TOXICITY OF PESTICIDES AND HEAVY METALS TO BACTERIAL ISOLATES FROM AQUATIC ECOSYSTEM.

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CERTIFICATION

This is to certify that this work "Toxicity of pesticides and heavy metals to bacterial isolates from aquatic ecosystem" was carried out by Frank-Ogu, Ngozi with Reg. No. 20174082208 in partial fulfillment for the award of Doctor of Philosophy Degree (Ph. D) in Environmental Microbiology in the Department of Microbiology of the Federal University of Technology, Owerri.

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This research work is dedicated to God Almighty for the grace to complete this work

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ABSTRACT

The research work was carried out to determine the effect of pesticides and heavy metals on bacterial isolates from an aquatic ecosystem using Otamiri River as a case study. Otamiri River is the major river in Owerri metropolis and its environs, thus most of the drainages empty into the River. A total of seventy-two (72) water and twelve (12) soil samples were collected from the River. The physiochemical and bacteria diversity of Otamiri River was analyzed using standard microbiological methods, equally the heavy metal and pesticides content of the River was analysed. Finally, the toxicity assay was carried out using the preponderant organisms. Among the
physiochemical parameters analyzed in the River water and soil samples, pH was between 5.3 and
6.11. The BOD was 3-5mg/L which was above the recommended standard by World Health Organization (WHO). From the heavy metals and pesticides content of the river, Cd and Pb recorded the highest value of 0.03mg/l and 0.1 mg/l respectively while Hg was absent in most sampling site, it has its highest deposit at Umezuruike hospital sample site (0.1mg/l). The pesticides content analyses reveals the presence of Athrazine, Butachlor, Glyphosate DDVP and Alachlor in the River, The results of the standard plate count showed that heterotrophic bacteria count ranged from $1.0\times10_6$ Cfu/ml to $9.7\times10_6$ Cfu/ml, fecal coliform count $2.0\times10_5$ Cfu/ml to $9.0\times10_6$ Cfu/ml, Salmonella/Shigella count $3.5\times10_5$ Cfu/ml to $1.5\times10_7$ Cfu/ml, Staphylococcus count $2.0\times10_5$ Cfu/ml to $1.8\times10_7$ Cfu/ml, coliform count $3.5\times10_5$ Cfu/ml to $4.6\times10_8$ Cfu/ml, Vibrio
count $1.5\times10_5$ Cfu/ml to $3.5\times10_7$ Cfu/ml. Anaerobic bacteria count ranged from $2.0\times10_5$ Cfu/ml to
$2.9\times10_6$ Cfu/ml. The percentage occurrence of the bacterial isolates in the water and soil samples showed that *Alcaligens* sp had the highest 68(94.44%), 24(100%), followed by *Lysinibacillus* 54(75%), 21(87.5%) while *Lactobacillus* sp had the least in water sample 6(8.33%), *Klebsiella* sp had the least in soil 6(25%). The five highest occurring isolates were subjected to molecular identification using 16SrRNA and were confirmed to be *Lysinibacillus macrolides* (OK298881), *Pseudomonas aeruginosa* (CP058331), *Klebsiella pneumonia* (MK641337), *Alcaligenes faecalis* (KX302624), *Proteus mirabilis* (MZ067158). *Lysinibacillus macrolides* and *Alcaligenes faecalis* were futher subjected to toxicity assay using five heavy metal ions (Pb(II), Zn(II), Co(II), Ni(II) and Cd(II)) and two pesticides fomulations (Glyphosate and DDVP -(2,2- dichlorovinyldimethyl phosphate)). This analysis was done in singles, as well as mixtures (binary, ternary and septenary) using inhibition of dehydrogenase activity as a response. The EC_{50} equieffect concentration ratio (EECR-50) and fixed ratio ray design (Arbitrary concentration ratio- ABCR) were used to evaluate
the toxicity of the mixtures to the bacterial isolates. The effects of the mixtures on the dehydrogenase activities of the preponderant organisms were assessed using toxic index (TI) and isobolographic analyses. The EC_{50s} were compared statistically by two way ANOVA using POSTHOC= TURKEY ALPHA at $P<0.05$ level of significance. The results of the experimental toxicity thresholds of single metal ions to the total dehydrogenase activity of *L. macrolides* (OK298881) and *A. faecalis* (KX302624) showed that the EC_{50s} of the toxicant on *L. macroides* (OK298881) ranged from 20.49 - 713.57mg/L while that of A. *faecalis* (KX302624) ranged from 58.87 – 624.41 mg/L. The order of increasing toxicity on *L. macroides* (OK298881) was Co (II) <
Cd (II) < Ni(II) < Pb (II) < Zn(II) while that of *A. faecalis*(KX302624) *was* Ni (II) < Cd(II) < Co(II)
< Pb(II) < Zn(II). Glyphosate had an EC_{50} value of 893.23mg/L. on *L. macroides* (OK298881) while on *A faecalis* (KX302624) it *had* an EC_{50} value of 593.98mg/L. The DDVP inhibited the total dehydrogenase activity of *L. macroides* (OK298881) and *A. faecalis* (KX302624) at EC_{50} values 893.23mg/L. and 593.98mg/L respectively. The statistical analysis indicates that the EC_{50} of the toxicant were significantly different from each other ($P<0.05$) and the order of decreasing toxicity is DDVP> GLY. The toxic index of the mixture analyses showed that the toxic effect was
increasing progressively as concentrations were increased.

Keywords: Heavy metals, Pesticides, Toxicity, Dehydrogenase, EC_{50} ratio, Toxic index

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Environmental pollution is a burning issue all over the world; recent trends of global warming have increased awareness on the impact of anthropogenic activities on the environment due to a continuous and rapid deterioration of the abiotic and biotic components. Surface water is grossly affected by pollution resulting from industrial, domestic and agricultural activities; the wholesomeness of this surface water has become an issue of great concern. Water is essential for survival and sustenance of all living forms on earth. Water is utilized for various purposes such as drinking, agriculture, power generation, navigation, as well as for industrial and recreational activities (Arora, 2007; Gupta & Gupta, 2008). It contributes to the environmental balance of the world. Over 70% of the earth surface is covered with water, which forms part of the hydrologic cycle. According to World Health Organization (2017), report on guidelines for drinking water, a safe drinking-water does not represent any significant health risk over a lifetime of consumption; including different sensitivities that may occur between life stages, for this reason, particular concern has become apparent regarding the adequacy of the quality of water resource; both surface and groundwater resources. The earths water according to Dana & Peter, (2022) is always in motion forming a cycle called water cycle. Some of the water cycle reservoirs are oceans, glaciers, groundwater, lakes, soil moisture, atmosphere, biosphere, streams and rivers (Anyadike & Obeta, 2012).

Otamiri River in Owerri, Imo State is a major source of water for the inhabitants of the community and its environs.The river is used for various purposes such as domestic activities, fishing, recreation and mining. The impact of human activities in the urban, municipal and populated area makes surface water like streams, river, lagoons and

ground water bodies to be susceptible to contamination and pollution. Rentier & Cammeraat, (2022) opined that the effects of human activities on water quality are both wide spread and varied in the degree to which they disrupt the ecosystem and restrict water use. River pollution could lead to some undesirable effects such as contamination of water supplies, restriction of its use for recreational activities, loss of aquatic lives, creation of nuisances, as well as hindrances to navigation (Dike, Nevoh & Uzoma, 2016).

Pollution of soil and water by heavy metal and pesticides occurs due to industrial wastes, application of fertilizer, other agricultural practices, smelting of iron, corrosion of sheeting, wires, pipes, and burning of coal and wood, (Nwankwoala & Ekpewerechi, 2007). Discharges from sources like runoffs, industrial waste waters (effluents), sewage treatment plants, chemical fishing activities, leachate from refuse dumps, agricultural fertilizer application, sand mining contribute to these pollution processes. Runoff of agricultural pesticides into aquatic environment poses significant toxicological risks to resident organisms (Abdulaziz, Jasmin, Sheeba, Gireeshkumar & Shanta, 2015).

Microorganisms are fundamental components of aquatic ecosystems where they perform crucial roles such as primary production, decomposition, and nutrient cycling. Aquatic microbial communities potentially include a host of waterborne bacterial, protozoan, and fungal pathogens. These pathogens may be natural (autothonous) inhabitants of aquatic ecosystems or allothonous/ intruders contributed by human or animal waste and they have been found to be sensitive to different types of pollutants: pesticides, antibiotics, xenobiotic, poly aromatic hydrocarbons (PAH), poly chlorinated biphenyls (PCBs) and heavy metals (Staley, Senkbeil, Harwood & Rohr, 2012).

Pesticides and heavy metals are amongst the group of potentially toxic substances that are capable of disrupting the microbial structure and function in aquatic habitats (Atieh, Ji, & Kochkodan, 2017) Every year, the world uses approximately 2.6 billion pounds of

pesticides and the U.S. accounts for 22% of the world's pesticide consumption, (Grube, Donaldson, Kiely, & Wu, 2011). Nearly 80% of these chemicals are used for agricultural purposes while a fraction of the remainder is devoted to the control of structural and public health pests, (Grube et al., 2011).

Many aquatic habitats are embedded within agricultural landscapes and may unintentionally be exposed to agricultural pesticides through spray drift, leaching and/or surface runoff. Whilst most pesticides are designed to target a specific pest or particular pest group, they often extend their effects to non-target species. In fact, the frequent detection of pesticides in both surface and ground water has been associated with loss of biodiversity and detrimental effects on human and wildlife health, (Bridges, 2000; Horrigan, Lawrence & Walker,2002).

Pesticides can cause direct toxic or beneficial effects on microbial communities similar to those reported for higher organisms. Some microbes particularly bacteria may utilize pesticides at low dose as a source of nutrients facilitating their growth and survival, while sensitive species may be impaired or decimated by pesticides, (DeLorenzo, Scott & Ross, 2010). These ecological alterations may trigger a cascade of indirect effects, for example: elimination or reduction of certain microbial populations by pesticides may release pesticide-tolerant microbes from competition for shared resources and thereby promote their growth and survival, (Johnsen, 2001). Similarly, some protozoan species prey on bacteria and their suppression by pesticides may facilitate survival of bacterial prey, (Staley, Harwood & Rohr, 2015). These processes may lead to dramatic shifts in microbial communities, ((Johnsen, 2001; Lupwayi, 2009)) or have little effect on microbial functions, (Johnsen, 2001; Widenfalk, Svensson & Goedkoop, 2004).

The majority of studies that investigate the impact of pesticides on microbes in aquatic ecosystems have mainly focused on individual pesticides using community-level

endpoints such as microbial activity, respiration, and total microbial biomass or analysis of microbial community composition using culture-dependent approaches or traditional culture-independent approaches such as quantitative real-time PCR and genetic fingerprinting, (Downing et al.,2004).

Heavy metal refers to metallic chemical elements that have relatively high density, toxic or poisonous at low concentration values. They are natural components of the Earth's crust that cannot be degraded or destroyed, which would mainly include the transition metals, some metalloids, lanthanides and actinides (Sengor, et al., 2009) examples include copper, zinc, selenium, iron, lead, mercury, cobalt, cadmium and silver etc. Heavy metals are also classified based on density, atomic weight, chemical toxicity in relation to living organisms. They can cause serious health effect with different symptoms depending on the nature and quality of the metal ingested, (Njiiar, Iwara, Offong & Deekor, 2012). Some heavy metals (such as Fe, Cu, Co, Ni, and Zn) are essential elements thus, are required for microbial growth at a specified low dose, while others (like Cd, Hg, As, Ag, Au) have no biochemical function and thus non-essential. The non-essential elements are toxic, (Bruins, Kapil & Oehme, 2000). The essential heavy metals are usually protein stabilizers, biochemical catalysts, regulators of gene expression and osmotic balance controllers in microbial membranes. The type, speciation, and concentration of the heavy metal and type of microorganism are determinants of toxicity of metals (Șengor et al., 2009; Gikas & Romanos, 2006)). Both essential and non- essential heavy metals (physiologically and non-physiologically important metals), at relatively high concentrations, cause harmful effects such as loss of membrane integrity, oxidation of vital enzymes, inactivation of microbial organelles and harmful effect on the genetic make-up of the cell by direct reaction with DNA, (Gikas, 2007). As an essential element, heavy metal such as zinc plays catalytic, structural and regulatory roles in living systems, (Chasapis, Loutsidou, Spiliopoulou, & Stefanidou 2012). Zinc is

also a component of many microbial enzymes where it is necessary for their catalytic function and structural stability, (Choudhury & Srivastava, 2001; Gikas, 2007). However, zinc can become toxic to cells at elevated/ high dose and concentrations. For instance, zinc is known to be inhibitory to respiratory electron transport system of bacteria and eukaryotic organisms, (Nweke & Orji, 2009). Cadmium (Cd) competes with cellular zinc for binding sites and bind non-specifically to DNA, inducing single strand breaks, (Roane & Pepper, 2000). Although nickel and cobalt are microelements, they are both microbial growth inhibitors, at relatively high concentrations, (Gikas, 2007; Gikas, 2008). Other heavy metals such as Cadmium Cd, Mercury Hg and lead Pb have no physiological function and are toxic even at low concentrations, (Nwanyanwu, Nweke, Orji, & Opurum, 2013 & Nweke, Umeh, & Ohale, 2018).

Heavy metals generally exert toxicity in microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active conformation of biomolecules, denaturing and inactivating enzymes and disrupting cell membrane integrity (Xu, Li & Wang 2011). However, in the environment heavy metals do not usually exist as individuals/ singles but as arrays of mixtures arising from many natural and anthropogenic sources. Therefore, studies of interactions among toxicants are of fundamental interest and practical importance in toxicological science. Thus, environmental microorganisms are exposed to multiple mixtures of metals which may have antagonistic, synergistic or additive effect. It is more suitable and advantageous to study their interactive effects in the environment. The combined toxic effects of multiple chemicals are recognized as an important consideration in ecotoxicology because mixtures of chemicals can possibly induce synergistic effects (McCarty & Borgert, 2006). Most estimate of the hazard of chemicals to aquatic organisms is based on evidence that depends on information derived from exposure to a constant concentration of a single chemical to a single species. However, most industries discharge mixtures of chemical

that may vary enormously in both concentration and composition due to various production processes and activities.

Traditionally, toxicant level in effluents, industrial and domestic wastes, agricultural inputs and several other human activities have been estimated by bioassays employing the micro and macro vertebrates. Lately, there has been an increase tendency to use microbial system for screening toxicants as an alternative to test with animals (Monavari & Guieysse, 2007). As prime mediators of biogeochemical cycling, bacteria are ubiquitous, diverse and adapted to exist on dissolved substances that are often present in the environment at very low concentrations (Monavari & Guieysse, 2007). Due to the versatility of bacterial populations, some strains are capable of tolerating or even thriving in the presence of high concentrations of a potentially inhibitory substance whereas others are limited or completely eliminated. Toxicity data involving microorganisms and pesticides are limited. Most studies have focused on microbial degradation of pesticides rather than impacts on natural microbial populations. In addition, studies of pesticide effects on soil microbes are far more common than studies of those in aquatic environments.

Aquatic ecosystems are typically exposed to a mixture of toxicants and understanding the response of microorganisms to these mixtures can improve our ability to predict the full impact of chemical disturbances on microbial processes and trophic interactions. In the interactions between pesticides and microorganisms, most have examined the role of both naturally occurring and laboratory-derived microorganisms in the biodegradation of pesticides (Cycoń, Wojcik & Piotrowska, 2009; Seffernick, *et al*., 2007; & Zeinat, Nashwa, Fetyan, Mohamed, & Sherif, 2008). As many microorganisms can breakdown pesticides, using them as a resource, and pesticides can be directly toxic to microorganisms, the effects of pesticides on microorganisms are difficult to predict a priori. Of the studies reviewed, some used pesticide concentrations that are ecologically

relevant, that is, levels that could be expected to be found in water bodies as a consequence of storm water or agricultural runoff from neighboring watersheds while others use concentrations consistent with direct applications. Finally, other studies test the concentrations that are not ecologically relevant, and thus are above the expected environmental concentrations based on application instructions, (Zachery, Valerie & Jason, 2015) but no substantial study has really been done on the interaction of the mixture of pesticides and heavy metals to bacterial isolates in aquatic ecosystem.

Parameters used to evaluate toxic effects of chemicals on bacterial populations include inhibitions of growth, respiratory activities, bioluminescence, activities and biosynthesis of specific and non-specific enzymes. The estimation of respiratory activities has been used primarily to assess toxicity of chemicals to bacteria (Okolo, Nweke, Nwabueze, Dike & Nwanyanwu, 2007) and reduction of redox indicators are followed spectrophotometrically. Redox indicators are used as artificial electron acceptor in dehydrogenase assay to determine intracellular flux of electrons from electron donors to acceptors in the presence of toxicants. Total dehydrogenase assay therefore is a good tool for assessing the toxicity effect of single and mixtures of metals on microbial diversity under different nutrient conditions. In this regard, toxicity assessment based on inhibition of dehydrogenase activity in natural microbial diversity using 2-(p-iodophenyl-3-(p-nitro phenyl)-5-phenytetrazolium chloride (INT) and 2, 3, 5-triphenyltetrazolium chloride (TTC) as redox indicator are widely used whilst the use of 3-[4, 5-dimethylthiazole-2yl]-2,5-diphenyltetrazolium bromide (MTT) is a promising tool. Redox indicators serve as artificial electron acceptors in dehydrogenase activity assays to measure intracellular flux of electrons. INT, TTC or MTT is among the tetrazolium salt in dehydrogenase activity assay. In this process, INT, TTC or MTT compete with the natural enzymes involved in microbial respiration, acting as electron acceptor and is reduced to red-coloured INT-formazan (INTF), red coloured 2, 3, 5-triphenyl formazan (TPF) or purple- coloured

MTT- formazan by a battery of microbial dehydrogenases that catalyze the movement of electrons from substrates to electron acceptors in respiratory chain (Trevors, 1984, Nweke, et al., 2014; Reuben Edna &Christian, 2020).

1.2 Problems Statement

Advances in industrialization and drive for sustainable agriculture have led to increasing discharge of heavy metals and pesticides into the environment, particularly in the aquatic ecosystem. Mining practice is known for its hazardous working condition. Waste materials deposited at the mining sites consequently pollute the environment, also the underground and surface water of the surrounding. Sand mining from aquatic sources can modify the structure and dynamics of the soft bottom community These toxicants pose serious environmental problems due to their toxicity, bioaccumulation, and reduction in nutrient cycling in aquatic environment and deaths of aquatic lives that provide a greater percentage of sea foods to man. Increasing levels of heavy metals and pesticides adversely affect metabolic and enzymatic activities of microorganisms thus impacting on their diversity and bio-geochemical functions. Organisms (micro and macro) in aquatic environments are confronted with mixtures of toxicants and the effects of combined toxicants may be quite different from individual toxicants due to their chemical interactions. Analytical experiments have shown that mixture of toxicants exposure may provoke combined effects and ignoring it will underestimate the resultant adverse outcome (Nweke, et al., 2014; Nweke, et al., 2018; Reuben, et al., 2020; Saxena, 2004). It is therefore of interest to conduct a study to determine the effects of heavy metals and pesticides in single and in mixtures to evaluate the level of possible interactions and effects on aquatic bacteria.

1.3 Aim of Study

The aim of this study is to determine the toxicity of pesticides and heavy metals on bacterial isolates from aquatic ecosystem using Otamiri River as a case study.

1.4 Objectives

The objectives of the study are to:

i. Determine the physicochemical parameters of the Otamiri River and sediment samples.
ii. Determine the heavy metals and pesticides content of the Otamiri River and sediment samples.
iii. Ascertain the bacteria diversity of water and sediments of Otamiri River.
iv. Determine the susceptibility of bacteria to some heavy metals in single and combined doses using effective concentrations.
v. Determine the susceptibility of bacteria to some pesticides in single and combined doses using effective concentrations.
vi. Ascertain the interactive effects of mixtures of toxicants (heavy metals and pesticide) on aquatic bacteria dehydrogenase enzyme using mathematical models.

1.5 Justification of the Study

Mechanic wastes, agricultural waste and domestic waste including used/unused heavy duty oil, spent engine oil, used carbide / other welding waste, metals, battery waste, fertilizers, pesticides, industrial waste, hospital waste, etc. which are heavily laden with different types of heavy metals and pesticides are typical pollutants of Otamiri River. In recent times, sand mining in Otamiri River has become a lucrative business in the area because of its use in construction industry. Indiscriminate production and uses of pesticides within our environs encourage spills and drift of pesticide into our river. Moreso, heavy metal and pesticides pollution of water due to indiscriminate industrial waste disposal, application of pesticides, fertilizer, and other agrochemical wastes by farmer along the banks of the river is equally a thing of great concern. Heavy metals are the most persistent in the environment and usually very toxic. Many studies have been

carried out on heavy metal contamination of Otamiri River but there has not been any substantial work on possible effects of the mixtures of these metals and pesticides on the microbiota of the river. Thus, this work will create a new perspective in pollution studies on the river.

1.6 Significance of the Study

Aquatic ecosystem serves as repositories for pollutants from upland sources. Industrialization, agricultural practices and other anthropogenic activities is very common in developing countries like Nigeria. These have led to indiscriminate disposal of untreated waste to the environment. Mining activity in water environment pollutes the water source of the area, thereby endangering the life of people and aquatic organisms in the area. Toxicity data involving microorganisms and pesticides are limited. Most studies have focused on microbial degradation of pesticides rather than impacts on natural microbial populations. Pesticides are designed to target a specific pest or particular pest group, the use of pesticides can have additional effects on non-target species (Rohr et al., 2006). In addition, studies of pesticide effects on soil microbes are far more common than studies of those in aquatic environments. Detrimental effects of pesticides and heavy metals on microbial species may have subsequent impacts to higher trophic levels and aquatic ecosystem serve as repositories for pollutants from upland sources (Nweke, Ahumibe & Orji, 2014).

1.7 Scope of the Study

This study covered the physicochemical analysis and estimation of both pesticides and heavy metal content of Otamiri River, the bacteriological analysis of samples from twelve different columns / sample points of the river, molecular identification of pure culture and antibiotic susceptibility testing of the isolates identified. Finally, the determination and estimation of the toxicity of pesticides and heavy metals on bacterial isolate from the river both as singles and mixtures using a mathematical model.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1. Bacterial Distribution in Otamiri Water and Soil:

Quantifying microbial species diversity is critical for an understanding of complex ecological systems since microorganisms are important participants in nutrient cycling, energy flow, and contaminant fate. Microbial diversity has been described for a variety of freshwater ecosystems using multiple techniques (Zeglin, 2015) and there exists a similarity in taxa across streams and within similar habitats (Jezbera, Jezberová, Kasalický, Šimek, & KHahn, 2013; Newton, Jones, Eiler, McMahon, & Bertilsson, 2011). In rivers, microbial communities are dominated by Actinobacteria, Cyanobacteria, and Bacteriodetes regardless of watershed location, suggesting a common community of organisms (Zeglin, 2015). Microbial communities in urban streams like Otamiri River are subjected to multiple environmental stressors related to land use change, and urban infrastructure such as sewage systems, stormwater control channels,indiscriminate dumping of rufues among others. These human influences have resulted in the occurrence of human-dominated taxa, such as Bacteroidetes, Acinetobacter, Aeromonas, Trichococcus, and Proteobacteria (Vandewalle, et al., 2012) in urban streams, and a focus on using stream microbial communities to develop indicators of stream health or human impacts (Simonin, et al., 2019).

Bacteriological water analysis is a method of analyzing water to estimate the amount of bacteria as well as the type of bacteria present. The bacteriological quality of water in most rural areas in sub-Saharan Africa is worrisome with water related diseases and illness such as typhoid fever, dysentery, cholera, meningitis and diarrhea amongst others being some of the outcomes (Amadi et al, 2012) River water can be influenced directly and indirectly by microbial processes which can transform both organic and inorganic constituents. According to Matthew (2006). Water polluted with human feaces may

contain potentially pathogenic microorganisms that causes disease in the aquatic environment. Single and multi-celled organisms have become adapted to using the dissolved materials and suspended solid matter in the aquifer in their metabolism and the presence of faecal coliforms (over 99% of which are Escherichia coli) and a water body is an indication of possible human animal waste contamination (Ogah, Ubaka, & Ogah,2018). The detection of Escherichia provides definite evidence of faecal contamination (WHO, 2017). However, in practice, the detection of thermotolerant (faecal) coliform bacteria is an acceptable alternative. According to World Health Organization (2017), standard faecal coliforms should be absent (zero colony forming units per 100ml water) in portable water while total coliforms should be less than 10 colony forming units in 100ml water sample. The work of Ogah, Ubaka & Ogah, (2018) on Bacterioloigical Assesment of Water from Otamiri River showed that, at the time of the study, Otamiri recorded the total mean viable count (TVC) ranged from 1.2x105 to 9.0 x 105 CFU/ml, while the total mean coliform count (TCC) was from 5.0x105 to 9.0x105 CFU/ml. Comparing with the guide on the natural history of freshwater bacteria, the microbial load of both the TVC and TCC were high which is a reflection of the input of microorganism from external sources and availability of growth supporting organic matter (Newton, Jones, Eiler, McMahon & Bertilsson, 2011). It also shows the level of water pollution as an indication of organic matter present. The mean total bacterial counts obtained in this study were very high but it is not surprising because Otamiri River serves the population within Owerri municipal and its environs for domestic purposes. The TCC of Otamiri River from mechanic village was very high; this may be as a result of so many anthropogenic activities going on in that area of the city. The study conducted by Amadi, Olasechinde, Okosun & Yisa (2011) on the Assessment of the water quality index of Otamiri reveals high TCC bacterial isolates from Otamiri River. Their presence in water indicates faecal contamination of the water. The coliform

isolated in their study is an indication of gross contamination of water. *Staphylococcus aurues*, *Bacillus* sp. *Pseudomonas* sp. and *Proteus* sp may have also come from current contamination of the river. Bacillus sp is a spore former and can survive in harsh environmental condition. Ogah, et al., (2018) also isolated *Staphylococcus aureus and Bacillus* sp in their study which may have come from contamination before or during this study. *Salmonella* sp and *Streptococci sp* observed by Ogah, et.al. (2018) in their study is a health concern because their presence in Otamiri water has made it prone to diseases such as Typhiod fever, Salmonellosis, choleraete on consumption. Okechi and Chukwura (2020) also recorded the presence of *Serratia marcescens*, *Staphylococcus* sp., *Pseudomonas* sp., *Sreptococcu*s sp., *Enterococcus* sp, *Escherichia coli, Klebsiella* sp, *Bacillus* sp and *Acinetobacter seifertii* in Otamiri water and sediments.

2.2 Heavy Metals

Heavy metal refers to metallic chemical elements that have relatively high density, toxic or poisonous at low concentration values. They are defined as elements in the periodic table having atomic number of more than 20 densities or more than 5g/cm^3 generally excluding alkali metals and alkaline earth metals (Jeyasingh, Somasundaram, Philip & Bhallamudi, 2010). The presence of heavy metals in the environment is an issue of global concern because of their toxicity, bio-accumulating tendency and threat to human, plant and microbial life (Jeyasingh et al*.,* 2010). Heavy metals are resistant to bacterial attack and other degradation processes in the environment hence, their concentration often exceed permissible levels. Heavy metal pollution of the environment is caused by various metals such as Cu, Cd, Ni, Pb, Co, Cr, and Hg, As etc. Some heavy metals such as Co, Cr, Ni, Cu, Fe and Zn are essential and are required by the organisms as micro nutrients. However, they are toxic at elevated concentrations (Hussein et al*.,* 2005). Metals however are naturally occurring in the aquatic and terrestrial environments;

significant levels of metals are introduced into the environment by anthropogenic activities (Onyekuru, Nwankwoala, & Uzor, 2017). Heavy metals are of primary concern since they are toxic and highly persistent thus, are bio accumulative and can biomagnify through the food chain. These metals are equally toxic to microorganisms via a number of mechanisms; Heavy metals bind to cellular molecules and displace essential metals from their normal binding sites, (Roane & Pepper, 2000). They also disrupt protein and DNA functions and may affect oxidative phosphorylation and negatively affect the physiology of microbes resulting in decreased biomass, (Chasapis, et al*.,* 2012). As stated above, some heavy metals (such as Fe, Cu, Co, Ni, Zn) are required for microbial growth, whilst others (like Cd, Hg, As, Ag, Au) have no biochemical function. The non-essential elements are toxic. The essential heavy metals are usually protein stabilizers, biochemical catalysts, regulators of gene expression and osmotic balance controllers in microbial membranes essential element, zinc plays catalytic, structural and regulatory roles in living systems, (Chasapis, et al*.,* 2012). Some heavy metals such as Zinc, is also a component of many microbial enzymes where it is necessary for their catalytic function and structural stability, (Choudhury & Srivastava, 2001). However, zinc can become toxic to cells at high / elevated concentrations and inhibit the respiratory electron transport system of bacteria and eukaryotic organisms. Cadmium competes with cellular zinc for binding sites and bind non-specifically to DNA, inducing single strand breaks, (Roane & Pepper, 2000). Although nickel and cobalt are microelements, they are both microbial growth inhibitors, at relatively high concentrations, (Roane & Pepper, 2000).

2.2.1 Heavy Metal Toxicity to Bacteria

Several works have studied the toxicity of heavy metals to pure bacterial cultures. (Nweke, *et al*., 2007; Nweke, et.al., 2014; Nweke et. at., 2018; Nwanyanwu *et al*., 2017, Reuben, Edna & Christian 2020). Although it is apparent that different species have

different responses (Mathew et al., 2017), some trends are evident. According to Waturangi et al., (2016) findings in the species they tested, actinomycetes were more tolerant to cadmium than Gram negative bacteria. These differences may be due to the different biochemical and morphological characteristics of the groups. This may be reflected in the distribution of metals in cellular fractions. During an investigation of the effects of inorganic lead salts on *Azotobacter sp*. and *Micrococcus luteus* by Puyen et al., (2012), 37.6% of the lead immobilised by *Azotobacter sp*. was found in the cell wall, compared to only 9.5% of that immobilised by *Micrococcus luteus*.

The outer layers of cells are probably very important in determining how much of a metal penetrates the cytoplasm. Of the lead extracted from a medium containing 600 mg/l lead bromide or lead nitrate by *Micrococcus luteus*, 75 to 82% was found in lipid extracts of the cells (Bishop, 2016). The analysis showed that no specific plumbated lipids were present, thus it appears that only a natural mixture of cell lipids had the capacity for lead retention, (Bishop, 2016).

The lead caused structural inconsistency of the cytoplasmic membrane, and attempts to prepare protoplasts by treatment of cells with lysozyme often resulted in protoplasmic lysis, (Gautam et al., 2018). Some other bacteria have been shown to undergo plasmolysis and changes in mesosomal structure indicative of membrane disruption. Treatment of the extracted lipid fraction with tris (hydroxymethyl) aminomethane and ethylenediaminetetracetate (EDTA) or reduction of thiol groups with p-chloromercuric phenylsulphonic acid had little effect on lead retention, (Saxena, 2004).

2.2.2 Metal-Microbe Interactions:

Understanding the distribution of toxic metals in aquatic ecosystems is important to our assessments of environmental and human health risks from natural waters. It is becoming increasingly apparent that microbial processes may be important and even dominating

factors in the distribution of specific metals (Ford & David, 2015). Our understanding of microbe-metal interactions has been limited by the complexity of both the microbiology and chemistry of natural systems. Laboratory studies, however, indicate the potential for significant interaction, at least within river water and soil ecosystems. There is considerable information on specific interactions between microorganisms and metal ions and on the importance of these interactions in the biogeochemical cycling of these elements (Nweke, et al., 2018; Reuben, Edna & Christian, 2020; Ford & David, 2015). The following discussion will focus on the cycling of toxic metals and the potential role of microbe-metal interactions in these process

Interactions between microorganisms and metals can be conveniently divided into three distinct processes (Ford & David, 2015), all of which may be important with respect to metal distribution in natural waters:

a) Intracellular interactions

b) Cell-surface interactions

c) Extracellular interactions.

2.2.2.1. Intracellular Interactions and sequestration:

Intracellular sequestration is the complexation of metal ions by various compounds in the cell cytoplasm. Assimilation of metals may be important to the microbe in detoxification, enzyme function, and physical characteristics of the cell. Probably the most widely recognized microbial interaction with toxic metals in the aquatic environment, is the microbial methylation of mercury. A considerable number of studies have addressed the importance of this interaction in the volatilization and subsequent bioaccumulation of the lipid-soluble, methylated form of mercury (Belden, Gilliom & Lydy, 2007; Benson, Anake & Olanrewaju, 2013). However, in the environment, sulfate-reducing bacteria

appear to dominate in this process (Eggleton J & Thomas, 2004). The mechanism is thought to involve intracellular methylation by non-enzymatic transfer of methyl groups from methylcobalamin (vitamin B12) (Dopp, von, Diaz-Bone, Hirner & Rettenmeier, 2010). For the microorganism, this is probably a detoxification mechanism, as it results in volatilization of the mercury, and hence removal from the immediate environment of the sulfate-reducing bacteria (Benson, Anake & Olanrewaju, 2013). The eventual fate of the methyl mercury is then dependent on rates of microbial demethylation, a process that occurs closer to the sediment-water interface. Although receiving less attention than mercury, methylation of other toxic metals, with subsequent volatilization, also occur in the aquatic environment (Grandjean, Weihe, Debes, Choi & Budtz-Jørgensen2014; Goldman, 2014) Methylation has been shown for tin, arsenic, lead, selenium, tellurium, thallium, and antimony (Bustaffa, Stoccoro, Abianchi & Migliore 2014; Boucher, (2014) correlated production of monomethyl tin in sediment samples with numbers of sulfate-reducing and sulfide-oxidizing bacteria. In addition, (Golding, Steer, Hibbeln, Emmett, Lowery &Jones,2013) isolated Desulfovibrio spp. from the sediments that were able to methylate tin in culture medium at rates similar to those for sediment methylation.

2.2.2.2 Cell-Surface Interactions:

A number of authors have shown that metal binding to cell surfaces is an important factor in the distribution of metals in natural waters (Dopp et al., 2010; Agrawal, Flora, Bhatnagar & Flora, 2014; Ahamed, Verma, Kumar& Siddiqui, 2005).

Gram-negative bacteria possess lipopolysaccharides and phospholipids in their cell walls, with phosphoryl groups as the most abundant electronegative sites available for metal binding (Basile et al., 2012). Gram-positive bacterial cell walls possess teichoic acids and peptidoglycan, providing carboxyl and phosphoryl groups that are potential sites for metal binding (Løkke, Ragas & Holmstrup, 2013). For both gram-negative and positive

bacteria, metal binding to cell-surface functional groups is thought to be an important step to intracellular accumulation of trace metals required for enzyme function (Nweke, et al., 2007). In addition, certain bacteria appear capable of using toxic metal species as electron acceptors, with both selenate and chromate reportedly reduced under anaerobic conditions (Nweke, et al., 2007; Dopp, et al., 2010).

2.2.2.3 Extracellular interactions and sequestration:

Extracellur sequestration is the accumulation of metal ions by cellular components in the periplasm or the outer membrane or complexation of metal ions insoluble compounds. The cell wall, pasma, membrane or capsule can prevent metal ions from entering the cell. Bacteria belonging to different taxonomical groups can adsorb metal ions by ionizable groups of the cell wall or capsule, (Kovacic & Somanathan, 2014) With toxic metals range from the potential to leach metals from sediments by production of acidic metabolites to the formation of colloidalsized extracellular polysaccharide (EPS) metal complexes implicated in mobilization and transport of toxic metals in soils (Baranowska-Bosiacka, 2009; Balistrieri & Mebane, 2014; Basile et al., 2012). Indirectly, toxic metals closely associated with iron oxide (Cd and Zn) have been shown to be solubilized by enzymatic reduction of the ferric iron (Ercal, Gurer-Orhan & Aykin-Burns, 2001). Insoluble complexes may also be formed by the activity of microorganisms.

2.2.3 Response of Microbes to Heavy Metal Toxicity

Microbes encounter metals and metalloids of various kinds in the environment and it is, therefore, not surprising that they should interact with them, sometimes to their benefit, at other times to their detriment. Of particular practical interest are the base metals including vanadium, chromium, manganese, iron, cobalt, nickel, copper, zinc, molybdenum, silver, cadmium and lead; the precious metals gold and silver; and the metalloids arsenic, selenium, and antimony. In nature, these metals and metalloids exist

mostly as cations, oxyanions, or both in aqueous solution, and mostly as salts or oxides in crystalline (mineral) form or as amorphous precipitates in insoluble form (Ehrlich,2017). All microbes, whether prokarotic or eukaryotic, employ metal species for structural functions and/or catalytic functions. The alkali metals Ca and Mg serve structural as well as catalytic functions. The metals V, Cr, Mn Fe, Co, Ni, Cu, Zn, Mo, and W, and the metalloid Se may participate in catalytic functions. For such uses, low envirmental concentrations are suffcient (Ehrlich, 2017).

Microorganisms in aquatic ecosystem are exposed to changes in the environment. To survive these unfavourable conditions, these microbes developed adaptive defence mechanisms or physiological and structural adaptations which resulted from evolution. The metabolic reaction known as stress response is included in the adaptive mechanism. Microbial stress response to heavy metal toxicity induced by the changes in the metabolic activity of cell leads to the repression of synthesis of most proteins that are found in normal physiological conditions and synthesis of specific proteins for cell survival in the new environment (Nweke et al., 2007; Reuben, Edina & Christian, 2020,) Meanwhile, changes that occur in gene expressions are linked to alteration that involves different sigma protein factors and catalytic core of RNA polymerase (Pranesha & Wan, 2016). According to findings from their review on microbial stress response to Heavy metal in the Environment, the rRNA polymerase is needed to identify genes that are required in a particular environmental condition and produce mRNA transcripts that later will be translated into a protein.

A microorganism responds differently to the toxicity of heavy metals. These responses are due to the levels and accumulation of the heavy metals in the environment. According to Nweke et al. (2007), on the effects of Zinc (Zn^{2+}) on the dehydrogenase activity of heterotrophic bacteria from tropical river sediment, they discovered that in *Bacillus sp*. SED1 dehydrogenase activity decreased with increasing concentration of

Zn^{2+}. In *Salmonella sp.* SED2, dehydrogenase activity was slightly stimulated at 0.02 mM Zn^{2+} and progressively inhibited at concentrations greater than 0.2 mM (0.4 – 1.0 mM). Also the stimulatory effect observed with *Salmonella sp*. SED2 according to them may be attributed to the use of zinc as trace element by this bacterium. The inhibition of dehydrogenase activity observed in their study is consistent with the reported toxic effect of zinc at high concentrations.

Although zinc is an essential element, it is an inhibitor of respiratory activities in microorganisms and a result of an in vitro study indicates that Zn^{2+} is potentially toxic to the sediment bacteria of New Calabar River, (Nweke et al., 2007b). Contamination and accumulation of Zn^{2+} in the sediment would likely impact negatively on carbon metabolism and respiratory activities of the bacterial strains, (Nweke et al*.,* 2007b). Likewise, Zn^{2+} inhibited glucose uptake and mineralization by water and sediment microbial communities of a contaminated stream. Elsewhere, Nweke et al*.* (2018) reported the effects of the individual metal ion on the dehydrogenase activity of the *Pseudomonas fluorescens*. The metal ions progressively inhibited the dehydrogenase activity as the concentrations were increased, giving percent inhibitions greater than 95% at 2 mM nickel, (Ni(II)) and zinc, (Zn(ii)), 1.5 mM cobalt, (Co(II)) and 0.2 mM cadmium (Cd(II)). The EC50 values of the metals ranged from 0.022 mM for cadmium (Cd (II)) to 0.356 mM for nickel (Ni (II)). The experimental dose-response relationships of the binary mixtures of nickel, zinc, cobalt and cadmium as reported by Nweke et al*.*(2018), indicated similar toxicities for the binary mixtures, especially for zinc, Zn(II) and cadmium, Cd(II) mixtures, and their dose-response curves were almost superimposed.

There is higher toxicity for 20% nickel, Ni(II): 80% cobalt, Co(II) mixture and accurately the toxicities of 50% nickel, Ni(II) + 50% cobalt, Co(II) and 80% nickel, Ni(II) + 20% Co(II) mixtures and zinc, nickel and cadmium ions were inhibitory to microbial activities at high concentrations, (Nweke et al*.* 2018). According to Nweke &

Okpokwasili (2012), cobalt, Co (II) inhibited 50% of dehydrogenase activity in *Pseudomonas species* from petroleum refinery wastewater at Co (II) concentrations ranging from 0.065 mM to 0.347 mM. Cadmium ion is known to be more toxic than zinc, cobalt and nickel ions. Median inhibitory concentrations (IC50s) ranging from 0.237 mM to 0.275 mM Cd(II) was reported by Nweke et al. (2007b) for the inhibition of dehydrogenase activity in microbial community extracted from soil. However, little reports have been published on the combined effects of Ni(II) + Co(II), Zn(II) +Cd(II) and Ni(II) + Co(II) + Cd(II), Zn(II)+Pb(II) mixtures on microorganisms.

Using isobolographic representation, Gikas (2007) reported synergistic toxicity of binary mixtures of Ni (II) and Co (II) against growth of activated sludge microbial community while Nweke et al. (2018) in their study, reported that Ni(II) and Co(II) mixture was antagonistic at the zone of decreasing stimulation. On the combined effects of metals and chlorophenols on dehydrogenase activity of bacterial consortium as reported by Nwanyanwu et al. (2017) reported that the order of increasing toxicity of the individual chemicals was Cd > 2,4-DCP > Zn > 4-CP.

Binary mixtures of cadmium and 2,4-DCP has been reported to show relatively high toxicity with IC_{50} values that were lower than the values observed for the 2,4-DCP alone, (Nwanyanwu et al., 2017). Also in their findings, as binary mixtures, IC_{50} obtained showed that 90%Zn + 10% Cd among the mixtures of zinc and cadmium had the highest toxicity with IC_{50} value of 0.098 ± 0.009 Mm while 25% Zn +75% Cd with IC_{50} OF 0.514 ± 0.055 Mm was least.For the quaternary mixtures, highest and lowest toxicity were observed in 10% 2,4-DCP + 15% Cd + 30% Zinc + 45% 4-CP and 15% 2,4-DCP + 15% Cd + 45% Zinc + 25% 4–CP with IC_{50} values of 0.179 ± 0.006 mM and 0.252 ± 0.011 mM respectively, (Nwanyanwu et al., 2017). The dehydrogenase enzyme activity exhibited hormetic response upon exposure to heavy metals and phenolic compounds.

Hormetic response to chemicals is a widely reported phenomena occurring in microorganisms and higher life, (Calabrese & Blain, 2005).

Nweke and co-workers reported low dose stimulation of dehydrogenase activity of *Rhizobium species* by glyphosate, 4-chlorophenol and 2, 4 - dichlorophenol, (Nweke, et al*.,* 2014). Christofi reported hormetic response of immobilized bioluminescent *Vibrio fischeri* to low doses of phenol and 3, 5 - dichlorophenol, (Christofi, 2002). Stimulation at low doses of zinc is attributed to the role of zinc as essential element, participating as enzyme co-factor. Cadmium do not exhibit low dose stimulation. This is attributable to the fact that cadmium has no physiological function and is more toxic to microorganisms, ((Nwanyanwu *et al.,* 2017). Antagonistic effect in the interaction of Zinc (II) and Cadmium (II) at the mixture ratio of 25% + 75% respectively observed by Nwanyanwu and coworkers is a proof that Zn (II) can reduce the toxicity of Cd (II) on dehydrogenase enzyme activity. Antagonistic interaction was observed by toxicity of Zinc and Cadmium binary mixtures on sea Urchin embryo while quaternary mixtures of Copper, Lead, Zinc and Cadmium were mainly addictive, (Xu, et al., 2011). Mowat and Bundy as reported by Sengor et al., opined the possibility of synergistic, additive and antagonistic interactions among binary and ternary mixtures of pollutants, (Şengor et al*.,* 2009). Toxicity interaction of zinc and cadmium on *Pseudomonas fluorescens* and *Escherichia coli* was synergistic, (Preston, et al*.,* 2000).

The environmental contaminants at increasing concentrations exert inhibitory effects on indigenous microorganisms of an ecosystem and thus disturb the ecosystem. The heavy metal analysis of Otamiri River in Imo state, South-Eastern Nigeria as reported by Onyekuru et al. (2017) indicated that iron (Fe) has the highest concentration followed by zinc (Zn) before copper (Cu) and finally mercury (Hg) and cadmium (Cd) with concentrations tending towards zero in all samples (Fe>Zn>Cu>Hg and Cd), with exceptions where copper (Cu) has higher concentration than zinc (Zn). The interaction of

ternary and quaternary mixtures of metal ions according to Nweke *et al.* (2018) described a dose response patterns similar to those of binary mixtures. This was justified when fitted in a 2-parameter logistic model and the EC_{50} of Zn (II) + Cd (II) + Ni (II) mixtures ranged from 0.035 mM to 0.122 mM and seem to be dependent on the relative amount of Cd (II) in the mixture. Also, the ternary mixtures of Zn (II), Cd (II) and Ni (II) were synergistic while the ternary mixture of Ni (II), Co (II) and Cd (II) were antagonistic. The quaternary mixtures were synergistic except the EECR-50 mixture, (Nweke et al*.,* 2018).

Kalantari & Ghaffari, (2008) evaluated the toxicity of heavy metals on *Escherichia coli* growth and reported that in a culture containing 0.1 mM/l and 0.5 mM/l concentration of iron, Fe (II and III), the bacteria grew approximately as well as control while the bacteria growth decreased to 31.3 % when 1 mM/l concentration of iron, Fe (III) was used and it did not grow when 1 mM/l concentration of iron, Fe (II) was used. In other part of their study bacteria growth was measured after treatment with cadmium chloride and combination of cadmium with iron. Their findings demonstrated that bacteria grew at 0.01 mM/l concentration of cadmium but reduced extremely at of 0.05 mM/l of this element. Bacteria did not enter to growth phase at 0.1 mM/l concentration of Cd^{+2}. Findings obtained from interaction between cadmium and iron in their findings showed that inhibitory effect of cadmium on bacteria growth was partially supported by Fe (III). For example, bacteria growth was reduced from 12.5% to 6 % at concentration of 0.1 mM/l of Fe^{3+} and 0.05 mM/l of Cd^{2+} in comparison with 0.05 mM/l of cadmium alone (OD control=0.8, OD 0.05 mM/l Cd^{2+} = 0.1 and OD 0.05 mM/l of Cd^{2+} and 0.1mM/l Fe^{3+}=0.05). In other hand, effects of cadmium in combination with iron (II) on *Escherichia coli* growth were also measured. Results obtained according to them showed that the bacteria growth was increased from 12.5% to 20% at 0.1 mM/l of Fe^{2+} and 0.05 mM/l of Cd^{2+} compared with 0.05 mM/l of cadmium alone.

Results obtained from supplementation of the bacteria culture medium with Cr^{3+} and in combination with Fe^{2+} or Fe^{3+} revealed that Cr^{3+} has partially inhibitory effects on growth of the bacteria and that the Bacterial growth was reduced to 77% and 38% using 0.1 and 0.5 mM/l concentration of chromium, respectively compared with control, (Kalantari & Ghaffari, 2008). It was also revealed that cultivation of microbes in a mixture containing chromium will have an inhibitory effect. The inhibitory effect of chromium on microbial growth was supported when combined with iron where the toxic effects of iron were removed by chromium. It seems that chromium salts act as an iron chelating agent which could precipitate it and thus removed the excess amounts of this elements, (Kalantari & Ghaffari, 2008). The results from the effect of combination of iron and cadmium on *Bacillus cereus* growth according to Narges, (2008) showed that they have antagonistic effects on the growth of bacteria and the antagonistic effects of cadmium with iron (II) were more in comparison with iron (III). They observed that from cultivation of *Bacillus cereus* at the mixture combination of chromium and iron showed that, the inhibitory effect of iron was partially removed by chromium. For example, the growth of bacteria was significantly increased from 52% to 85% and from 10.5% to 68% and the bacterial growth was increased to 62% using 0.5 mM/l Fe^{3+} and 0.1 mM/l Cr^{3+} (Narges, 2008). They also found out that in the mixture combination of cadmium and chromium, the inhibitory effect of cadmium on *Bacillus cereus* growth was partially removed by chromium. Growth of the bacteria increased to 51% when 0.01 mM/l Cd^{2+} and 0.5 mM/l Cr^{3+} was used, (Narges, 2008). The effects of the heavy metal ions on the dehydrogenase activities of the rhizoplane microbial community according to Nweke, et al., (2007) reported that apart from zinc and iron, which had stimulatory effects on dehydrogenase activities at low concentrations of 0.2 and 0.4 mM respectively, progressive inhibition of dehydrogenase activity were observed with all the metal ions studied. For cobalt, this inhibition is linearly dependent upon the concentration of the

metal ion up to 0.6 mM. At 0.8 mM, there was almost total inhibition of dehydrogenase activity. For cadmium, at 0.2 and 0.4 mM, sharp inhibitions of dehy drogenase activity were observed. However, at higher concentrations, the relative inhibition became less pronounced. Like zinc, nickel sharply inhibited dehydrogenase activity at 2 and 4 mM. The inhibitory effect became less severe at concentrations greater than 4 mM. This reduced relative inhibition of dehydrogenase activity at high concentrations could be ascribed according to them as the saturation of active sites in the microbial cells.

