected were isolated from areas with very minimal exposure to chemical as environmental pollutants (open space soils in a university campus and an organic farm with history of non-chemical treatments). Chorpyrifos is a widely used pesticide in Nigeria. It is therefore advocated that the treatments of chlorpyrifos on soil microbiota (especially industrially important microorganisms) be further examined and researched upon to comprehensively determine the biomechanistic effects on microbial physiologies.

Corresponding Author:
Audu O. Jemilatu
Department of Laboratory Technology
Modibbo Adama University of Technology, Yola.
Adamawa State, Nigeria.
Telephone: +60102122159
E-mail: j3suwa@yahoo.com

References

1. Armburst, K. L. (2001). Chlorothalonil and chlorpyrifos degradation products in golf course leachate. *Pest Management Science* 57 (9): 797-802.
2. Boone, J. C. and Pine, L. (1968). Rapid Method for Characterisation of Actinomycetes by Cell Wall Composition. *Applied Microbiology* 16(2): 279-284.
3. Caceres, T., He, W., Naidu, R. and Megharaj, M. (2007). Toxicity of chlorpyrifos and TCP alone and in combination to Daphnia carinata: the influence of microbial degradation in natural water. *Water Research* 41 (19): 4497-4503.
4. Dhananjeyan, V., Selvan, N. and Dhanapal, K. (2010). Isolation, charcaterisation, screening and antibiotic sensitivity of actinomycetes from locally (near MCAS) collected soil samples. *Journal of Biological Sciences* 10: 514-519.
5. Feng, Y., Racke, K.D. and Bollag, J. (1997). Isolation and characterization of a chlorinated-pyridinol-degrading bacterium. Applied Environmental Microbiology 63 (10): 4096-4098.
6. Goodfellow, M. and Donnell, A.G. (1989) Search and discovery of inductrially significant actinomycetes, *Soc. Gen. Microbio. Symp.* 44: 343-383.
7. Gulve, R.M. and Deshmukh, A.M. (2012). Antimicrobial activity of the marine actinomycetes. International Multidisciplinary Research Journal 2(3): 16-22/.
8. Howard, P.H. (1991). *Handbook of Environmental Fate and Exposure Data for Organic Chemicals*, Vol. 3. Pesticides, Lewis Publishers pp 5-13.
9. Kalyani, A.L.T., Ramya Sravani, K.M., Annapurna, J. (2012). Isolation and characterization of antibiotic producing actinomycetes from marine soil samples. *International Journal of Current Pharmaceutical Research* 4(2): 110-112.
10. Karalliedde, L. and Senanayake, N. (1989). Organophosphorus insecticide poisoning British *Journal of Anaesthesia* 63: 736-750.
11. Lu, P., Li, Q., Liu, H., Feng, Z., Yan, X., Hong, Q. and Li, S. (2013). Biodegradation of chlorpyrifos and 3,5,6-trichloro-2-pyridinol by *Cupriavidus* sp. DT-1. *Biorescource Technology* 127: 337-342.
12. Manclus, J.J. and Montoya, A. (1995). Development of immunoassays for the anlaysis of chlorpyrifos and its major metabolite 3,5,6-trichloro-2-pyridinol in the aquatic environment. *Anal. Chim. Acta* 311 (3): 341-348.
13. McConnell, L.L., Nelson. E., Rice, C.P., Baker, J.E., Johnson, W.E., Harman, J.A. and Bialek, K. (1997). Chlorpyrifos in the air and surface water of Chesapeake Bay: predictions of atmospheric deposition fluxes. *Environ. Sci. Technol*. 31 (5): 1390-1398.
14. Rajesh, R.O., Helen, P.A.M. and Sree, S.J. (2013). Screening of Antibiotic Producing Actinomycetes from Coconut Husk Retting Sample. *International Journal of Research in Pharmaceutical and Biomedicals* 4(1): 67-74.
15. Singh, B.K., Walker, A., Morgan, J.A.W., Wright, D.J. (2003). Effects of soil pH on te biodegradation of chlorpyrifos and isolation of a chlorpyrifos-degrading bacterium. *Appl. Environ. Microbiol*. 69(9): 5198-5206.
16. Thawaii, C., Tanasupawat, S., Itoh, T., Suwanborirux, K., Suzuki, K.I., Kudo, T. (2005). Microminospora eburnean sp. nov., isolated from a Thai peat swamp forest. *Int. J. Syst. Evol. Microbiol*.55:417-422.
17. Tomlin, C.D.S. and Council, B.C.P. (1994). *The Pesticide Manual*. British Crop Protection Council Farnham, Surrey, UK.

6/13/2016