2.2.4 Toxicity of metal mixtures:

In the environment, living organisms are exposed to multiple xenobiotics such as metals, pesticides and organic substances through different routes as a result of environmental and occupational variability. Though there are varied health effects associated with exposure to single metals, information on toxicity and associated mechanisms for metal mixtures especially in low doses is scanty. Considering binary mixtures of low concentration heavy metals, Anochie & Ike (2016) assessed the effect of concentration of Pb and Cd on Bacillus Species Isolated from river water. In their study, they observed that the mixture induced significant changes on the viability and growth of the organism. Nweke, Ahumibe & Orji, (2014) in their study on Toxicity of Binary Mixtures of Formulated Glyphosate and Phenols to Rhizobium Species Dehydrogenase Activity observed that interactions between the binary mixtures were Synergistic and additive. Similarly, Yuan et al. (2014) in their study on Toxicological assessment of combined lead and cadmium: acute and sub-chronic toxicity study in rats recorded strong additivity in the metal interaction. Similar observations made by Hambach et al. (2013) showed that co-exposure of Pb and Cd increases the association between Cd and renal biomarkers. Interactions between the mixtures of Hg and Cd with erythrocyte biomembranes revealed that the mixture showed changes in the lateral lipid packing as indicated by area expansion as well as enhanced film rigidity (Le et al. 2013). Apoptosis

was identified by Hernández-García et al. (2014) to be the main cell death when a mixture of Pb and Cd was exposed to isolated red blood cells of common Lizard. Wu et al. (2012) also studied the combined exposure of Pb and Cd on earthworm and noted that the combination of both metals significantly inhibited cellulase activity. They concluded that the combined toxicological effects between Pb and Cd were complex and might be influenced by the competitive adsorption of both metals and their bioavailability (Wu et al. 2012). More antagonistic interactions were observed by Smith et al., (2012), when they concluded in their study that Pb uptake was mediated by the presence of Cd in co-contaminated soils. They observed that the presence of Cd in contaminated soils had a major effect on the Pb in the kidney of mice (Smith et al. 2012). In their study on the effects of low doses of eight water contaminating metals (including Pb, Hg, As, and Cd), Jadhav et al. (2007) noted that exposure to the metal mixtures decreased body weight and water consumption and increased weights of the brain, liver, and kidneys of rats. They concluded that the general health of the rats was affected by altering the functional and structural integrity of the liver, brain, and kidney (Jadhav et al. 2007). In a similar study using metal mixtures including Pb, Hg, As, and Cd, Jedhav et al. observed that the hematopoietic and immune systems of male rats were toxicologically sensitive to joint mixtures. They concluded that this could lead to anemia and suppression of humoral and cell-mediated immune responses (Jadhav et al. 2007b). Whittaker et al. (2011) also established that low-dose concentrations of mixtures of Pb, Cd, and As resulted in increases in delta-aminolevulinic acid (ALA), iron, and copper in rat. Martínez-Pacheco et al. (2014) exposed other studies on exposure to mixtures of metals to bacterial isolate showed low dose effects of mixtures of toxicants. Nweke et al., (2018) on their study on Toxicity of four metals and their mixtures to Pseudomonas fluorescens recorded co-contamination of the metal ions as their effects were generally synergistic.

2.3 Pesticides

Pesticide is any substance or mixture of substance intended for prevention, destroy, or controlling any pest, including vectors of human or animal diseases, unwanted plants or animals causing harm during, or otherwise interfering with the production, processing, storage, transport, or marketing of food (Erhunmwunse, Dirisu & Olomukoro, 2012) Pesticide can also be used as vector control and agriculture control agent in public health programmes (WHO, 2017). The development of pesticides became widespread after the Second World War; they were introduced to avert the problems of plant diseases and pest control (Erhunmwunse, Dirisu & Olomukoro, 2012)

2.3.1 Types of pesticides:

The name "Pesticides" is a general nomenclature or name for all substances or mixture of substances used to kill, repel, deter or control certain forms of plant or animal life that are considered to be pests (Erhunmwunse et al., 2012)

There are different types of pesticides according to World Health Organization (WHO), each meanth to be effective against specific pest. The term "-cide" comes from the Latin word "to kill". According to WHO, types of pesticides include;

1. Algaecides are used for killing and/or slowing the growth of algae.
2. Herbicides kill or inhibite the growth of unwanted plants or weeds.
3. Insecticides are used to control insects.
4. Fungicides are used to control fungal problems like molds, mildew and rust.
5. Defoliants causes plants to drop their leaves.
6. Desiccants are used to dry up living plant tissues.
7. Antimicrobials control germs and microbes such as bacteria.
8. Miticides control mites that feed on plant and animals.
9. Ovicides are used to control eggs of insects.

These pesticides are further classified according to Delorenzo et al., (2010) based on their chemical family and on biological functions.

1. Classification based on chemical family: they are classified into the; Organochlorine, Organophosphate, Carbamates and Pytheriods.
2. Classification based on biological functions includes; Stomach poisoning, Contact poisoning and Systemic poisoning.

Global pesticide usage is increasing, and the presence of pesticides, which include herbicides, fungicides, and insecticides, has become pervasive in freshwater and marine ecosystems. A survey on pesticides usage in Nigeria indicated that about 15,000 metric tons annually of pesticides comprising about 135 pesticide chemicals marketed locally under 200 different produce brands and formulation were imported during 1983-1990 thus making Nigeria one of the largest pesticides users in sub-Sahara Africa (Osibanjo, 2002). Although the benefits of pesticides cannot be overemphasized, their uses raise a number of environmental concerns such as potential toxicity to humans and other organisms. The adverse effects of pesticides contamination are not limited to the environment but, extended to human health through the food chain (Ize-Iyamu, Abia & Egwaikhide, 2007). A large number of the populace / farmers can't read and understand herbicide label. This has resulted in the contamination of streams, rivers and ground water which is an important natural resource (Baran, Mouvet & Negrel 2007). These contaminations do not pose danger to only the non-target organisms and the environment but exposes human beings to many health implications. Hence, the need to study the effects of some of these herbicides which are commonly used in Ghana in order to assess their inhibitory effects on some of the beneficial microorganisms in the soil (Michael & Stephen, 2016)

Microorganisms are important inhabitants of aquatic ecosystems, where they fulfill critical roles in primary productivity, nutrient cycling, and decomposition. Aquatic environments receive direct and indirect pesticide inputs, inevitably exposing microorganisms to pesticides. While pesticides elicit a variety of acute and chronic toxicity effects in microorganisms, microorganisms also have the capability to accumulate, detoxify, or metabolize pesticides to some extent (DeLorenzo, Scott & Ross, 2010). Detrimental effects of pesticides on microbial species may have subsequent impacts to higher trophic levels. For example, changes in the macromolecular composition of phytoplankton species or shifts in community composition can affect the growth rate of zooplankton grazers, (Debenest, Silvestre, Coste &Pinelli, 2010). Estuaries serve as critical feeding and nursery grounds for many aquatic organisms, including commercially and recreationally important fish and shellfish species. These productive, diverse ecosystems are particularly vulnerable to pollution because they serve as repositories for pollutants from upland sources. Millions of pounds of active pesticide ingredients are applied in coastal watersheds each year.

Runoff of agricultural pesticides into estuaries poses significant toxicological risks to resident organisms, (Downing, *et al*., 2004). Toxicity data involving microorganisms and pesticides are limited. Most studies have focused on microbial degradation of pesticides rather than impacts on natural microbial populations. In addition, studies of pesticide effects on soil microbes are far more common than studies of those in aquatic. Global pesticide usage is increasing and the presence of pesticides, which include herbicides, fungicides and insecticides, has become pervasive in freshwater and marine ecosystems. Aquatic microbial communities potentially include a host of waterborne bacterial, protozoan and fungal pathogens. These pathogens may be natural (autochthonous) inhabitants of aquatic ecosystems, e.g. *Vibrio species,* or they may be allochthonous intruders contributed by human or animal waste. Therefore, any pesticide impact

resulting in alteration of microbial communities could result in dramatic changes and damage to an impacted water body, as well as posing a risk to human health. As farmers continue to realize the usefulness of herbicides, larger quantities are applied to the soil but the fate of these compounds in the soils is becoming increasingly important since they could be leached; in which case ground waters is contaminated or immobile thus persist on the top soil (Downing, *et al.,* 2004).

2.3.2 Mechanisms of pesticide action

Pesticides can be classified according to their mechanisms of action. For example, organochlorine, organophosphate, and carbamate insecticides act primarily by disrupting nervous system function, while herbicides target mainly photosynthesis pathways as described on table 1 below. The mechanism of pesticide action in microbial species may not be the same as for the target

Table 2.1 Summary of pesticide mechanisms of action on target organisms:

Pesticide class	Groups included	General toxic effect	Specific site of action
Organophosphates	Carbamates	Nervous system inhibition	Acetylcholinesterase
Organochlorines	Cyclodienes	Nervous system inhibition	GABA receptor
Herbicides	Ureas, cyclic ureas, triazines, acylanilides, phenylcarbamates, triazinones	Photosynthesis inhibition	Hill reaction of electron transport
		Photosynthesis inhibition	Reducing side of photosystem
	Bipyridiniums Pyridazinones, Dinitroanilines, phosphoric amides,	Biosynthesis inhibition	Carotene accumulation
			Fatty acid synthesis
	chlorthaldimethyl,	Biosynthesis inhibition	Microtubule formation
	propyzamide,	Biosynthesis inhibition	
	cholchicine, terbutol		
Broad-spectrum biocides	Chlorophenols	Multiple inhibiting actions	Phophosphorylation, protein synthesis, lipid biosynthesis
	Tributyl tins, trialkyl tins	Respiratory system inhibition	Mitochonrial ATPase

(Source: Delorenzo *et al*., 2010)

2.3.3 Effects of insecticides on aquatic microorganisms

The general mode of action of most commercial insecticides is nervous system disruption. Organophosphate insecticides (i.e., parathion, diazinon, Malathion, chlorpyrifos, etc.) and carbamate insecticides (i.e., carbaryl and carbofuran) hydrolyze acetylcholine and inhibit acetylcholinesterase, resulting in a buildup of the neurotransmitter acetylcholine and eventual death of the organism (Azizullah, Richter, & Hader 2011). In their work, Pyrethroid insecticides and organochlorine insecticides (i.e., dichlorodiphenyltrichloroethane [DDT], aldrin, dieldrin, and endrin) act by binding to g-aminobutyric acid or GABA receptors, preventing chloride anions from entering nerve cells.

2.3.4 Effects of Organophosphates on microorganisms:

Dichlorvos also known as DDVP- 2, 2- dichlorovinyldimethyl phosphate, is an organophosphate insecticide cum pesticide (USEPA, 2007). It is widely used in Nigeria to control household pests. Dichlorvos (CAS No. 62-73-7) according to them is a broad-spectrum organophosphorus insecticide used primarily for controlling household pests and for protecting stored productsfrom insects. It is no longer approved for use in some jurisdictions because of concerns over its acute toxicity. Dichlorvos is expected to be very mobile in soils. It is rapidly degraded by microbial activity and hydrolysis in soil, and does not adsorb to sediments. Degradation in water occurs primarily through hydrolysis. There are relatively few studies on its occurrence in source waters. Exposure from food varies widely, depending on local circumstances and usage.

2.3.5 Effects of Herbicides on microorganisms:

As part of the systemic and broad spectrum herbicide, glyphosate had the place of honor as the best-selling and most widely used herbicides globally (Okada, et al., 2020) its application is mainly by spraying so that a high percentage is deposited directly on soil

(Grandcoin, 2017) and dispersion to other environmental compartments like water is dependent upon its interaction with the soil component. Extensive usage and improper waste management practices has led to high distribution of this chemical in the aquatics. According to World Health Organisation guidelines on its permitted limit in drinking water, glyphosate should not exceed 280 µg/ L in Canada, 1mg/L in Australia, 0.1 µg/L in European Union. 700µg/L in USA and in Nigeria among others (WHO, 2017)

Glyphosate, at a concentration of 500 μgL^{-1}, reduced the growth of heterotrophic bacteria (Rai, 2009). Glyphosate at 500 μgL^{-1} significantly decreased zoosporangia concentration and zoospore production of the pathogenic fungi *Batrachochytrium dendrobatidis* (Hanlon & Parris, 2012). Nweke et al*.,* (2014) in their study on "Toxicity of binary mixtures of formulated glyphosate and phenols to *Rhizobium Species* dehydrogenase activity" revealed that dehyrogenase activity (DHA) were stimulated at low doses (hormesis) and inhibited at high doses. As individual substances, glyphosate, phenol, 4-chlorophenol and 2,4-dichlorophenol stimulated the enzyme activity at concentrations up to 400 mg/l, 600 mg/l, 60 mg/l and 20 mg/l respectively. At concentrations above the hormetic range, glyphosate and phenols progressively inhibited dehydrogenase activity of the *Rhizobium species*, reaching saturations at 1200 mg/l for glyphosate and 4-CP, 400 mg/l for 2, 4-DCP and 3000 mg/l for phenol (Nweke et al., 2014). As mixtures, the substances stimulated Dehydrogenase activity at concentrations ranging from 20 mg/l to 80 mg/l for 2,4-DCP/glyphosate mixtures, 20 mg/l to 60 mg/l for 4-CP/glyphosate mixtures and 20 mg/l to 600 mg/l for phenol/glyphosate mixtures, (Nweke et al*.,* 2014). They equally reported that at concentrations above the hormetic range, the mixtures also progressively inhibited DHA, reaching saturations at concentrations between 80 mg/l and 400 mg/l for 2,4-DCP/glyphosate mixtures, 1000 mg/l and 1200 mg/l for phenol/glyphosate mixtures and at 400 mg/l for 4-CP/glyphosate mixtures. In the case of 4-CP/glyphosate mixtures, synergistic effects were observed at mixture concentration

ratios of 20% 4-CP/80% glyphosate, 50% 4-CP/50% glyphosate and 80% 4-CP/20% glyphosate. At a percentage mixture concentration ratio of 60% 4-CP/40% glyphosate, the effects were slightly antagonistic. Similarly, mixtures of phenol and glyphosate mixtures indicated both synergistic and antagonistic effects at different concentration ratios, (Nweke et al*.,* 2014). According to WHO, (2017), Glyphosate (CAS No. 1071-83-6) is a broad-spectrum herbicide used in both agriculture and forestry and for aquatic weed control. Microbial biodegradation of glyphosate occurs in soil, aquatic sediment and water, the major metabolite being aminomethylphosphonic acid (AMPA) (CAS No. 1066-51-9). Glyphosate is chemically stable in water and is not subject to photochemical degradation. The low mobility of glyphosate in soil indicates minimal potential for the contamination of groundwater. Glyphosate can, however, enter surface and subsurface waters after direct use near aquatic environments or by runoff or leaching from terrestrial applications. (Zain, Rosli, Kamaruzaman, NurMasirah & Yahya, 2013**)**

The work of Michael & Stephen, (2016) on the Effects of Some Commonly Used Herbicides on Soil Microbial Population revealed that, at low dose, microorganisms utilizes pesticides as substrate. In their study, the mean bacterial population against fifteen exposure periods for sample soil treated with Paraquat recorded the highest bacterial population and subsequently declined gently from the first five days to day fifteen. The first five days after Paraquat application revealed an increased bacterial population beyond the entire treated sample halved the recommended field rate, but it sharply declined after day five to day fifteen. This decreasing trend in all the treatment towards the fifteen days suggests a decline in carbon source that supported the initial population of the bacteria (Michael & Stephen, 2016).

2.4: Effects of Mixtures of heavy metals and pesticides to organisms in aquatic environment

Heavy metal mixtures may have joint effects that are significantly different from their individual effects. Many studies have revealed the individual toxicity of these metals, but no study has shown the mixture effects, which actually represent the real-life situation in the world. In the need to mimic the real-life situation using multiple heavy metal exposure, there three types of joint action that chemicals in mixtures may show, namely:

(1) Independent joint action, where chemicals act independently of one another and have different modes of toxic action.

(2) Similar joint action, where chemicals have similar effects but do not interact (i.e. dose addition)

(3) Synergistic or antagonistic action, where the toxicity of a mixture may be greater than or less than the toxicity that would be predicted from the individual constituents. Studies have established that the toxicity arises from exposure to metal mixtures on various organs and tissues in the body system including cytogenicity, oxidative stress, neurotoxicity, and bladder cancer, toxicity on embryogenesis, immunotoxicity and mortality (Nweke et al., 2014).

2.5: MATHEMATICAL MODELS

The toxic unit (TU) and toxic index (TI):

The Toxic Index (TI) of each mixture are calculated as the sum of toxic unit (TU) for all the component of the mixture (Nweke, et al., 2018) as shown in equation below:

$$TU_i = \frac{Cmix_i}{IC_{50i}} \qquad \ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 2.1)$$

Where *Cmix* is the concentration of the component in the mixture at the EC_{50} of the mixture and EC_{50} is the concentration of the component that elicited 50% decrease in dehydrogenase activity when tested as an individual.

The Toxic Index (TI) of each mixture calculates the sum of TU for all the components of the mixture.

$$TI = \sum_{i=1}^{n} TU \quad \text{.....} \quad (Equation\ 2.2)$$

Where *n* is the number of components in the mixture. TI = 1 shows additivity, TI > 1 shows antagonistic interaction and TI < 1 shows synergistic interaction (Boillot & Perrodin, 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of Study Area

The Otamiri River is one of the main rivers in Imo State, Nigeria. The river takes its name from Ota Miri, a deity who owns all the waters that are called by its name, and who is often the dominating god of Mbari houses. The river runs south from Egbu to Owerri and through Nekede, Ihiagwa, Eziobodo, Olokwu Umuisi, Mgbirichi and Umuagwo to Ozuzu in Etche, in the Rivers State, from where it flows to the Atlantic Ocean. The length of the river from its source to its confluence at Emeabiam with the Uramiriukwa River is 30 kilometres (19 mile). The Otamiri watershed covers about 10,000 square kilometres (3,900 sq mi) with annual rainfall of 2,250 to 2,500 millimetres (89 to 98 in). The watershed is mostly covered by depleted rain forest vegetation, with mean temperatures of 27 °C (81 °F) throughout the year. The Otamiri is joined by the Nworie River at Nekede in Owerri, a river about 9.2 kilometres (5.7 mi) long. The Nworie River is subject to intensive human and industrial activities, and is used as a source of drinking water by the poor when the public water system fails. Waste management in Owerri is inefficient and contributes to pollution of the river. Most of the wastes from Owerri are dumped at the Avu landfill in Owerri West along the Port Harcourt highway, which creates a high concentration of toxicants in the river. Apart from that, the river is also polluted with toxicants such as heavy metals, pesticides, phenols, hydrocarbons, polycyclic aromatic hydrocarbons (PAH), linear alkyl benzene sulfonate and other toxic chemicals as a result of human and industrial activities in Owerri local government areas and beyond in Imo State and more importantly at Egbu abattoir and mechanic village where the river transits to other communities. Figure 3.1 showed the pictorial view of Otamiri River while figure 3.2 is the location map of the study area.

Figure 3.1: Pictorial view of the Otamiri River

3.2 Collection of Samples

The methods of Ogbonna, (2014) was adopted for samples collection along the course. Samples were collected from twelve (12) different sampling locations (sites) at the interval of approximately two thousand meters (2000m) from each site. Three different water samples were collected at the interval of one hundred meters (upstream, mid-stream and downstream) and one soil sample at the river bank.

Sample locations are:

i. Egbu Abattior
ii. Aba road bridge
iii. Akachi
iv. Umezurike hospital
v. Mechanic village
vi. West end bridge
vii. Nekede
viii. Inland Bridge
ix. FUTO
x. Ihiagwa
xi. Mgbirichi
xii. Umuagwo

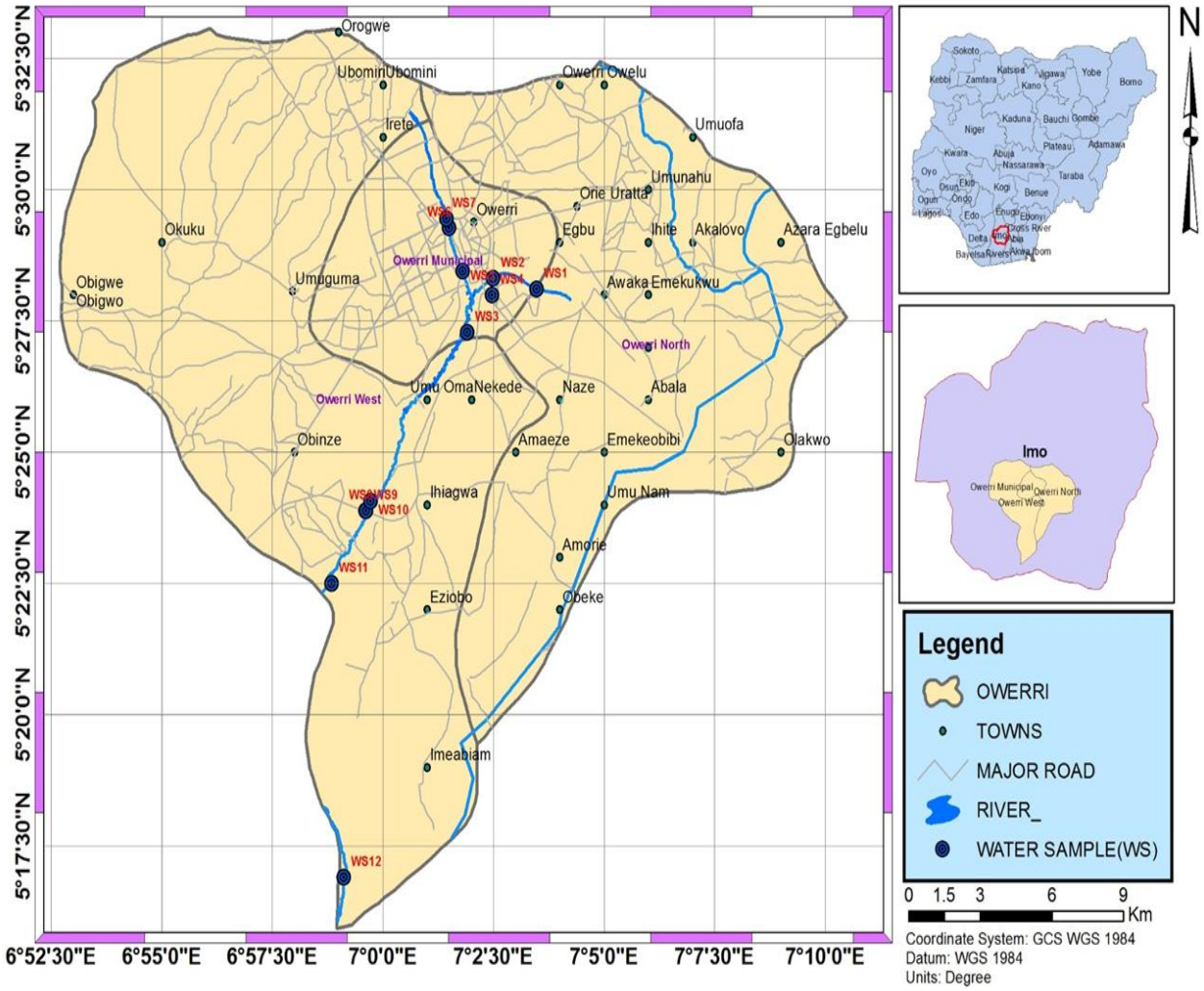

Figure 3.2: Location Map of the Study Area

Location Sample	ID	Latitude	Longitude
Egbu Abattior	WS1	5.468454167	7.0579694
Akachi	WS2	5.47177	7.041655
Mechanic Village	WS3	5.454669	7.0319396
Aba Road	WS4	5.4664851	7.0412746
Nekede Westend	WS5	5.474175	7.030155
Umezurike Hospital	WS6	5.4877883	7.0250167
Inland Bridge	WS7	5.4907867	7.024105
Nekede	WS8	5.3980043	6.9934721
Ihiagwa	WS9	5.3980504	6.9937335
FUTO	WS10	5.4010048	6.995505
Mgbirichi	WS11	5.350636111	6.967972222
Umuagwo	WS12	5.281780556	6.985272222

A total of forty-eight (48) samples were collected in duplicate which is (48×2=96 samples) from four (4) different points (viz; Up stream, Mid-stream, Downstream and Soil at river bank) of twelve (12) sampling location.

Geographical coordinates of the sampling points signify the points of sampling. Samples were collected with sterile wide mouth one (1) liter capacity sampling bottles. A canoe was used during sample collection. Prior to the day of collection of samples, the sample containers were washed thoroughly and sterilized by soaking for 30minutes in 70% ethanol and rinse with sterile water then stored in a sterile cooler to avoid contamination.

At the point of collecting the sample, the containers were rinsed three (3) to four (4) times with the river water. For surface water collection, while mounting on the canoe, samples were collected from the points mentioned above using the sample containers held at the base with one hand, the sample containers were plunged at an elbow depth (about 50cm) below the water surface, positioning the mouth of the container at the direction of water flow. A gap of about three (3 cm) was left in the container. The containers were covered and then pulled out. The sample was stored in a cooler and taken to the laboratories for analysis. The samples were stored in the refrigerator at 4^{o}C, until required.

For soil sample, samples were collected at the river bank. The soil sample was scooped with the grab spatula and transferred into the sterile sample bag, covered and placed in ice block pack and transported to the laboratory for analysis.

3.3 Physicochemical Analyses of Samples

Standard methods were used for physicochemical parameter analysis of the samples. The parameters assayed for includes: temperature, pH, turbidity, dissolved oxygen (DO), conductivity, total hardness, total dissolved solids (TDS), biological oxygen demand (BOD), salinity, Presence of chlorine, phosphate, NH_3, NO_2, SO_3 and SO_4.

Heavy metals and pesticides content of the river water were also analyzed.

3.3.1 Temperature

The temperature of the river sample was determined according to AOAC, (2010). After collecting the sample, the thermometer was immersed immediately into the water sample and the reading was taken. The instrument was calibrated in degree Celsius with standard range of 0-100^{o}c.

3.3.2 pH:

The pH was determined using a pH meter (HANNA 1910 meter) which was calibrated using a standard buffer of pH 4 and pH 7. The method of AOAC, (2010) was adopted where the electrode of the standard buffer was rinsed with distilled water and dipped into the sample to take the reading. It was cleansed with tissue paper before dipping it into the next sample.

3.3.3 Determination of the turbidity

Determination of the turbidity using APHA, (2005) methods, the turbidity was determined with a spectrophotometer at wavelength of 750 nm. Twenty-five milliliter (25ml) of deionized water was measured in a 25ml bottle as blank. The essence of the blank is to zero the spectrophotometer before same quantity of sample in another curvette is placed into the light shield after removing the blank. Then the read button was pressed to take readings. The results gotten were recorded in Nephrometric Turbidity Unit (NTU).

3.3.4 Dissolved oxygen determination

Dissolved oxygen (DO) was determined using a DO meter. This was calibrated using 5% sodium sulphate solution before inserting the probe of the meter into the sample. Readings were taken and recorded in mg/L. (AOAC, 1996.)

3.3.5 Determination of conductivity

The conductivity of the sample was determined using a standard conductivity meter as described by APHA, (2005). The meter was calibrated with a standard solution; the conductivity cell was cleaned with a lint free tissue before dipping the cell into the sample and the readings were obtained from the read out. The cell was rinsed with deionized water.

3.3.6 Determination of Total hardness

The method of APHA, (2005) was adopted where twenty-five milliliter of the sample was measured into a conical flask; one milliliter of ammonium chloride was added. Three drops of an indicator were also added to the flask, using a micro burette, the solution was titrated with a 0.01M EDTA. The conductivity was determined using a formula:

$$\text{Total hardness (mg/1CaCO}_3) = \frac{vol, EDTA(titer) \times m(EDTA) \times 100 \times 1000}{Vol\ of\ Sample} \quad \text{…….. (Equation 3.1)}$$

3.3.7 Determination of biological oxygen demand (BOD)

The BOD was determined using a DO meter. The DO was calibrated using 5% sodium sulphate solution as described by APHA, (2005). The probe of the meter was inserted into the sample after switching it on for about 10 mins then the reading was taken. The sample was incubated for five days in a Winkler bottle at temperature of 20^{o}c. At the fifth day, another reading was recorded by inserting the probe into the sample again. The difference between the two DO results was recorded as the BOD.

$$BOD = DO\ (0) - DO\ (1) \text{ ----------(Equation 3.2)}$$

The method involved filling the samples to overflowing, in an airtight bottle of the specified size and incubating it at the specified temperature for 5days. Dissolved oxygen (DO) was measured initially and after incubation and the BOD were computed from the difference between initial and final (DO). Because the initial (DO) was determined shortly after the dilutions was added, all oxygen uptake occurring after this measurement

was included in the BOD measurement. One Millimeter (1ml) of $MgSO_2$, $CaCl_2$ phosphate buffer, $FeCl_3$ were added to 1L of water. The solution was then shaken thoroughly to saturate the dissolved oxygen. This solution was used to dilute samples. One hundred millimeters (100 ml) of the samples were measured into different one liter flasks and were made up to (1L) mark with the dilution water previously prepared. The dilution sample solution was then poured into BOD bottles and subsequently incubated at 20^oC in the dark for 5 days.

Determination of initial dissolved oxygen**:** Three hundred millimeters (300ml) BOD bottles were filled with the diluted samples previously prepared and the initial dissolved oxygen (DO) was determined using the Winkler's method

Determination of Final Dissolved Oxygen**:** After incubation for 5days, the final dissolved oxygen (DO) was determined using the same procedure above

BOD (mg/L) = $[DO^0-DO^1]$ /B

Where DO^0= initial dissolved oxygen (immediately after preparation)

DO^1= final dissolved oxygen (after 5days of incubation)

B = Fraction of sample used

3.3.8 Determination of chloride

Twenty-five milliliter (25ml) of the water sample was pipette into a conical flask then 1ml of potassium chromate indicator was added. The solution was titrated with 0.02 m silver nitrate to a reddish brown end point using a micro burette and a blank titration was done using deionized water. Then chloride was estimated according to APHA, (1998) method.

$$\text{Chloride (mg/1)} = \frac{(Sample\ titer - Blanc\ titre) \times 0.02m \times 35.5 \times 1000}{Vol\ of\ Sample} \text{ ----------(Equation 3.3)}$$

3.3.9 Determination of phosphate

The method of US-EPA, (1993) was adopted, where 6.0 g of ammonium heptamolybdate was measured into250 ml conical flask and dissolved with 150 ml of distilled water. Then, 2.6 g of ascorbic acid was dissolved in 50ml of distilled water and added in the 1-liter volumetric flask to give 0.0007 M. Afterwhich, 0.4g of potassium antimony tartrate was weighed and dissolved in 20ml of distilled water (0.0000086 M) in a separate flask. In another flask, 0.1M stock of sulfuric acid was prepared by dissolving 10ml of the stock in 50ml of distilled water.

One thousand milligram per liter (1000 mg/L) phosphate stock was prepared by weighing 1.532 mg of potassium phosphate trihydrate in 250 ml of distilled water. Then, 0.5, 1.5, 2.0 and 2.5 ppm PO_5 were prepared by proper dilution with distilled water for calibration curve. In a 50 ml volumetric flask, 12.4 ml of the ammonium molybate solution was pipetted then 10 ml sulphuric acid was added and swirled then 2.3ml of antimony potassium tartrate was added. The mixture was swirled to mix before making up the mixture to the mark with distilled water. Then, 0.4 ml molybdate reagent was added and it was swirled to mix before adding 0.4 ml of L-Ascorbic acid. It was also mixed properly by swirling. Then the light absorption of the solution was determined at 850 nm wavelength spectrophotometrically.

3.3.10 Heavy metal analysis

The method of APHA, (1995) was adopted using Atomic Absorption spectrophotometer. (AAS). Both water and soil samples were digested following the method of Adrian, (1973). For soil sample digestion, 2 g of the dried soil sample was weighed into a digestion flask and 20 ml of acid mixtures was added. (650 ml concentrated HNO_3; 80 ml perchloric acid; 20 ml concentrated H_2SO_4). The flask was heated until a clear digest was obtained. The digest was then diluted with 100 ml of distilled water.

The water sample was thoroughly mixed by shaking and 100 ml of it was transferred into a 250ml beaker to which 5 ml of concentrated nitric acid was added and heated to boil till the volume was reduced to about 15ml by adding 5 ml increment of concentrated nitric acid till all the residue was completely dissolded. The mixture was cooled, transferred into a 100 ml volumetric flask and made up to 100 ml using metal free distilled water. The sample was aspirated into the oxidizing air- acetylene flame. When the aqueous sample was aspirated, the sensitivity for 1% absorption was observed.

Reference solution: A series of standard metal solutions in the optimium concentration range were prepared, the reference solutions were prepared daily by diluting the single stock element solutions with water containing 1.5 ml concentrated nitric acid/ litre. A calibration curve for each metal was prepared by plotting the absorbance of standards versus their concentrations.

The digested samples were analyzed for heavy metals using Atomic Absorption Spectrophotometer at each metals specific wavelength.

3.3.11 Pesticides content determination

The method as described by AOCA (1996) was adopted for the determination and evaluation of the presence of pesticides in the river sample. A gas chromatography coupled to a mass spectrometry (GC-MS) was used.

Both water and soil samples were mixed together and extracted. About 5g of the homogenized sample was mixed with 20 g of sodium sulphate in agitation morter to absorb moisture. The homogenate was then placed in extraction cellulose thimble covered with Whatman filter paper and inserted into the extraction chamber unit. Extraction was carried out with 200 ml ethanol for 4hours The extracts were concentrated by evaporation and 1 ml of it used for GC analysis.

3.4 Bacteriological Analysis of the Samples

1. Direct count method:

The method of Fawole & Oso, (2004) was adopted for bacteriological analysis of the samples. For surface water samples, one milliliter (1ml) of the sample was diluted with the normal saline while for soil sample; one gram (1g) of the oven dried sample was diluted with the normal saline. Briefly, a tenfold serial dilutions of the samples were carried out. One milliliter (1ml) of the stock was collected aseptically using a Pasteur pipette and placed in a nine milliliter (9ml) of distilled water in a test-tube. This was shaken vigorously for 60 second, from this tube, 1ml of the dilution was transferred into the next tube containing 9ml of water, this was continued till the last tube, and thereafter 1ml of the dilution was discarded from the last tube.

From the dilutions factor three and four (10^{-3} and 10^{-4}) for water sample, 0.1ml of the samples were inoculated into freshly prepared sterile duplicate plate of agar plates (Nutrient Agar (NA), Mannitol salt agar (MSA), MacConkey agar(MA), Salmonella-shigella agar (SSA), Thio Sulphate Citrate Bile Salt Sucrose agar(TCBS) and Eosin methylene blue agar(EMB))

For the soil sample, 1ml from dilution five and six (10^{-5} and 10^{-6}) were used for the analysis. The inoculums were spread with sterile bent glass rod. The plates were left on the bench for 20 minutes such that the media absorbs the inoculums before been inverted and incubated at 28^0C for 24hrs in an incubator. Colonies which developed on the plates were counted using digital colony counter and counts expressed as colony forming unit per milliliter or grams (cfu/ml or cfu/g) of the samples analyzed (surface waters and soil respectively).

2. Membrane filtration method:

Methods as described by Cheesbrough, (2006) was adopted: The membrane filter paper was placed on the funnel assembly of the membrane filtration vaccum pump using a well flamed foreceps. From 10^{-5} dillution, 100 ml ot the sample was pipetted into a beaker

connected to the funnel of the pump. The vacuum was switched on and allow the sample to draw completely. A forecep was flamed againto remove the filter from the vacuum and planted (placed) on a well prepared media and incubated at room temperature for 22hours. The media (TCBS, EMB and MacConkey) were used.

3. Culturing of Anaerobes

An anaerobic condition is needed for the cultivation and isolation of strict anaerobes such as *Clostrisium species*, anaerobic *streptococci, Campylobacter* etc. It also helps to differentiate pathogens and to isolate facultative anaerobes from samples containing commensals.

An anaerobic jar will be required with the availability of oxygen removing system to produce anaerobic condition. To achieve this, the jar was air tight with an oxoid Gas Pak. The prepared and inoculated culture plates were incubated in this condition for 24hrs.

3.5. BACTERIAL COUNT:

After incubation at the specified time, the bacterial isolates were counted to determine the total bacterial counts.

Total no. of colonies = no of colonies × dilution factor (CFU/ml)

Result obtained were expressed:

i. Total heterotrophic bacterial count (THBC)

ii. Total coliform count (TCC)

iii. Total vibrio count (TVC)

iv. Total staphylococcal count (TSC)

v. Total salmonella shigella count (TSSC)

vi. Total counts on Eiosin methylene Blue (TCEMB)

vii. Total anaerobic bacterial count

3.6 PURIFICATION / PRESERVATION OF PURE CULTURES:

i. **Morphological Characteristics:**

Colonies which develop on the agar plates were sub cultured onto freshly prepared nutrient agar plates using their colonial morphological appearance and incubated at room temperature for 24hrs.

ii. **Gram Staining**: The gram reactions of the isolates were determined by conducting a gram staining. This helped to differentiate the Gram positive organisms from the Gram negative ones.

The method as described in Cheesbrough, (2006) was adopted. A smear of each of the bacterial isolate were made on a grease free slide and fixed by air drying. The smear was passed through a burnsen burner flame two to three times. The fixed smear was covered with crystal violet for 30-60 seconds then, washed with clean running water. The smear was covered with lugol's iodine for 60seconds thereafter washed off. The smear was decolorized with acetone alcohol for 30 secs and then washed off. The smear was flooded with safranin for 2 minutes and then washed off. The back of the slide was clean and placed on a dry rack to air dry after which the smear was viewed under the microscope with oil immersion at objective lens. Gram positive gives dark purple whilst gram negative showed pale to dark red.

iii. **Spore Staining**: Smears of each bacterial isolate were made on a clean grease free slide and was fixed by air drying. The smears were then be covered with malachite green stain and place over heat for 5 minutes ensuring that the slide did not get dried by flooding the smear with more malachite green stain. At the end of the 5 minutes the stain was rinsed off with clean water and counter

stained with safranin for 2 minutes then wash off. The smear was allowed to air dry and viewed under the microscope with oil immersion. Spore positive appeared green while negative pink (Cheesbrough, 2006).

iv. **Capsule Formation:** A capsule is a gelatinous outer layer secreted by bacterial cell that surrounds and adheres to the cell wall. Most capsule are composing of polysaccharides while others are of polypeptides. A drop of India ink was placed on a clean slide; a loop full of bacteria isolate was added and smeared. Another slide was used to drag the ink-cell mixture into a thin film and was allowed standing for 5mins to air dry. It was flooded with crystal violet; this stains the cell and not the capsule for 1mins. The crystal violet was drained by tilting the slide at angle 45^{o}. it was observe under objective lens. A clear zone around the cell indicates a positive result.

v. **Motility Test**: This test was used to differentiate motile organisms from non-motile ones based on the presence and absents of flagella respectively. This was done using Agar- Agar NO 1. A loop full of each isolate was aseptically inoculated into the agar tube using stab method and was incubated at 28^{o}c for 24hrs. After incubation, it was observed for growth by the level of extension of growth in the incubation tubes. Extension from the stabbed position indicates positive otherwise negative. (Cheesbrough, 2006).

Vi. Biochemical Test:

The necessary biochemical tests were done for characterization of the bacterial isolates. The biochemical test that was adopted includes: catalase, citrate, nitrate utilization, coagulase, oxidase, Indole, Methyl Red (MR), voges proskauer (VP), Urease, H_2S, Mannitol and sugar fermentation test (lactose, sucrose, maltose and glucose).

i. **Catalase:** This test was used to differentiate bacteria that produce enzyme catalase such as *Staphylococci* from non-catalase producing bacteria. Two milliliter (2 ml) of hydrogen peroxide solution was poured into tubes according to the number of the isolate. A sterile wire loop was used to introduce / immerse each isolate in the tubes containing the solution. Active bubbling within 10seconds indicates a positive test while none indicates a negative test (Cheesbrough, 2006).

ii. **Citrate test:** The test will serve as a tool in the identification of Enterobacteriaceae. Loops full of fresh culture of each test organisms were inoculated onto a sterile agar slope of Simon citrate agar using a stab inoculation technique. The inoculated agar slope was incubated at 27^{o}C for 24hours. A bright blue coloration indicates a positive result otherwise negative (Cheesbrough, 2006).

iii. **Nitrate Reduction test**: Many gram negative bacteria uses nitrate as the final electron acceptor. This test determines the production of an enzyme called nitrate reductase which results in the reduction of nitrate (NO_3). Bacterial species may be differentiated by the ability of them to reduce nitrate to nitrite or nitrogen gas (Sager, 2019). The procedure entails, inoculating a bacterial isolate in a nitrate broth in a test tube containing Durham's tube. Then the tube was incubated at 28^{o}C for 24hrs, after which it was observe for N_2 gas before adding the reagent. 6-8 drops of the nitrate reagent A were added, and then 6 drops of nitrate reagent B were added. The mixture was shaken and observes for 2 mins. If no colour change, zinc powder was added and observes for 3 mins. Red colour change after addition of reagent A and B indicates positive result. Equally, absence of red colour after adding zinc is positive. Negative test is determined by development of red colouration after addition of zinc.

iv. **Coagulase test**: This is the ability of the organism to react with fibrinogen found in the plasma to form agglutination. Here, a loop full of the test organism was emulsified on a clean grease free slide using a normal saline; a loop full of human plasma was added. A control without plasma was set up. The slides were rock for 10seconds and observe for agglutination. Clumping of cells indicates positive test while absence is negative (Cheesbrough, 2006).

v. **Oxidase test:** The method of Cheesbrough, (2006) was adopted. A piece of whatman's No 1 filter paper was placed in a clean petri dish and three drops of oxidase reagent was added in each of the test organism. With a sterile loop, each colony of the test organism was smeared on the oxidase reagent soaked paper. The blue purple coloration indicates a positive test otherwise negative (Cheesbrough, 2006).

vi. **Indole Test:** Some microorganisms have the capability of hydrolyzing the amino acid Tryptophan to produce indole. The test organism was inoculated in a tube containing 3 ml of peptone preparation and incubated for 24 hrs at 30^{o}C thereafter 0.5ml of kovac's reagent was added and shake gently. A red coloration on the surface layer within 10mins indicates positive otherwise negative (Cheesbrough, 2006).

vii. **Methyl Red/ Voges Proskauer Test:** For MR, the test is used to determine if an organism produce acetylmethyl carbinol from glucose fermentation. If present, acetylmethyl carbinol is converted to diacetyl in the presence of α- naphthol, strong alkali (40%KOH) and atmospheric oxygen (Sager, 2018). The method updated by Sager, (2018) was adopted. Prior to inoculation, the medium was allowed to equilibrate the room temperature. A fresh broth culture of the isolate was prepared and incubated at 35^{o}C for 24hrs. From the aliquote, 2 ml of the inoculum was transferred in a sterile testube. 5-6 drops of 5% α- naphthol was

added and mix well. 2 drops of 40%potassium hydroxyl was added and mix to aerate. Then the pink-red colour change after 30mins indicates positive result, ensuring that the tube is shaking well within the 30mins.

For VP test, some bacterial isolates have the ability to utilize glucose and convert it to a stable acid like acetic acid, lactic acid and formic acid as the end products. After preparing the inoculum broth, 2-3 drops of MR indicator was added to it. Appearance of red color immediately indicates a positive result.

viii. **Sugar fermentation test:** Each colony of the different test organisms was inoculated onto sterile agar slopes of triple sugar iron agar using stab inoculation method. Thereafter the inoculated agar slopes were incubated at37^{o}C for 24hr. The different colors of the slopes and butts in addition to the presence of gas production and hydrogen sulphide (H_2S) blackening was indicative of the type of bacteria present (Cheesbrough, 2006)

Once confirmed, pure colonies were preserved by sub culturing into culture bottles in slants and were kept in the refrigerator at 4^0C for future analysis. The percentage of occurrence of each isolate in each sample was determined as follows:

$$Occurrence(\%) = \frac{Number\ of\ colonies\ of\ isolate\ A}{Total\ number\ of\ isolate} \times 100 \ldots\ldots\ldots (Equation\ 3.3)$$

3.7 Molecular Identification of Pure Culture of Bacteria Isolates

The isolates with the highest percentage of occurrence from the river and soil samples were selected for the toxicity test. The strain identities of these preponderant bacteria were further confirmed through molecular identification. The identification was done by Inqaba Biotechnological laboratory, Pretoria South Africa.

3.7.1. DNA extraction (Boiling method)

Five milliliters of an overnight broth culture of the bacterial isolate in Luria Bertani (LB) was spun at 14000 rpm for 3 min. The cells were re-suspended in 500 ul of normal saline and heated at 95^0C for 20 min. The heated bactererial suspension was cooled on ice and spin again for 3 min at 14000 rpm. The supernatant containing the DNA was transffered to a 1.5 ml microcentrifuge tube and stored at -20ºC for other downstream reactions.

3.7.2. DNA quantification

The extracted genomic DNA was quantified using the Nanodrop 1000 spectrophotometer. The software of the equipment was lunched by double clicking on the Nanodrop icon. The equipment was initialized with 2 µl of sterile distilled water and blanked using normal saline. Two microlitre of the extracted DNA was loaded onto the lower pedestal; the upper pedestal was brought down to contact the extracted DNA on the lower pedestal. The DNA concentration was measured by clicking on the “measure” button.

3.7. 3 16S rRNA Amplification

The 16s rRNA region of the rRNA gene of the isolates were amplified using the 27F: 5'-AGAGTTTGATCMTGGCTCAG-3’ and 1492R: 5'-CGGTTACCTTGTTACGACTT-3’ primers on a ABI 9700 Applied Biosystems thermal cycler at a final volume of 40 microlitres for 35 cycles. The PCR mix includes: The X2 Dream taq Master mix supplied by Inqaba, South Africa (taq polymerase, DNTPs, MgCl), the primers at a concentration of 0.5µM and the extracted DNA as template. The PCR conditions follows thus: Initial denaturation, 95ºC for 5 minutes; denaturation, 95ºC for 30 seconds; annealing, 52ºC for 30 seconds; extension, 72ºC for 30 seconds for 35 cycles and final extention, 72ºC for 5 minutes. The product was resolved on a 1% agarose gel at 130V for 30 minutes and visualized on a blue light transilluminator.

3.7.4. Sequencing

Sequencing was done using the BigDye Terminator kit on on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. The sequencing was done at a final volume of 10 µl, the components included 0.25 µl BigDye® terminator v1.1/v3.1, 2.25µl of 5 x BigDye sequencing buffer, 10µM Primer PCR primer, and 2-10ng PCR template per 100bp. The sequencing condition were 32 cycles of 96°C for 10 seconds, 55°C for 5seconds and 60°C for 4 minutes.

3.7. 5. Phylogenetic Analysis

Obtained sequences were edited using the bioinformatics algorithm. Trace edit, similar sequences was downloaded from the National Center for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using MAFFT. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 (Saitou & Nei, 1987). The bootstrap consensus trees inferred from 500 replicates (Felsenstein, 1985) were taken to represent the evolutionary history of the taxa analyzed. The evolutionary distances were computed using the Jukes-Cantor method (Jukes & Cantor, 1969).

3.8 Toxicity Assay

3.8.1 Preparation of bacterial inoculum

The highest occurring organisms in both Otamiri water and soil were used for the toxicity assay.

The method of Nwanyanwu *et al.,* (2017) was adopted for bacteria inoculum preparation for the toxicity. Nutrient broth was prepared according to the manufacturer's instruction; 1.3 was weighed into a 250ml conical flask and 100ml of sterile deionized water was added and shook to dissolve. The medium was dispensed into two different conical flasks, 50ml each. The flasks were corked and sterilized by autoclaving at 121^{o}C 15 psi

for 15 minutes. The flask was removed and allow to cool to body temperature. When cool, the flasks were aseptically inoculated with the test organisms (*Lysinibacillus macrolides* and *Alcaligenes* faecalis) and then incubate on a rotary shaker (150 rpm) at room temperature (28±2ºC) for 24 hours.

3.8.2 Harvesting the bacterial cells:

The method of Nweke *et al*., (2014) was adoptedin this process. The bacterial cells were harvested by centrifugation at 3000 rpm for 15 minutes. Then it was washed with a sterile deionized water and centrifuge again at the same revolution per minute. The cells were washed again as stated above. The reason of this repeatation (washing twice) was to avoid nutrient carry over. The washed cells were transferred into a sterile curvette and the cell density were adjusted to 0.1 at 540nm wavelenghtin a spectrophotometer.

3.8.3 Test toxicants used:

Heavy Metals: All metals used were procured from Sigma- Aldrich (Germany) Lead, (Pb(II)), cadmium, (Cd(II)), cobalt, (Co(II)), zinc, (Zn(II)) and Nickel, (Ni(II)) were used as:

$Pb(NO_3)_2$ = Lead (II) nitrate.

$CdSO_4.8H_2O$ = Cadmium sulphate hydrate.

$Co(NO_3)_2$ = Cobalt (II) nitrate.

$Zn\ NO_3.6H_2O$ = Zinc nitrate hexahydrate.

$NiSO_4.\ 6H_2O$ = Nickel (II) sulphate hexahydrate.

Pesticides: The pesticides used were fomulations sourced from local dealer with Owerri metropolis. The pesticides were Glyphosate and Diclovors fomulations (2, 2-dichlorovinyldimethyl phosphate) DDVP.

3.8.4 Preparation of pesticides and heavy metal stock:

The heavy metals were prepared by dissolvingthe required quantities of the metals in 250ml of deionized water. That is 0.744 gof Zn(II), 0.641 g of Cd(II), 0.828 g of Pb(II), 0.657 g of Ni(II) and 0.7257 g of Co (II) in separate conical flasks containing 50ml of deionized water and allowed to dissolve by shaking gently. The flasks were made up to 250ml by adding extra 200ml of deionized water.

The pesticides were prepared by dissolving 350 g glyphosate in 1 litre of water (according to the manufacturer's manual) while DDVP was 100 g in 1 liter of deionized water.

Both the heavy metals and pesticides were converted to microgram per litre (mg/l) as shown in Appendix 6. They were sterilized by membrane filteration. The membrane filter paper usedhad a pore size of 0.45µm.

3.8.5. Preparation of the indicator stock:

The indicator used was 3-(4,5-dimethyl-2- thiazolyl) – 2,5- diphenyl- 2H- Tetrazolium Bromide (MTT indicator) stock.

The 0.02 % solution of MTT indicator stock was prepared by dissolving 0.02g of MTT IN 20ml of sterile deionized water and made up to 100ml, then sterilized by membrane filteration and stored ina 100ml – conical flask wrapped in aluminum foil.

Trial runs of the test were carried out with different concentration range of the individual toxicant (pesticides and metal ions) against 0.02% MTT- indicator to ascertain potency of the quantity of MTT used.

3.8.6 Design of experimental protocol:

A protocol is a table that shows the volume and concentration of reagents (toxicant, water, nutrient broth, and indicator) and the test organisms used in the right proportion to make up the 1ml final volume as expressed on appendix7.

The volume of MTT and the test organisms were constant at 100 µl (0.1ml) each, while that of nutrient broth was constant at 250 µl (0.25ml). Different protocols were prepared for individual toxicants as well as their mixtures.

3.8.7 Design of Experiment

The single, binary, ternary and septenary mixtures of the heavy metals and pesticides were evaluated using EC_{50} fixed ratio ray experimental design. In each case, the EC_{50} ratio was kept constant, while the total concentration of the mixture was varied around the EC_{50} to obtain a complete dose-response relationship of the mixtures. The toxicity tests determine the toxicity of individual heavy metals and pesticides and their mixtures. This design has been used by other scholars; (Nweke, *et al*., 2014; Nweke, *et al*., 2017; Nweke *et al*., 2018; Reuben, Edna & Christian, 2020) The Equieffect Concentration Ratio(EECR) and Arbitrary Concentration Ratio (ABCR) of Binary, Ternary and Septenary mixtures for both organisms were as shown in Appendix 5

3.8.8 Dehydrogenase activity assay

The total dehydrogenase enzyme activity inhibition was ascertained using 3-[4, 5-dimethylthiazole-2yl]-2, 5-diphenyltetrazolium bromide (MTT) as the artificial electron acceptor which was reduced to purple-colored MTT-formazan (MTTF). The inhibition of dehydrogenase activity was done in 1ml final volume with; nutrient broth (pH 7). MTT, water and different concentrations of individual heavy metals, pesticides and their mixtures.

A 0.25 ml portion of X4-strength of nutrient broth and appropriate volumes of sterile distilled water and stock solution of heavy metals and pesticides were added to each 20 ml screw capped test tube in order to obtain the different individual toxicant concentration and mixture ratios or different concentrations of individual toxicants. Thereafter, 0.1 ml each of 0.02% w/v solution of MTT and standardized bacterial suspension was added into each tube. Each concentration of individual heavy metals and their mixtures were prepared in triplicates. Controls were prepared which consists of the inoculated medium without the toxicants. Triplicate control tubes were prepared. The cultures were incubated at room temperature for 24 hours. After incubation the formazan that was produced in each tube was extracted with 4 ml of butanol. Absorbance of the extract was determined with a spectrophotometer at 540 nm.

3.8.9 Design of experiment for individual toxicant:

The experiment set-up is done in 1ml final volume as well as in duplicate. In each 15ml screw capped culture tubes containing 0.25ml portion of ×4- strength nutrient broth (pH 7.0) requisite volumes of stock solution of respective toxicant was added, then the amount of sterile water to make up the final volume was added. Thereafter 0.1 ml of MTT and 0.1ml of standardize bacterial suspension were added. Controls were prepared without the toxicants. The cultures were incubated at room temperature for 24 hours. At the end of the incubation time, the reaction was stopped by adding 4ml of n- butanol and then shook for 5- 10 minutes. The MTT- formazan (MTTF) produced was extracted into the n-butanol and the absorbance of the extract was determined spectrophotometrically at 540nm.

3.8.10 Design of experiments for mixtures:

The binary, ternary and septenary of two pesticides and five metal ions were studied using the same EC_{50} fixed ray ratio experimental design. The 50% equi effect concentration ratio (EECR-50) was determined from the EC_{50} while arbitrary

concentration ratios (ABCR) were choosen. The mixture was combine; metal + metal and metals + pesticides. Meaning that the requisite volume of the stock concentrations of metal was tested with another metal and metal was equally combined with pesticide.

The set- up was as in the individual toxicants above where 1ml final volume was used and the experiment done in duplicate. In each 15ml screw capped culture tubes containing 0.25ml portion of ×4- strength nutrient broth (pH 7.0) requisite volumes of stock solution of respective toxicant mixtures were added, then the amount of sterile water to make up the final volume was added. Thereafter 0.l ml of MTT and 0.1ml of standardize bacterial suspension were added. Controls were prepared without the toxicants. The cultures were incubatedat room temperature for 24 hours. At the end of the incubation time, the reaction was stopped by adding 4ml of n- butanol and then shook for 5- 10 minutes. The MTT- formazan produced was extracted into the n-butanol and the absorbance of the extract was determined spectrophotometrically at 540nm

3.9 Data Analysis

3.9.1 Response estimation

The inhibition of dehydrogenase activity at different concentrations of the toxicants (as single, binary, ternary and septenary mixtures) was calculated as indicated in Equation (1) below. The inhibition (%) was generated as mean and standard deviation from triplicate determinations.

$$Inhibition(\%) = \frac{C_A - T_A}{C_A} \times 100 \ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 3.5)$$

Where: CA is the absorbance of MTTF extract in the control (without toxicants); TA is the absorbance of MTTF extract in the tests with different concentrations of the toxicants or their mixtures, (Nweke et al*.,* 2014).

3.9.2 Determination of EC_{50} (Effective concentration):

The dose-response data was fitted with 2-parameter logistic model (Equitation 2, below) to obtain their respective EC_{50} which is defined as the concentrations of the toxicants that inhibited the dehydrogenase activity of the bacterial isolate by 50%.

$$Inhibition(\%) = \frac{100}{1 + \left(\frac{x}{IC_{50}}\right)^{b}} \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 3.6)$$

Where: x is the concentration of the toxicant, IC_{50}(or EC_{50}) is the concentration that caused 50% inhibition; b is parameter determining the relative slope at EC_{50}, (Nweke et al., 2014)

3.9.3 Isobolographic analysis:

The estimated EC_{50s} of the binary mixture was used in isobolographic analysis of the mixtures toxicity to determine the combined effects of the tested chemicals. From the concentration of each component at $EC_{50,}$ (Cmix) was calculated, which was used to compute the isoboles. The isoboles generated were plotted in an isobologram, (Nweke et al., 2014).

3.9.4 Determination of toxic index:

Toxic index (TI) model was used to analyze the combined effect of the mixtures. The TI values were calculated using the equation (3) below:

$$TI = \sum_{i=1}^{n} TU_i \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 3.7)$$

TUi is the toxic unit of ith component in the mixture. Each toxic unit was computed using equation (4) given below:

$$TU_i = \frac{Cmix_i}{IC_{50i}} \ldots\ldots\ldots\ldots\ldots\ldots (Equation\ 3.8)$$

Where: $Cmix_i$ is the concentration of the ith toxicant in the mixture and IC_{50i} is the IC_{50} of the same toxicant when tested as an individual. TI = 1 signifies additive interaction, TI > 1 signifies antagonistic interaction and TI < 1 signifies synergistic interaction, (Boillot et al., 2008).

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULT

4.1.1 Physicochemical characteristics of Otamiri River

Table 4.1 below shows the physical and chemical parameters of Otamiri River. From the twelve sample sites analyzed, the temperature ranges from 23-27^{o}C with Egbu abattoir and Akachi bridge recording the highest (27^{o}C) while the least temperature was recorded at Umuagwo river column (23^{o}C). The pH was between 5.3 and 6.11 with the highest acidity recorded at Egbu abattoir with the pH of 5.3. The dissolved oxygen (DO) was within the range of 4.11 to 7.2 mg/L while the highest turbidity was recorded at the Inland Bridge with the turbidity of 21.6 NTU. The total hardness and the Total suspended solid (TSS) were 104 and 341mg/l respectively. The Electrical conductivity of Otamiri River was high with Aba road and west end recording 154 and 138 µS/cm respectively. The biological Oxygen demand (BOD) was 3.9 mg/l. The Mg recorded 43.1 mg/l while chloride, calcium and PO_4 were 8.9, 63.9 and 0.1 mg/l respectively. Table 4.2 showed that Iron (Fe), Nickel (Ni), Zinc (Zn). Copper (Cu). Cadmium (Cd) Lead (Pb) and Mercury (Hg) were among the heavy metals recorded in Otamiri River. From the table, Cd and Pb recorded the highest of 0.03 and 0.1 mg/l at Inland Bridge and Westend Bridge respectively. While Hg is said to be absent in most sampling site, it has its highest deposit at Umezuruike hospital sample site as 0.1mg/l.

Table 4.1: Physiochemical parameters of the Otamiri river water samples analyzed and compared with WHO (2017) standards.

Parameters	Egbu abattior	Akachi bridge	Mechanic village	Aba road	Westend	Umezuruike hospital	Inland bridge	Nekede	FUTO	Ihiagwa	Mgbirichi	Umuagwo	WHO Std (2017)
Temperature(°c)	27	27	26	24	24	25	25	24	24	26	25	23	27-28
Ph	5.3	5.6	5.5	6.4	5.41	6.02	6.11	5.65	6.05	5.39	5.22	5.4	6.5-8.5
Alkalinity	40	50	54	46	48	51	55.9	45.7	55.5	42	39	46	100
Chloride(mg/L)	7.646	8.354	8.921	5.911	7.08	7.821	6.872	8.884	7.055	7.611	6.9	8.003	5
BOD(mg/L)	3.9	3.05	2.2	2.45	3.3	3.09	3.4	3.25	2.9	3.2	2.9	3.5	5
DO(mg/L)	4.9	5.1	4.9	5.8	6.7	7.07	7.3	6.11	6.5	6.5	6.8	5	>5
Total hardness(mg/L)	96	88	104	65	50	69	53.2	45	71	81	76	60.6	500
E/conductivity(µS/cm)	98.8	97.25	99	54	97	100	98	98.6	82	95	99	100	100
Acidity	27.68	28.9	28.05	28	32.04	32.45	29	33	31	27	29	31.2	0
Turbidity(NTU)	10.55	10.8	12.2	12.8	22	18.5	21.6	20	10.8	11.8	12.5	10.8	50
TSS(mg/L)	205	211	112	115	211	341	245	115	121	129	234	111	250-500
PO_4(mg/L)	0.286	0.31	0.411	0.261	0.331	0.29	0.196	0.223	0.303	0.331	0.119	0.263	3.5
Ca(mg/L)	50.6	42	63.9	39.5	31	49	33	30	40	50.9	46	40.5	<250
Mg(mg/L)	35,4	36	43.1	20.5	19	20	20.2	15	31	29.1	31	20.1	<50
SO_4(mg/L)	1.9	1.98	2.1	1.89	2.2	2.21	2.1	1.98	1.99	1.9	1.19	1.79	250
NH_4 (mg/L)	0.008	0.009	0.109	0.18	0.003	0.001	0.004	0.1	0.105	0.1	0.12	0.004	0.2

Table 4.2: Heavy metal analyses of Otamiri river water sample

Parameters	Egbu abattior	Akachi bridge	Mechanic village	Aba road	Westend	Umezuruike hospital	Inland bridge	Nekede	FUTO	Ihiagwa	Mgbirichi	Umuagwo	WHO stds (2017)
Fe(mg/L)	0.14	1	1.9	0.98	0.99	0.67	1.89	1.98	1.8	1.7	1.99	1.09	<0.3
Ni(mg/L)	0.121	0.132	0.141	0.086	0.089	0.11	0.111	0.079	0.068	0.045	0.062	0.066	3
Zn(mg/L)	0.106	0.114	0.122	0.079	0.068	0.086	0.059	0.066	0.049	0.054	0.076	0.049	0.002
Cu(mg/L)	0.121	0.139	0.211	0139.	0.12	0.211	0.13	0.109	0.067	0.112	0.103	0.069	2
Cd(mg/L)	0.062	0.078	0.096	0.064	0.051	0.039	0.036	0.044	0.052	0.056	0.053	0.049	0.003
Pb(mg/L)	0.148	0.169	0.211	0.089	0.106	0.13	0.082	0.021	0.063	0.059	0.03	0.116	0.01
Hg(mg/L)	0	0.005	0.01	0	0.005	0.01	0.007	0.005	0	0	0	0.001	0.001

4.1.2 Physicochemical characteristics of Otamiri Soil

The Table 4.3 shows the physicochemical parameters of Otamiri soil among the twelve sample sites analyzed. The sites / river columns analyzed were Egbu abattoir, Akachi Bridge, Mechanic village, Aba Road Bridge, Westend Bridge, Umezuruike Hopital, Inland Bridge, Nekede Bridge, FUTO under bridge, Iheagwa, Mgbirichi and Umuagwo. The pH ranges between 5.2 and 6.6 with the highest acidity at mgbirichi with the pH of 5.2. The PO_4 has it highest at Umuagwo and Aba road with the record of 18.6mg/l. The heavy metals analyzed showed that Fe is between 19.81-17mg/l. while the mechanic village recorded the highest, Umuagwo had the least. As Nickel had its highest record at Westend Bridge at 0.12mg /l and Zinc recorded its highest of 0.1 mg/l at Aba Road. Among all the heavy metals, lead was shown to be more predominant.

Table 4.3: Physiochemical parameters of the Otamiri river soil samples analyzed and compared with WHO (2017) standards.

Parameters	Egbu abattior	Akachi bridge	Mechanic village	Aba road	Westend	Umezuruike hospital	Inland bridge	Nekede	FUTO	Ihiagwa	Mgbirichi	Umuagwo	WHO stds (2017)
Ph	5.4	5.8	5.5	5.87	6	6	6.6	6	6	5.5	5.2	5.4	6.5-8.5
PO_4	17	18.4	18.2	18.6	17.8	17.9	18	18.5	18.2	17.8	18.3	18.6	3.5
Fe	18	18.5	19.818	18.8	19.5	17.5	17.8	19	18	18	17.71	17	0.3
Ni	0.139	0.155	0.17	0.123	0.12	0.133	0.132	0.123	0.099	0.069	0.071	0.084	3
Zn	0.144	0.16	0.146	0.116	0.139	0.142	0.094	0.082	0.065	0.08	0.088	0.089	0.02
Co	0.139	0.141	0.211	0.138	0.12	0.211	0.13	0.114	0.088	0.144	0.119	0.062	NONE
Cd	0.082	0.116	0.118	0.119	0.089	0.128	0.094	0.082	0.071	0.093	0.12	0.073	0.003
Pb	0.162	0.176	0.261	0.129	0.16	0.165	0.114	0.114	0.080	0.084	0.06	0.152	0.01
Hg	0.85	1.5	1.89	0.905	1.67	1.44	1.62	1.33	0.89	1.2	0.98	0.6	0.001

4.1.3 Pesticides content of otamiri river

The pesticide content of Otamiri River water analysis showed the following pesticides were present in the water: Athrazine, Butachlor, glyphosate, Malathion and Alachlor with glyphosate and Malathion more prevalent. As shown in Table 4.3 below, the glyphosate had its highest records at the FUTO, Ihiagwa, Mgbirichi and Umuagwo as 3500 mg/l and the least as 1560mg/l at Egbu abattoir. The DDVP recorded its highest of 325mg/L Egbu abattoir with the least record of 120 mg/l at Akachi Bridge. Other pesticides analyzed had it highest record as: Athrazine; 2.2 mg/l at Akachi, Butachlor; 0.1 mg/l at Akachi and Umuagwo and Alachlor; 0.1 mg/l at Aba Road, Westend, FUTO, Ihiagwa and Nekede.

Table 4.4: Pesticides Content of Otamiri River compared with WHO Standards

SAMPLE SITES/PARAMETERS	Athrazine (mg/l)	Butachlor (mg/l)	Glyphosate (mg/l)	DDVP (mg/l)	Alachlor (mg/l)
Egbu abattior	1	0.2	1560	325	0.25
Akachi bridge	2.2	0.1	2500	120	0.24
Mechanic village	1.2	0.01	2800	190	0.22
Aba road	0.1	0.01	3000	455	0.1
Westend	0.1	0.01	3100	200	0.1
Umezuruike hospital	0.15	0.01	2340	180	0.001
Inland bridge	0.1	0.01	2220	370	0.02
Nekede	0.2	0.02	2.18	160	0.1
FUTO	0.15	0.03	3500	170	0.1
Ihiagwa	0.1	0.01	3500	100	0.1
Mgbirichi	0.2	0.01	4200	180	0.001
Umuagwo	0.2	0.1	5000	170	0.05
WHO standards (2017)	**0.1**	**0.1**	**3500**	**NONE**	**0.02**

4.1.4 Bacteriological quality of Otamiri River and sediment

4.1. 4.1. The mean bacterial counts of the bacterial isolates

The bacterial counts in this present study as presented in figure 4.1, showed that the Total heterotrophic bacteria count ranged from 4.50E+05 Cfu/ml to 2.20E+07 Cfu/ml with upstream water samples from umuagwo sampling stations recording highest counts of 2.20E+07 Cfu/ml. At Egbu abattoir, the counts were 9.70E+06 Cfu/ml for upstream, 1.20E+07Cfu/ml mid stream, 1.27E+07 Cfu/ml down stream and 4.40E+06 for the soil sample. The least microbial load was recorded at West End Bridge up stream with the mean value of 4.50E Cfu/ml. (Appendix 2a)

The total Faecal *coliform* mean counts ranged from 2.00E+05 Cfu/ml to 3.40E + 07 Cfu/ml with the soil samples from Nekede sampling stations recorded the highest counts and upstream of Inland bridge had the least count (Figure 4.2). The midstream counts from Umezurike sampling area were 9.00E+ 06 Cfu/ml while the down stream is 1.15E+ 07Cfu/ml (Appendix 2a)

Figure 4.3 showed the *Salmonella Shigella* mean counts were the counts ranged from 3.50E+ 05 Cfu/ml to 1.52E + 07 Cfu/ml with midstream and downstream water samples of Inland Bridge recording the least count while downstream water samples from Egbu abattoir had the highest count. The up stream of Akachi Bridge and Mechanic village sampling stations recorded higher microbial load (1.14E+07Cfu/ml and 3.2E+Cfu/ml) than their down streams with bacterial counts of 6.00E+06Cfu/ml and 1.65E+06Cfu/ml respectively (see appendix 2a)

Figure 4.4 represent the *Staphylococcal* count downstream from Egbu abattoir. Soil sample from Nekede had the highest count of 1.79E + 07 Cfu/ml while downstream water sample from Nekede Bridge sampling station, the midstream of Ihiagwa sampling station and upstream water sample from Mgbirichi station had the least count of 2.00E+05 Cfu/ml. At almost all the

sampling stations, the downstreams tends to have higher *Staphylococcal* load. This was witnessed at all sampling stations apart from Nekede Bridge and Umuagwo where the highest *staphylococcal* load was recorded at upstream and midstream respectively. The *staphylococcal* loads at the soil samples were higher compared to other class of bacteria monitored.

The mean *coliform* counts ranged from 3.50E+05 to 4.59E+08 Cfu/ml. The soil sample from Nekede had the highest count of 4.59E+08Cfu/ml (Figure 4.5)

Figure 4.6 is the mean *Vibro* count of all the sample sites analyzed. The *Vibrio* count ranged from 1.50E+05 Cfu/ml to 3.52E+ 07 Cfu/ml. However, upstream water sample from FUTO sampling station had the highest count of 3.52E+07Cfu/ml while midstream water sample from Nekede sampling station had the least count of 1.50E+05Cfu/ml.

The Anaerobic bacterial count as shown in figure 4.6 revealed that the mean anaerobic count ranged from 2.00E+ 05 Cfu/ml to 2.85E+06Cfu/ml. upstream water sample from Ihiagwa sampling station had the least count of 2.00E+06 Cfu/ml while the soil sample from Egbu abattoir had the highest 2.85E+06Cfu/ml (see Appendix 2a).

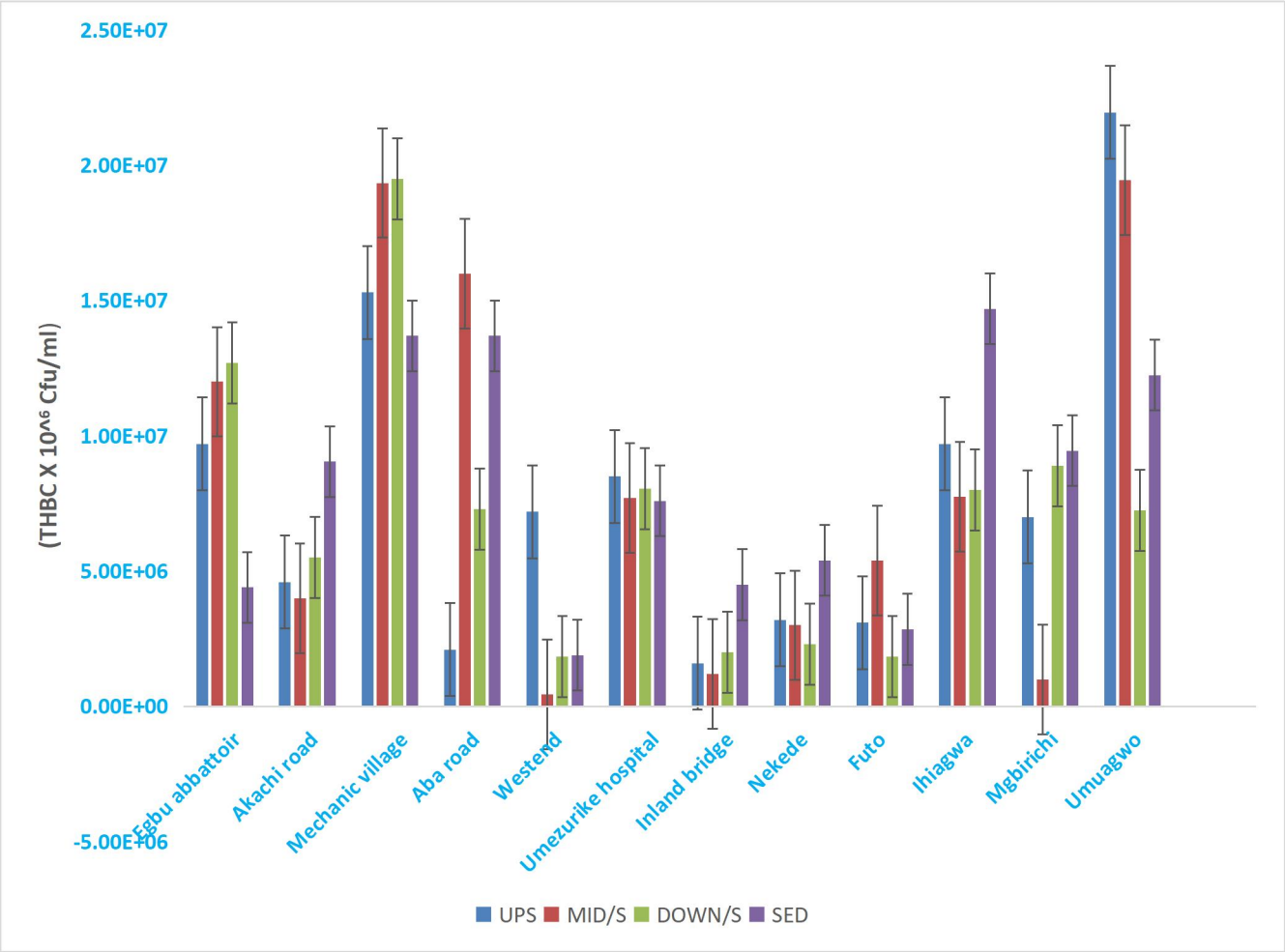

Figure 4.1: Mean heterotrophic bacteria counts Cfu/ml of samples analyzed

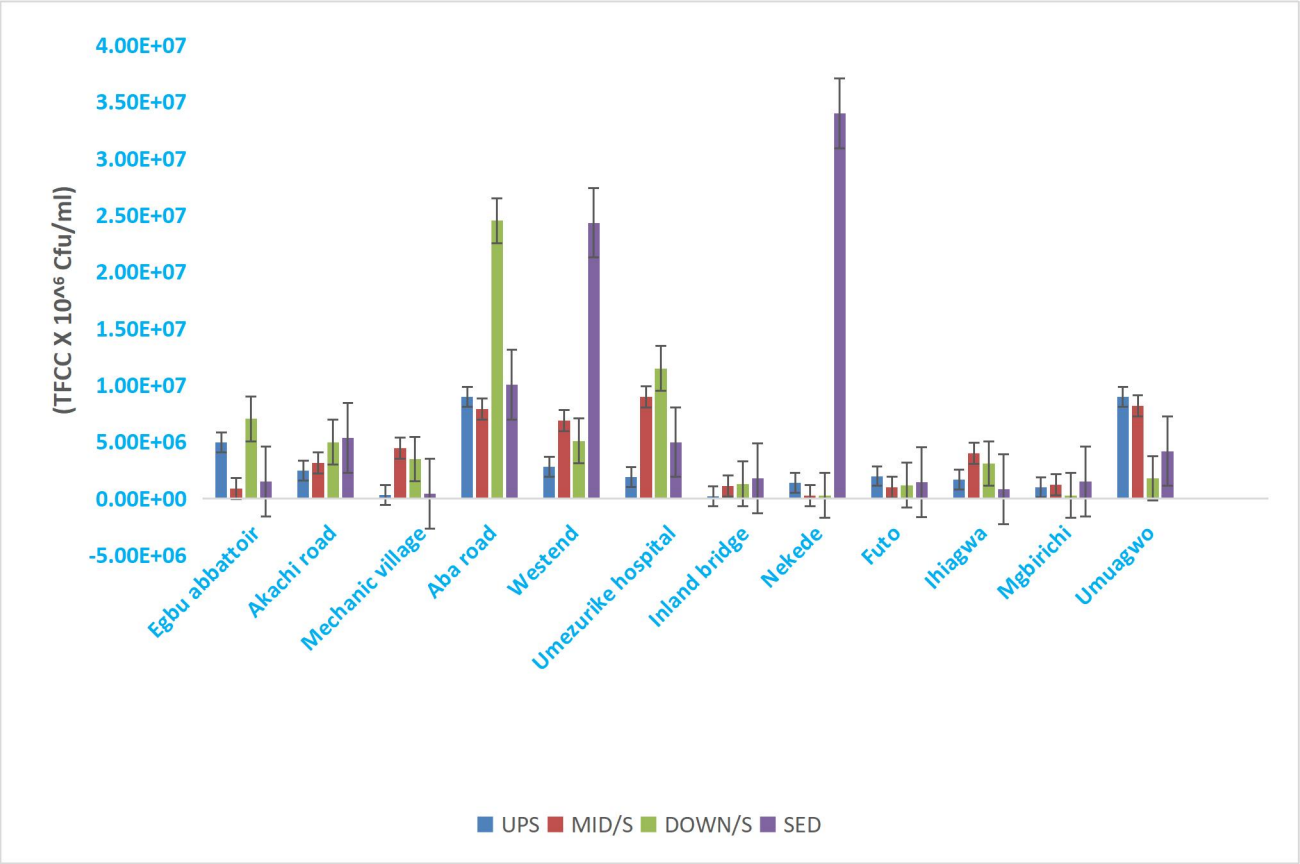

Figure 4.2: Mean coliform counts Cfu/ml of the samples analyzed

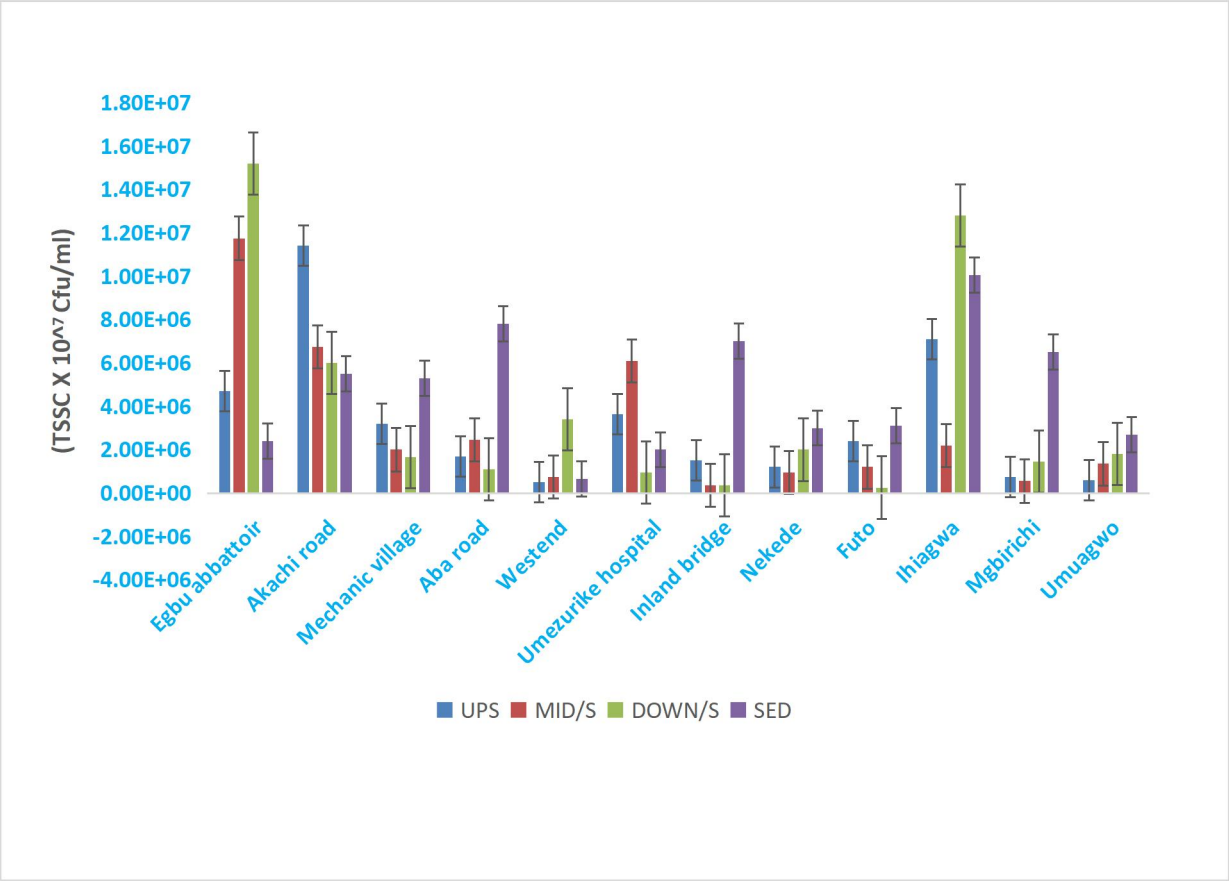

Figure 4.3: Mean Salmonella / Shigella counts Cfu/ml of samples analyzed

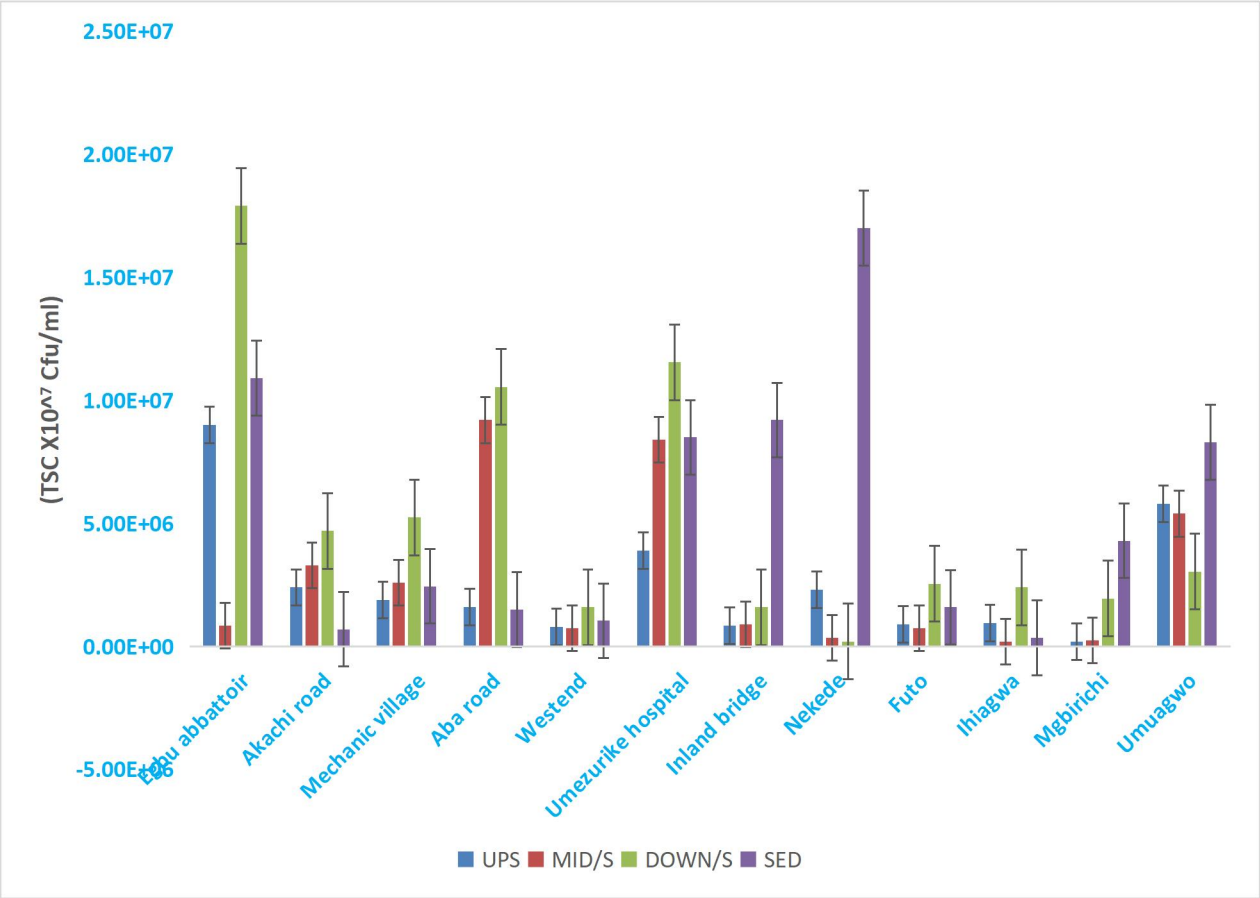

Figure 4.4: Mean Staphylococcus counts Cfu/ml of samples analyzed

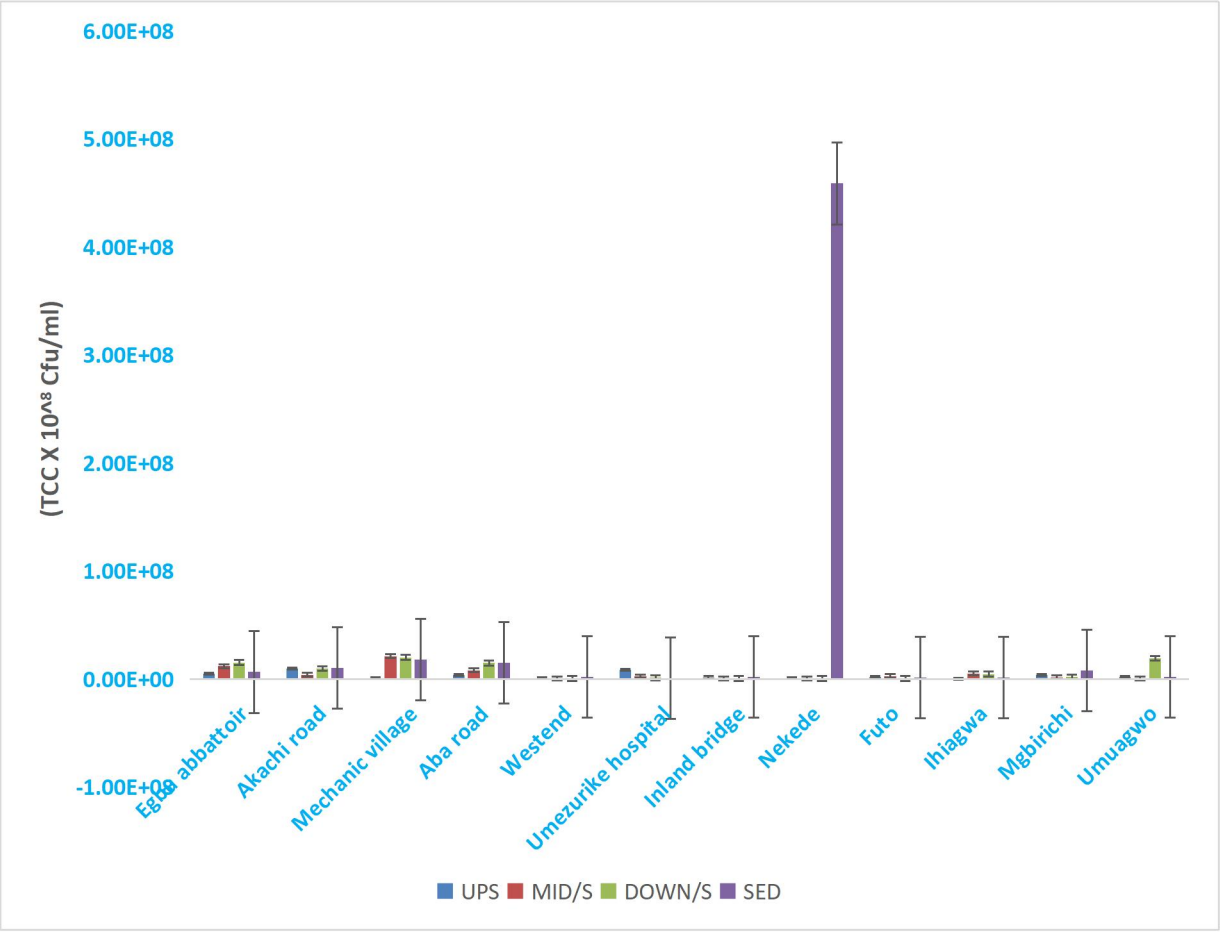

Figure 4.5: Mean coliform counts Cfu/ml of samples analyzed

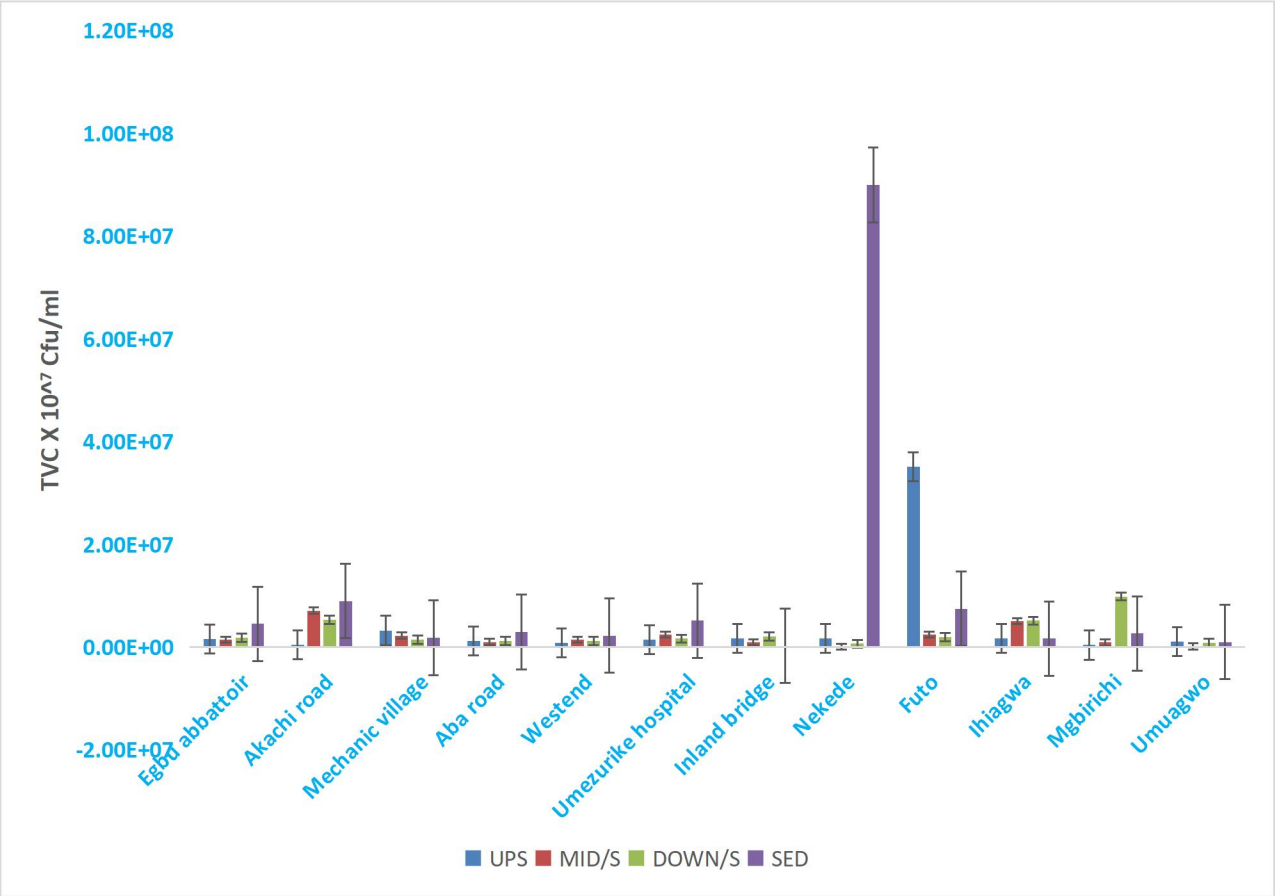

Figure 4.6: Mean Vibrio count Cfu/ml of samples analyzed

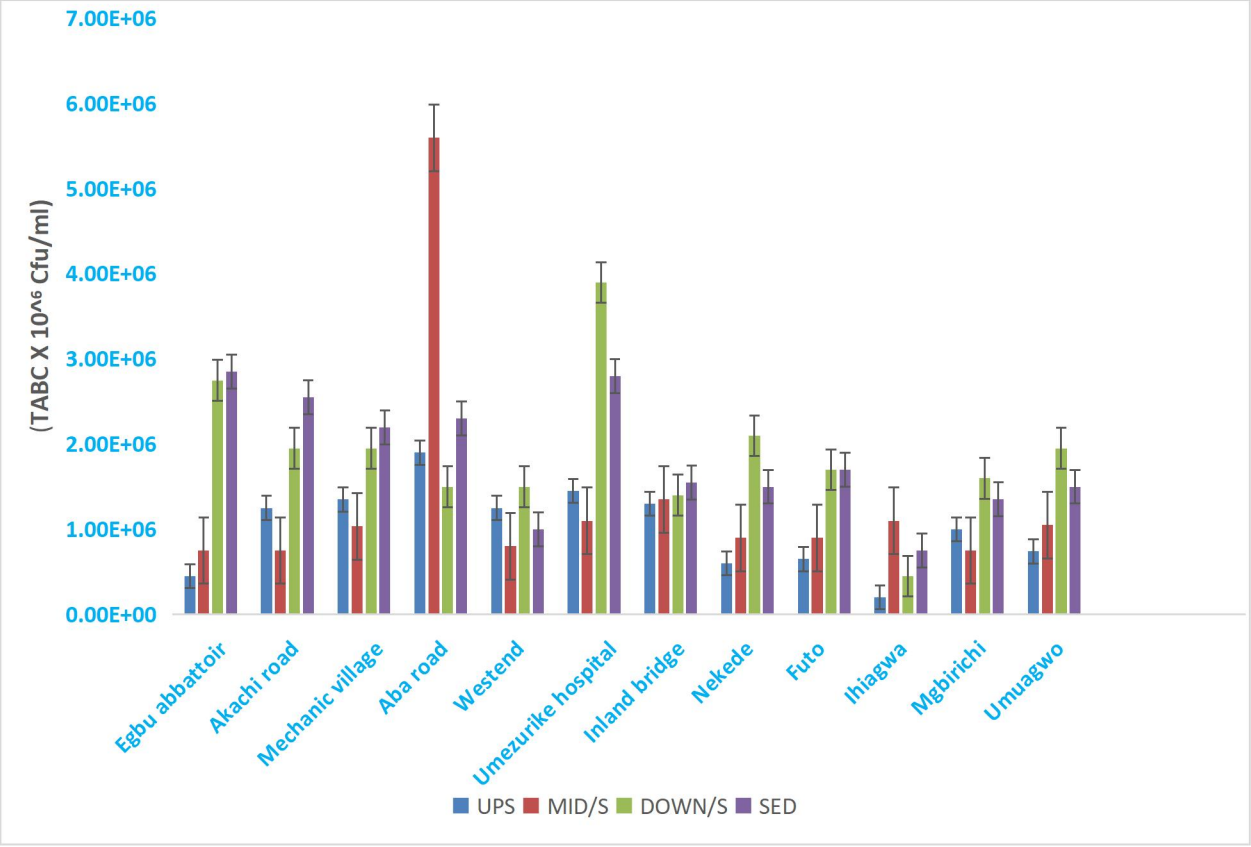

Figure4. 7: Mean anaerobic bacteria count Cfu/ml of samples analyzed

4.1. 4.2. The Bacterial Identification and Frequency of Occurrence:

The result of the biochemical test and identification of the bacteria isolates revealed that the following bacteria were present viz: *Klebsiella* sp*., Klebsiella* sp*., Eschericia coli. Enterobacter* sp*., Enterococcus* sp*., Alcaligenes* sp*., Staphylococcus* sp*., Vibrio cholera, Bacillus* sp*, Salmonella* sp*, Shigella* sp*., Citrobacter* sp*, Pseudomonas* sp*, Bacillus* sp*., Serratia* sp*., Proteus* sp*, Clostridium* sp*, Lysinibacillus* sp*, Streptococcus* sp*, Micrococcus* sp*., Lactobacillus* sp (see Appendix 3). The frequency of occurrence of these bacterial isolates from sampling stations for both water and soil is presented in tables 4.5 and 4.6 respectively. From the water samples, *Alcaligens* sp. and *Lysinibacillus* sp had the highest percentage of occurrence of 68(94.44%) and 54(75%) respectively while *Lactobacillus* sp and *Proteus* sp were the least occurring isolates; 6(8.33%) for both isolates. The percentage occurrence of bacteria isolates from the soil samples revealed that *Alcaligenes* sp (24(100%)) and *Lysinibacillus* sp (21(87.5%)) had the highest percentage of occurrence while *Vibrio cholera* was the least occurring isolates (3(12.5%)). In water sample, *Klebsiella* sp spread in the entire sample sites and occurred most at umezurike hospital where it recorded 5(83.33%). *E. coli* occurred in all the sites with an overall percentage of occurrences of 81.94%. *Shigella* sp had its highest occurrence of 5(83.33%) spread across the entire sites with an overall percentage of 48(66.66%) while *Salmonella* sp had an overall percentage of 39(54.16%). *Vibro cholera* was recorded only as umezurike hospital.

In the soil samples, *Staphylococcus* sp. were recorded 100% in all the sites except mechanic village and West End Bridge that had no growth. *Pseudomonas* sp. has 75% occurrence with counts spreading in all the sampling stations.

The highest occurring organisms in Egbu abattoir water sample were *E. coli*, *Salmonella* sp., *Shigella* sp., and *Lysinibacillus* sp. that had their percentage occurrences as 83.33%. Soil samples

recorded 100% occurrence for *Alcaligenes* sp., *Enterobacter* sp., *Lysinibacillus* sp., *E. coli, Pseudomonas* sp., *Clostridium* sp., and *Staphylococcus* sp.

Table 4.5: Percentage occurrence of the bacterial isolates from the sampling stations at Otamiri river

Isolates	Egbu abattoir N=6 n=%	Akachi bridge N=6 n=%	Mechanic village N=6 n=%	Aba road N=6 n=%	Westend N=6 n=%	Umezuruike hospital N=6 n=%	Inland bridge N=6 n=%	Nekede N=6 n=%	FUTO N=6 n=%	Ihiagwa N=6 n=%	Mgbirichi N=6 n=%	Umuagwo N=6 n=%	Overall % of occurrence N=72 n=%
Klebsiella sp.	4(66.66)	3(50)	2(33.33)	1.(16.66	3(50)	5(83.33)	3(50)	3(50)	1.(16.66	4(66.66)	4(66.66)	4(66.66)	37(51.38)
Eschericia coli	5(83.33)	5(83.33)	4(66.66)	5(83.33)	6(100)	5(83.33)	4(66.66)	6(100)	5(83.33)	4(66.66)	5(83.33)	5(83.33)	59(81.94)
Enterobacter sp.	2(33.33)	1(16.66)	1(16.66)	4(66.66)	4(66.66)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	24(33.33)
Enterococcus sp.	2(33.33)	0(0)	0(0)	0(0)	1(16.66)	2(33.33)	3(50)	4(66.66)	2(33.33)	3(50)	3(50)	3(50)	21(29.16)
Alcaligenes sp.	6(100)	6(100)	6(100)	5(83.33)	5(83.33)	5(83.33)	5(83.33)	6(100)	6(100)	6(100)	6(100)	6(100)	68(94.44)
Staphylococcus sp.	0(0)	0(0)	0(0)	0(0)	0(0)	3(50)	5(83.33)	0(0)	5(83.33)	4(66.66)	4(66.66)	4(66.66)	25(34.72)
Vibro cholerae	3(50)	3(50)	1(16.66)	3(50)	2(33.33)	4(66.66)	4(66.66)	4(66.66)	4(66.66)	3(50)	3(50)	3(50)	37(51.38)
Bacillus sp.	0(0)	0(0)	0(0)	0(0)	4(66.66)	0(0)	0(0)	1(16.66)	0(0)	1(16.66)	1(16.66)	1(16.66)	8(11.11)
Salmonella sp.	5(83.33)	4(66.66)	2(33.33)	3(50)	4(66.66)	3(50)	3(50)	3(50)	3(50)	3(50)	3(50)	3(50)	39(54.16)
Shigella sp.	5(83.33)	5(83.33)	2(33.33)	4(66.66)	0(0)	5(83.33)	3(50)	4(66.66)	5(83.33)	5(83.33)	5(83.33)	5(83.33)	48(66.66)
Citrobacter sp.	2(33.33)	3(50)	2(33.33)	3(50)	3(50)	1(16.66)	4(66.66)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	28(38.88)
Pseudomonas sp.	1(16.66)	0(0)	0(0)	2(33.33)	0(0)	2(33.33)	1(16.66)	1(16.66)	1(16.66)	1(16.66)	1(16.66)	1(16.66)	11(15.27)
Bacillus sp.	0(0)	0(0)	0(0)	0(0)	3(50)	2(33.33)	4(66.66)	0(0)	1(16.66)	5(83.33)	5(83.33)	5(83.33)	25(34.72)
Serratia sp.	2(33.33)	3(50)	2(33.33)	1(16.66)	0(0)	2(33.33)	1(16.66)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	2(33.33)	21(29.16)
Proteus sp.	0(0)	0(0)	0(0)	0(0)	5(83.33)	0(0)	0(0)	0(0)	0(0)	1(16.66)	0(0)	0(0)	6(8.33)
Clostridium sp.	4(66.66)	0(0)	0(0)	4(66.66)	3(50)	3(50)	4(66.66)	4(66.66)	2(33.33)	4(66.66)	4(66.66)	4(66.66)	40(55.55)
Lysinibacillus sp.	5(83.33)	5(83.33)	5(83.33)	6(100)	0(0)	5(83.33)	5(83.33)	5(83.33)	3(50)	5(83.33)	5(83.33)	5(83.33)	54(75)
Streptococcus sp.	1(16.66)	0(0)	1.(16.66	3(50)	1.(16.66	3(50)	0(0)	1.(16.66	1.(16.66	1.(16.66	1.(16.66	1.(16.66	14(19.44)
Micrococcus sp.	1.(16.66)	1.(16.66	1.(16.66	1.(16.66	0(0)	3(50)	3(50)	1.(16.66	1.(16.66	1.(16.66	1.(16.66	1.(16.66	15(20.83)
Lactobacillus sp.	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	2(33.33)	0(0)	4(66.66)	6(8.33)

Legend: N= total number of samples in each sampling station, n= percentage occurrence of bacteria isolates

Table 4.6: Percentage occurrence of the bacterial isolates from the sampling stations of Otamiri soil.

Isolates	Egbu abattoir N=2 n=%	Akachi bridge N=2 n=%	Mechanic village N=2 n=%	Aba road N=2 n=%	Westend N=2 n=%	Umezuruike hospital N=2 n=%	Inland bridge N=2 n=%	Nekede N=2 n=%	FUTO N=2 n=%	Ihiagwa N=2 n=%	Mgbirichi N=2 n=%	Umuagwo N=2 n=%	Overall % of occurrenc N=24 n=%e
Klebsiella sp.	0(0)	0(0)	2(100)	0(0)	0(0)	0(0)	2(100)	0(0)	0(0)	0(0)	0(0)	2(100)	6(25)
Klebsiella sp.	1(50)	1(50)	0(0)	1(50)	1(50)	2(100)	2(100)	2(100)	1(50)	2(100)	2(100)	2(100)	16(66.66)
Alcaligenes sp.	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	24(100)
Enterobacter sp.	0(0)	0(0)	1(50)	0(0)	0(0)	0(0)	2(100)	0(0)	0(0)	0(0)	2(100)	2(100)	7(29.16)
Enterococcus sp.	2(100)	2(100)	0(0)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	2(100)	1(50)	0(0)	13(54.16)
Lysinibacillus sp.	2(100)	0(0)	2(100)	2(100)	2(100)	1(50)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	21(87.5)
Eschericia coli	2(100)	1(50)	0(0)	1(50)	1(50)	2(100)	0(0)	1(50)	2(100)	2(100)	2(100)	2(100)	16(66.66)
Vibro cholera	0(0)	0(0)	1(50)	0(0)	0(0)	2(100)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	3(12.5)
Lactobacillus sp.	1(50)	1(50)	0(0)	1(50)	1(50)	0(0)	0(0)	2(100)	0(0)	1(50)	1(50)	1(50)	9(37.5)
Salmonella sp.	1(50)	1(50)	2(100)	0(0)	2(100)	2(100)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	13(54.16)
Shigella sp.	1(50)	2(100)	2(100)	2(100)	1(50)	2(100)	0(0)	0(0)	2(100)	1(50)	1(50)	1(50)	15(62.5)
Citrobacter sp.	0(0)	0(0)	2(100)	1(50)	2(100)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	12(50)
Pseudomonas sp.	2(100)	2(100)	0(0)	1(50)	1(50)	2(100)	1(50)	2(1000	2(100)	2(100)	2(100)	2(100)	18(75)
Bacillus sp.	1(50)	1(50)	0(0)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	11(45.83)
Serratia sp.	0(0)	0(0)	2(100)	1(50)	1(50)	0(0)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	10(41.66)
Proteus sp.	1(50)	1(50)	0(0)	1(50)	2(100)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	12(50)
Clostridium sp.	2(100)	2(100)	0(0)	1(50)	1(50)	2(100)	2(100)	2(100)	1(50)	2(100)	2(100)	2(100)	19(79.16)
Bacillus sp.	1(50)	1(50)	2(100)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	1(50)	13(54.16)
Streptococcus sp.	1(50)	1(50)	0(0)	1(50)	0(0)	1(50)	0(0)	1(50)	1(50)	1(50)	1(50)	1(50)	9(37.5)
Micrococcus sp.	0(0)	0(0)	1(50)	1(50)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	18(75)
Staphylococcus sp.	2(100)	2(100)	0(0)	2(100)	0(0)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	2(100)	20(83.33)

Legend: N= total number of samples in each sampling station, n= percentage occurrence of bacteria isolates

4.1. 5.3. Molecular identification:

Three preponderant bacterial genera from the water (*Lysinibacillus* sp*., Pseudomonas* sp*. and Klebsiella* sp.) and two from soil (*Alcaligenes* sp. *and proteus* sp) were subjected to further screening with tag number B2, B5, B1, B3, B4. From the molecular analysis as shown on plate 1, the isolates were identified to be:

B1= *Klebsiella pneumonia* (MK641337)

B2 = *Lysinibacillus macrolides* (OK298881)

B3= *Alcaligenes faecalis* (KX302624)

B4= *Proteus mirabilis* (MZ067158)

B5= *Pseudomonas aeruginosa* (CP058331)

Figure 4.8 displayed the phylogenic tree of molecular identified bacteria isolates. This figure shows the evolutionary history of the taxa under study and it's represented by the bootstrap consensus tree generated from 500 replicates.

Plate 1 is the Agarose gel electrophpsis of the 16S RNA gene of some selected bacterial isolates. Lane B1-B5 represents the 16SrRNA gene bandsat 1500bp while the lane l represents the 1000bp molecular ladder.

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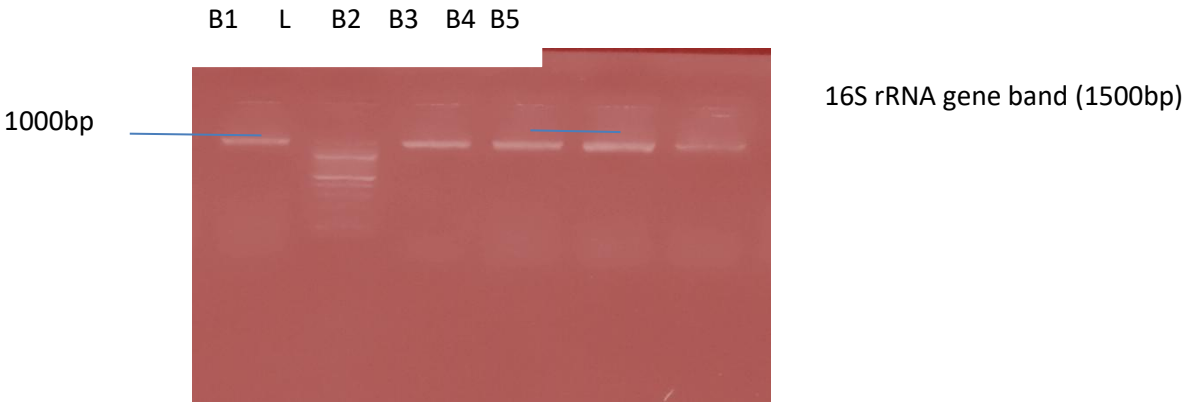


Plate 1: Agarose gel electrophoresis of the 16S rRNA

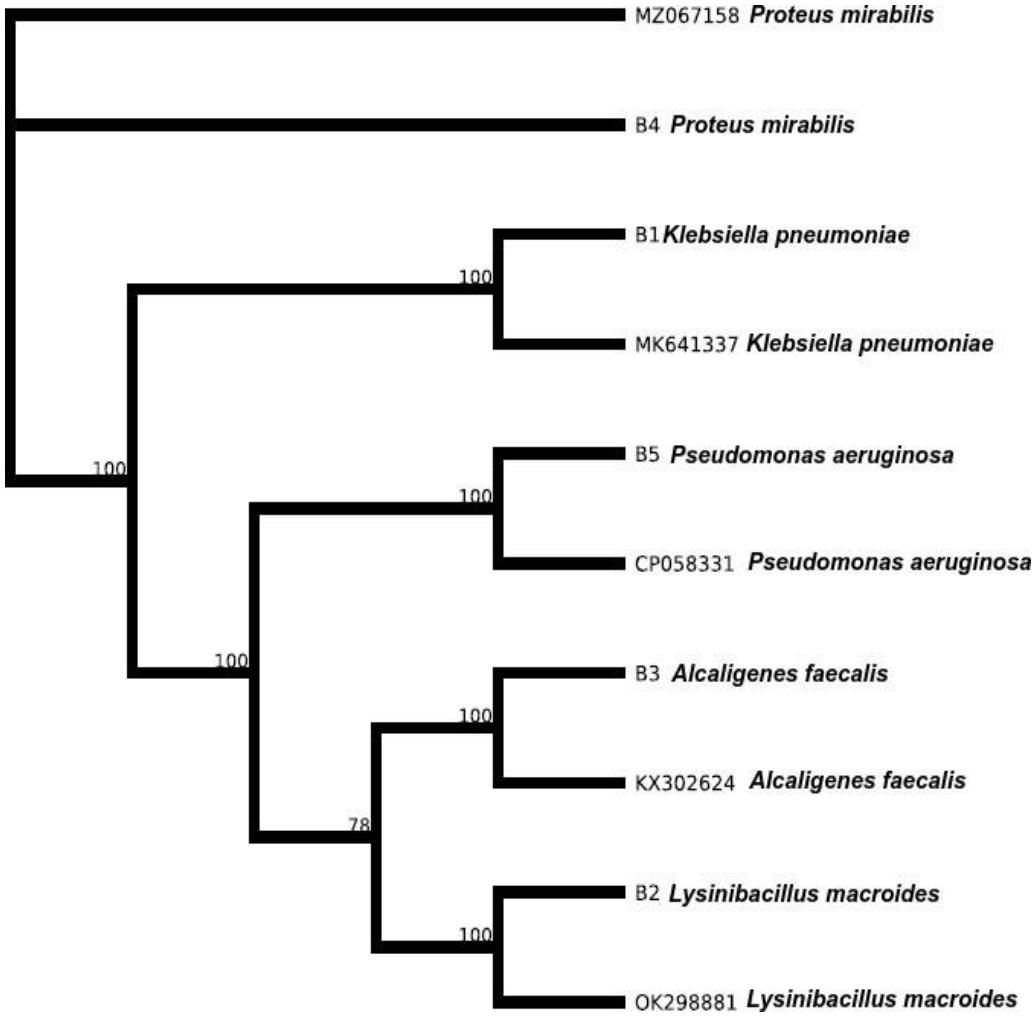


Figure 4.8: Phylogenic tree of molecular identified bacteria isolates.

4.1.6.1 Toxicity thresholds of single metal ions(Pb(II), Co(II), Ni(II), Cd(II) and Zn(II)) to *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity

The result presented in table 4.7 shows the experimental toxicity thresholds of single metal ions to the total dehydrogenase activity of *L. macrolides* (OK298881) and *A. faecalis* (KX302624) isolated from Otamiri river water and soil. The EC_{50S} of the toxicant on *L. macroides* (OK298881) ranged from 20.49 - 713.57mg/L while that of A. *faecalis* (KX302624) ranged from 58.87 – 624.41 mg/L. The order of increasing toxicity on *L. macroides* (OK298881) was Co (II) < Cd (II) < Ni(II) < Pb (II) < Zn(II) while that of *A. faecalis*(KX302624) *was* Ni (II) < Cd(II) < Co(II) < Pb(II) < Zn(II).

Lead (II) ion showed a dose dependent inhibition of total dehydrogenase activity of both isolates. The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) closely fitted into a logistic four parameter equation (R^2=0.978) with EC_{50} value of 493.38 mg/L. while the inhibition of the total dehydrogenase activity of *A faecalis* (KX302624) fitted into a logistic three parameter equation (R^2= 0.985) with EC_{50} value of 509.75mg/L.

Cobalt (II) ion showed a dose dependent inhibition of total dehydrogenase activity of both isolates. The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) *and A. faecali* (KX302624) isolate closely fitted into a logistic three parameter equation with R^2 values of 0.956 and 0.986 and EC_{50} value of 20.49mg/L. and 107.90mg/L respectively.

The toxicity effect of Nickel (II) ion on the total dehydrogenase activity of *L. macrolides* (OK298881) progressively increased with concentration of the metal ion. Moreover, the toxicity was more rapid against *A. faecalis* (KX302624) than *L. macroides* (OK298881). The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) *and A. faecalis* (KX302624) were describable by Sigmoid, 3 parameter equations, where they

had an R^2 values of 0.887and 0.947, and EC_{50} values of 164.37mg/L, and 58.87mg/L respectively.

The Cadmium (II) ion showed an inhibitory response to *L. macrolides* (OK298881) between the concentration range of 0 – 82mg/. Beyond this concentration, there was a rapid inhibition of total dehydrogenase activity of both isolates. The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) and *A. faecalis* (KX302624) *c*losely fitted into a sigmoidal three parameter equation had R^2 values of 0.981 and 0.971and EC_{50} values of both isolates were 67.70mg/L. and 89.06mg/L respectively. Zinc (II) ion equally showed a dose dependent inhibition of total dehydrogenase activity of both isolates. The inhibition of the total dehydrogenase activity of *L. macroides*(OK298881) **and** *A. faecalis* isolates closely fitted into a sigmoid three parameter equation with R^2 values of 0.976 and 0.935 and EC_{50} values of 713.27 mg/L. and 624.41mg/L respectively.

Table 4.7.: Experimental Toxicity (EC_{50}) Thresholds of Individual Metals on *L. macroides* (OK298881) and *A. faecalis (*KX302624):

Toxicant	R^2 value	EC_{50} on *L. macrolides* (OK298881) (mg/L)	R^2 value	EC_{50} on *A. faecalis* (KX302624) (mg/L)
Pb(II)	0.9781	493.38 ± 29.60	0.9851	509.75 ± 30.58
Co(II)	0.9565	20.49 ± 1.22	0.9850	107.90 ± 6.47
Ni (II)	0.8877	164.37 ± 9.86	0.9474	58.87 ± 3.53
Cd(II)	0.9816	67.70 ± 4.06	0.9693	89.06 ± 5.34
Zn(II)	0.9763	713.57 ± 42.79	0.9395	624.41 ± 37.46

The experimental EC_{50} values of the toxicants are significantly different from each other ($p<0.05$) values are represented as Mean ± STD

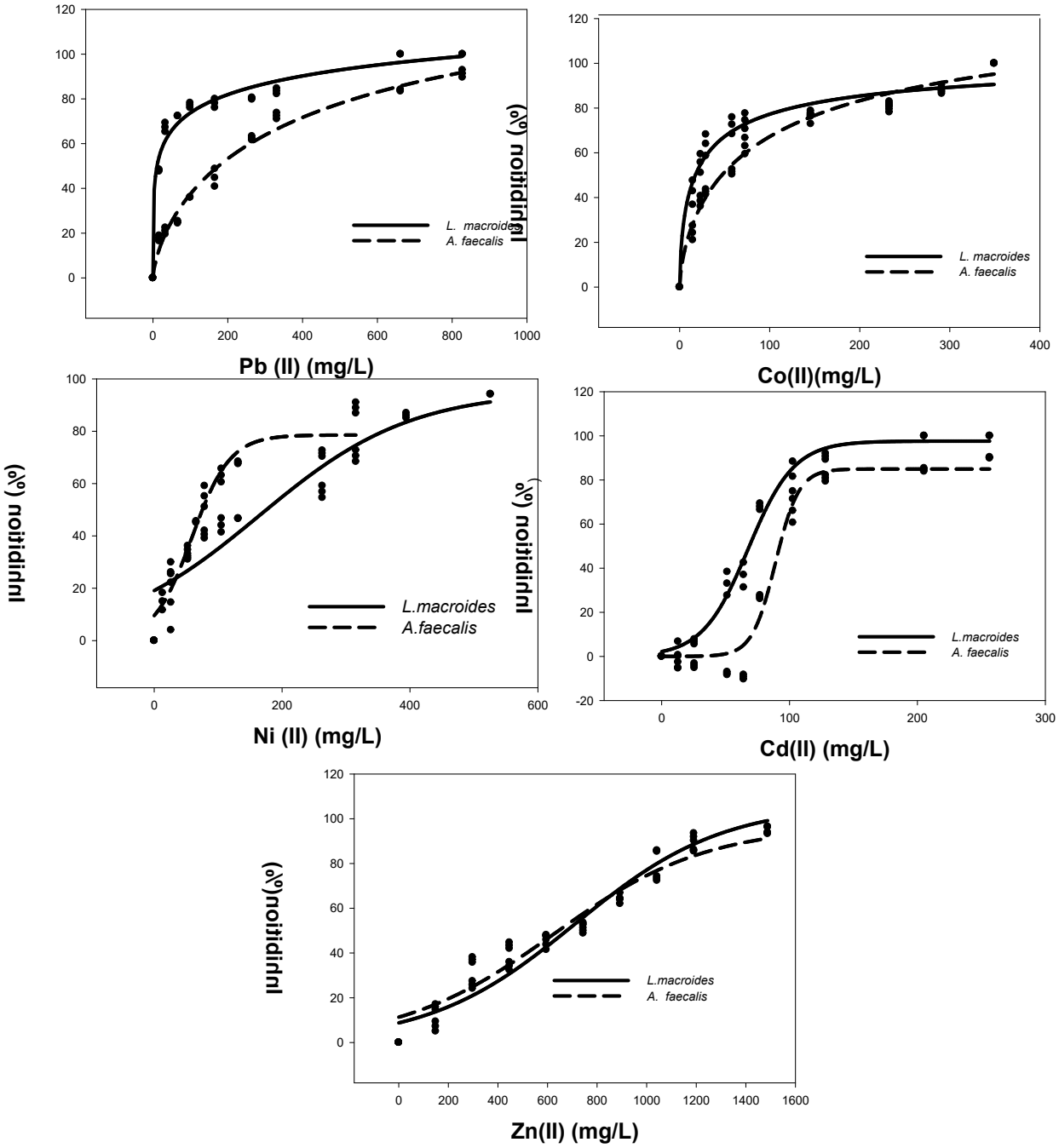


Figure 4.9: Response of *L. macroides* (OK298881) and *A. faecalis* (KX302624) total dehydrogenase activity to single metal (Pb (II), Co (II), Ni (II) Cd (II) and Zn (II)) ions toxicity. The data points represent the experimental dose-response data while the lines represent toxicities obtained by fitting experimental data to logistic model. Dashed line represents *A. faecalis* while solid lines represent *L. macroides.*

4.1.6.2 Toxicity threshold of single pesticides to L. *macroides* (OK298881) and *A. faecalis* (KX302624) total dehydrogenase activity.

The figure 4.2.2 represents the results obtained from the toxicity of individual pesticides (glyphosate and DDVP) to the total dehydrogenase activity of *L. macroides* (OK298881) and *A. faecalis* (KX302624) isolated from Otamiri river water and soil.

Glyphosate mg/L showed a hormetic response to *A. faecalis within* the range of 0- 4000 mg/L followed by an inhibition of total dehydrogenase activity of both isolates with further increase in concentration. The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) closely fitted into a sigmoidal three parameter equation (R^2= 0.915) with EC_{50} value of 893.23mg/L. while the inhibition of the total dehydrogenase activity of *A faecalis* (KX302624) *fitted* into a sigmoidal four parameter equation (R^2= 0.985) with EC_{50} value of 593.98mg/L. The mixtures with glyphosate exhibited a biphasic toxicity trend thus stimulatory and hormesis withnessed at concentration 0-2500mg/L as shown in Figure 4.11.

The DDVP showed hormetic response to L. macrolides between the concentration ranges of 0 – 400mg/L. At elevated concentrations, there was rapid inhibition of total dehydrogenase activity of both isolates. The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) and *A. faecalis*(KX302624) closely fitted into a sigmoidal four parameter equation with R^2 values of 0.850and 0.985. The EC_{50} values of both isolates were 893.23mg/L. and 593.98mg/L respectively. The statistical analysis indicates that the EC_{50} of the toxicant were significantly different from each other ($P<0.05$) and the order of decreasing toxicity is DDVP> GLY.

Table 4.8: Experimental Toxicity (EC_{50}) Thresholds of Individual Pesticides on *L. macroides* (OK298881) and *A. faecalis* (KX302624)

Toxicant	R^2 -value	EC_{50} on *L. macrolides* (OK298881) (mg/L)	R^2 - value	EC_{50} on *A. faecalis*(KX302624) (mg/L)
Glyphosat	0.9155	9735.04±584.10	0.9569	7965.91± 477.90
DDVP	0.8587	893.23 ± 35.64	0.9858	593.98 ± 35.64

The experimental EC_{50} values of the toxicants are significantly different from each other ($P<0.05$) values are represented as Mean ± STD

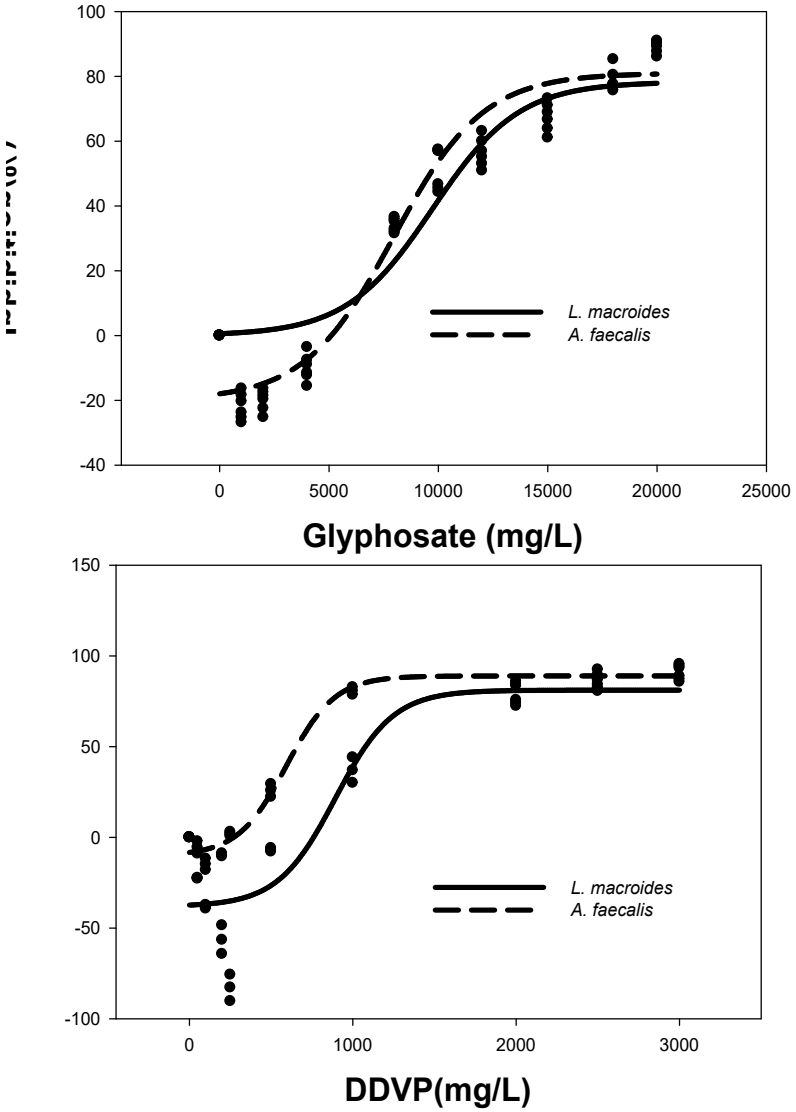


Figure 4.10: Response of *L. macroides* (OK298881) and *A. faecalis* (KX302624) total dehydrogenase activity to Glyphosate and DDVP formulation toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model. Dashed line represent *A. faecalis* while solid lines represent *L. macroides* (OK298881)**.**

4.1.7.1. Toxicity threshold of binary mixtures of metal ions and pesticides to *Lysinibacillus macroides* (OK298881) total dehydrogenase activity

Table 4.9 below shows the experimental toxicity threshold (EC_{50}) and the arbitrary toxicity threshold of the binary mixtures of metals ions and pesticides on total dehydrogenase activities of *L. macrolides* (OK298881).

The experimentally derived EC_{50} in the binary mixture of Pb (II) and Co (II) showed that the mean EC_{50} of ABCR 2 (84.09±4.20 mg/l) was higher than that of ABCR 1(38.98±1.94 mg/l). Among the experimentally derived EC_{50s}, the EECR-50 mixture ratios were statistically different from ABCR1 and ABCR 2 ($P<0.05$). The toxic index (TI) ranges from 1.350 ± 0.081 to 0.535 ± 0.032. In mixtures of Lead (Pb) with other toxicants (Ni, Cd, Zn and DDVP) the first arbitrary concentration ratios (ABCR 1) were higher than the second arbitrary concentration ratio. (ABCR 2). The mixture of Pb (II) + Ni (II), the ABCR 2 was not determined while the ABCR 1 was 82.65±4.13 mg/l. The EECR-50 was 47.6259± 2.38 mg/l and had an R^2 value of 0.7948 which was closely fitted in a sigmoidal logistic three parameter model as shown in figure 4. 12. These interactions were purely synergistic. There were similar interactive effects when Pb was combined with Zn (figure 4. 14) and DDVP (figure 4.15). The combination of Pb with Cd showed an antagonistic toxic effect on both the EECR and ABCR1&2. The mixture of Pb (II) +Cd (II) at a mixture ratio of 20:80 for ABCR1 and 25:75 for ABCR 2 showed that the ABCR1 was higher than the ABCR2 (642.80±32.14 mg/l and 300.42±15.02 mg/l) respectively. Their EECR-50 was 191.39±9.56. They had an R^2 values of (EECR= 0.9698, ABCR1 =0.9823 and ABCR 2 = 0.9711) and was closely fitted into a sigmoidal four parameter model as shown in figure 4.13

The mixtures of Cobalt and other toxicants showed both synergistic and antagonistic effects. In the mixture of Co (II) + Cd (II), both the EECR-50 and the ABCRs showed antagonistic effects. The ABCR 1 had a mean EC_{50} value of 104.34±5.21 mg/l which is

less than that of ABCR2 (490.62±24.53 mg/l). It was fitted in logistic three parameter model with R^2 values of 0.9886 and 0.9922 (figure 17). Mixtures of Co (II) +Ni (II) and that of Co (II) + DDVP had synergistic toxic effects: Co (II) +DDVP, the mean EC_{50} value of ABCR 2 was higher than that of ABCR 2 and EECR-50 (see table 4.9).

In mixtures of nickel with other toxicants, there were both antagonistic and synergistic toxic effects. The mixture of Ni (II) + Cd (II) showed that the mean EC_{50} of ABCR2 was higher than ABCR 1(261.06 ±3.05 mg/l and 200.46± 10.0 mg/l respectively). Mixture of Ni (II) + Zn (II) was antagonistic in both experimental and arbitrary concentration mixtures with Toxic index of (EECR=1.707±0.1022, ABCR1 = 2.58±0.155 and ABCR 2= 1.446±0.087). Using a sigma plot, this interaction was closely fitted in a sigmoidal three parameters as shown in figure 4.21. The R^2 value was 0.9594, 0.9963 and 0.9807. The entire EC_{50} equieffect concentration ratio (EECR – 50) and the ABCR were statistically different from each other however significantlly different from each other, ($P<0.05$).

The mixtures of DDVP with other metal ions other than Cadmium (Co, Zn, Ni and Pb) showed complete synergistic effects in both the EECR and ABCRs. The mixture of DDVP with cadmium were additive with toxic index of 0.995±0.059 for EECR and 1.095±0.065 for ABCR 1. The mixture at EECR-50 had EC_{50} at 518.78±25.93 mg/l while the ABCR1 and ABCR2 were 701.0431± 35.05mg/l and 676.01± 33.80mg/l as shown in table 4.9. These interactions were closely fitted into a mathematical sigmoidal relationships using Sigma plot as shown in figure 4.23.

When DDVP was combined with nickel (Ni (II) + DDVP), their EC_{50} at experimental equieffect concentration ratio of 12.83% and 87.17% was 167.65±8.38 mg/l. their ABCR1 and ABCR2 were 166.88± 8.34mg/l and 166.18±8.30mg/l. This interaction showed a strong positive relationship as they had R^2 values of 0.9858 and 0.9819 but

were statistically different as ($p < 0.05$). This was fitted into a sigmoidal parameter four model using sigmaplot 10.0 as shown in figure 4.22.

The mixture of Zn(II) + DDVP were synergistic at both EECR and the ABCRs as their TI values were (0.234±0.014, 0.106±0.006 and 0.102±006) and they had a strong positive relationship as R^2 values tends to one (0.9261, 0.9972 and 0.9781). The inhibitory effect of zn (II) + DDVP to the total dehydrogenase activity of *L. macrolides* was shown on figure 4.24, where they exhibited a dose response relationship.

TABLE 4.9: The Mean EC50, Toxic Index and Toxic Effects of binary mixtures of metal ions and Pesticides on *L. macrolides* (OK298881)

Toxicant mixtures	R^2 value	Mean EC_{50} (mg/l)	Toxic index(TI)	Toxic effect
Pb (II) +Co(II)				
Pb(II)73.05% + Co(II) 26.95% (EECR-50)	0.97.81	37.36 ±1.86	0.547 ±0.032	Synergistic
Pb (II)75% + Co(II) 25% (ABCR 1)	0.9934	38.98 ±1.94	0.535 ±0.032	synergistic
Pb(II) 70% +Co(II) 30% (ABCR 2)	0.9458	84.09 ±4.20	1.350 ±0.081	Antagonistic
Pb(II) + Ni(II)				
Pb(II) 9.17 % + Ni (II)90.82 % (EECR- 50)	0.7948	47.6259 ± 2.38	0.272 ±0.016	synergistic
Pb (II)10 % + Ni(II) 90 % (ABCR 1)	0.8484	82.65 ± 4.13	0.469 ± 0.028	synergistic
Pb(II) 5 % + Ni (II)95 % (ABCR 2)		ND	ND	
Pb(II) + Cd(II)				
Pb (II)19.31% + Cd(II)80.69 % (EECR- 50)	0.9698	191.39 ± 9.56	2.356 ± 0.141	Antagonistic
Pb (II)20% + Cd(II) 80% (ABCR 1)	0.9823	642.80 ± 32.14	7.855 ± 0.471	Antagonistic
Pb (II)25% + Cd(II) 75% (ABCR 2)	0.9711	300.42 ± 15.02	3.480 ± 0.208	Antagonistic
Pb(II) + Zn(II)				
Pb (II)2.27% + Zn(II) 97.73% (EECR- 50)	0.9965	107.75 ± 5.38	0.153 ± 0.009	synergistic
Pb(II) 5 % + Zn(II) 95 % (ABCR 1)	0.9662	88.417 ± 4.42	0.128 ± 0.008	synergistic
Pb (II)10 % + Zn (II)90% (ABCR 2)	0.8389	50.21 ± 2.51	0.155 ± 0.009	synergistic
Pb(II) + DDVP				
Pb (II)1.47% + DDVP 98.53 % (EECR- 50)	0.9963	87.12 ± 4.36	0.099 ± 0.0060	synergistic
Pb (II)2 % + DDVP 98 % (ABCR 1)	0.9950	91.44 ± 4.57	0.104 ± 0.006	synergistic
Pb (II)3% + DDVP 97% (ABCR 2)	0.9977	16.35 ± 0.82	0.019 ± 0.001	synergistic
Co(II)+ Ni (II)				
Co (II)3.56 % + Ni (II)96.40 % (EECR – 50)	0.9861	93.21 ± 4.66	0.710 ± 0.042	synergistic
Co (II)5% + Ni(II)95% (ABCR 1)	0.9876	768.10 ± 38.40	6.314 ± 0.379	Antagonistic
Co (II)10% + Ni (II)90% (ABCR 2)	0.9237	45.63 ± 2.28	0.473 ± 0.0284	synergistic
Co (II)+ Cd(II)				
Co(II) 8.11% + Cd(II) 91.89% (EECR-50)	0.9014	193.88 ± 9.69	3.399 ± 0.204	Antagonistic
Co (II)10 % + Cd(II) 90 %(ABCR 1)	0.9886	104.34 ± 5.21	1.89 ± 0.114	Antagonistic
Co(II) 5 % + Cd (II)95% (ABCR 2)	0.9922	490.62 ± 24.53	8.080 ± 0.484	Antagonistic
Co(II)+ Zn(II)				
Co(II) 0.85% + Zn(II) 99.15% (EECR-50)	0.9881	581.52 ± 29.07	1.050 ± 0.063	Additive
Co (II)0.5% + Zn(II) 99.5 % (ABCR 1)	0.9624	543.23± 27.16	0.890 ± 0.053	synergistic
Co(II) 2% + Zn (II)98% (ABCR 2)	0.9874	643.49 ± 32.17	1.512 ± 0.091	Antagonistic
Co (II) + DDVP				
Co 0.55% + DDVP 99.45 %(EECR-50)	0.9904	518.76 ± 25.94	0.716 ± 0.043	synergistic
Co(II) 1 % + DDVP 99% (ABCR 1)	0.9802	655.08 ± 32.75	0.327 ± 0.020	synergistic
Co (II)0.25 + DDVP 99.75 % (ABCR2)	0.9930	676.01 ± 33.80	0.837 ± 0.050	synergistic
Ni(II) + Cd(II)				
Ni(II) 70.31 % +Cd(II) 29.69% (EECR-50)	0.9961	128.40 ± 6.42	1.112 ± 0.067	Antagonistic
Ni(II) 75 % + Cd (II)25% (ABCR 1)	0.9404	200.46 ± 10.0	1.66 ± 0.099	Antagonistic
Ni(II) 80 % + Cd (II)20 % (ABCR 2)	0.9898	261.06 ± 3.05	0.477 ± 0.029	synergistic
Ni (II) + Zn(II)				
Ni (II)18.71 % + Zn(II) 81.29 (EECR-50)	0.9594	749.47 ± 37.47	1.707 ± 0.102	Antagonistic
Ni (II)20 % + Zn(II) 80% (ABCR 1)	0.9963	1101.78 ± 55.08	2.58 ± 0.155	Antagonistic
Ni (II)15% + Zn (II)85 % (ABCR 2	0.9807	687.433 ± 34.37	1.446 ± 0.087	Antagonistic
Ni(II) + DDVP				
Ni (II)12.83 % + DDVP 87.17 % (EECR-50)	0.9858	167.65 ± 8.38	0.294 ± 0.018	synergistic
Ni (II)15 + DDVP 85 % (ABCR 1)	0.9819	166.88 ± 8.34	0.338 ± 0.020	synergistic
Ni (II)10 % + DDVP 90 % (ABCR 2)	0.9328	166.18 ± 8.30	0.269 ± 0.016	synergistic
Cd(II)+ DDVP				
Cd (II)5.85 % + DDVP 94.15% (EECR-50)	0.9904	518.76 ± 25.93	0.995 ± 0.059	Additive
Cd (II)3 % + DDVP 97% (ABCR 1)	0.9887	701.04 ±35.05	1.095 ± 0.065	Additive
Cd(II) 8 % + DDVP 92 %(ABCR 2)	0.9930	676.01 ± 33.80	1.495 ± 0.089	Antagonistic
Zn (II)+ DDVP				
Zn(II) 39.01 % +DDVP 60.99% (EECR-50)	0.9261	190.94 ± 9.54	0.234 ± 0.014	synergistic
Zn (II)35% +DDVP 65%(ABCR1)	0.9972	87.00 ± 4.35	0.106 ± 0.006	synergistic
Zn(II) 45 % +DDVP 55 %(ABCR2)	0.9781	82.42 ± 4.12	0.102 ± 0.006	synergistic

Values are represented as Mean ± STD., Pb= lead, Zn=zinc, Co = Colbalt, Ni= Nickel, Cd= Cadmium, Gly= Glyphoate, DDVP = Diclovores. EECR-50 = EC 50 Experimental equieffect concentration ratio, ABCR = Arbitrary concentration ratio

4.1.7.2: Toxicity threshold of binary mixtures of metal ions and pesticides to Alcaligenes *faecalis* (KX302624) total dehydrogenase activity

Table 4.10 below shows the Experimental toxicity threshold (EC_{50}) and the arbitrary toxicity threshold of the binary mixtures of metals and pesticides on total dehydrogenase activities of *Alcaligenes faecalis* (KX302624). The experimentally derived toxic index (TI) ranges from 2.35 ± 0.141mg/L to 0.02 ± 0.0060 mg/L. The first arbitrary concentration ratio (ARCR 1) and the second arbitrary concentration ratio (ABCR 2) range from 3.05± 0.183 mg/l to 0.01±0.001 mg/l.

The interaction of lead with other toxicants had synergistic toxic effects; Pb (II) + Co (II), Pb(II)+Ni(II), Pb (II) + Cd (II), Pb (II) + Zn(II) and Pb (II) + DDVP.

The mixture of Pb (II) + Co (II) had a mean EC_{50} of 92.85±6.499 mg/l at EECR-50 while the ABCR1 and ABCR2 were 62.34±4.364 mg/l and 46.99±3.285 mg/l respectively. Their R^2 values were 0.9220, 0.9469 and 0.9054 which closely fitted into a sigmoidal four parameter model with a biphasic response. The toxic indexes (TI) were 0.22±0.013, 0.14±0.008 and 0.12±0.007 as shown in figure 4.11.

Mixture of Pb (II) + Ni (II) equally showed a synergistic toxic effect. The ABCRs at concentration ratio of 85:15 for ABCR 1 and 90:10 for ABCR 2 had mean EC_{50} values of 6.85± 0.479 mg/l and 8.01± 0.561 mg/l with R^2 values of 0.9889 and 0.9799 respectively. The EC_{50} value of their EECR was 10.03±0.702 mg/l (figure 4.12)

Mixture of Pb (II) +DDVP were equally synergistic at EECR and ABCRs. At equal concentration of 50:50, the ABCR 1 had a mean EC_{50} value of 165.89±11.612 mg/l and R^2 value of 0.9824 with TI value 0.30±0.018. At an elevated concentration of 45:55 there was no significant difference as their toxic effect was stll synergistic. In the EECR were the concentration of lead (Pb) was reduced (52.25:49.75), their interaction effect was still

synergistic. The mean EC_{50} values were 514.05±35.983 mg/l for EECR and 165.89±11.612 mg/l and 309.00±21.630 mg/l for ABCR1 and ABCR2 respectively. They were closely fitted in sigmoidal four parameter model as shown in figure 4.15.

The mixtures of Co (II) + Cd (II), Co (II) +Zn, Ni (II) +Cd (II) and Ni (II) + Zn (II) showed an antagonistic effect as their toxic indexes were greater than one. Their mean EC_{50} were shown on table 4.10; the ABCR 1 and ABCR 2 of Co (II) + Cd (II) had mean EC_{50} of 72.63±5.086 mg/l and 220.82±15.457mg/l respectively. Their R^2 values were closely relatedand both fitted in logistic three parameter model as shown in figure 17. Mixtures with DDVP showed synergistic effect as observed in (Cd (II) +DDVP, Zn (II) + DDVP and Co (II) +DDVP). Some level of Additivity was witnessed in the combination of Nickel with DDVP as well between Cadmium and DDVP at ABCR2. Interaction of mixtures with DDVP exhibited a biphasic toxicity trend with hormetic response of the isolates to the mixtures at low dose. However, inhibition of dehydrogenase activity sets in as the thresholds of concentrations were elevated and closely fitted into mathematical sigmoidal relationships as shown in figures 4. 22, figure 4.23 and figure 4.24. The EECR-50 EC_{50} of Ni (II) + DDVP and Zn (II) + DDVP were 349.87±24.491 mg/l and 190.94 ± 13.366mg/l while that of Cd (II) + DDVP was 187.70 ± 13.139 mg/l.

TABLE 4.10. Mean EC50, Toxic Index and Toxic Effects of Metals and Pesticides Binary Mixtures on *A. faecalis* (KX302624):

Toxicant mixtures	R^2 value	Mean EC_{50} (Mg/l)	Toxic index(TI)	Toxic effect
Pb(II) + Co(II)				
Pb(II)93.68 % + Co (II)6.32 (EECR- 50)	0.9220	92.85 ± 6.499	0.22 ± 0.013	Synergistic
Pb (II)95% + Co(II) 5% (ABCR 1)	0.9469	62.34 ± 4.364	0.14 ± 0.008	Synergistic
Pb(II) 90% + Co(II)10 % (ABCR 2)	0.9054	46.94 ± 3.285	0.12 ± 0.007	Synergistic
Pb(II) + Ni(II)				
Pb(II) 88.42% + Ni (II) 11.58%(EECR-50)	0.9911	10.03 ± 0.702	0.02 ± 0.001	Synergistic
Pb(II) 85% + Ni(II) 15% (ABCR1)	0.9889	6.85 ± 0.479	0.01 ± 0.001	Synergistic
Pb(II) 90% + Ni(II) 10% (ABCR2)	0.9799	8.01 ± 0.561	0.01 ± 0.001	Synergistic
Pb(II) + Cd(II)				
Pb(II)87.69% + Cd (II)12.28% (EECR- 50)	0.8734	101.09 ± 7.076	0.36± 0.021	Synergistic
Pb(II)90 % + Cd (II)10% (ABCR 1)		ND	ND	
Pb(II)85% + Cd (II)15 %(ABCR 2)		ND	ND	
Pb(II) + Zn(II)				
Pb(II)50.25% + Cd (II)49.75%(EECR- 50)	0.9652	30.12 ± 2.108	0.05 ± 0.003	Synergistic
Pb(II)60% + Cd (II)40%(ABCR 1)		ND	ND	
Pb(II)55% + Cd (II)45%(ABCR 2)		ND	ND	
Pb(II) + DDVP				
Pb(II)52.62% + DDVP 47.38%(EECR- 50)	0.8583	514.05 ± 35.983	0.95 ± 0.056	Synergistic
Pb(II)50% + DDVP 50% (ABCR1)	0.9824	165.89 ± 11.612	0.30 ± 0.018	Synergistic
Pb(II)45% + DDVP 55% (ABCR2)	0.9935	309.00 ± 21.630	0.56 ± 0.033	Synergistic
Co(II) + Ni(II)				
Co(II)34.02% + Ni (II)65.98%(EECR- 50)	0.8603	56.69 ± 3.968	0.81 ± 0.048	Synergistic
Co(II)25% + Ni (II)75%(ABCR1)	0.9877	87.26 ± 6.108	1.31 ± 0.078	Antagonistic
Co(II)30% + Ni (II)70%(ABCR2)	0.9515	170.39 ± 11.927	2.49 ± 0.149	Antagonistic
Co(II) + Cd(II)				
Co(II)32.56% + Cd (II)67.44%(EECR- 50)	0.9558	188.22 ± 13.175	1.99 ± 0.119	Antagonistic
Co(II)30% + Cd (II)70%(ABCR1)	0.9809	72.65 ± 5.086	3.05 ± 0.183	Antagonistic
Co(II)25% + Cd (II)75%(ABCR2)	0.9904	220.82 ± 15.457	2.37 ± 0.142	Antagonistic
Co(II) + Zn(II)				
Co(II)6.38% + Zn (II)93.62%(EECR- 50)	0.9944	789.30 ± 55.251	1.65 ± 0.099	Antagonistic
Co(II)5% + Zn (II)95%(ABCR 1)	0.9880	623.98 ± 43.679	1.25 ± 0.075	Antagonistic
Co(II)10% + Zn (II)90%(ABCR 2)	0.9832	464.2± 32.494	1.09 ± 0.065	Additive
Co(II) + DDVP				
Co(II)6.97% + DDVP 93.03%(EECR- 50)	0.9857	267.32 ± 18.710	0.59 ± 0.035	Synergistic
Co(II)10% + DDVP 90%(ABCR1)	0.9573	187.70 ± 13.139	0.47 ± 0.028	Synergistic
Co(II)5% + DDVP 95%(ABCR2)	0.9544	302.51 ± 21.176	0.62 ± 0.037	Synergistic
Ni(II) + Cd(II)				
Ni(II)48.35% + Cd(II) 51.65%(EECR- 50)	0.9577	167.95 ± 11.756	2.35 ± 0.141	Antagonistic
Ni(II)50% + Cd(II) 50%(ABCR 1)	0.9799	88.53 ± 6.197	1.24 ± 0.074	Antagonistic
Ni(II)60% + Cd(II) 40%(ABCR 2)	0.9680	98.53 ± 7.197	2.21 ± 0.064	Antagonistic
Ni(II) + Zn(II)				
Ni(II)11.68% + Zn(II) 88.32%(EECR- 50)	0.9556	604.60 ± 42.322	2.05 ± 0.123	Antagonistic
Ni(II)10% + Zn(II) 90%(ABCR 1)	0.9897	623.49 ± 43.644	1.95 ± 0.117	Antagonistic
Ni(II)15% + Zn(II) 85%(ABCR 2)	0.9855	780.52 ± 54.636	3.05 ± 0.183	Antagonistic
Ni(II) + DDVP				
Ni(II)12.69% + DDVP 87.31%(EECR- 50)	0.9408	349.87 ± 24.491	1.26 ± 0.076	Antagonistic
Ni(II)15% + DDVP 85%(ABCR 1)	0.9784	228.23 ± 15.976	0.91 ± 0.054	Synergistic
Ni(II)10% + DDVP 90%(ABCR 2)	0.9939	272.37 ± 19.066	0.98 ± 0.052	Additive
Cd(II) + DDVP				
Cd(II)13.44% + DDVP 86.56%(EECR- 50)	0.9573	187.70 ± 13.139	0.55 ± 0.033	Synergistic
Cd(II)10% + DDVP 90%(ABCR 1)	0.9573	187.70 ± 13.139	0.4± 0.029	Synergistic
Cd(II)15% + DDVP 85%(ABCR 2)	0.9544	302.51 ± 21.176	0.95 ± 0.056	Additive
Zn(II) + DDVP				
Zn(II)52.36% + DDVP 47.64%(EECR- 50)	0.9261	190.94 ± 13.366	0.31 ± 0.018	Synergistic
Zn(II)50% + DDVP 50%(ABCR 1)	0.9972	87.00 ± 6.090	0.14 ± 0.008	Synergistic
Zn(II)55% + DDVP 45%(ABCR 2)	0.9781	82.41 ± 5.769	0.13 ± 0.008	Synergistic

Values are represented as Mean ± STD. Pb= lead, Zn=zinc, Co = Colbalt, Ni= Nickel, Cd= Cadmium, Gly= Glyphoate, DDVP = Diclovores. EECR-50 = EC 50 Experimental concentration ratio, ABCR = Arbitrary concentration ratio. ND=Not determined

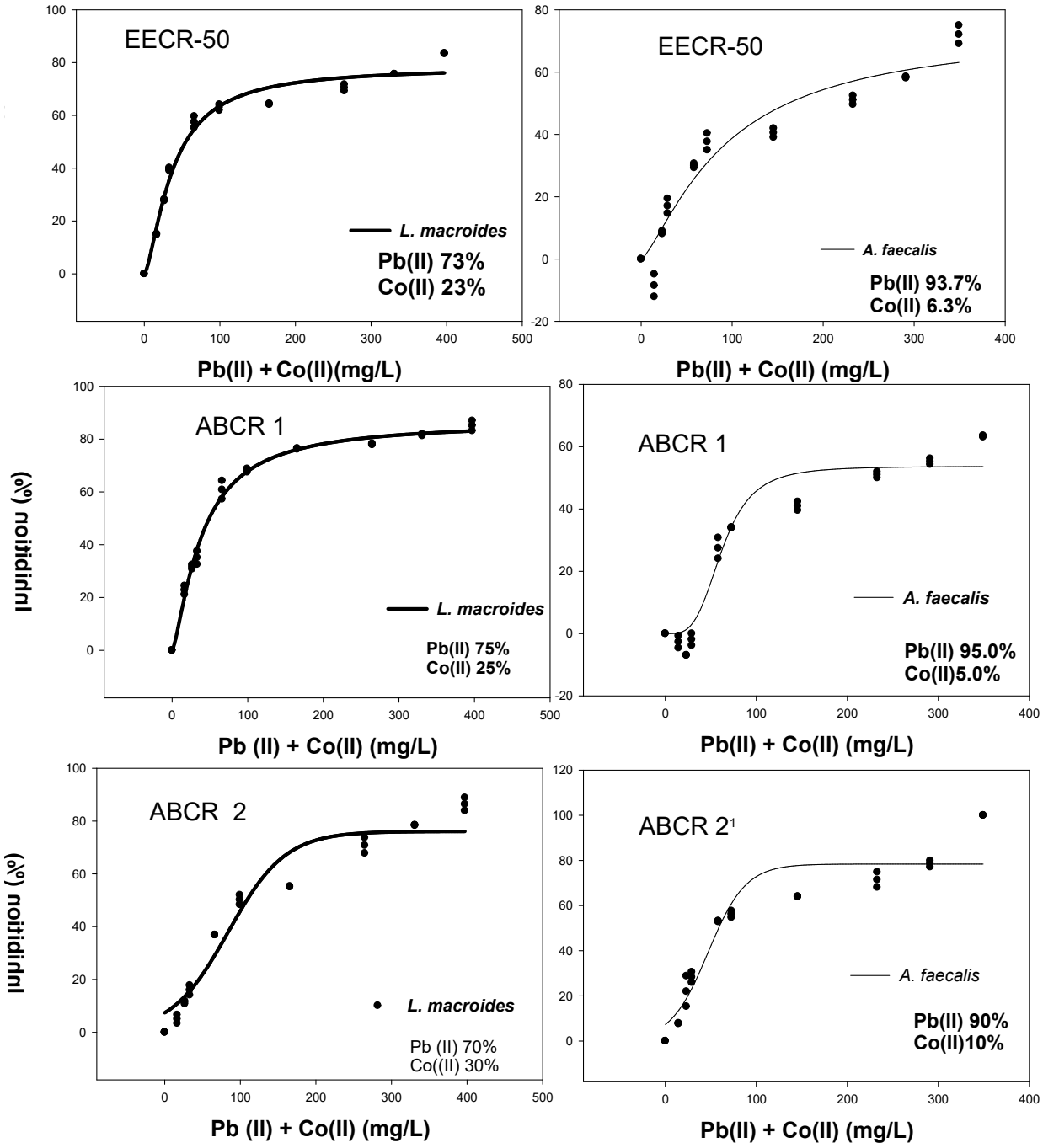


Figure 4.11: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Binary Mixtures of metal ions (Pb (II) and Co(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model.

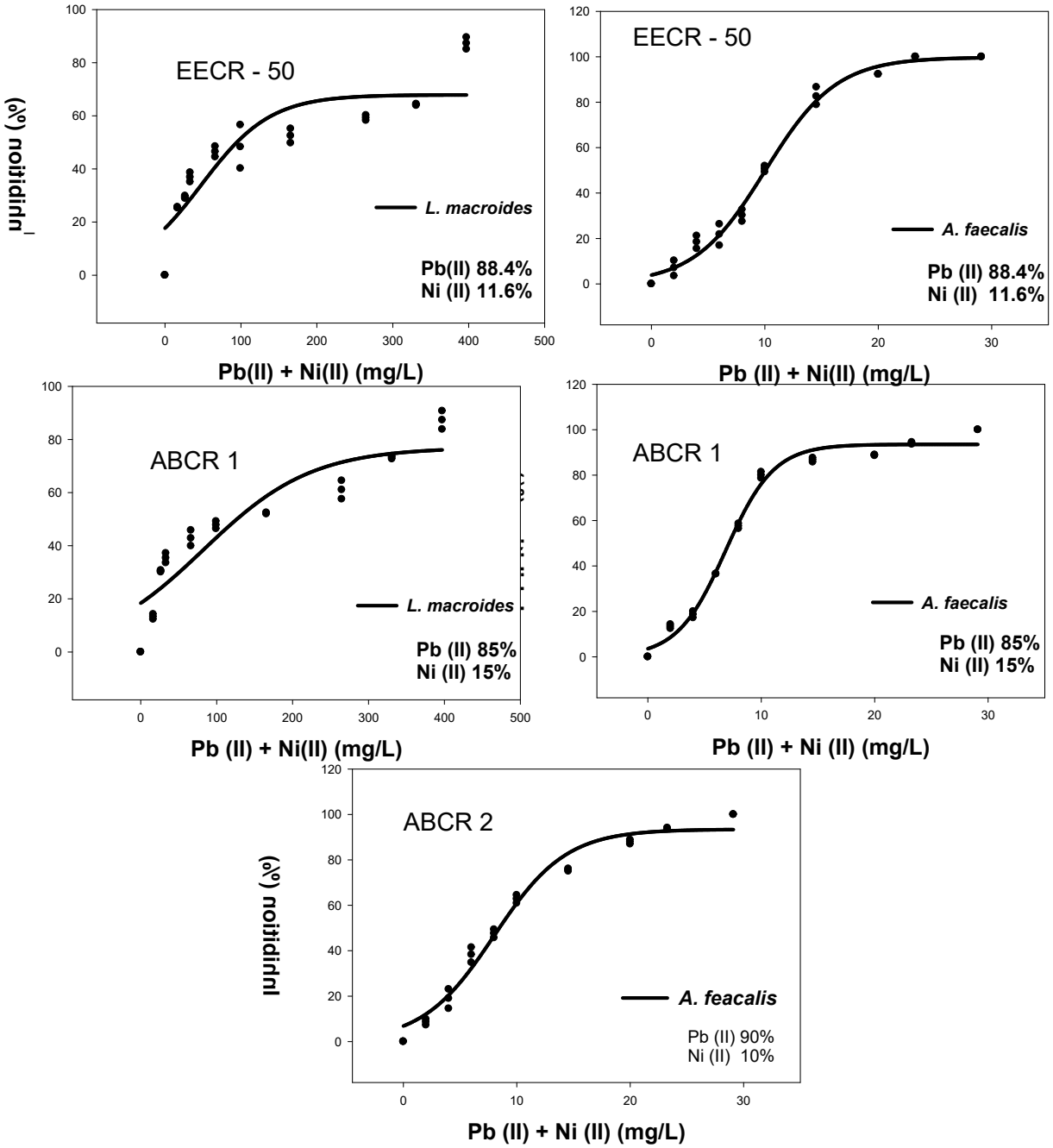


Figure 4.12: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Binary Mixtures of metal ions (Pb (II) and Ni(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

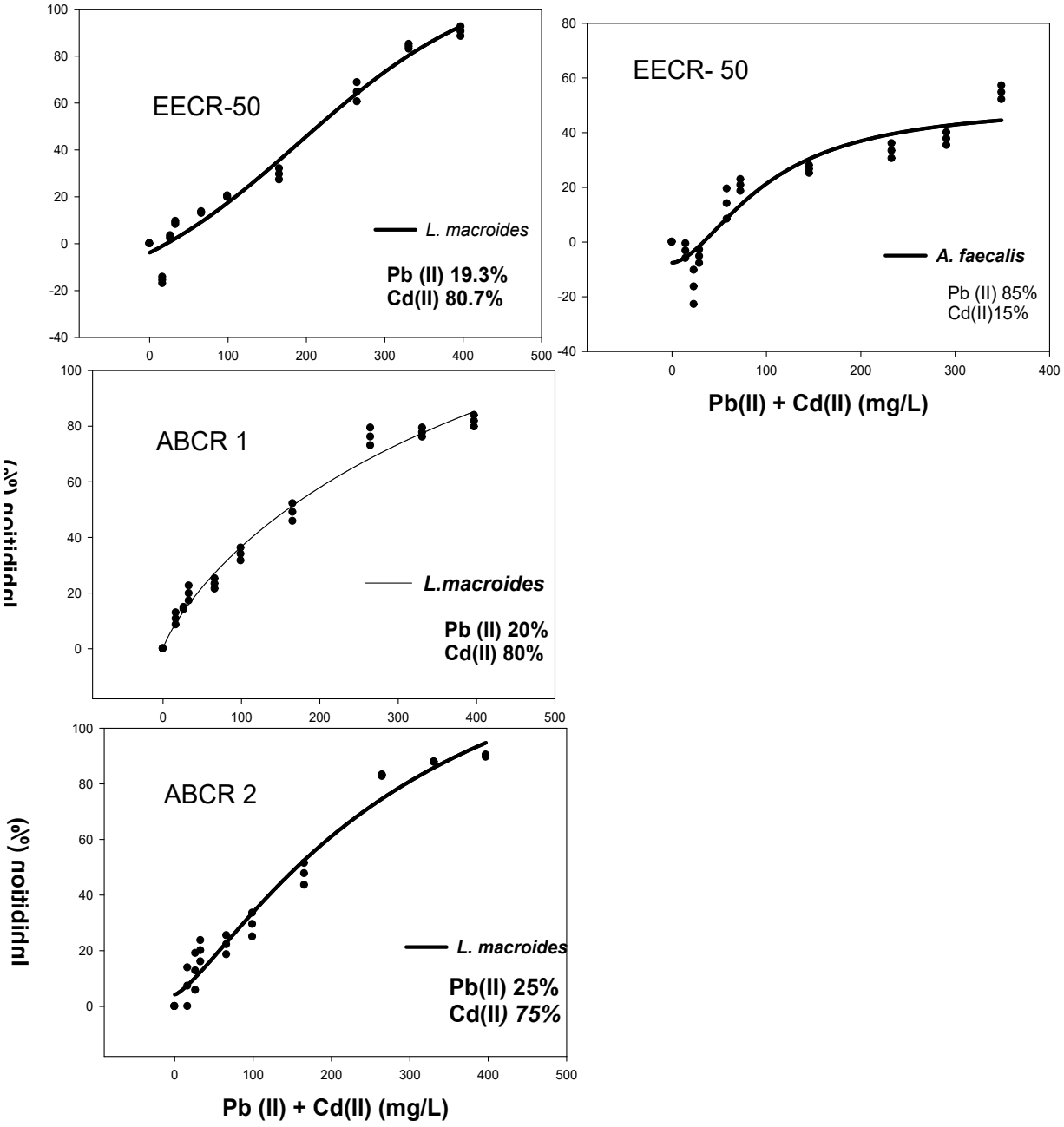


Figure 4.13: Response of *Lysinibacillus macroides*(OK298881) *and Alcaligenes faecalis*(KX302624) *total* dehydrogenase activity to Binary Mixtures of metal ions (Pb (II) and Cd(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

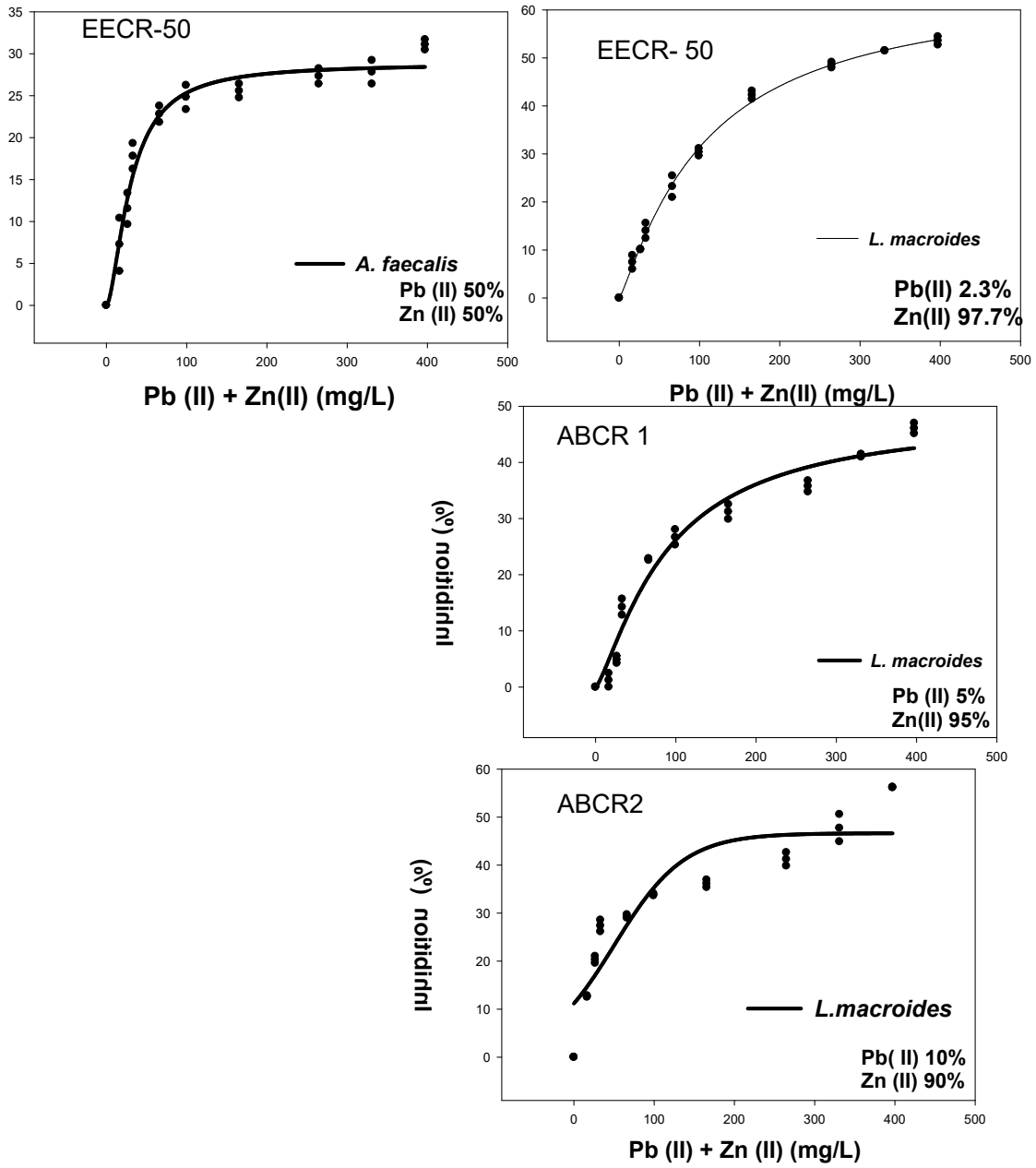


Figure 4.14: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Binary Mixtures of metal ions (Pb (II) Zn(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

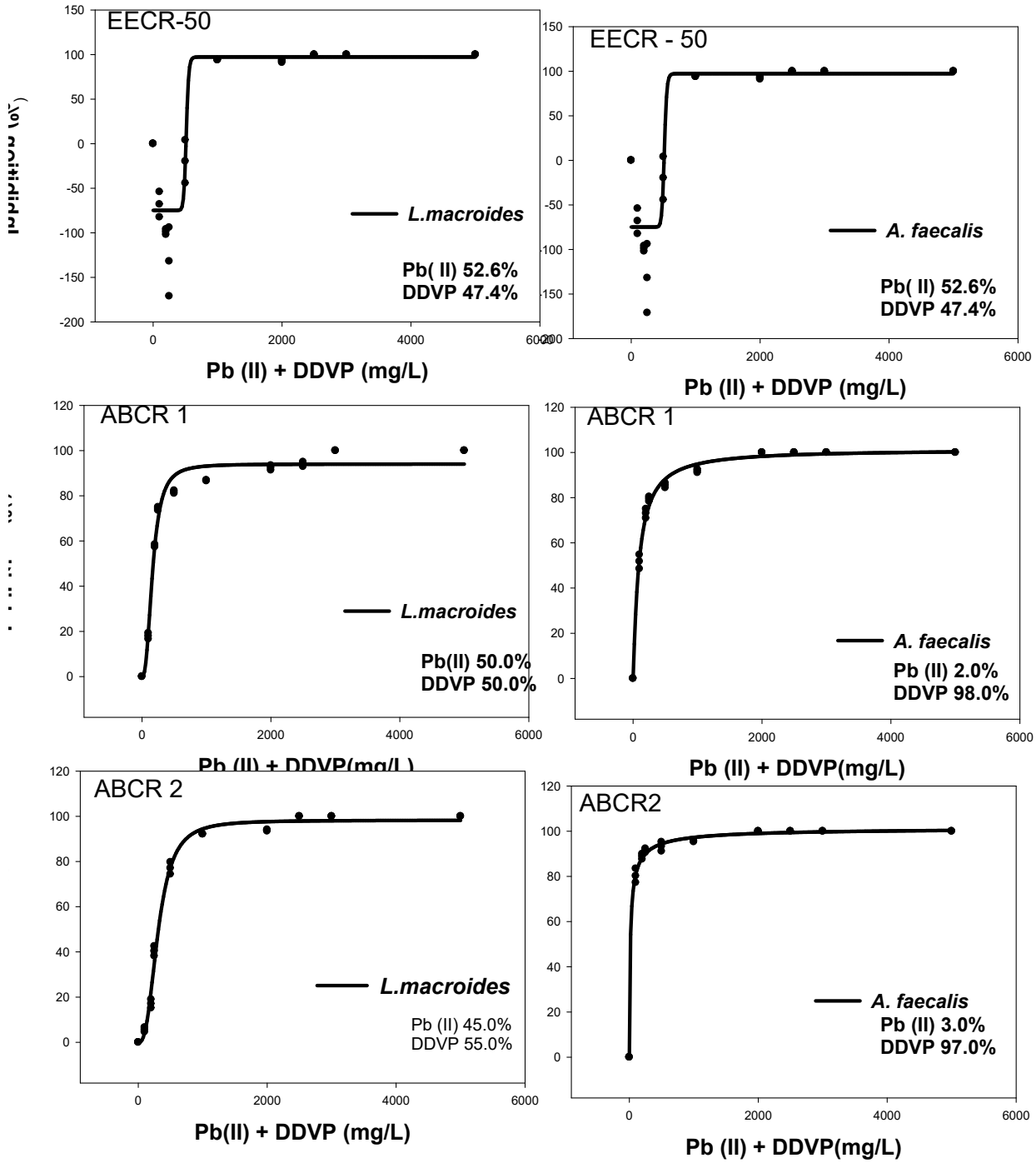


Figure 4.15: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Binary Mixtures of metal ion (Pb (II)) and pesticide (DDVP.). The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

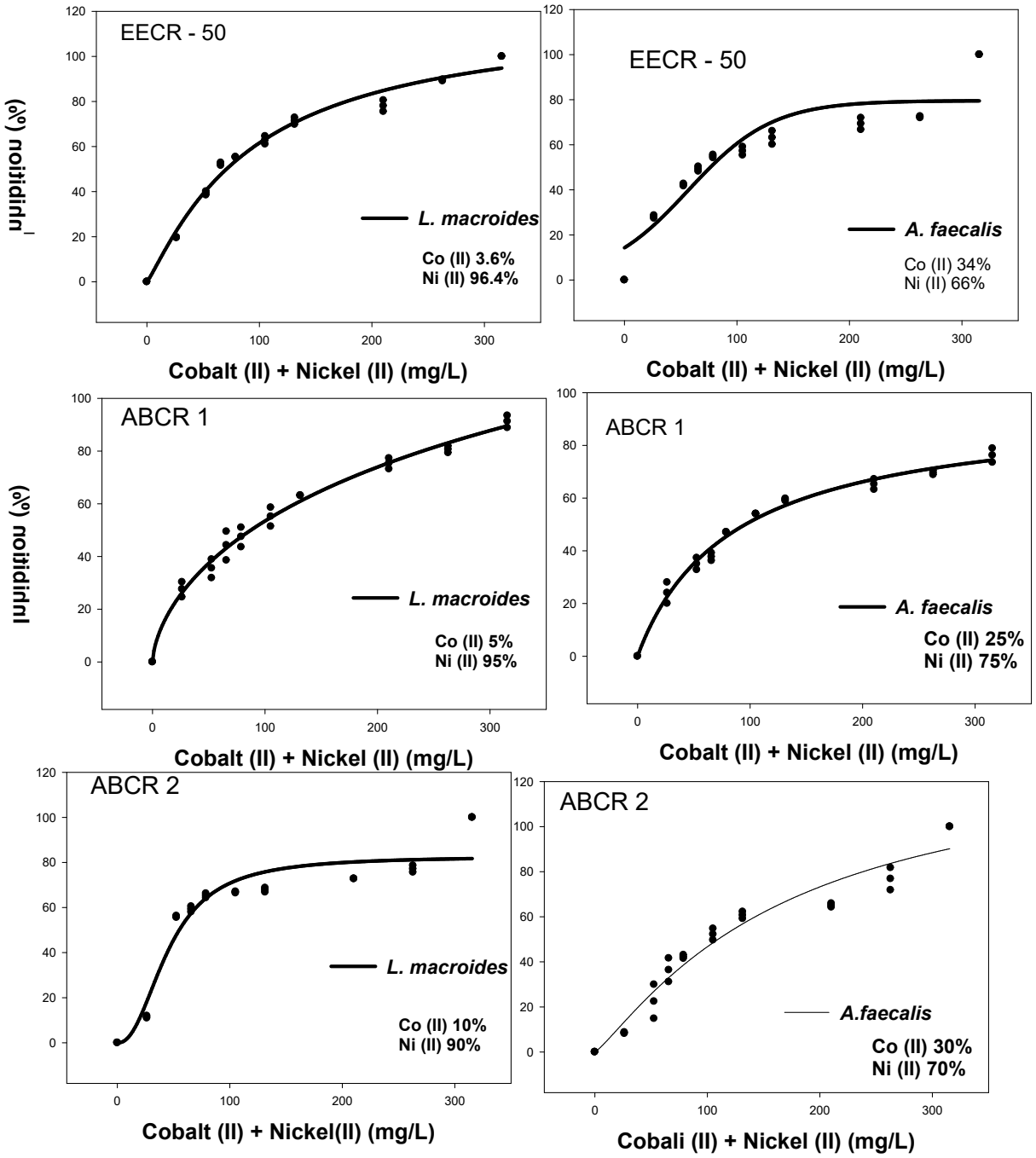


Figure 4.16: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Binary Mixtures of metal ions (Co (II) and Ni(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model**)**

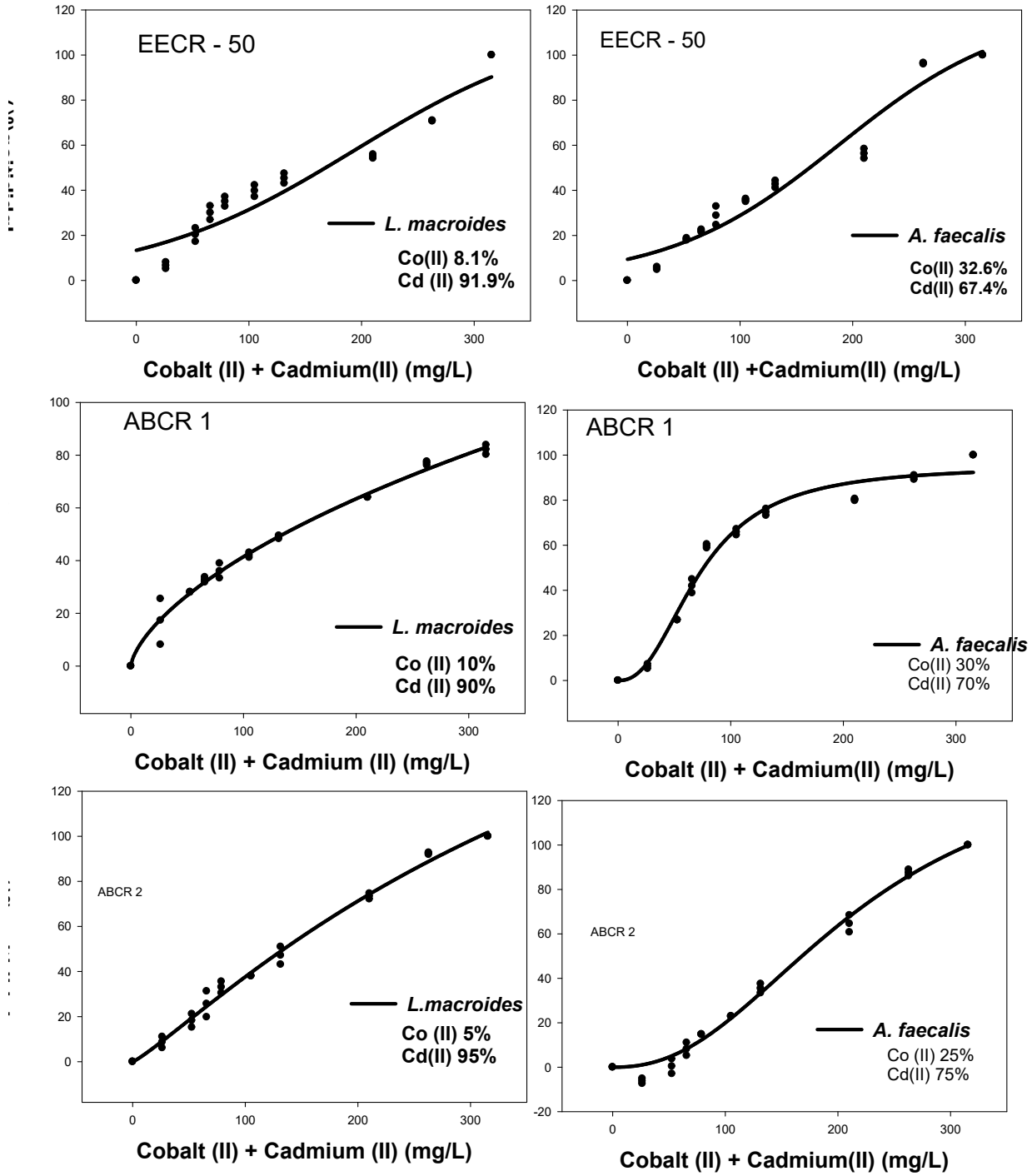


Figure 4.17: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* t(KX302624) otal dehydrogenase activity to Binary Mixtures of metal ions (Co (II) and Cd(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

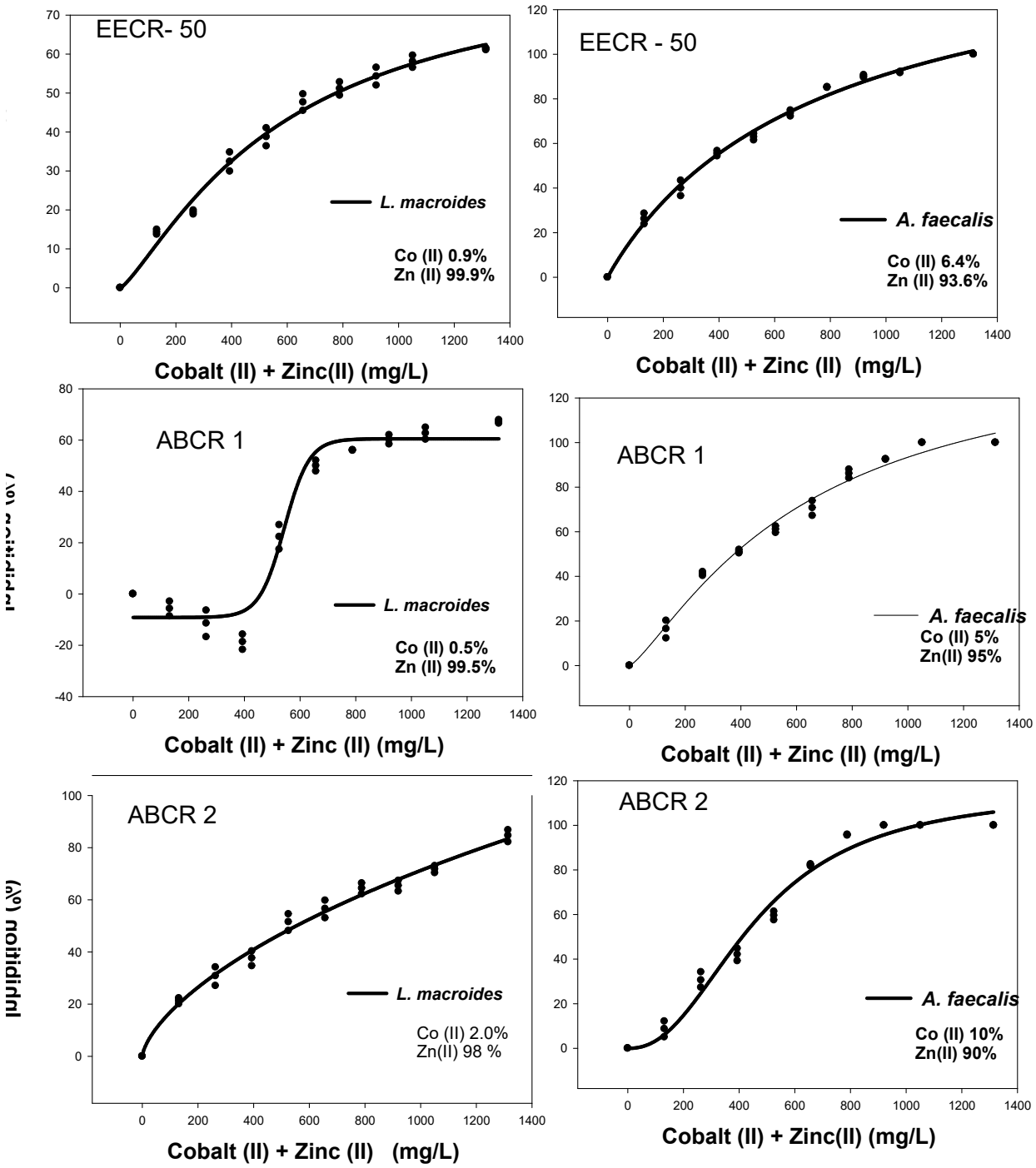


Figure 4.18: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Binary Mixtures of metal ions (Co (II) and Zn(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

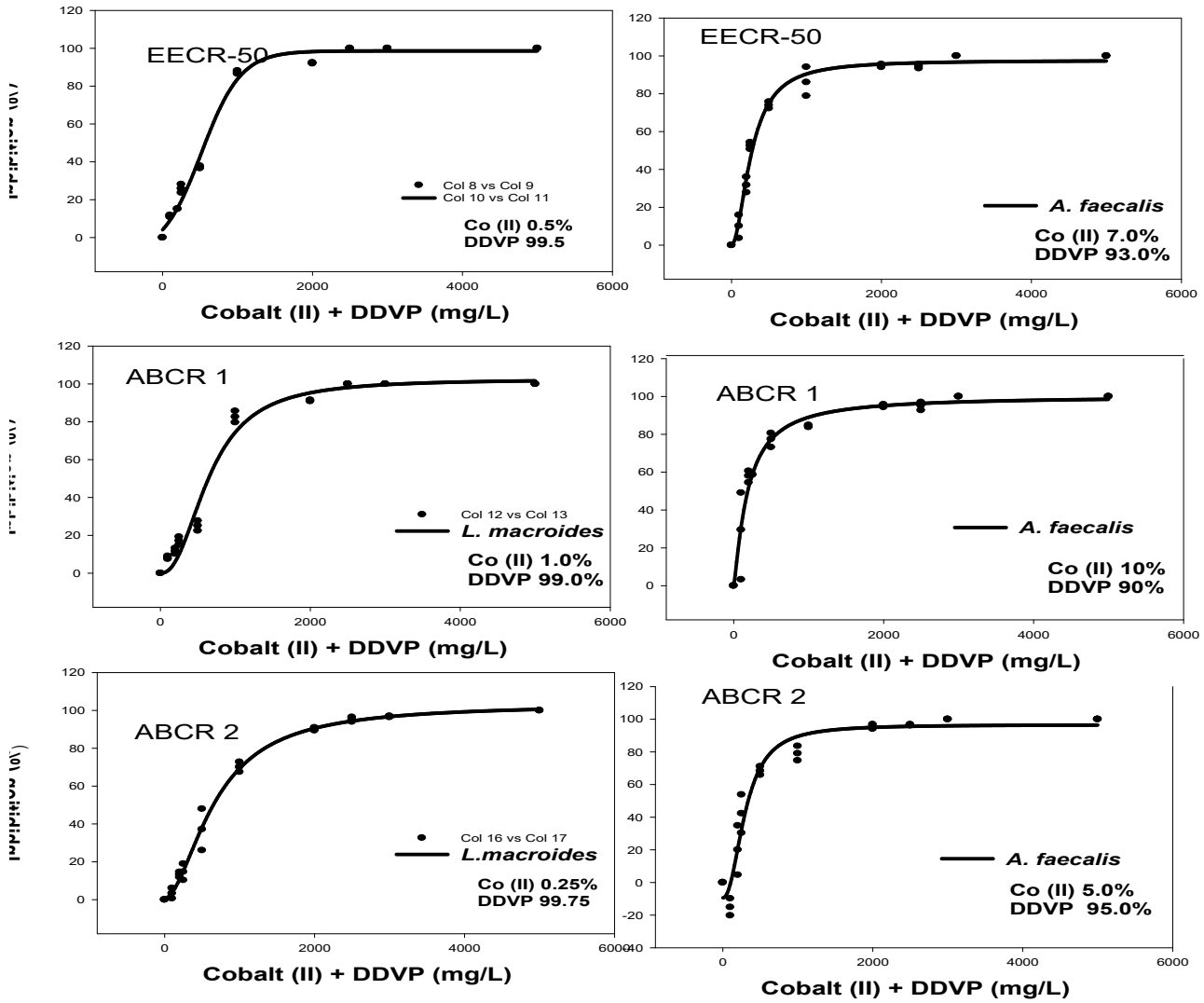


Figure 4.19: Response of Lysinibacillus macroides (OK298881) and Alcaligenes faecalis(KX302624) total dehydrogenase activity to Binary Mixtures of metal ion (Cb (II)) and pesticide (DDVP) The data points represents the experimental dose–response data While the lines represent toxicities obtained by fitting experimental data to logistic model

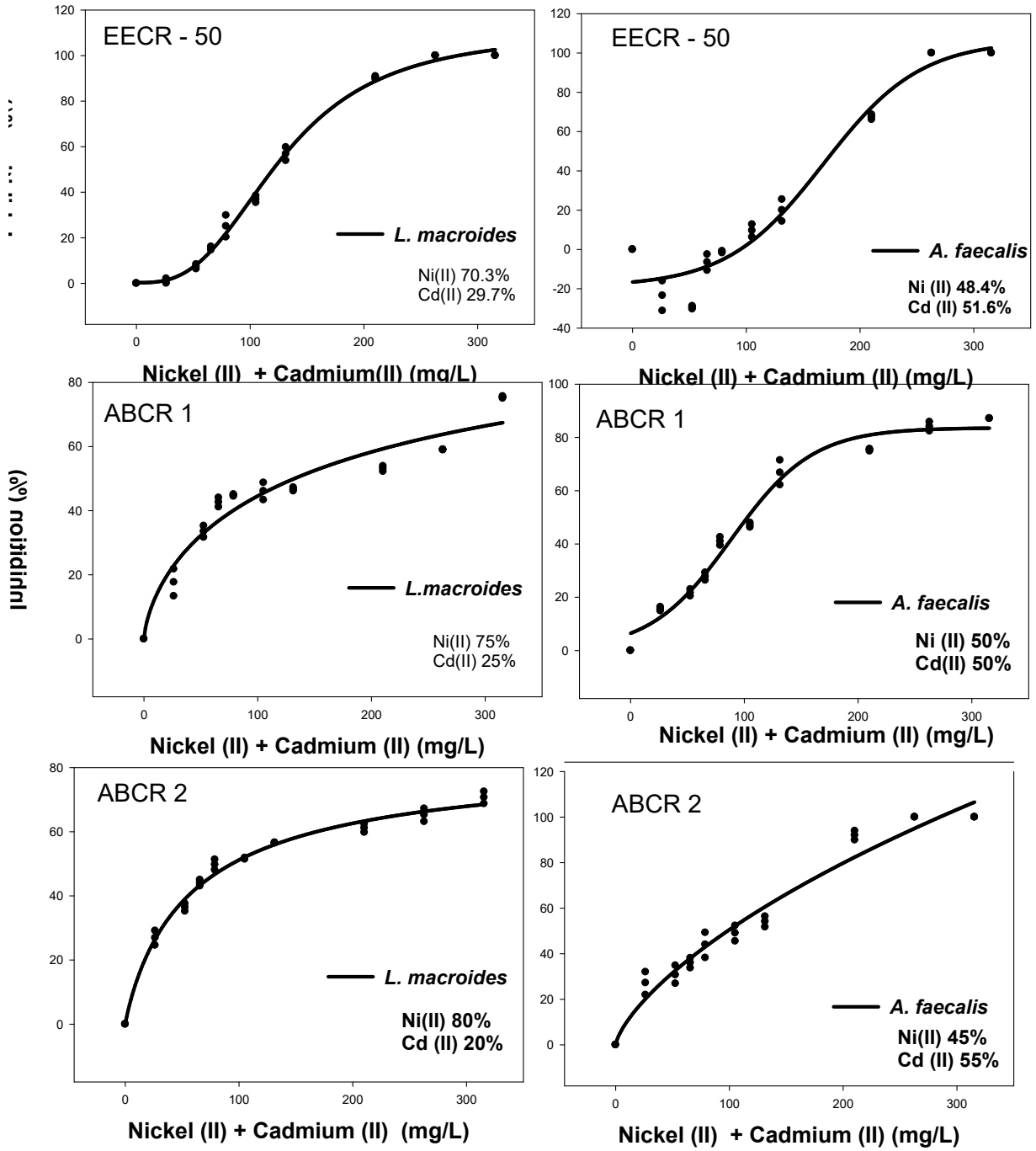


Figure 4.20: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Binary Mixtures of metal ions (Ni (II) and Cd(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

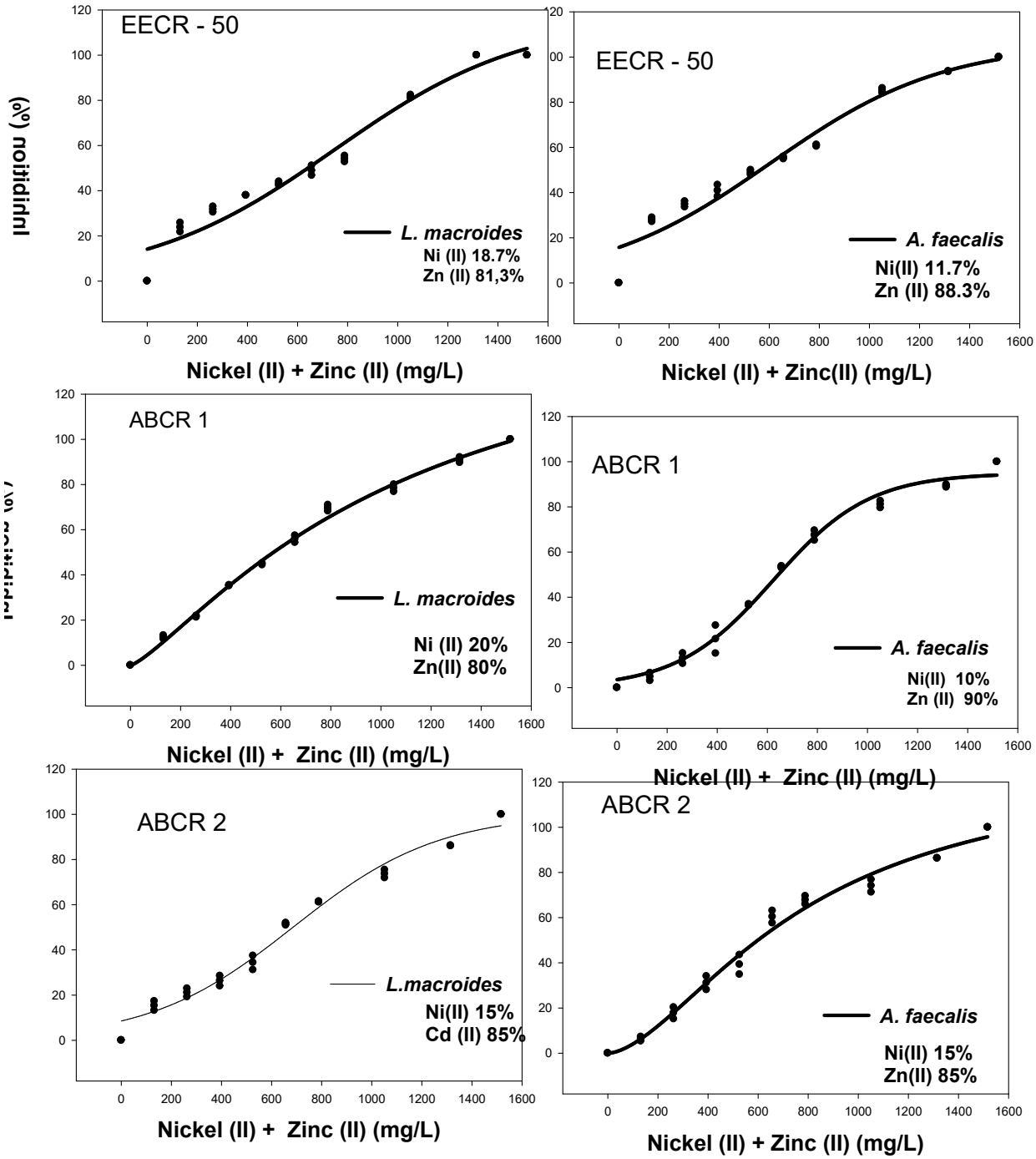


Figure 4.21: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Binary Mixtures of metal ions (Ni (II) and Zn(II)) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

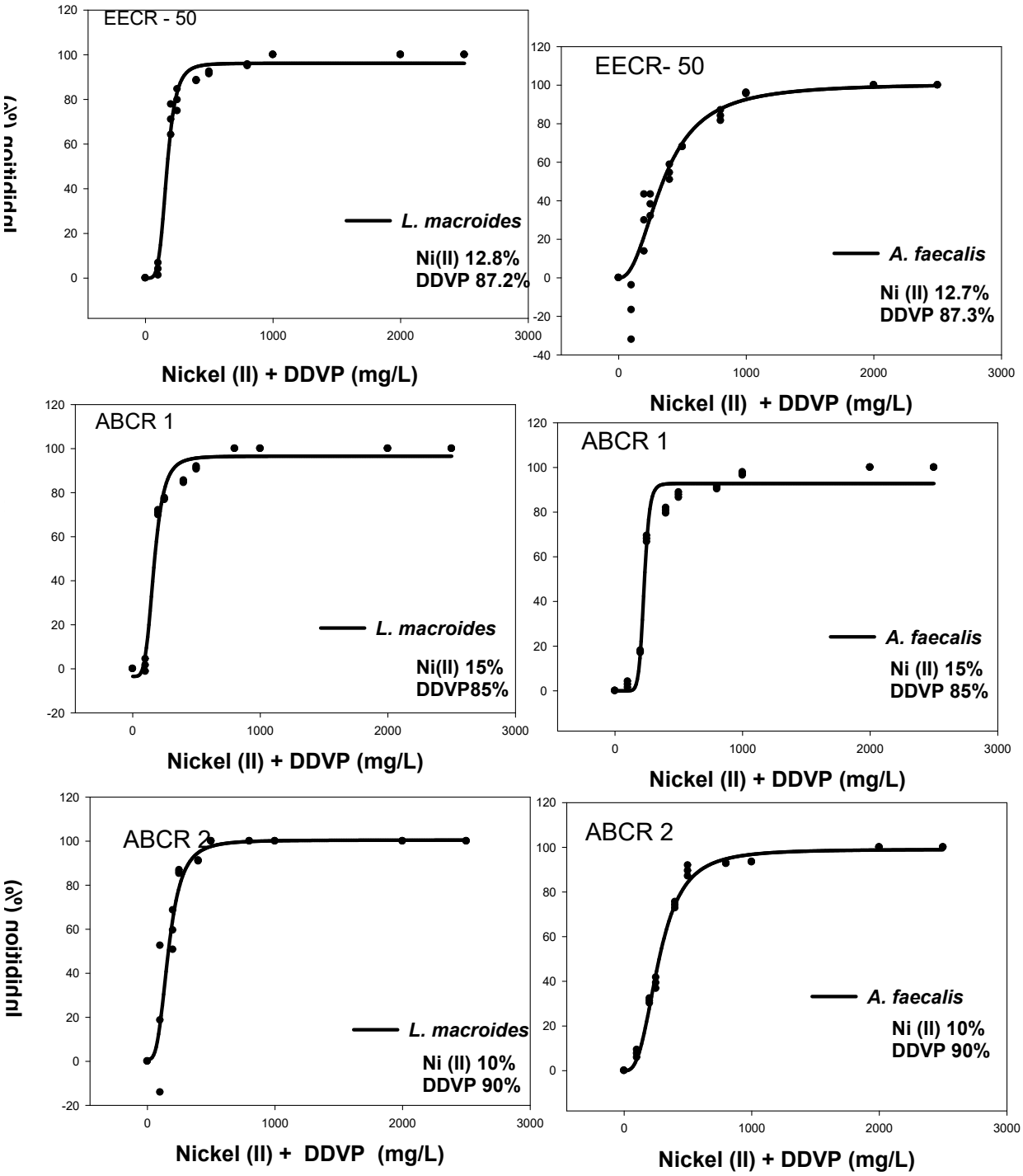


Figure 4.22: Response of Lysinibacillus macroides (OK298881) and Alcaligenes faecalis (KX302624) total dehydrogenase activity to Binary Mixtures of metal ion (Ni (II)) and pesticide (DDVP). The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

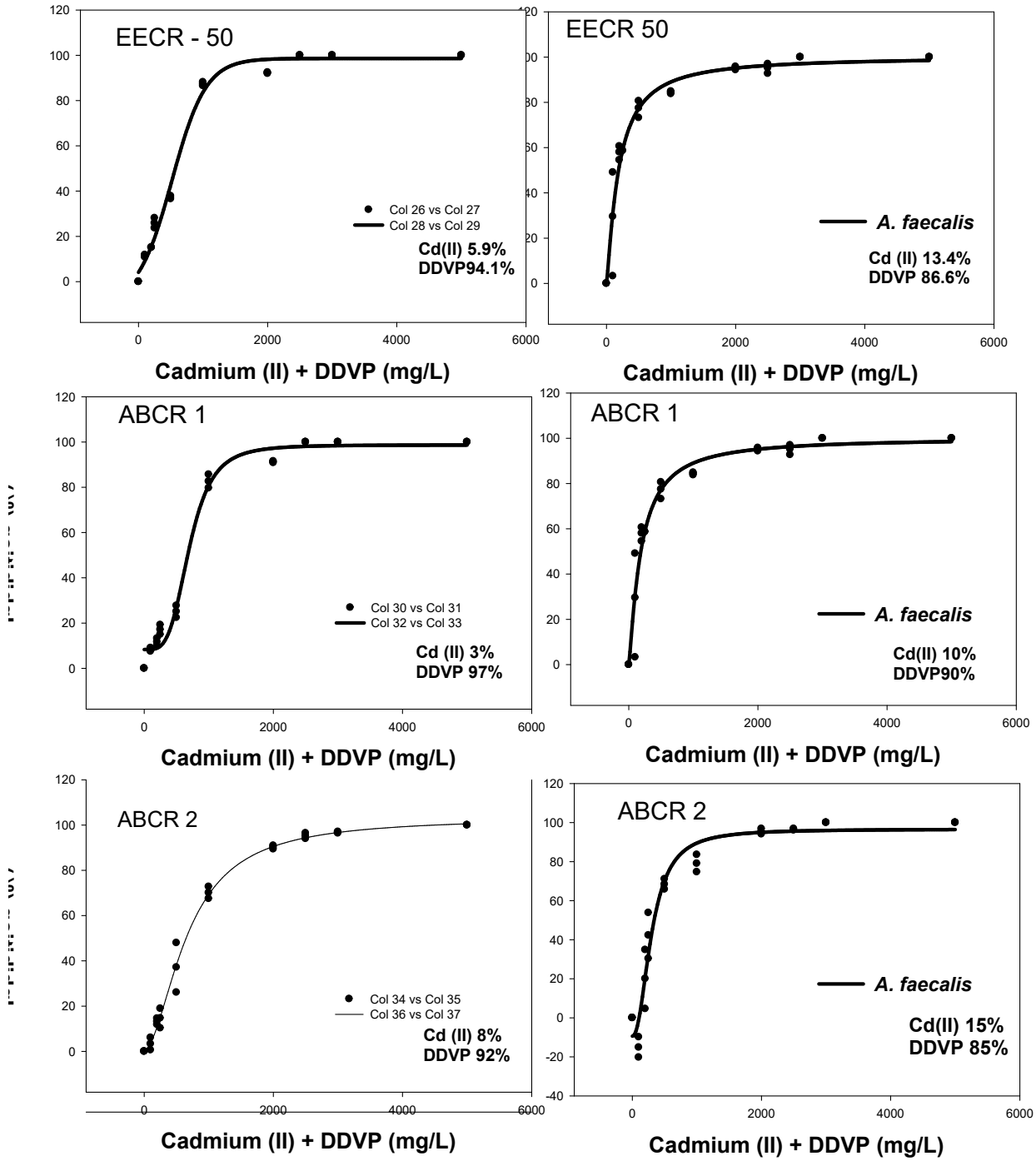


Figure 4.23: Response of *Lysinibacillus macrolides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Binary Mixtures of metal ion (Cb (II)) and pesticide (DDVP) The data points represents the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

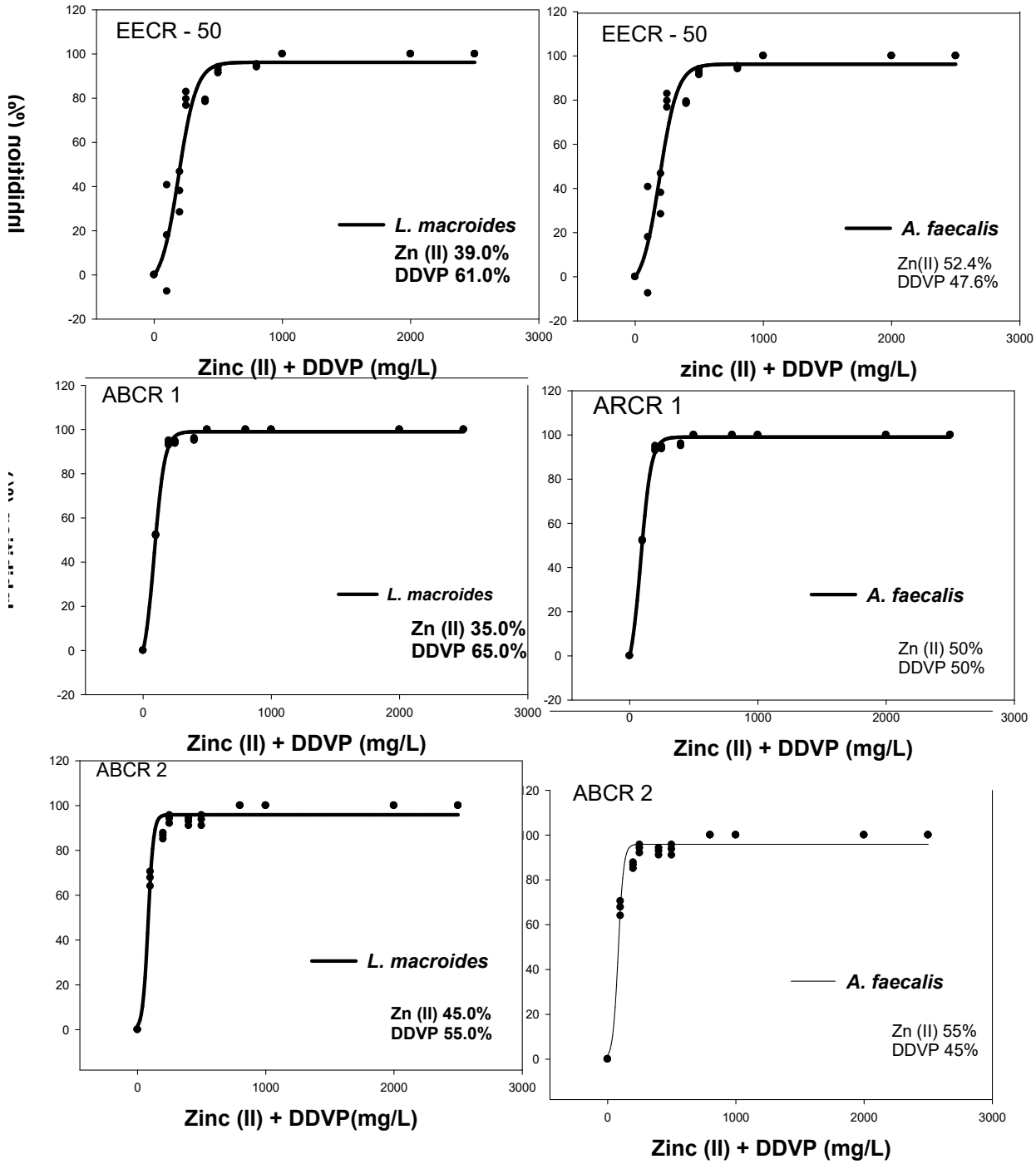


Figure 4.24: Response of Lysinibacillus macroides (OK298881) and Alcaligenes faecalis(KX302624) total dehydrogenase activity to Binary Mixtures of metal ion (Zn (II)) and pesticide (DDVP). The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

4.1.7.3 The Isobolograph analysis of the binary mixtures of metal ions and DDVP against the dehydrogenase activity of *L. macrolides* and *A. faecalis.*

The isobolograph analysis of the binary mixtures of metal ions and DDVP based on the EC_{50} values against the dehydrogenase activity of *L. macroides* (OK298881) are shown in figure 4. 25. The isobologram indicated a synergistic effect of the binary mixtures some of the metal ions and DDVP (Pb (II) + DDVP, Co (II) + DDVP, Ni (II) + DDVP and Zn (II) + DDVP) but additive effect of Cd (II) + DDVP on the dehydrogenase activity.

The figure 4. 26 showed the isobologram on *A. faecalis* dehydrogenase activity. The isobologram indicated synergistic effect of some of the metal ions and DDVP binary mixtures (Pb (II) + DDVP, Co (II) + DDVP, Ni (II) +DDVP and Zn (II) + DDVP) but additivity effect of Cd (II) + DDVP on the dehydrogenase activity.

The toxic unit (TU) values of the binary mixtures are plotted in an isobologram as described by Boillot & Perrodin, (2008). The straight line joining the TU of component A and TU of component B is called an additivity line representing the additive effect of the mixture. When the TU data point (TU of component A versus TU of component B) plotted in isobologram is below or above the additivity line, the interactions are taken to be synergistic or antagonistic.

This observation was corroborated by the toxic index result on table 4.9 and table 4.10.

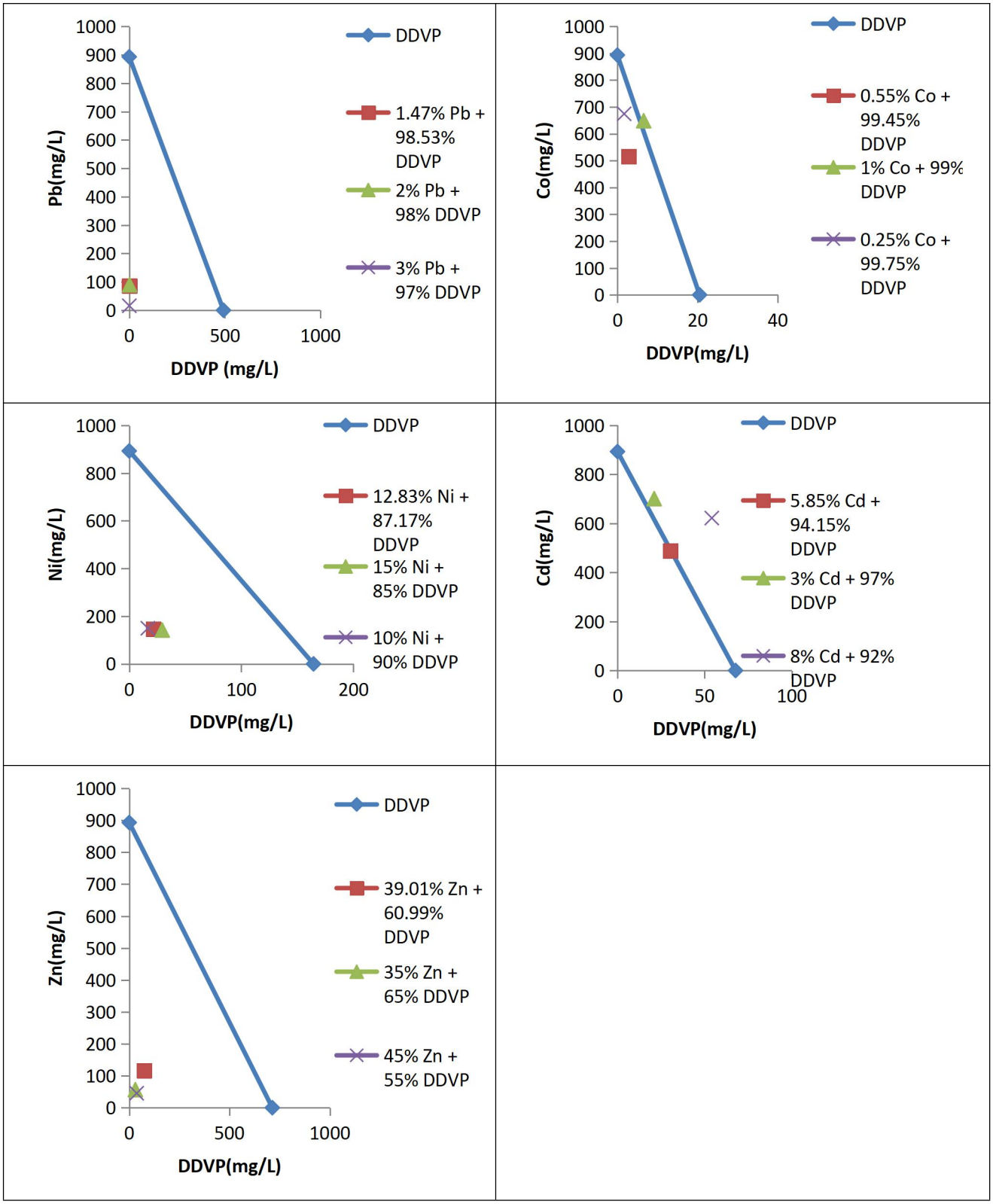


Figure 4.25: The EC_{50} isobole for DDVP and metal ions against dehydrogenase activity of *L. macrolides* (OK298881). The solid line represents additivity line.

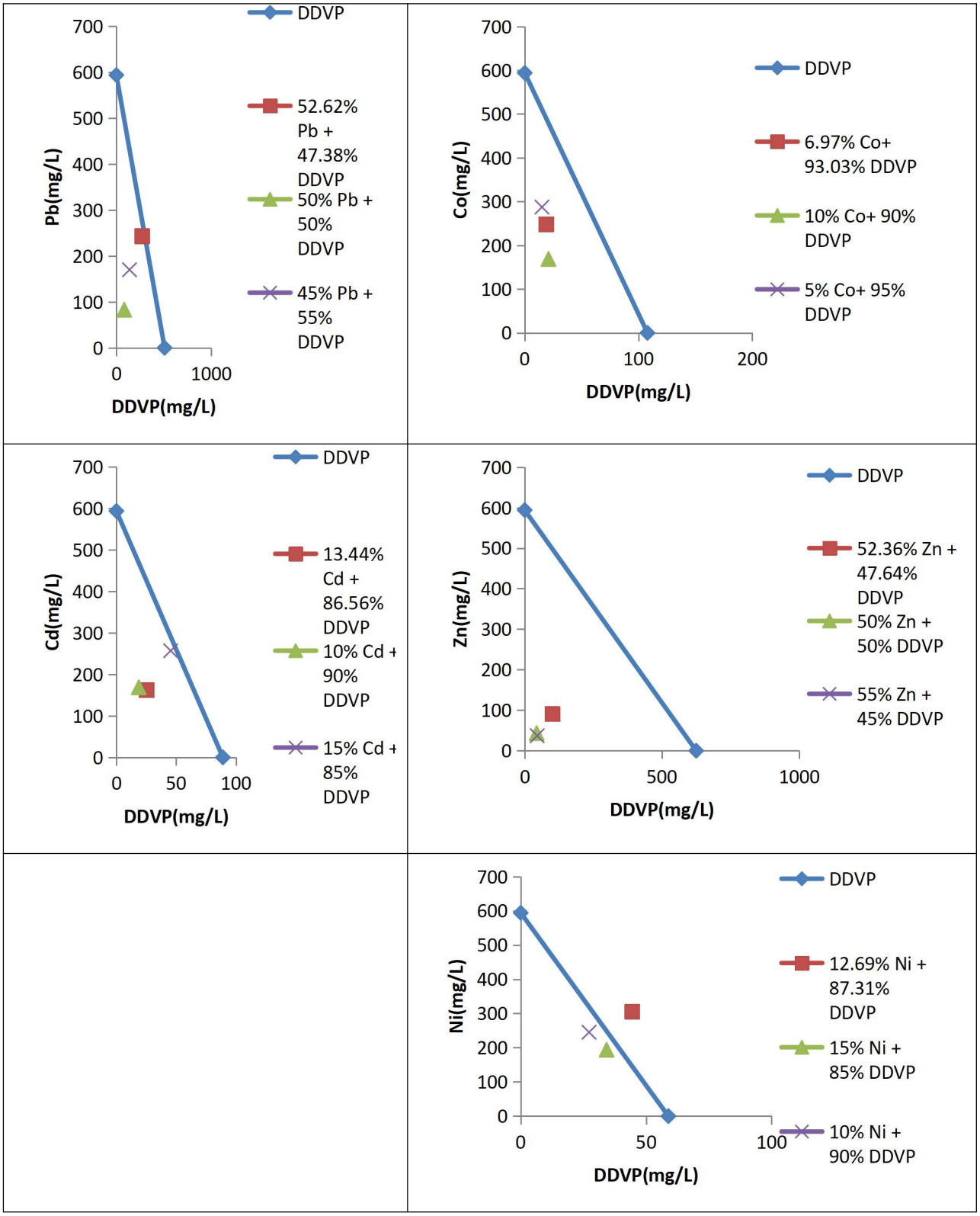


Figure 4.26: The EC50 isobole for DDVP and metal ions against dehydrogenase activity of *A. faecalis* (KX302624). The solid line represents additivity line.

4.1.8.1 Toxicity threshold of Ternary mixtures of Metal ions and pesticides to *Lysinibacillus macroides* (OK298881) total dehydrogenase activity.

Table 4.11 shows the experimental and arbitrary toxicity thresholds (EC_{50}) of ternary mixtures of metal ions and pesticides on *L. macrolides* (OK298881). The Experimentally derived EC_{50s} of lead, cobalt and cadmium (Pb (II) +Co (II) + Cd (II)) showed that the EECR-EC_{50} mixture ratio had the highest EC_{50} (320.3359 ±19.22015mg/L) while the arbitrary concentration ratio (ABCR) EC_{50} was less (191.427± 11.48562mg/L). Their R^2 values were 0.9592 and 0.9635, respectively which shows that they had very strong positive relationship but statistically, the analysis showed that they are different from each other ($P< 0.05$). The interaction had a dose dependent response when fitted in a logistic three parameter model using sigmaplot 10.0 tool as shown in figure 4.27.

The mixture of Pb (II) + Co (II) + Ni (II) at EECR and ABCR had a strong positive relationship as R^2 values (0.9766 and 0.9643) tends to one. Their mean EC_{50s} were 135.9528±8.157168 mg/l and 161.1928± 9.671568 mg/l and their inhibitory effects were dose dependent as they exhibited low dose inhibitory effect as shown in figure 4.28 . The toxicity effects as justified by the toxic index (TI) were additive for the EECR (1.0458± 0.0627) and synergistic (0.29183 ± 0.01751) for the ABCR.

Contrarily, the mixture of Pb (II) + Co (II) + DDVP was antagonistic at EECR-50 and ABCR as they had TI values of (1107.175 ± 66.43049 and 1054.844 ±63.29063) respectively. They inhibited total dehydrogenase activity of *L. macrolides* even at low dose.

The interaction of Pb (II) + Co (II) + Glyphosate were similarly, antagonistc as they had a TI values of (6.7785± 0.4067 and 1.1890± 0.0713) for both EECR and ABCR.They had a weak positive relationship as proved by the R^2 values (0.9375 and 0.8747) and had

a linear relationship when closely fitted into a sigmoidal logistic three parameter model as shown in figure 4.30

Mixtures Pb (II) +Cd (II) + Zn (II) showed a synergistic effect both in EECR-50 and ABCR. The interaction had an R^2 – values 0f 0.8832 and 0.7935 for both EECR and ABCR which showed a strong negative relationship and were statistically different from each other ($p< 005$). The EC_{50} of (217.3685±13.04211mg/l and 116.3359 ± 6.9801 mg/l) for EECR and ABCR were derived by Pb (II) + Cd (II) + Zn (II) interaction were they exhibited total inhibition of dehydrogenase activity of the isolate under study as closely fitted into logistic three parameter model as shown in figure 4.31.

Similarly, inhibitory response was recorded in the combination of Pb (II) + Cd (II) + DDVP where the EC_{50} values of 384.9027 ± 23.09416 mg/l and 333.9037 ± 20.0342 mg/l for both EECR and ABCR respectively and was fitted in sigmoidal three parameters as shown in figure 4.49 while the mixture of Pb(II) + Cd(II) + Glyphosate had a biphasic response. It was stimulatory at concentration 0- 2500 mg/l and became inhibitory as the concentration was elevated as shown in figure 4.33.

The mixtures of Pb (II) + Ni (II) + DDVP and Pb (II) + Ni (II) + Glyphosateshowed a synergistic reponses as their TI values were less than one; (0.37006 and 0.642686). The R^2 values for both the EECR and ABCR of both mixtures had strong positive relationships as R^2 tends to 1 (0.9942 and 0.9923) for Pb (II) + Ni (II) + DDVP and (0.9874 and 0.9917) for Pb (II) + Ni (II) +Glyphosate. They had a low dose stimulatory effect in *L. macrolides* dehydrogenase activity and inhibited it as the concentration was increased as shown in figures 4.34 and figure 4.35.

Some of the metal mixtures with pesticides showed antagonistic effect as their TI values were more than one: Zn(II) + DDVP + Pb(II), Ni(II)+Zn+Glyphosate, Cd(II)+Ni(II) +DDVP, Cd(II)+Ni(II) +Glyphosate, Co(II) + Ni(II) +Glyhosate, Co(II) + Cd(II) +Glyhosate and Pb(II) + Co(II) + DDVP. They exhibited biphasic interaction responses.

While mixtures of Pb (II) + Zn (II) + Glyphosate, Ni (II) + DDVP + Cd (II), Ni (II) + DDVP + Glyphosate and DDVP + Glyphosate + Cd exhibited additive toxicity effect, mixtures of DDVP + Glyphosate + Pb (II) and DDVP+Glyphosate + Co were synergistic.

TABLE 4. 11 Mean EC50, Toxic Index and Toxic Effects of Metal ions and Pesticides Ternary mixtures on *L. macrolides* (OK298881)

Toxicant mixtures	R^2-Value	Mean EC50	Toxic index	Toxic effect
Pb(II)+Co(II)+ Cd(II)				
Pb(II) 18.03%+ Co(II) 6.65% + Cd(II) 75.32% (EECR- 50)	0.9592	320.3359 ±19.22015	4.720377 ±0.283223	Antagonistic
Pb(II) 20.03% + Co(II) 9.65%+ Cd(II) 70.32% (ABCR 1)	0.9635	191.427±11.48562	3.277627 ±0.196658	Antagonistic
Pb(II)+Co(II)+ Ni(II)				
Pb(II) 15.88%+ Co(II) 5.28% + Ni(II) 78.84% (EECR- 50)	0.9766	135.9528 ±8.157168	1.045892 ±0.062754	Additive
Pb(II) 2.26%+ Co(II) 0.83% + Ni(II) 96.91% (ABCR1)	0.9643	161.1928 ±9.671568	0.29183 ±0.01751	Synergistic
Pb(II)+Co(II)+ DDVP				
Pb(II) 1.46%+ Co(II) 0.54% + DDVP 98% (EECR- 50)	0.9644	1107.175 ±66.43049	1.538227 ±0.092294	Antagonistic
Pb(II) 8.46%+ Co(II) 2.54% + DDVP 80% (ABCR1)	0.9284	1054.844 ±63.29063	2.538325 ±0.1523	Antagonistic
Pb(II)+Co(II)+ GLY				
Pb(II)7.15%+ Co(II) 2.05% +GLY 90.80% (EECR- 50)	0.9375	5465.258 ±327.9155	6.778574 ±0.406714	Antagonistic
Pb(II)2.56 %+ Co(II) 28.93% +GLY 68.51% (ABCR 1)	0.8747	122.8213 ±7.369278	1.189902 ±0.071394	Antagonistic
Pb(II)+Cd(II)+ Zn(II)				
Pb(II)2.08%+ Cd(II) 8.68% + Zn(II) 89.24%(EECR -50)	0.8832	217.3685 ±13.04211	0.559682± 0.033581	Synergistic
Pb(II) 46.95% + Cd(II) 6.56% + Zn(II) 36.48% (ABCR 1)	0.7935	116.3359 ±6.980154	0.335733 ±0.020144	Synergistic
Pb(II)+Cd(II)+ DDVP				
Pb(II)1.38%+ Cd(II) 5.77% + DDVP 92.85%(EECR -50)	0.9735	384.9027 ±23.09416	0.73903 ±0.0443420	Synergistic
Pb(II) 8.38% + Cd(II)7.77% + DDVP 83.85% (ABRC1)	0.9747	333.9037 ± 20.03422	0.753472± 0.045208	Synergistic
Pb(II)+Cd(II)+ GLY				
Pb(II)0.14% + Cd(II) 0.60% + GLY 99)%(EECR-50)	0.9886	5287.509 ± 317.2506	1.025289± 0.061517	Additive
Pb(II) 7.15%+ Cd(II) 2.60%NGly 90.25% (ABCR 1)	0.9937	4503.885 ± 270.2331	2.801108± 0.168066	Antagonistic
Pb(II)+Ni(II)+ DDVP				
Pb(II) 1.28%+ Ni(II) 12.67% + DDVP 86.05%(EECR-50)	0.9942	210.2529 ± 12.6151	0.370061± 0.0222040	Synergistic
Pb(II) 8.28%+ Ni(II) 14.67% + DDVP 77.05%(ABCR 1)	0.9923	201.7278 ± 12.10367	0.387896 ± 0.023274	Synergistic
Pb(II)+Ni(II)+GLY				
Pb(II) 0.14%+ Ni(II) 1.42% +GLY 89.44%(EECR-50)	0.9874	3379.894 ± 202.7936	0.642686 ± 0.038561	Synergistic
Pb(II) 7.14%+ Ni(II) 3.42% +GLY 89.44%(ABCR 1)	0.9917	2929.367 ± 175.762	1.486597 ± 0.089196	Additive
Pb(II)+Zn(II)+GLY				
Pb(II) 0.14%+ Zn(II) 5.87% +GLY 93.99%(EECR-50)	0.9621	7361.775± 441.7065	1.337175± 0.080231	Additive
Pb(II) 6.19%+ Zn(II) 6.13% +GLY 87.68%(ABCR1)	0.9287	3960.973± 237.6584	1.355883± 0.081353	Additive
Co(II)+Cd(II)+Zn(II)				
Co(II) 0.78%+Cd(II) 8.79% +Zn(I) 90.43% (EECR-50)	0.9690	728.6103± 43.71662	2.14592± 0.128755	Antagonistic
Co(II) 7.77% + Cd(II) 10.79% + Zn(II) 81.43(ABCR 1)	0.9748	668.728± 40.12368	4.366862± 0.262012	Antagonistic

Co(II)+Cd(II)+DDVP				
Co(II) 0.51% + Cd(II) 5.82% + DDVP93.66% (EECR-50)	0.9955	340.9402± 20.45641	0.736309± 0.044179	Synergistic
Co(II) 7.51% + Cd(II) 7.82% + DDVP84.66% (ABCR 1)	0.9963	302.3384± 18.1403	1.744476± 0.104669	Antagonistic
Co(II)+Ni(II)+DDVP				
Co(II) 0.48% + Ni(II) 12.77% + DDVP 86.75 %(EECR-50)	0.9813	513.3585± 30.80151	1.016805± 0.061008	Additive
Co (II) 7.48% +Ni(II) 14.77% + DDVP 77.75%(ABCR1)	0.9858	454.7168± 27.28301	2.463326± 0.1478	Antagonistic
Co(II)+Ni(II)+Pb(II)				
Cd(II) 3.28% + Ni(II) 87.84% +Pb(II) 8.87% (EECR -50)	0.9192	173.0896± 10.38538	1.039929± 0.062396	Additive
Cd(II) 6.28%+ Ni(II) 85.84% + Pb(II) 7.87%(ABCR1)	0.8583	131.0622± 7.863732	0.826896± 0.049614	Synergistic
Cd(II)+Ni(II)+Co(II)				
Cd(II) 28.93% + Ni(II) 68.51% + Co(II) 2.56%(EECR-50)	0.9801	103.0969± 6.185814	0.99881± 0.059929	Additive
Cd(II) 31.93% + Ni(II) 66.51% +Co(II) 1.56% (ABCR 1)	0.9792	67.108± 4.02648	0.638971± 0.038338	Synergistic
Cd(II)+Ni(II)+Zn(II)				
Cd(II) 7.32% + Ni(II) 75.33% + Zn(II) 17.35% (EECR-50)	0.9859	141.6856± 8.501136	0.83706± 0.050224	Synergistic
Cd(II) 14.32% + Ni(II) 77.33% + Zn(II) 8.34%(ABCR 1)	0.9921	164.1354± 9.848124	1.119446± 0.067167	Additive
Cd(II)+Ni(II)+DDVP				
Cd(II) 12.17% + Ni(II) 7.14%+ DDVP 82.68% (EECR-50)	0.9937	663.6454± 39.81872	2.015154± 0.120909	Antagonistic
Cd(II) 19.17% + Ni(II)7.14% + DDVP 73.69%(ABCR 1)	0.9858	756.5875± 45.39525	3.095407± 0.185724	Antagonistic
Cd(II)+Ni(II)+GLY				
Cd(II) 0.60% + Ni(II) 1.40% + GLY98% (EECR-50)	0.9829	12126.39± 727.5832	3.326304 ± 0.199578	Antagonistic
Cd(II) 7.60%+ Ni(II) 3.40%+ GLY89%(ABCR 1)	0.9664	7381.4± 442.884	10.486± 0.62916	Antagonistic
Ni(II)+ Zn(II) + Pb(II)				
Ni(II) 18.37% + Zn(II) 78% + Pb(II) 1.86%(EECR-50)	0.9654	430.5555± 25.83333	0.97884± 0.05873	Additive
Ni(II) 13.37% +Zn(II) 85.58) %+ Pb(II)1.05%(ABCR 1)	0.9747	370.7335± 22.24401	0.754203± 0.045252	Synergistic
Ni(II)+ Zn(II) + Co(II)				
Ni(II) 80.72% + Zn(II) 18.58%+ Co(II) 0.69(EECR-50)	0.9877	245.8075± 14.74845	1.751928± 0.105116	Antagonistic
Ni(II) 80.72%+ Zn(II) 18.58%+ Co(II) 0.70%(ABCR 1)	0.9845	196.9675± 11.81805	1.034507± 0.06207	Synergistic
Ni(II)+ Zn(II) + DDVP				
Ni(II) 55.97%+Zn(II) 35.79%+ DDVP 8.24 %(EECR-50)	0.9654	181.8456± 10.91074	0.727183± 0.043631	Synergistic
Ni(II) 55.97%+Zn(II) 35.79%+ DDVP 8.24%(ABCR 1)	0.9679	149.5206± 8.971236	0.632919± 0.037975	Synergistic
Zn(II)+ DDVP+Pb(II)	0.9694	834.2545± 50.05527	1.031866± 0.061912	Additive
Zn(II) 38.66% + DDVP 60.44 % + Pb(II) 0.90%(EECR-50)	0.9636	1750.462± 105.0277	2.199709± 0.131983	Antagonistic
Zn(II) 45.66% + DDVP 53.44 % + Pb(II) 0.90% (ABCR 1)				
Zn(II)+ DDVP+Co(II)				
Zn(II) 60.79% + DDVP 33.88 % + Co(II) 0.33% (EECR-50)	0.9875	2393.257± 143.5954	3.471112± 0.208267	Antagonistic
Zn(II) 65.79% + DDVP 33.88% + Co(II) 0.33% (ABCR 1)	0.9935	2802.058± 168.1235	4.1036± 0.246216	Antagonistic

Ni(II)+ DDVP+Cd(II)				
Zn(II) 37.58 % + DDVP 58.76 % + Cd(II) 3.65%(EECR-50)	0.9970	674.4151± 40.46491	1.16297± 0.069778	Additive
Zn(II) 37.58%+ DDVP 60.76% + Cd(II) 1.65%(ABCR 1)	0.9876	761.4312± 45.68587	1.105151± 0.066309	Additive
Ni(II)+ DDVP+GLY				
Zn(II) 5.38% + DDVP 8.42% + Gly 81.20% (EECR-50)	0.9895	3097.258± 185.8355	0.799992± 0.048	Synergistic
Zn(II) 12.38% + DDVP 10.42% + Gly 77.20% (ABCR 1)	0.9916	2906.025± 174.3615	1.073993± 0.06444	Additive
DDVP+GLY +Pb(II)				
DDVP 8.89%+ Gly 90.98% + Pb(II) 0.13%(EECR-50)	0.9970	2630.279± 157.8167	0.514556± 0.030873	Synergistic
DDVP 15.39% + Gly 84.48% + Pb(II) 0.13)(ABCR 1)	0.9876	2432.013± 145.9208	0.636506± 0.03819	Synergistic
DDVP+GLY +Co(II)				
DDVP 8.89% + Gly 91.05% + Co(II) 0.04%(EECR-50)	0.9942	2812.372± 168.7423	0.610117± 0.036607	Synergistic
DDVP 10.89 % Gly 88.06%+ Co(II) 1.04%(ABCR 1)	0.9935	2866.848± 172.0109	2.076175± 0.124571	Antagonistic
DDVP+GLY +Cd(II)				
DDVP 10.89% +Gly 88.06 % + Cd(II) 1.05% (EECR-50)	0.9974	4012.312± 240.7387	1.096995± 0.06582	Additive
DDVP 10.85 % + Gly 85.60% + Cd(II) 3.55% (ABCR 1)	0.9979	3520.679± 211.2407	2.583289± 0.154997	Antagonistic

Values are represented as Mean ± STD. Pb= lead, Zn=zinc, Co = Colbalt, Ni= Nickel, Cd= Cadmium, Gly= Glyphoate, DDVP = Diclovores. EECR-50 = EC 50 Experimental concentration ratio, ABCR = Arbitrary concentration ratio. ND=Not determined

4.1.8.2 Toxicity threshold of ternary mixtures of metal ions and pesticides to *Alcaligenes faecalis* (KX302624) total dehydrogenase activity.

Table 4.12 shows the Experimental toxicity thresholds (EC_{50}) of ternary mixtures of metal ions and pesticides on *Alcaligenes faecalis* (KX302624). The Experimentally derived EC_{50s} of lead, cobalt and cadmium (Pb (II) +Co (II) + Cd (II)) showed that the EECR-EC_{50} mixture ratio had the highest EC_{50} (376.3074± 18.81537mg/L) while the ABCR was less (94.4121 ±4.720605mg/L) with R^2 values of 0.9778 and 0.9828, respectively. The statistical analysis showed that they are different from each other ($P< 0.05$). As these interactions were closely fitted into a logistic three parameter mathematical model using sigmaplot 10.0 software as shown in figure 4.29, it showed that it had a low dose inhibitory effect on yhe total dehydrogenase activity of *A. faecalis.*

Mixtures Pb(II)+Co(II)+Ni(II) and Pb(II)+Co(II)+ Zn (II) showed a synergistic effect both in EECR-50 and ABCR as their TI values were less than 1. They showed a strong positive relationship thus the R^2 values for both EECR and ABCR tend to 1. The mean EC_{50} of the first mixture; (Pb (II) + Co (II) + Ni (II)) were 135.9528± 6.7976 mg/l for the EECR and 75.8832± 3. 6942 mg/l for the ABCR. This was closely fitted into a logistic three parameter mathematical model as shown in figure 4.28. The mean EC_{50} of the mixture (Pb (II) + Co (II) + Zn (II)) were 354.4471± 17.7224 mg/l for EECR and 289.0502±14.4525 mg/l. this was closely fitted into equally fitted into a logistic three parmetera mathematical model as shown in figure 4.29.

Mixtures of Pb (II) +Co (II) + DDVP and Pb (II) + Co (II) + Glyphosate were antagonistic with a TI values more than 1. Their R^2 values for the EECR and ABCR had a strong positive relationship (EECR = 0.9800 and 0.9655, ABCR = 0.9873 and 0.9892). The mixture; Pb(II) + Co (II) + Glyphosate fitted in a logistic three parameter

mathematical model using sigmaplot 10.0 software had an R^2 value of 0.9873 as shown in figure 4.30.

The mixtures of Pb (II) + Cd (II) + DDVP as justified by TI values was antagonistic with R^2 value of 0.9880 while Pb (II) + Cd (II) + Glyphosate was additive with TI of 0.9954. These were fitted both fitted into logistic three parameter model as shown in figure 4.32 and figure 4.33. They had an EC_{50} of 3013.905± 150.6953 mg/l and 2997.205 ± 1498603 mg/l.

The EECRs of Pb (II) + Ni (II) + DDVP and Pb (II) + Ni (II) Glyphosate were antagonistic as their TI values were (2.1696±0.151869 and 1.1422± 0.0799). Their R^2 value had strong positive relationships and was closely fitted in a logistic three parameter model as shown in figure 4.34 and 4.35. Their EC_{50} values at EECR were 773.034± 38.6517 mg/l and 2928.367 ±146.4683mg/l.

Additivity was recorded when DDVP and Glyphosate interacted with cobalt and lead. In the mixture of DDVP + Glyphosate + Pb (II), TI values of 1.03633± 0.0725 and 1.05229 ± 0.0946 were recorded for both the EECR and ABCR. The R^2 values had a strong positive relationship but were statistically different as P<005. This was applicable to the mixture of DDVP + Glyphosate + Co as shown in figure 4.40 and figure 4. 54.

When these two pesticides interacted with other metals like cadmium and nickel, their toxicity effects were antagonistic. The mixture of DDVP + Glyphosate + Cd (II) had an EC_{50} value of 5543.097± 277.1549 mg/l at the EECR and 5091.917± 254.5958 mg/l at the ABCR. This was fitted in a logistic three parameters as shown in figure 4.56 and their R^2 values were 0.9953 and 0.9856. Mixture DDVP + Glyphosate + Ni (II) equally exhibited an antagonistic toxic effect as TI values were greater than one. The TI values of both EECR and ABCR were 1.817557± 0.12723 and 4.7029± 0.3292, respectively. Their R^2 values were 0.9885 and

0.9900, it was closely fitted into logistic three parameters mathematical model were it shows to have very low dose inhibitory effect to the dehydrogenase activity of *A. faecalis*.

TABLE 4.12 Mean EC50, Toxic Index and Toxic Effects of Metals and Pesticides Ternary Mixtures on *A. faecalis* (KX302624)

Toxicant mixtures	R^2 value	Mean EC_{50}	Toxic index	Toxic effect
Pb(II) + Co(II) + Cd(II)				
Pb(II) 82.83% + Co(II) 5.59% Cd(II) 11.58% (EECR-50)	0.9778	376.3074± 18.81537	1.295759 ±0.090703	Additive
Pb(II) 84.83% + Co(II) 8.59% + Cd(II) 6.58% (ABCR 1)	0.9828	94.4121 ±4.720605	0.302041 ±0.021143	Synergistic
Pb(II)+ Co(II) + Ni(II)				
Pb(II) 83.44% + Co(II) 5.63 % + Ni(II) 10.93% (EECR-50)	0.9884	135.9528 ±6.79764	0.545762 ±0.038203	Synergistic
Pb(II) 90.44% +Co(II) 7.63% + Ni(II) 1.92% (ABCR 1)	0.9721	73.8832 ±3.69416	0.207487 ±0.014524	Synergistic
Pb(II)+ Co(II) + Zn(II)				
Pb(II) 48.60 % +Co(II) 3.28% +Zn(II) 48.12% (EECR-50)	0.9745	354.4471 ±17.72236	0.718838 ±0.050319	Synergistic
Pb(II) 55.60% + Co(II) 5.28% + Zn(II) 39.12%(ABCR 1)	0.9837	289.0502 ±14.45251	0.637813 ±0.044647	Synergistic
Pb(II)+ Co(II) + DDVP				
Pb(II) 50.81% +Co(II) 3.43% + DD VP) 45.76% (EECR-50)	0.9800	929.2408 ±46.46204	1.93745 ±0.135621	Antagonistic
Pb(II) 57.81 % + Co(II) 5.43% + DDVP) 37.76% (ABCR 1)	0.9655	1749.797 ±87.48983	3.947756 ±0.276343	Antagonistic
Pb(II)+ Co(II) + GLY				
Pb(II) 6.57 %+ Co(II) 0.44%+ Gly 93%(EECR-50)	0.9873	7790.247 ±389.5123	2.232716 ±0.15629	Antagonistic
Pb(II) 13.56 % + Co(II) 2.44% + Gly 84% (ABCR 1)	0.9892	7746.049 ±387.3024	4.631879 ±0.324232	Antagonistic
Co(II) +Cd(II)+ Ni(II)				
Co(II) 19.96%+Cd(II) 41.34% +Ni(II) 38.70% (EECR-50)	0.9361	131.87 ±6.5935	1.722919 ±0.120604	Antagonistic
Co(II) 26.96% + Cd(II) 43.34% + Ni(II) 29.79% (ABCR 1)	0.8480	85.8269 ±4.291345	1.065098 ±0.074557	Additive
Pb(II)+ Cd(II) + Zn(II)				
Pb(II) 46.95% + Cd(II) 6.56% + Zn(II) 46.48%(EECR-50)	0.9410	107.135 ±5.35675	0.257406 ±0.018018	Synergistic
Pb(II) 53.95% + Cd(II) 8.56% +Zn(II) 37.48%(ABCR 1)	0.9410	107.135 ±5.35675	0.280735 ±0.019651	Synergistic
Pb(II)+ Cd(II) + DDVP				
Pb(II) 49% + Cd(II) 6.85% + DDVP 44.15%(EECR-50)	0.9880	3013.905 ±150.6953	7.456264 ±0.521938	Antagonistic
Pb(II) 56% +Cd(II) 8.85% + DDVP 35.15%(ABCR 1)	0.9141	613.3511 ±30.66756	1.646434 ±0.11525	Antagonistic
Pb(II)+ Cd(II) + GLY				
Pb(II) 5.53% + Cd(II) 0.92% + Gly 92.55% (EECR-50)	0.9954	2997.205 ±149.8603	1.03992 ±0.072795	Additive
Pb(II) 13.53% + Cd(II) 2.92% +Gly 83.55%(ABCR 1)	0.9921	2345.45 ±117.2725	1.636084 ±0.114526	Antagonistic
Pb(II)+ Ni(II)+ DDVP				
Pb(II) 49.22% + Ni(II) 6.44% + DDVP 44.33%(EECR-50)	0.9491	773.034 ±38.6517	2.169552 ±0.151869	Antagonistic
Pb(II) 56.22% +Ni(II)8.44% + DDVP 35.33% (ABCR 1)	0.8813	576.7677 ±28.83839	1.806468 ±0.126453	Antagonistic

Pb(II)+ Ni(II)+ GLY				
Pb(II) 6.54% + Ni(II) 0.86% + Gly 92.60% (EECR-50)	0.9917	2929.367 ±146.4683	1.142167 ±0.079952	Antagonistic
Pb(II) 13.54% + Ni(II) 2.86% + Gly 83.60 %(ABCR 1)	0.9923	4418.984 ±220.9492	3.781046 ±0.264673	Antagonistic
Toxicant mixtures	**R^2 value**	**Mean EC_{50}**	**Toxic index**	**Toxic effect**
Pb(II)+ Zn(II)+GLY				
Pb(II) 6.19 % +Zn(II) 6.13% + Gly 87.68%(EECR-50)	0.9799	8852.51 ±442.6258	2.918349 ± 0.204284	Antagonistic
Pb(II) 13.19% + Zn(II) 8.13% + Gly 78.68% (ABCR 1)	0.9309	6630.037 ±331.5019	3.233568 ±0.22635	Antagonistic
Co(II)+ Cd(II)+ Zn(II)				
Co(II) 5.64% + Cd(II) 11.68% + Zn(II) 82.68% (EECR-50)	0.9766	609.1586 ±30.45793	1.923643 ±0.134655	Antagonistic
Co(II) 12.64% + Cd(II) 13.68 %+Zn(II) 73.68%(ABCR 1)	0.9634	628.8253 ±31.44127	2.444242 ±0.171097	Antagonistic
Co(II)+ Cd(II)+ DDVP				
Co(II) 6.09 % + Cd(II)12.62% + DDVP 81.29%(EECR-50)	0.9599	507.0499 ±25.3525	1.698705 ±0.118909	Antagonistic
Co(II) 13.09% +Cd(II) 14.62% + DDVP 72.29% (ABCR 1)	0.9652	619.3457 ±30.96729	2.521924 ±0.176535	Antagonistic
Co(II)+ Ni(II)+Pb(II)				
Co(II) 5.63%+ Ni(II) 10.92%+ Pb(II) 83.44% (EECR-50)	0.9422	224.2232 ±11.21116	0.900111 ±0.06300	Additive
Co(II) 12.43% + Ni(II) 7.12 % + Pb 80.44%(ABCR 1)	0.8832	173.7899 ±8.689495	0.684768 ±0.047934	Synergistic
Co(II)+ Ni(II)+DDVP				
Co(II) 6.14% + Ni(II) 11.91% + DDVP(II) 81.95%(EECR-50)	0.9767	759.0663 ±37.95332	3.01506 ±0.211054	Antagonistic
Co(II) 13.14% +Ni(II) 13.91% +DDVP 72.94%(ABCR 1)	0.9804	722.1747 ±36.10874	3.472902 ±0.243103	Antagonistic
Co(II)+ Ni(II)+GLY				
Co(II) 0.47% + Ni(II) 0.91% + Gly 98.61%(EECR-50)	0.9536	5921.268 ±296.0634	1.907724 ±0.133541	Antagonistic
Co(II) 7.47% + Ni(II) 2.91% + Gly 89.61%(ABCR 1)	0.9054	4491.315 ±224.5657	5.835513 ±0.408486	Antagonistic
Cd(II)+ Ni(II)+Pb(II)				
Cd(II)5.63% +Ni(II) 10.92% + Pb(II) 83.44%(EECR-50)	0.9584	218.1428 ±10.90714	0.899802 ±0.062986	Synergistic
Cd(II) 8.63% + Ni(II) 8.92% + Pb(II) 82.44%(ABCR 1)	0.8818	154.628 ±7.7314	0.634339 ±0.044404	Synergistic
Cd(II)+ Ni(II)+Co(II)				
Cd(II) 41.33% + Ni(II) 38.70 % +Co(II) 19.97%(EECR-50)	0.9912	117.6818 ±5.88409	1.537546 ±0.107628	Antagonistic
Cd(II) 44.34% + Ni(II) 36.70% + Co(II) 18.96% (ABCR 1)	0.9918	80.3648 ±4.01824	1.042312 ±0.072962	Additive
Cd(II)+ Ni(II)+Zn(II)				
Cd(II) 11.10% + Ni(II) 78.52% +Zn(II) 10.38%(EECR-50)	0.9836	326.5972 ±16.32986	4.817211 ±0.337205	Antagonistic
Cd(II) 18.10%+Ni(II) 80.52 % + Zn(II) 1.38% (ABCR 1)	0.9707	280.5185 ±14.02593	4.412913 ±0.308904	Antagonistic
Cd(II)+ Ni(II)+DDVP				
Cd(II) 11.18% + Ni(II) 11.94)% + DDVP 78.88%(EECR-50)	0.9842	642.7227 ±32.13614	2.941698 ±0.205919	Antagonistic
Cd(II) 18.18% + Ni(II) 13.94% + DDVP 67.88%(ABCR 1)	0.9656	726.1165 ±36.30583	4.030759 ±0.282153	Antagonistic
Ni(II)+ Zn(II)+Pb(II)	0.9473	1019.566 ±50.9783	2.774134 ±0.194189	Antagonistic
Ni(II) 6.17% + Zn((II) 46.68% + Pb(II) 47.15%(EECR-50)	0.8897	1005.621 ±50.28106	1.959763 ± 0.137183	Antagonistic
Ni(II) 1.17% + Zn(II) 52.48% +				

Pb(II) 46.35%(ABCR 1)

Toxicant mixtures	R^2 value	Mean EC_{50}	Toxic index	Toxic effect
Ni(II)+Zn(II)+Co(II)	0.9907	327.9372± 16.39686	4.870712 ±0.34095	Antagonistic
Ni(II) 83.30% + Zn(II) 11.02% + Co(II) 5.68%(EECR-50)	0.9926	278.7988 ±13.93994	3.904981 ±0.273349	Antagonistic
Ni(II) 77.80% +Zn(II) 16.51% + Co(II) 5.68% (ABCR 1)				
Ni(II)+Zn(II)+DDVP				
Ni(II) 44.55% + Zn(II) 48.97% + DDVP 6.48%(EECR-50)	0.9452	17.20216 ±344.043	2.910784 ±0.203755	Antagonistic
Ni(II) 49.55% +Zn(II) 43.97% + DDVP 6.48%(ABCR 1)	0.9786	275.7354 ±13.78677	2.544963 ±0.178147	Antagonistic
Zn(II)+ DDVP+Pb(II)				
Zn(II) 34.25 % + DDVP31.16% + Pb(II)34.59%(EECR-50)	0.9260	1214.918 ±60.74592	2.128142 ±0.14897	Antagonistic
Zn(II) 41.24% +DDVP 24.16% +Pb 34.60%(ABCR 1)	0.8628	1371.122 ±68.55608	2.281047 ±0.159673	Antagonistic
Zn(II)+ DDVP+Co(II)				
Zn(II) 45.99% + DDVP 50.56% + Co(II) 3.45%(EECR-50)	0.9916	963.0842 ±48.15421	1.836822 ±0.128578	Antagonistic
Zn(II) 50.99% + DDVP45.56% +Co(II) 3.45% (ABCR 1)	0.9845	668.342± 33.4171	1.271939 ±0.089036	Additive
Zn(II)+ DDVP + GLY				
Zn(II) 6.17% + DDVP 5.61% + Gly(88.22)(EECR-50)	0.9956	2068.249 ±103.4125	0.62865 ±0.044006	Synergistic
Zn(II) 13.17 % +DDVP 7.61% +Gly(II) 79.22% (ABCR 1)	0.9819	1927.104 ±96.35519	0.8449 ±0.059143	Synergistic
DDVP+ GLY+Pb(II)				
DDVP 5.61% +Gly(II) 88.17%+ Pb(II) 6.22%(EECR-50)	0.9919	158.371 ±3167.42	1.036332 ±0.072543	Additive
DDVP 12.11% +Gly 81.67% + Pb(II) 6.23%(ABCR 1)	0.9938	3157.067 ±157.853	1.052661 ±0.094686	Additive
DDVP+ GLY +Co(II)				
DDVP 5.95% + Gly 93.60 % + Co(II) 0.45%(EECR-50)	.9913	4167.943 ±208.3972	1.079678 ±0.075577	Additive
DDVP 7.95% + Gly 90.60% + Co(II) 1.45%(ABCR 1)	0.9968	3373.269 ±168.6635	1.087302 ±0.090111	Additive
DDVP+ GLY+ Cd(II)				
DDVP 5.92% +Gly 93.16% + Cd(II) 0.92%(EECR-50)	0.9953	5543.097 ±277.1549	1.773405 ±0.124138	Antagonistic
DDVP 7.92% + Gly 88.16% + Cd(II) 3.92%(ABCR 1)	0.9856	5091.917 ±254.5958	3.483781 ±0.243865	Antagonistic
DDVP+ GLY+ Ni(II)				
DDVP 5.93% +Gly 93.21% + Ni(II) 0.86%(EECR-50)	0.9885	4778.476 ±238.9238	1.817557 ±0.127229	Antagonistic
DDVP 11.42%+Gly 84.21% + Ni(II) 4.36%(ABCR 1)	0.9900	4526.675 ±226.3337	4.702881 ±0.329202	Antagonistic

Values are represented as Mean ± STD. Pb= lead, Zn=zinc, Co = Colbalt, Ni= Nickel, Cd= Cadmium, Gly= Glyphoate, DDVP = Diclovores. EECR-50 = EC 50 Experimental concentration ratio, ABCR = Arbitrary concentration ratio. ND=Not determined

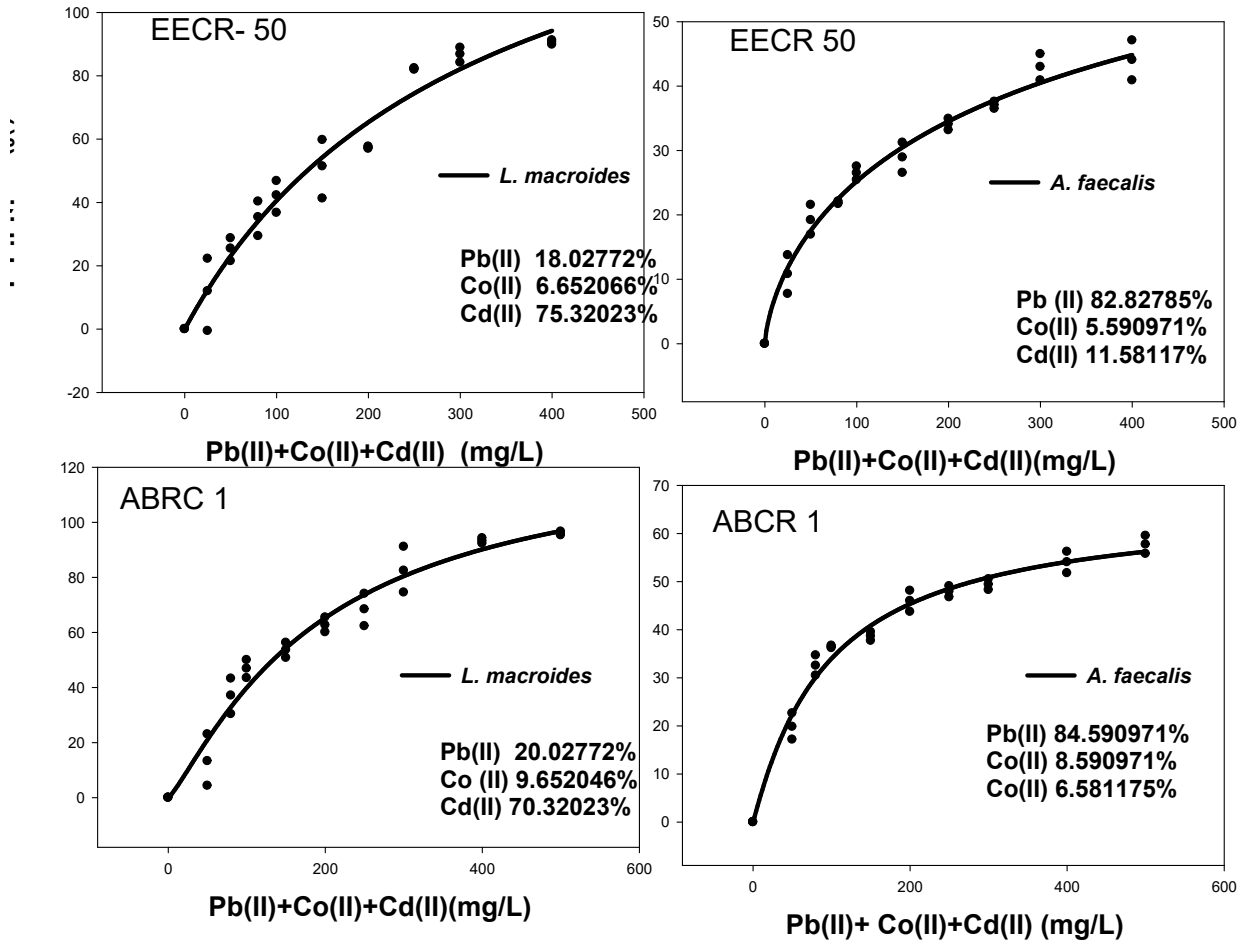


Figure 4.27: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624)total dehydrogenase activity to Ternary Mixtures of metal ions (Pb (II),)and pesticides (Glyphosate and DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model.

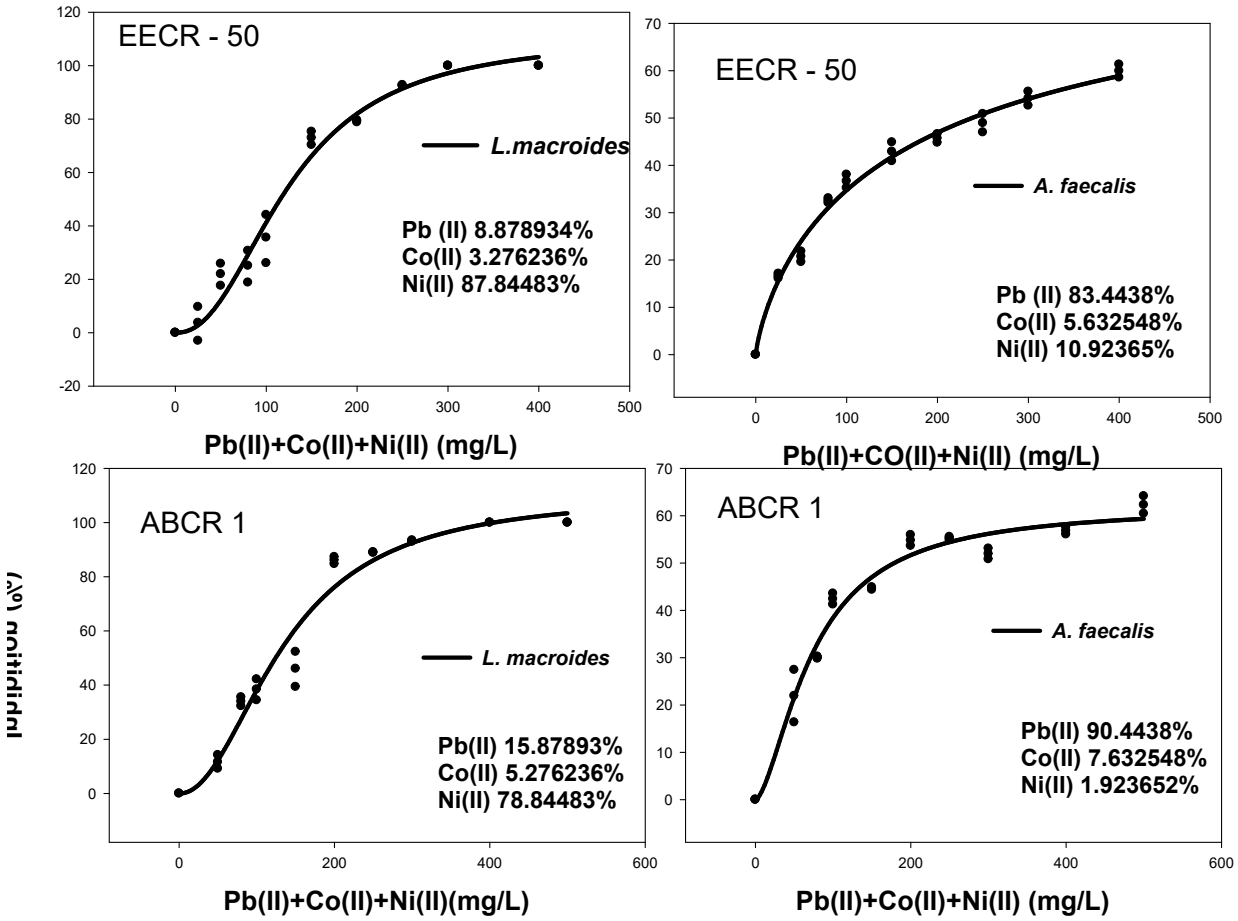


Figure 4.28: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Pb (II) +Co (II) + Ni(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

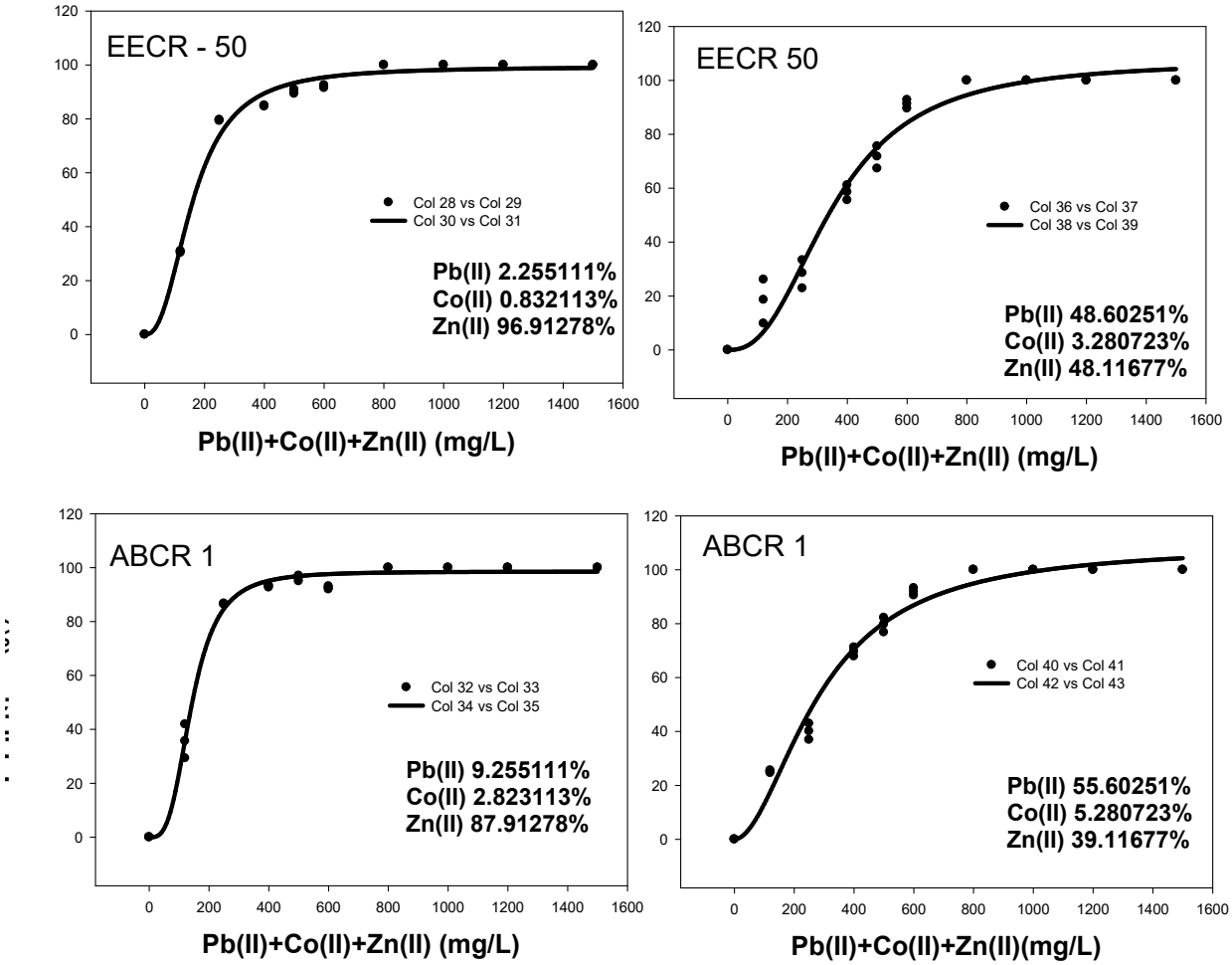


Figure 4.29: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Pb(II)+Co (II) + Zn(II))toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

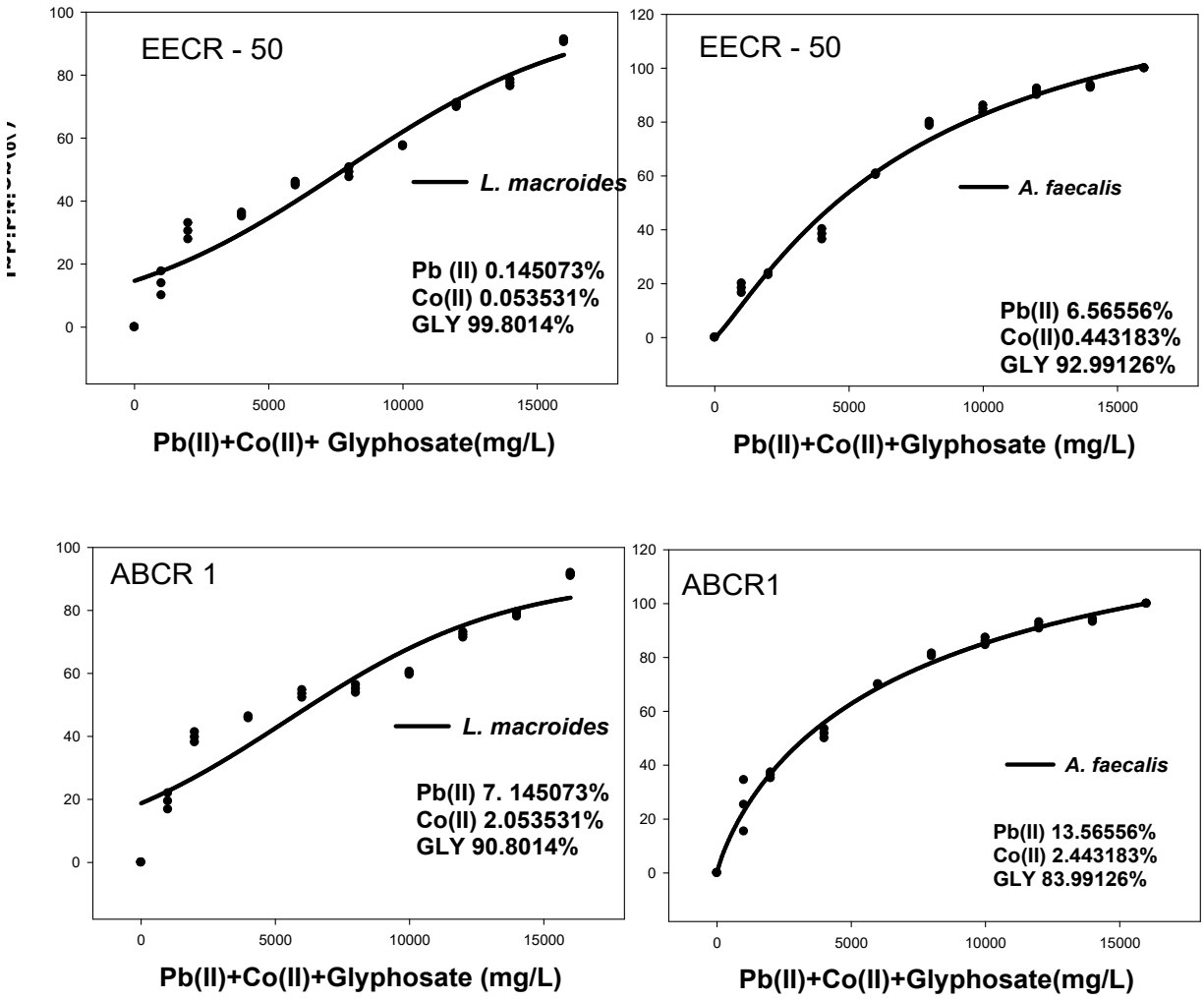


Figure 4.30: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Co (II) + Pb (II)) and pesticides (GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

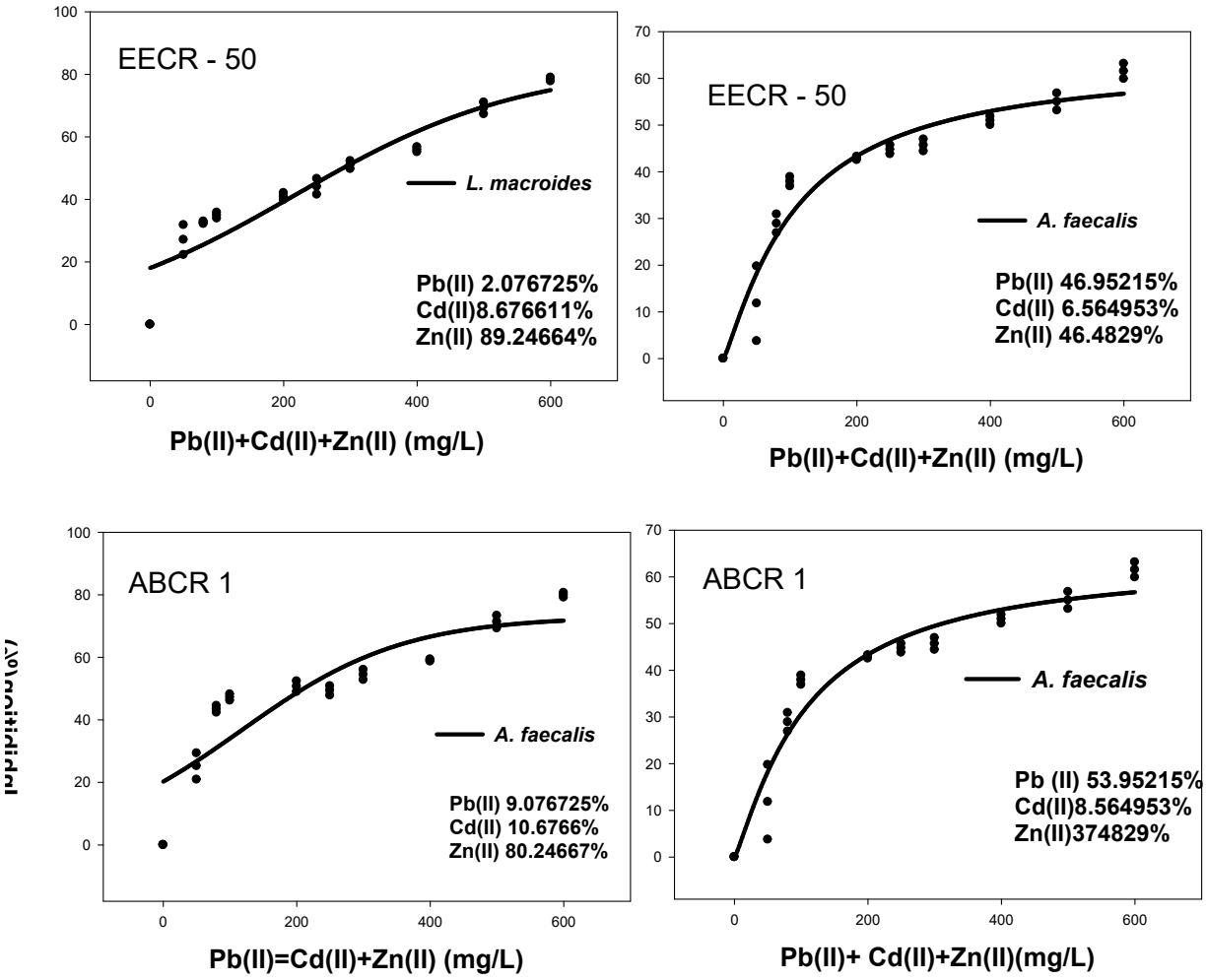


Figure 4.31: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Pb (II) +Cd (II) + Zn(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

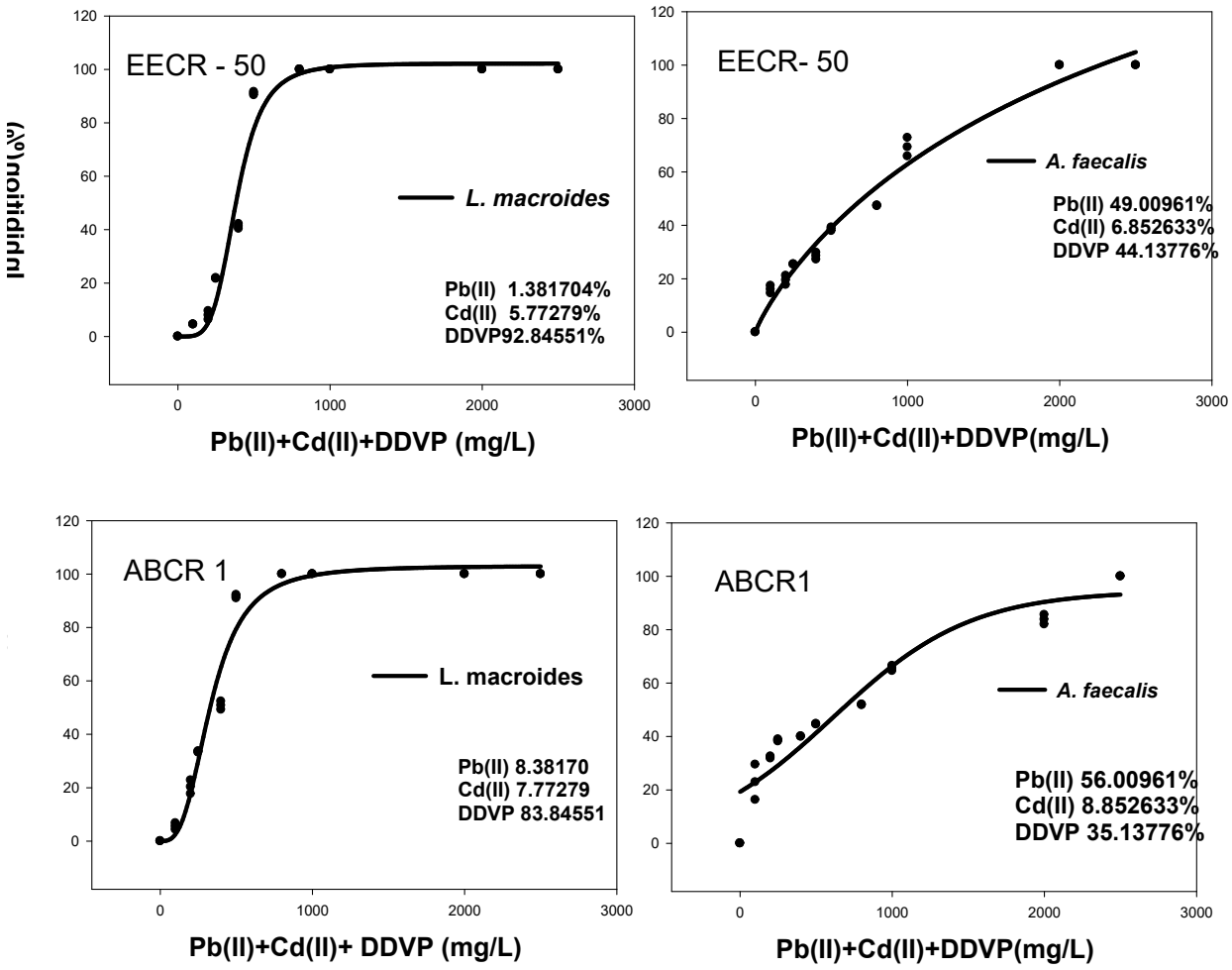


Figure 4.32: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Pb (II))and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

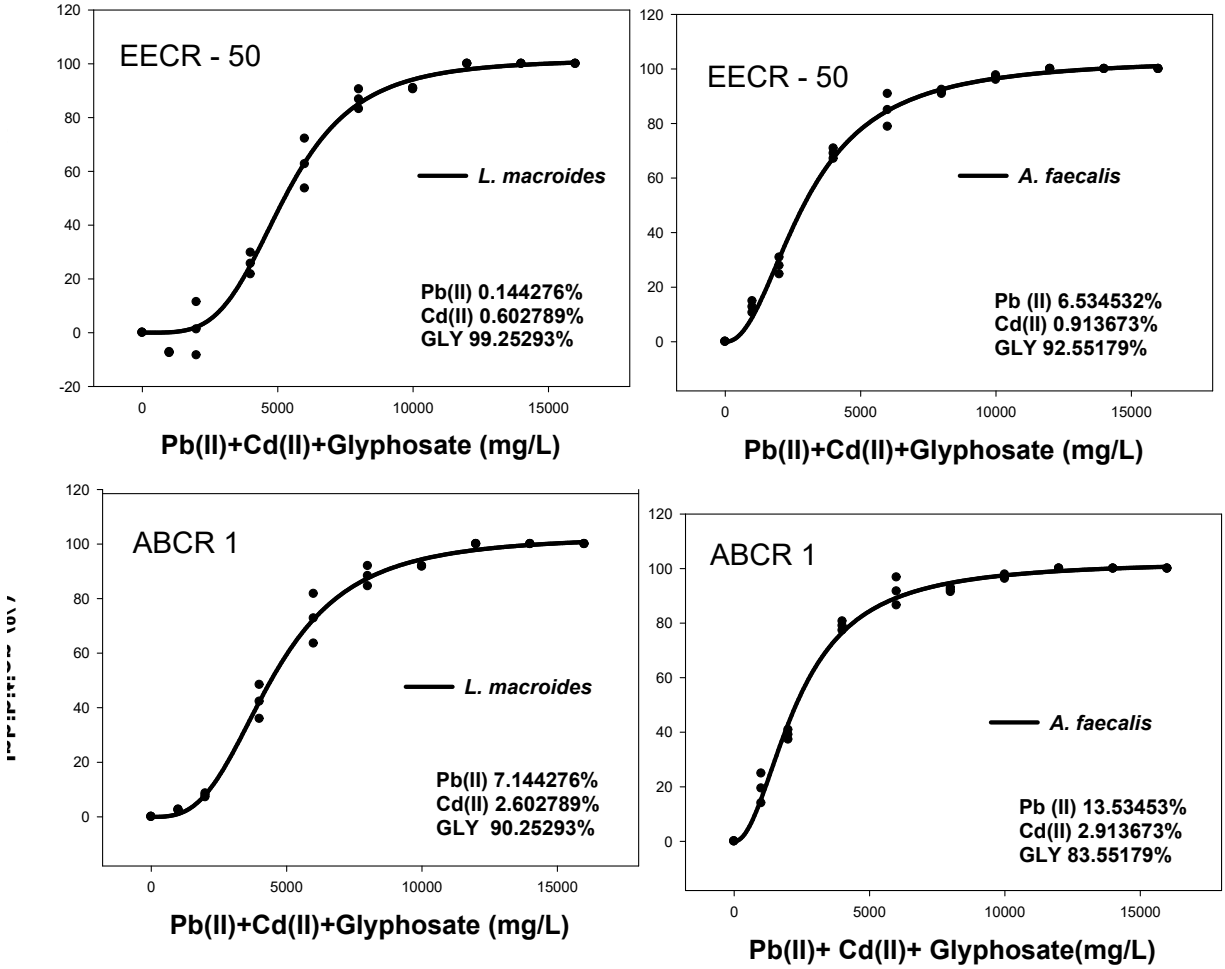


Figure 4.33: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Pb (II)) and pesticides (GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

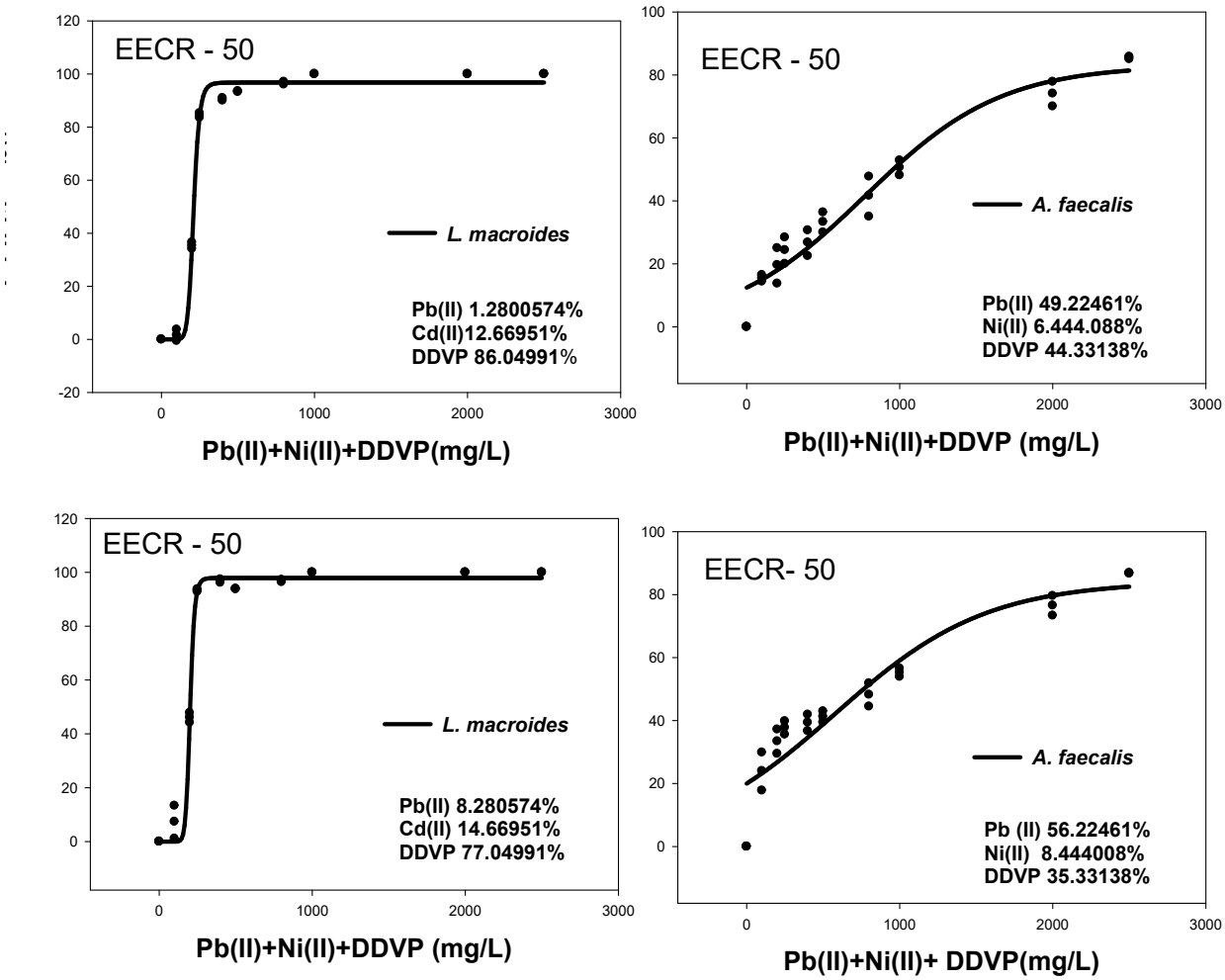


Figure 4.34: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Pb (II) + Ni (II)) and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

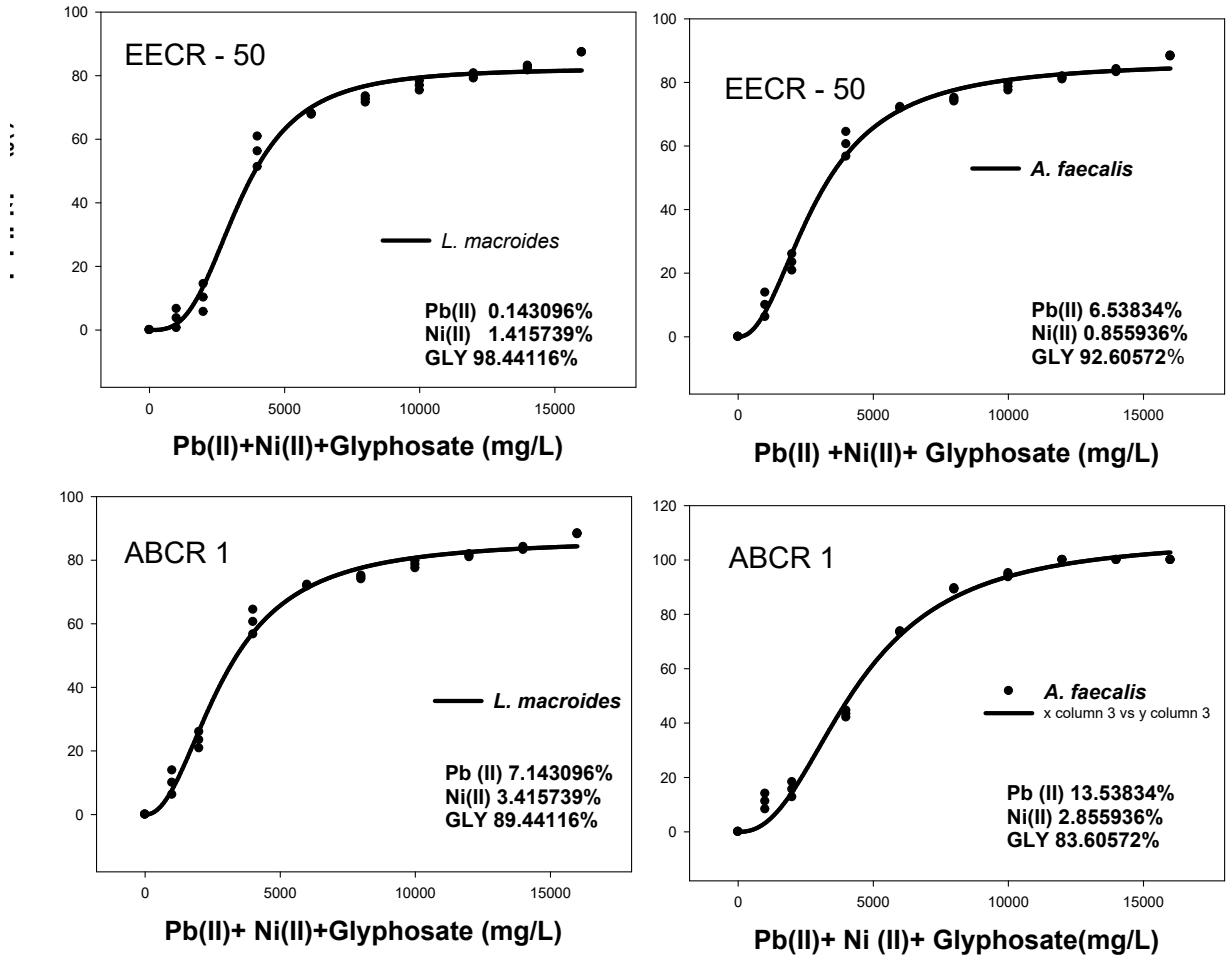


Figure 4.35: Response of Lysinibacillus macroides (OK298881) and Alcaligenes faecalis (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Pb (II) + Ni(II))and pesticides (GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

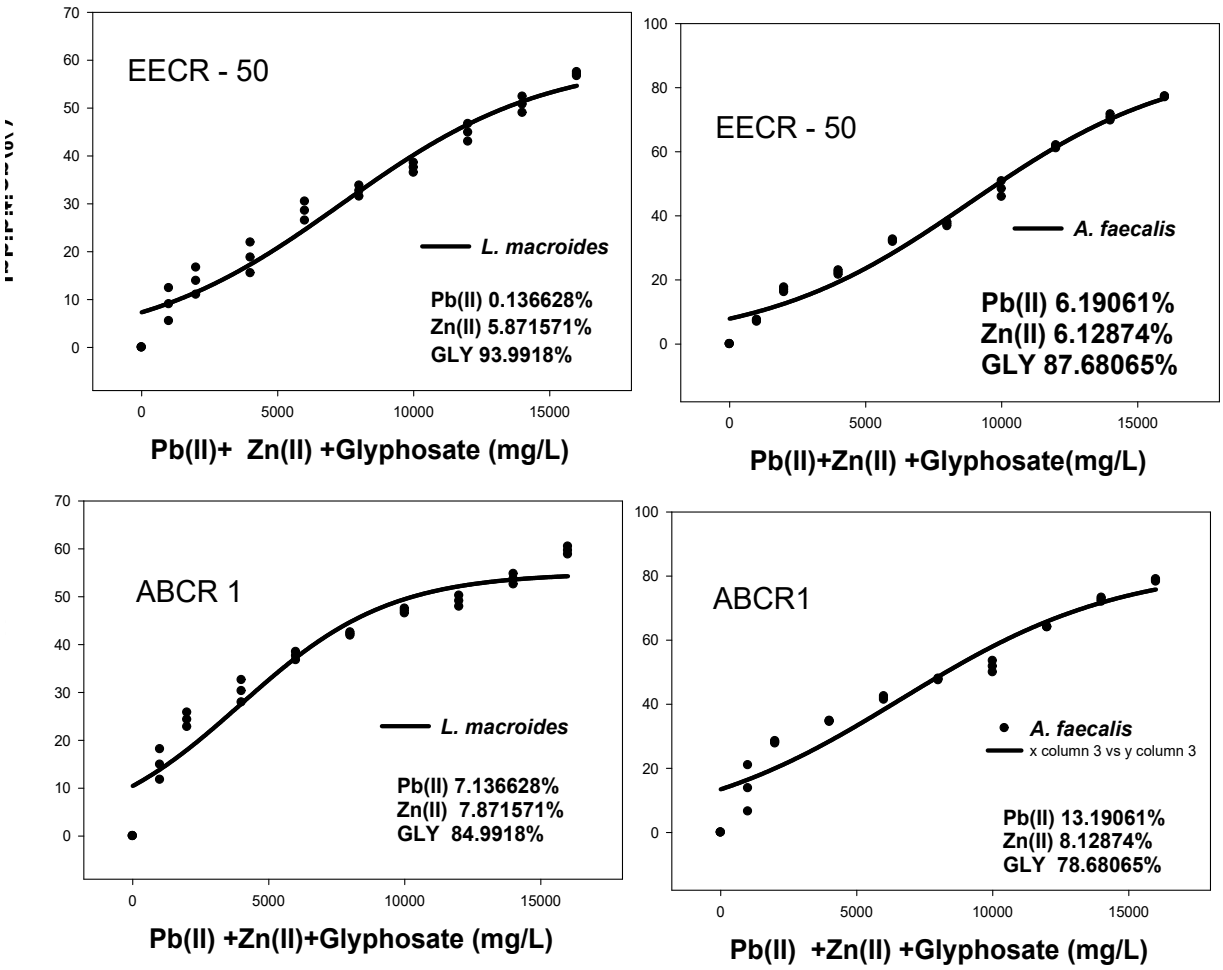


Figure 4.36: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Pb (II) + Zn(II))and pesticides (GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

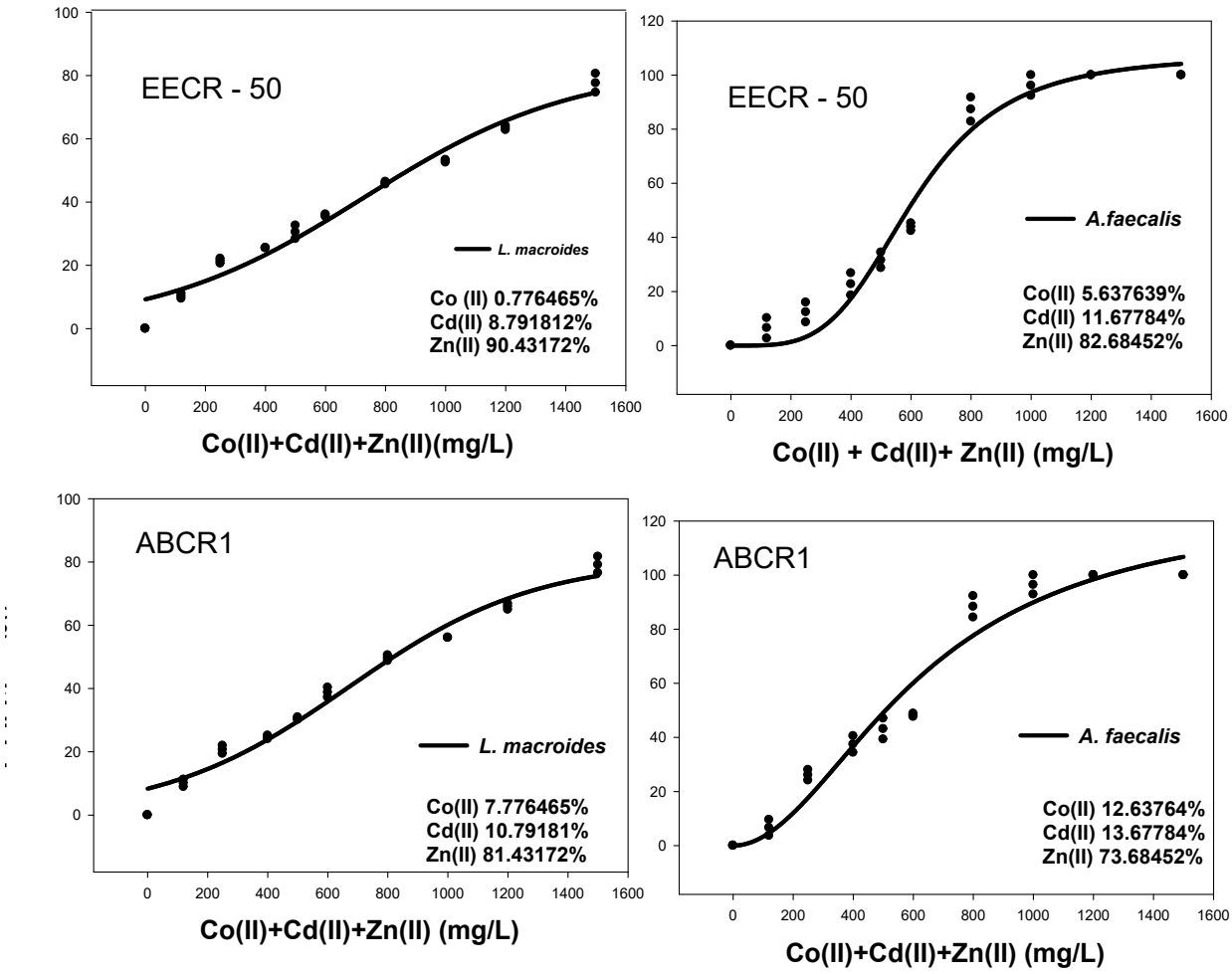


Figure 4.37: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Co(II)+Cd (II) + Zn(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

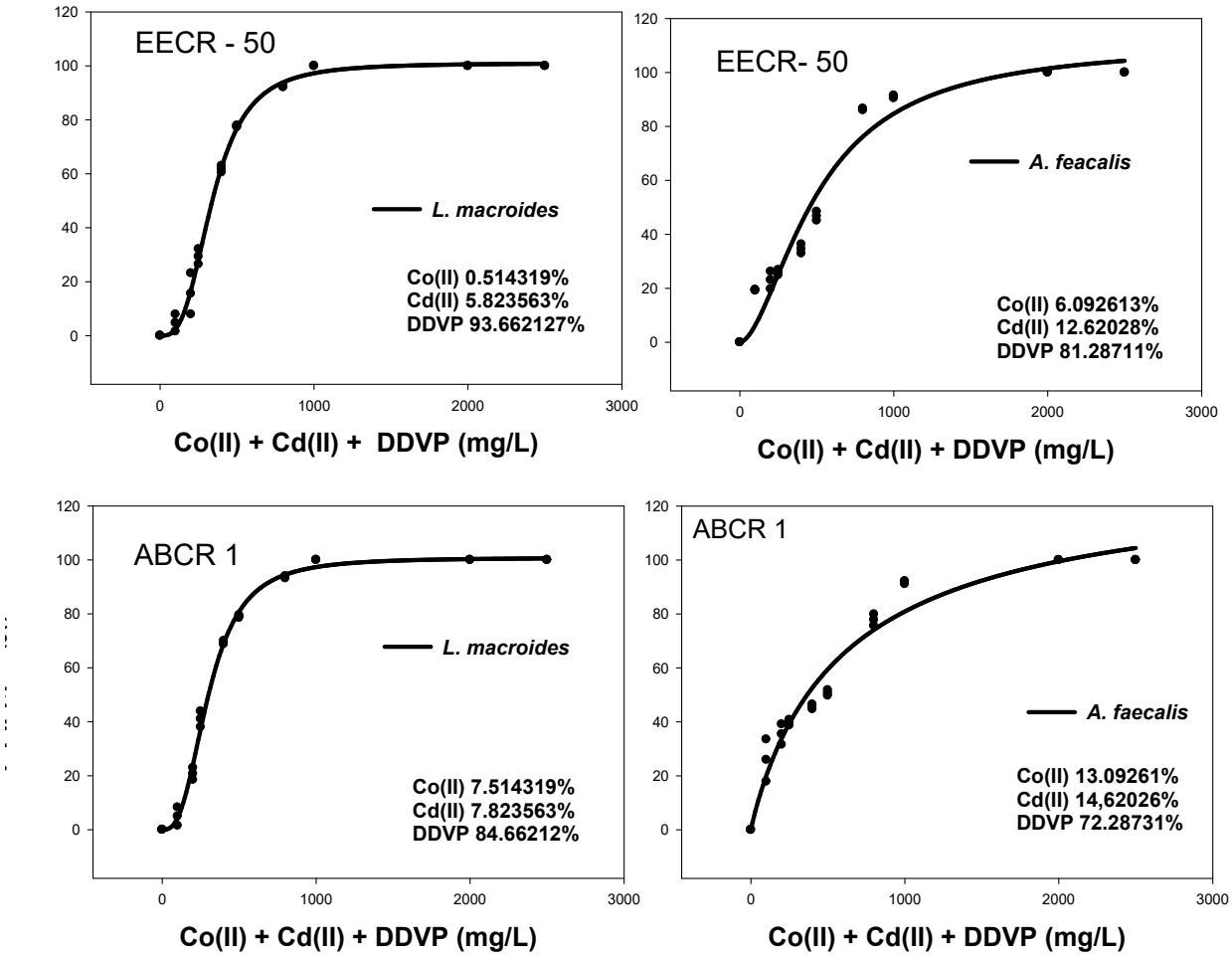


Figure 4.38: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Coi(II))and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

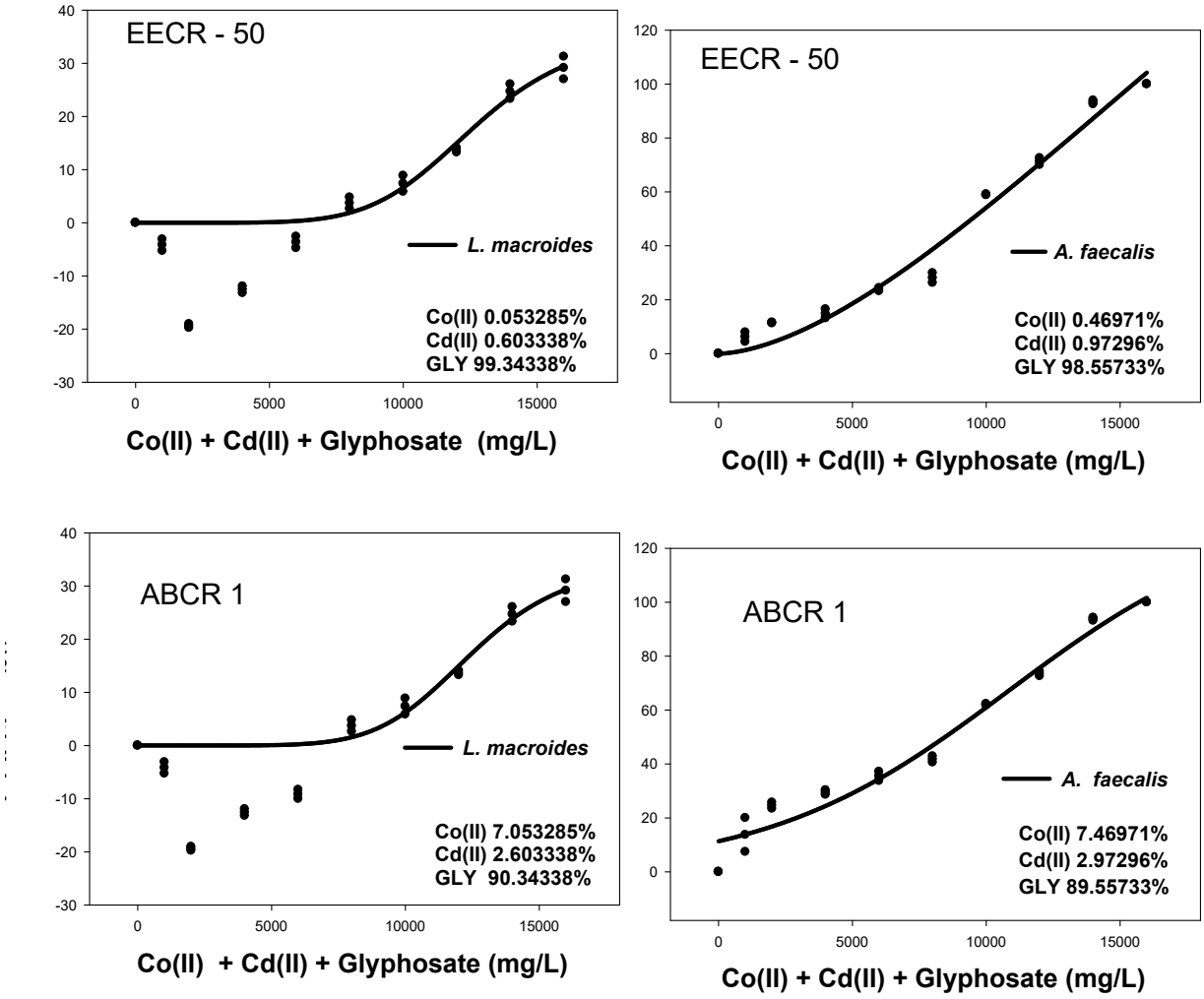


Figure 4.39: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Co(II))and pesticides (GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

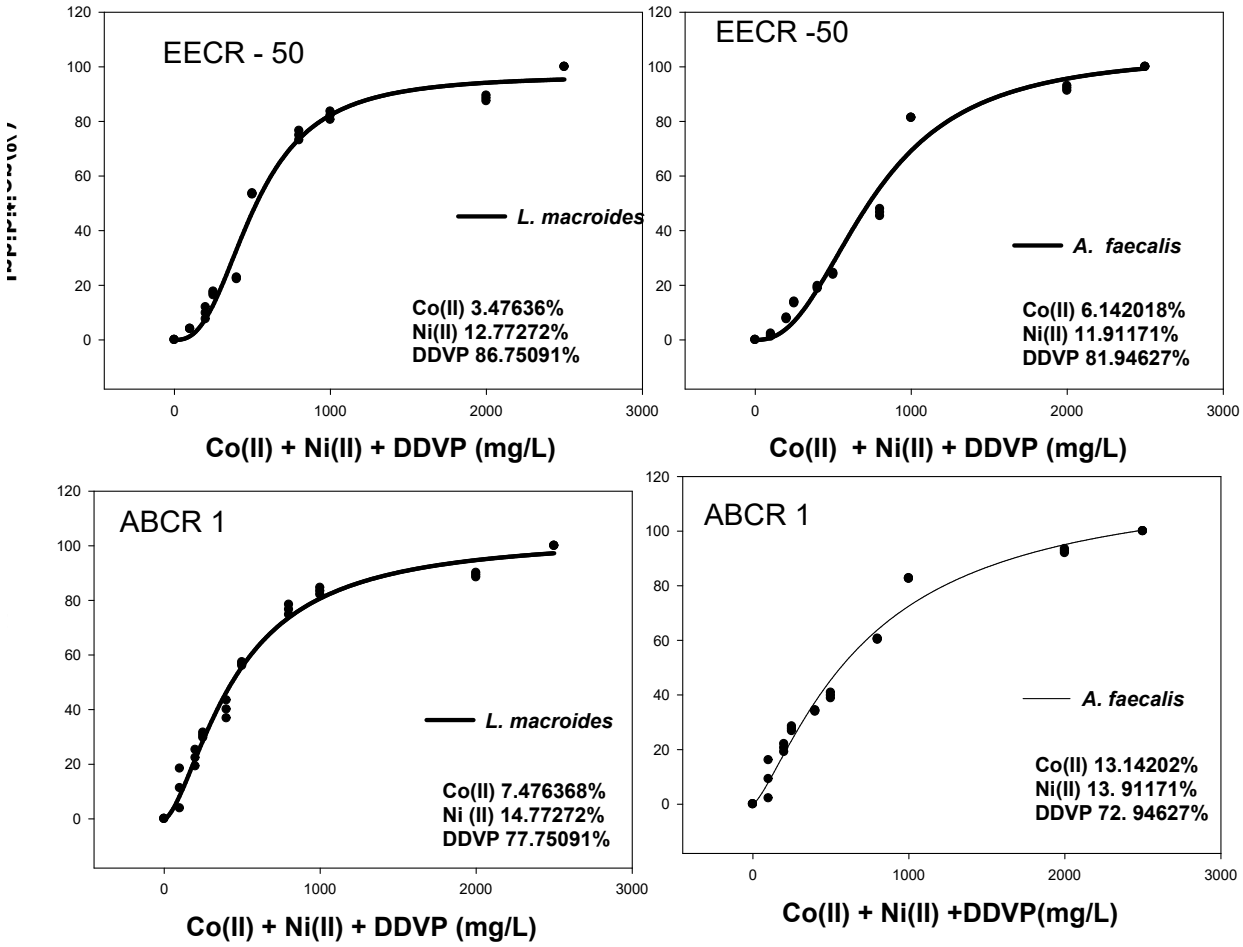


Figure 4.40: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Co (II) + Ni(II))and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

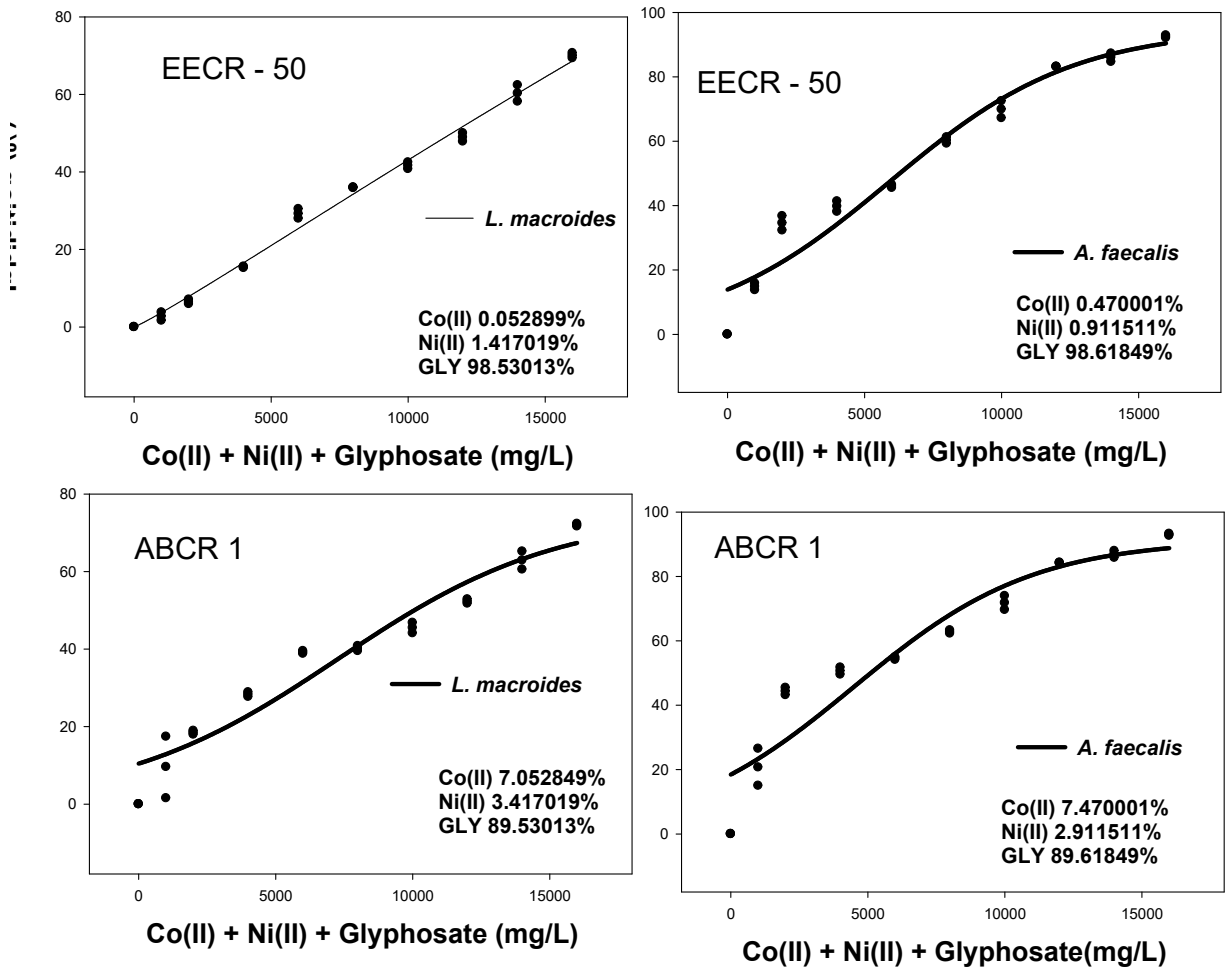


Figure 4.41: Response of Lysinibacillus macroides(OK298881) and Alcaligenes faecalis(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Co (II) + Ni(II))and pesticides (GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

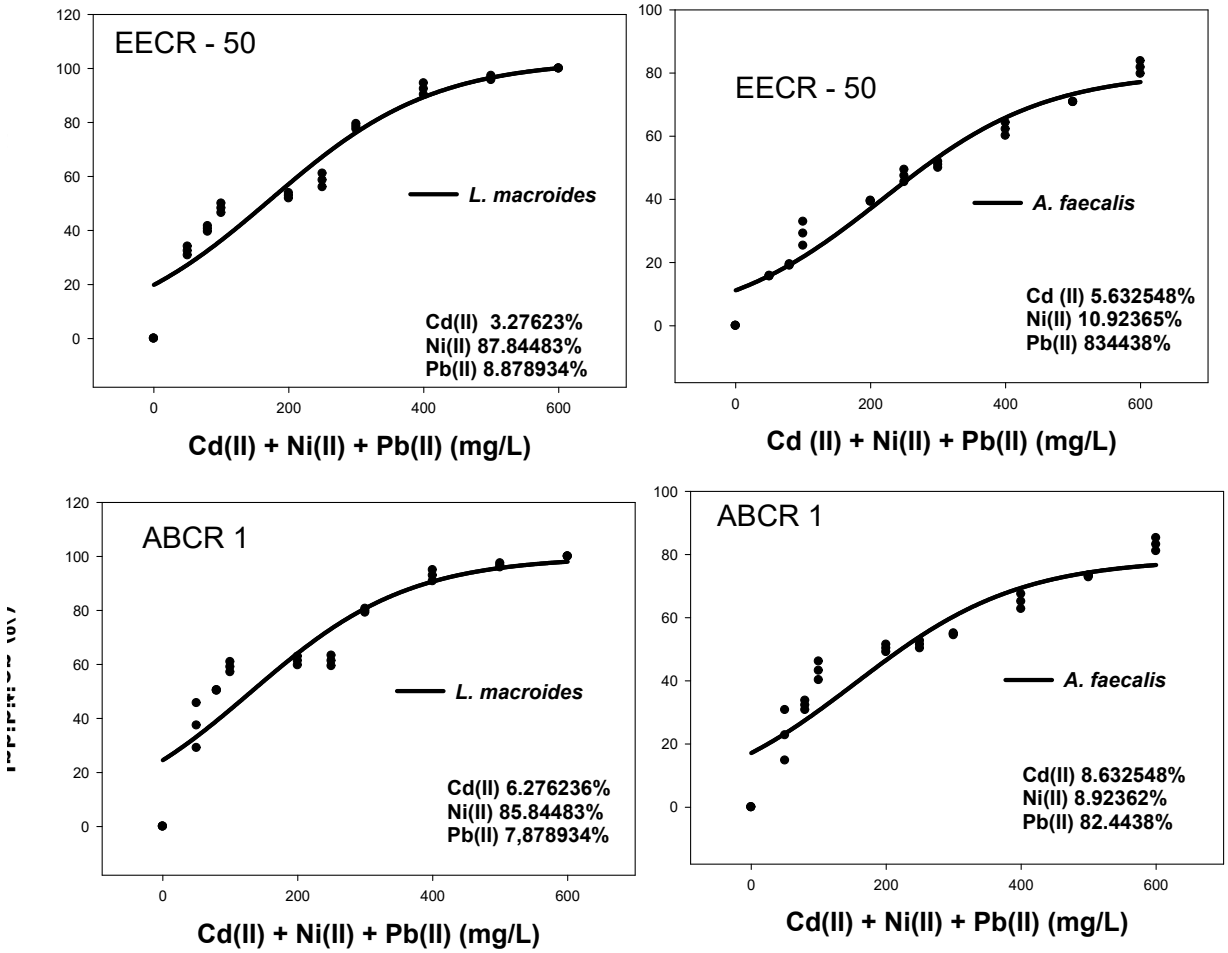


Figure 4.42: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis* (KX302624)total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Ni(II)+ Pb(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

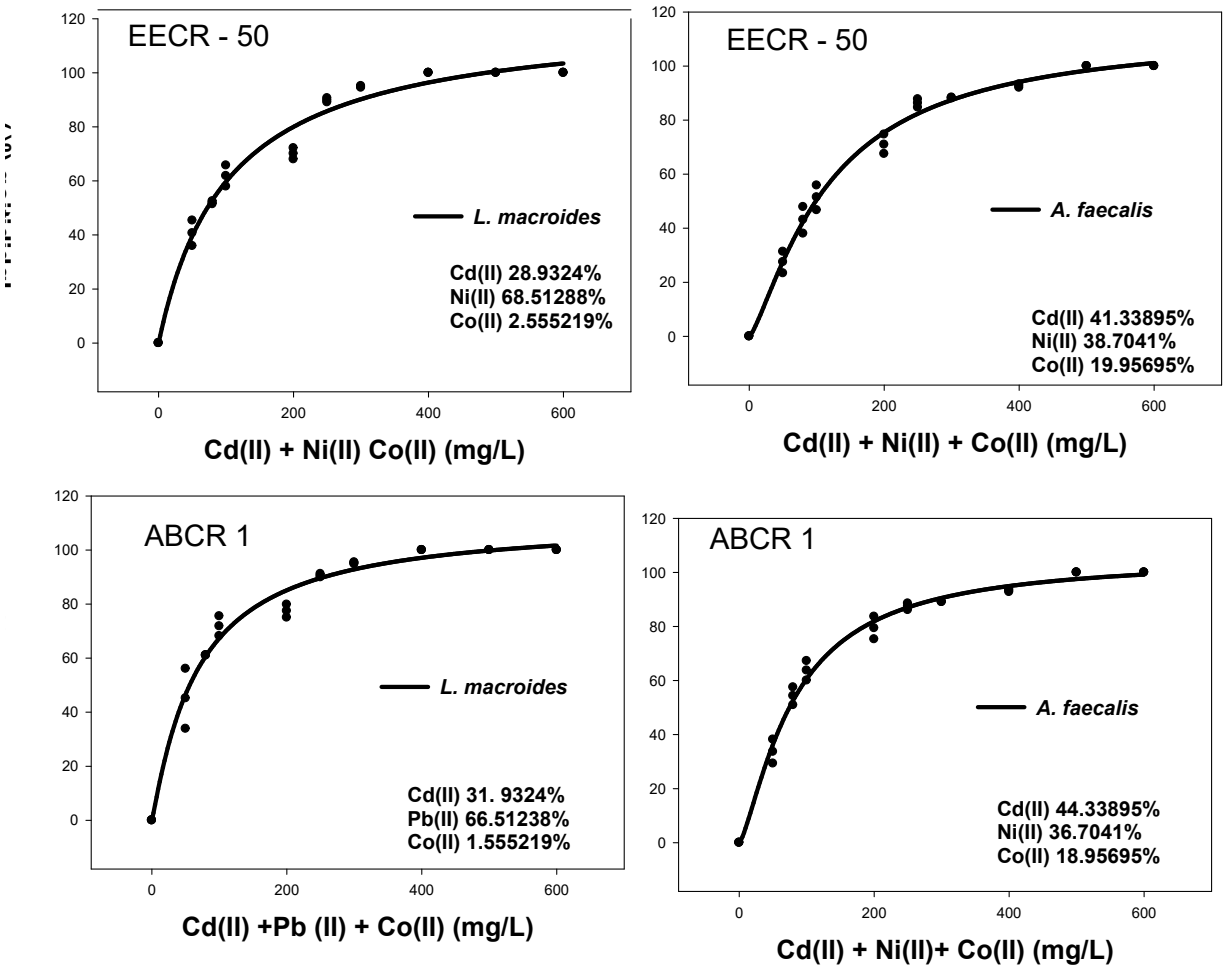


Figure 4.43: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Ni(II) + Co(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

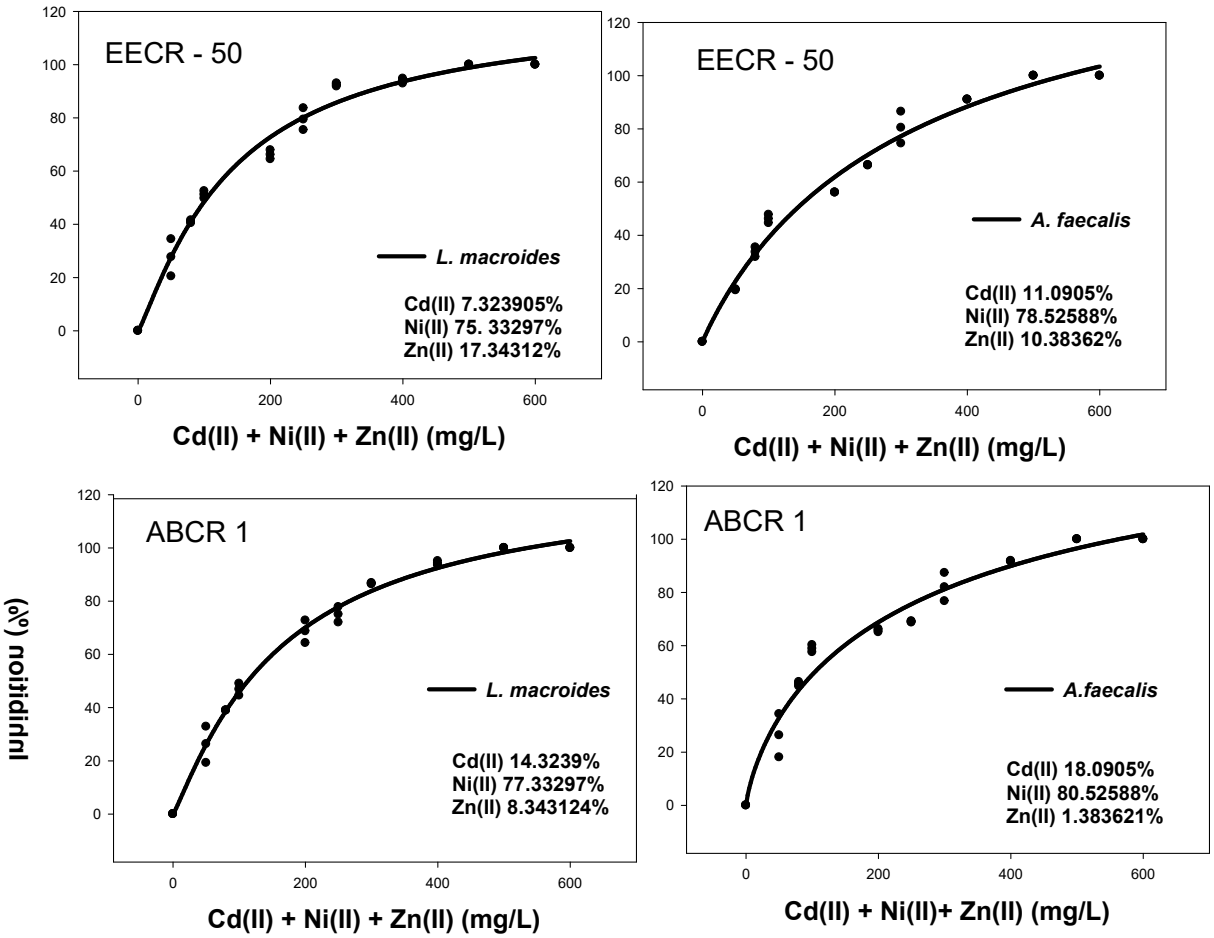


Figure 4.44: Response of Lysinibacillus macroides(OK298881) and Alcaligenes faecalis(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Ni(II)+Zn(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

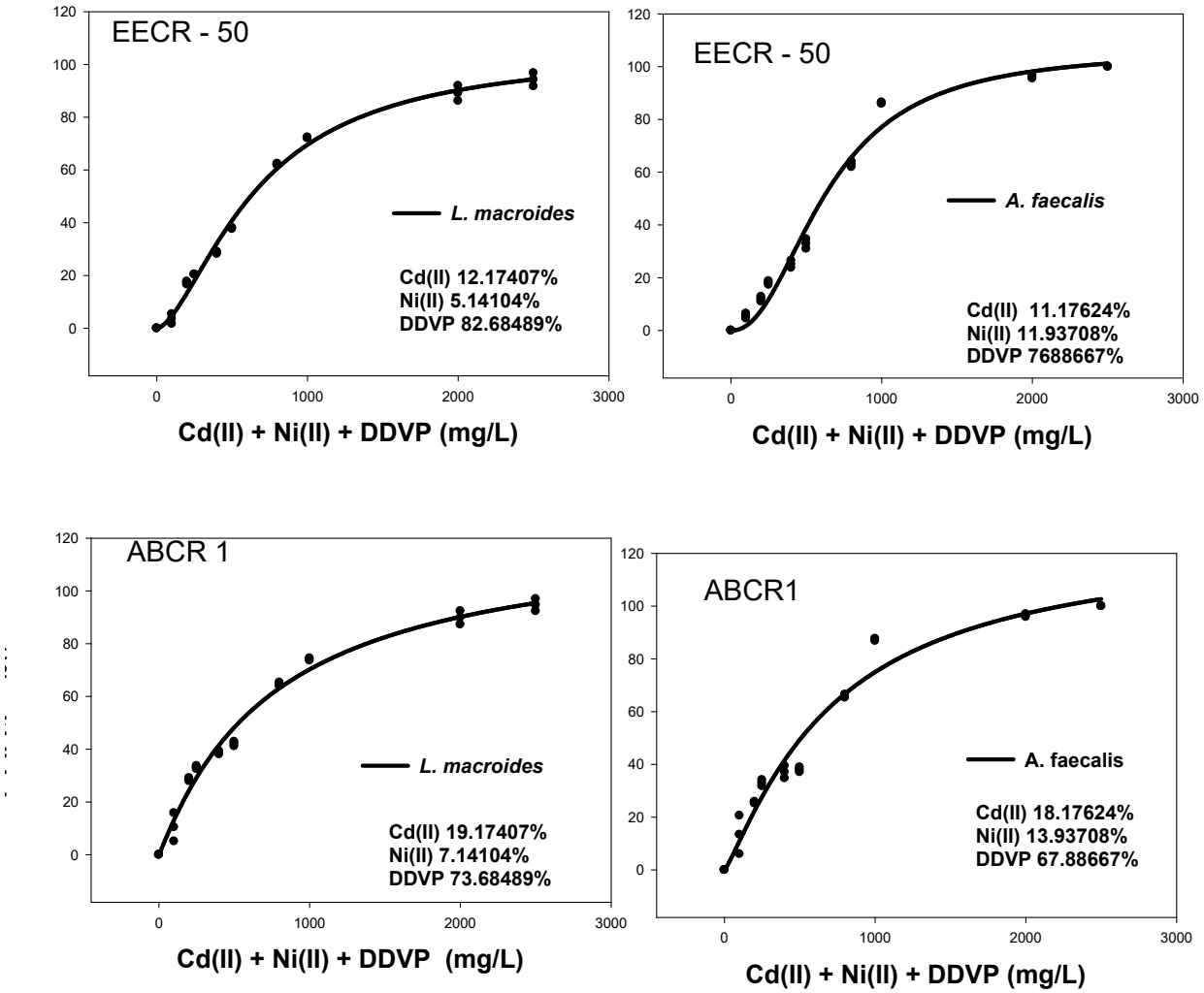


Figure 4.45: Response of Lysinibacillus macroides (OK298881) and Alcaligenes faecalis(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Ni(II)) and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model.

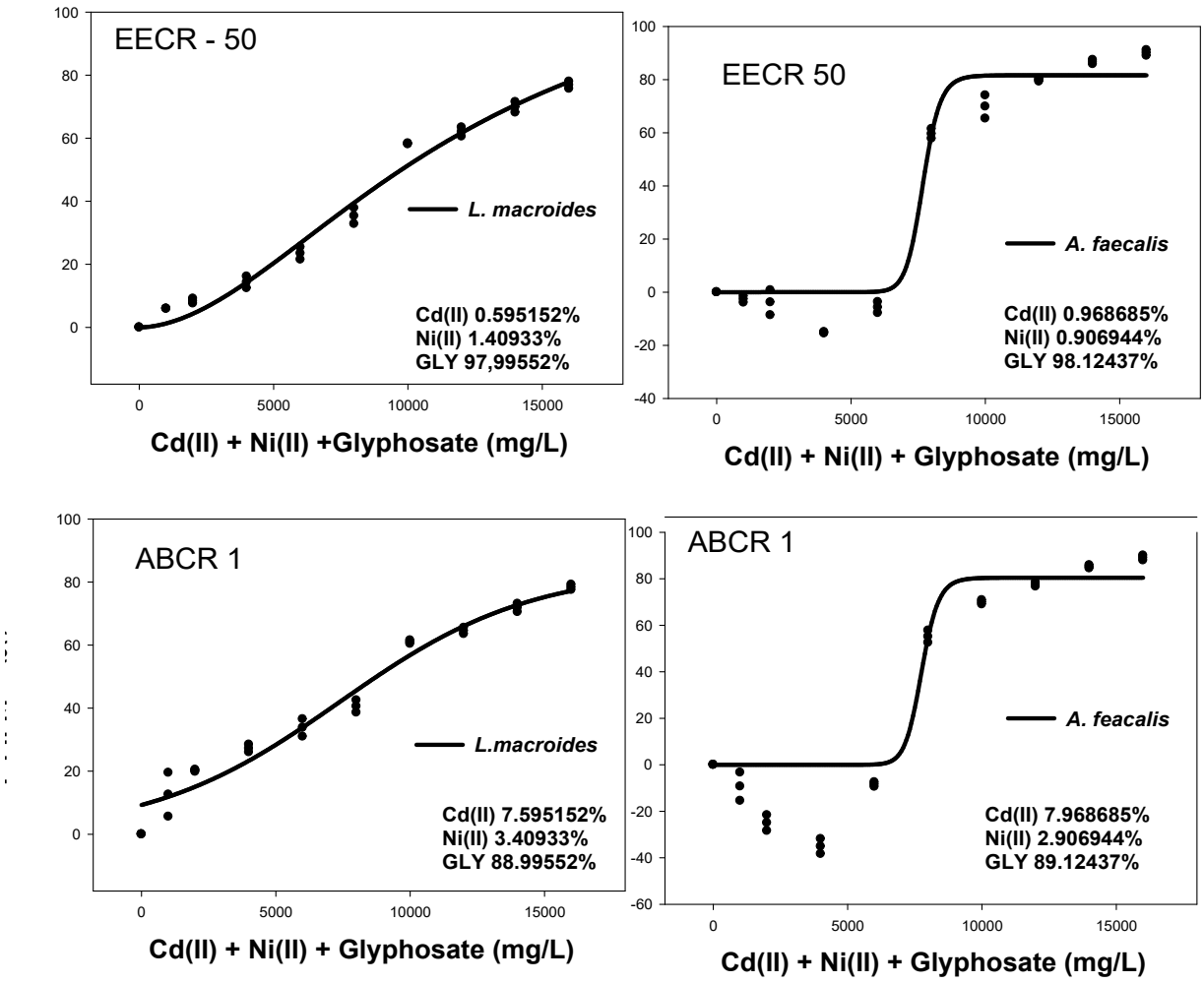


Figure 4.46: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Cd (II) + Ni(II))and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

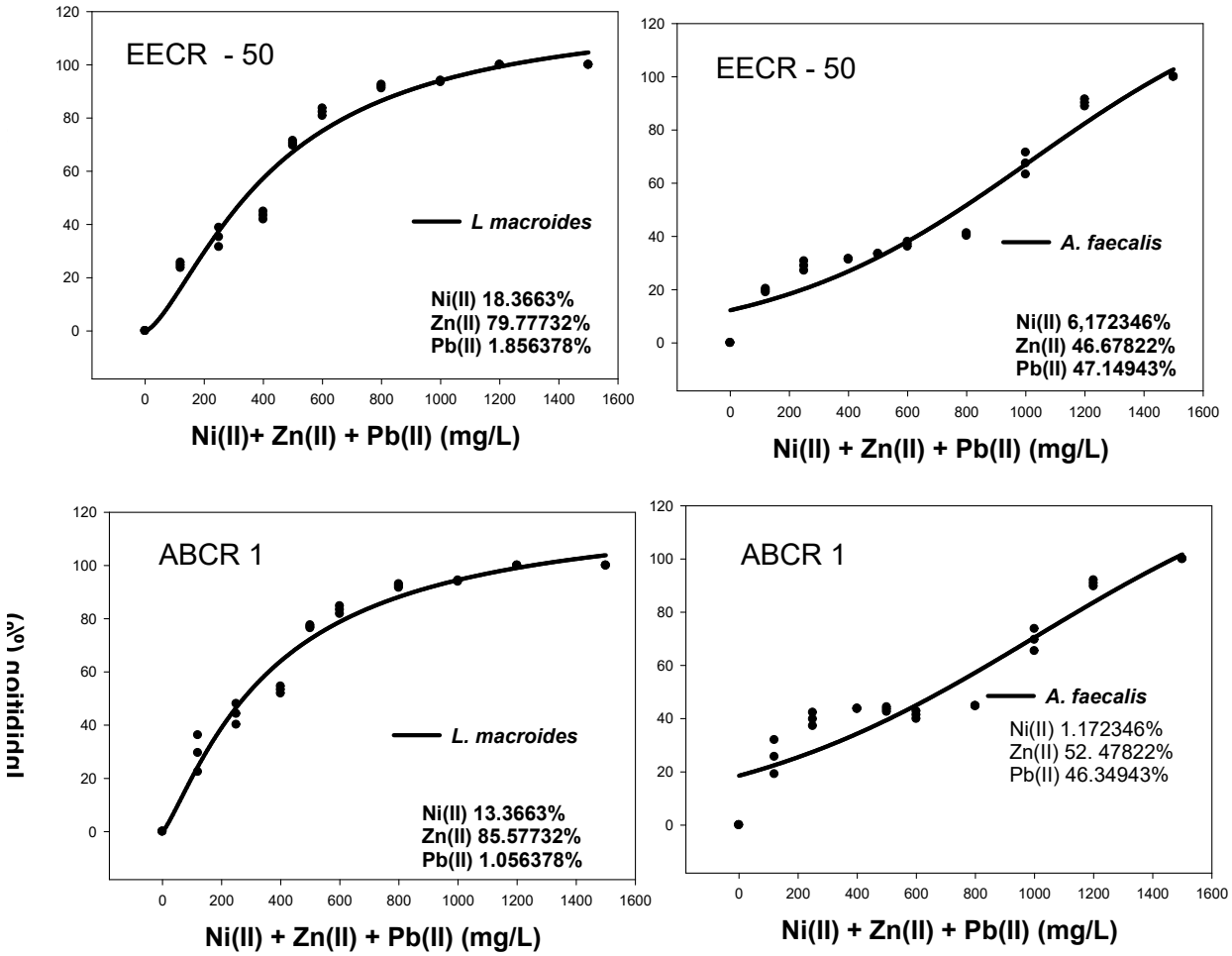


Figure 4.47: : Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Ni(II) + Zn(II)+ Pb(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

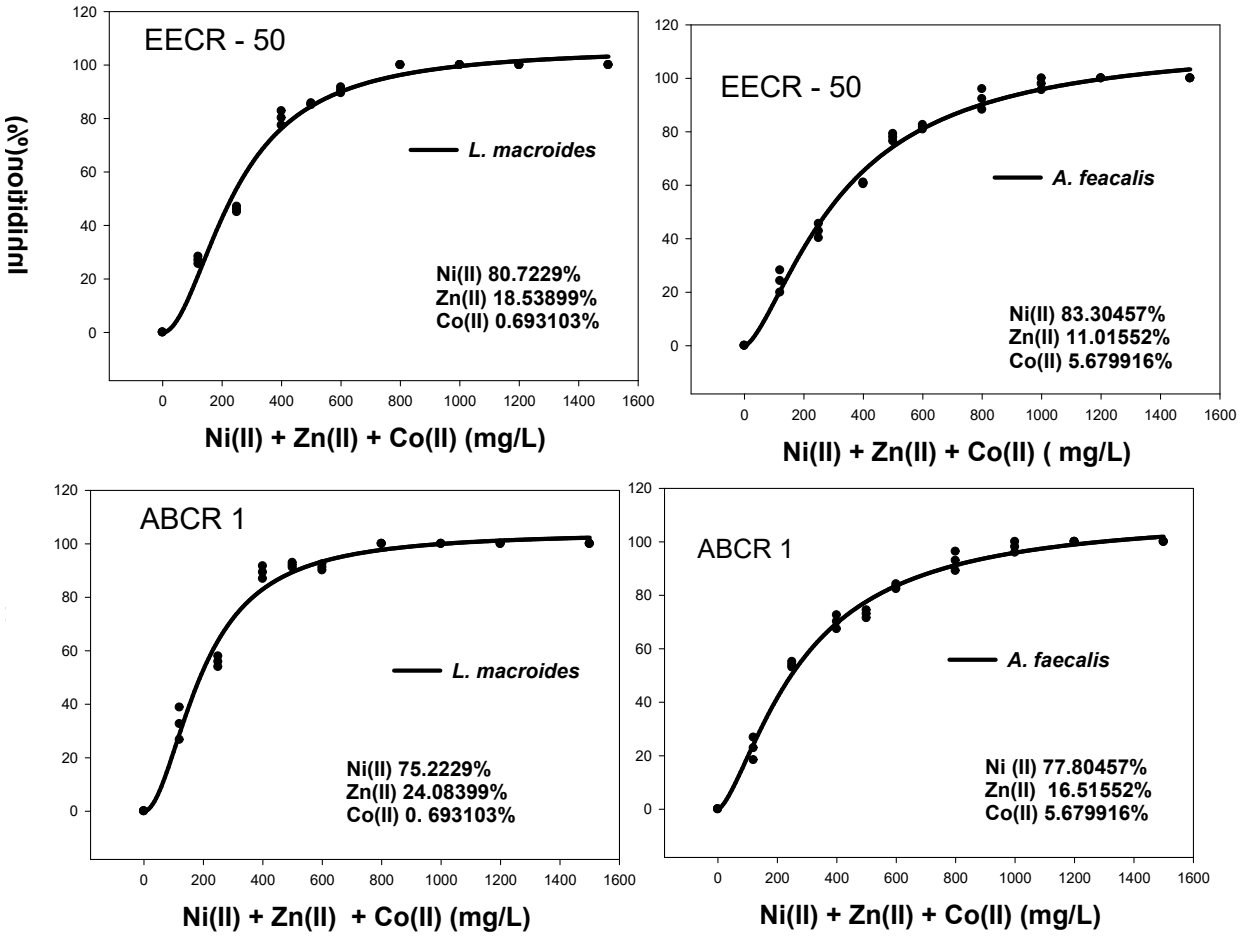


Figure 4.48: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Ni(II) + Zn(II)+ Co(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

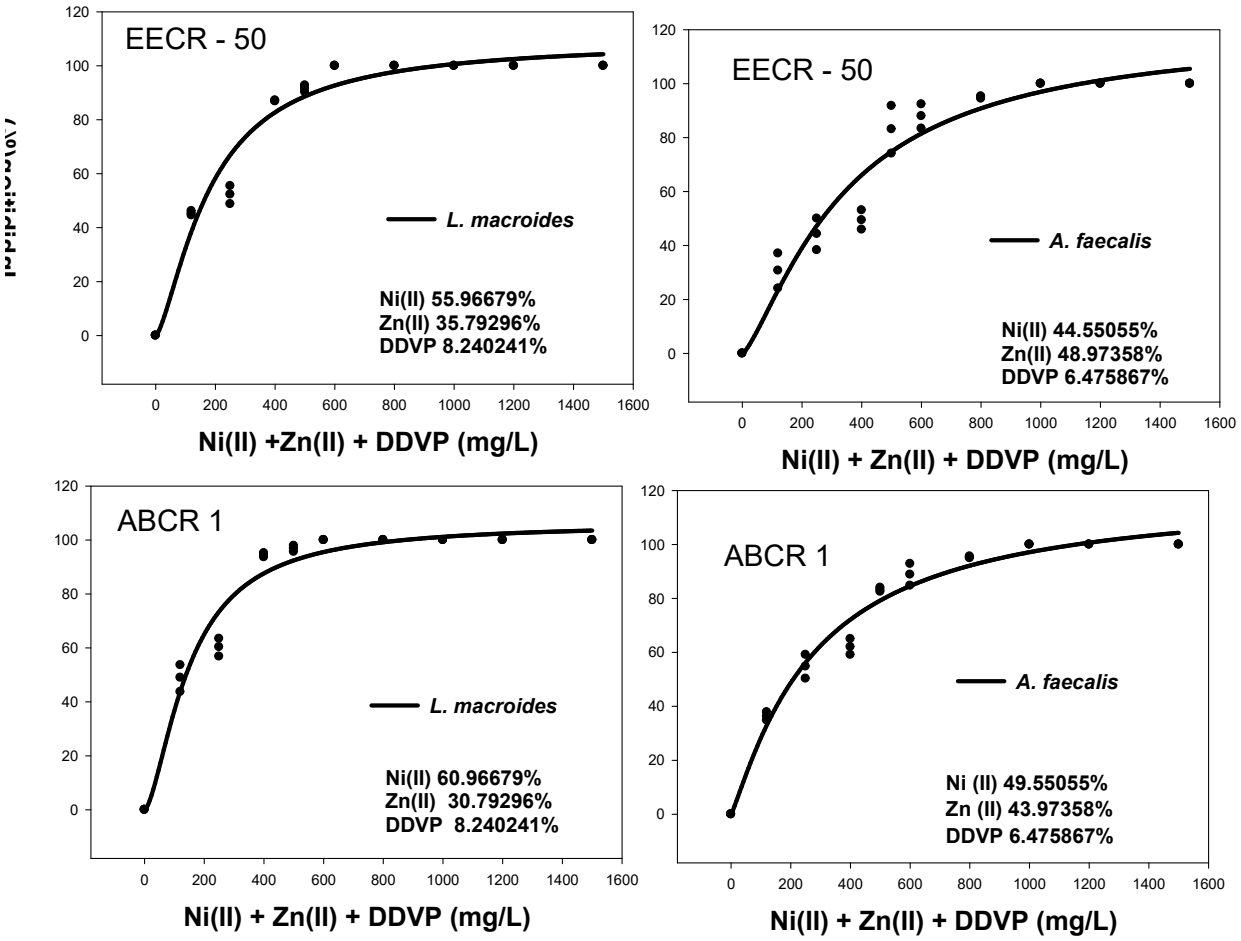


Figure 4.49: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Ni(II)+ Zn(II))and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

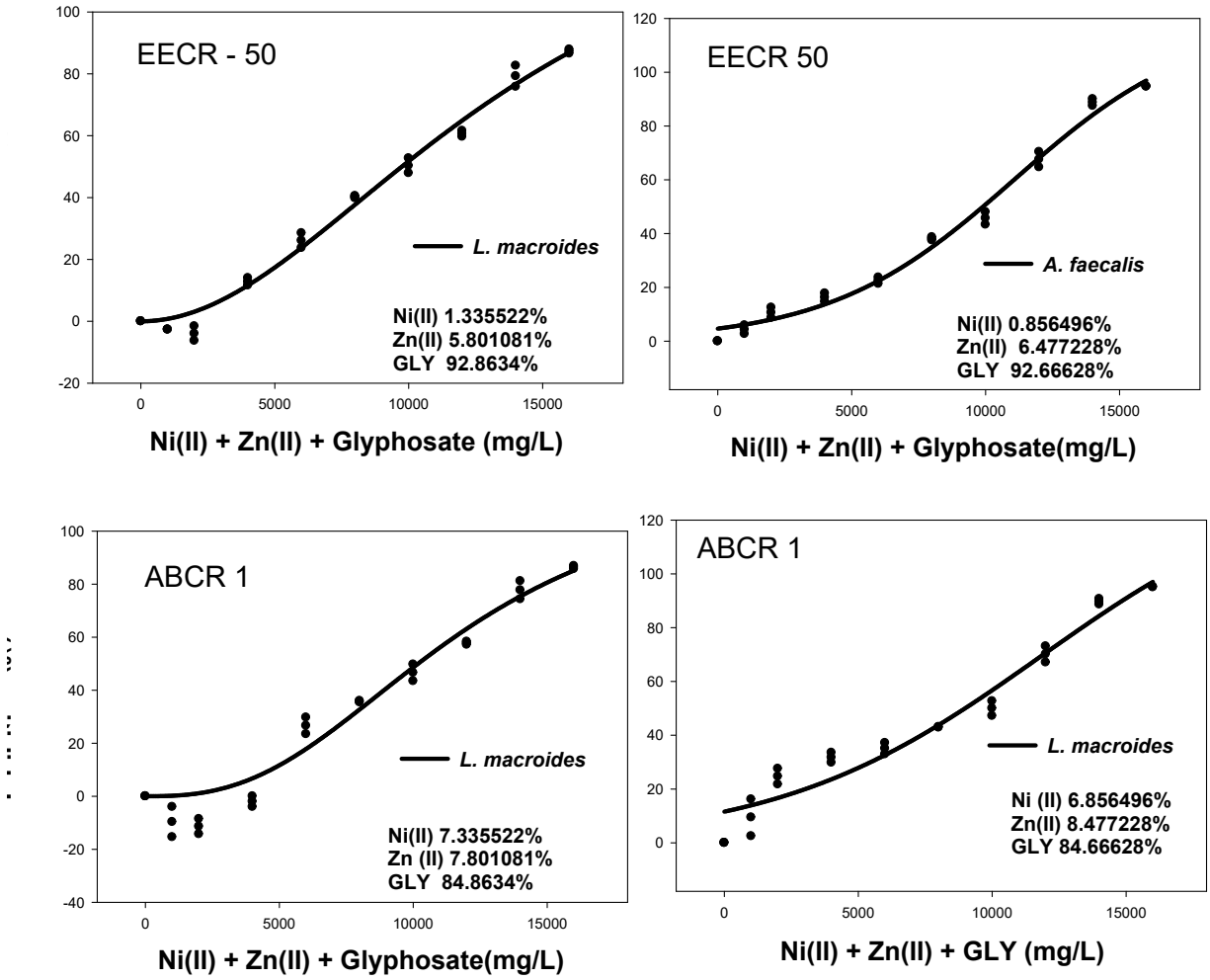


Figure 4.50: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Ni(II) + Zn(II))and pesticides (GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

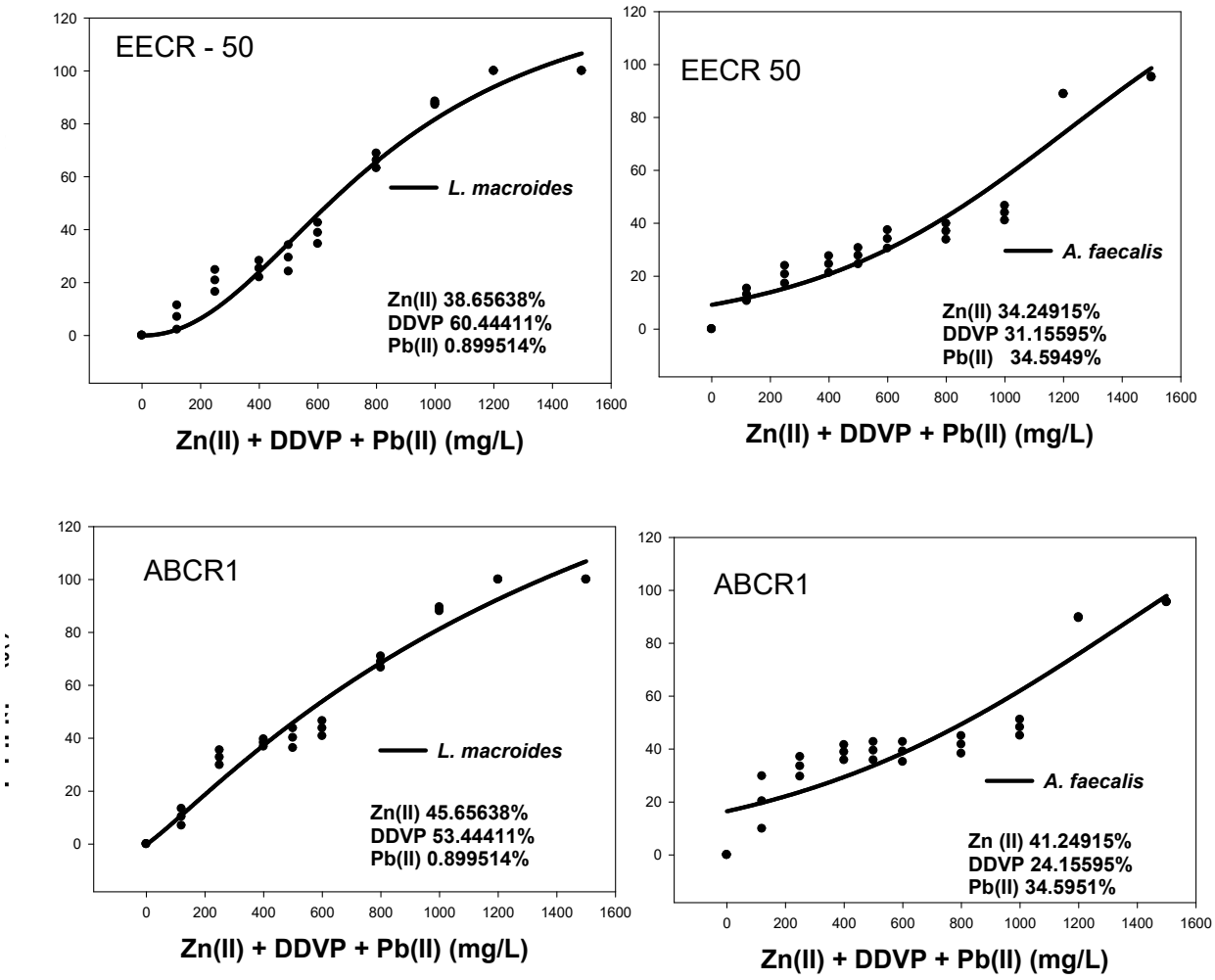


Figure 4.51: Response of *Lysinibacillus macroides* (OK298881) *and Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Zn (II) + Pb (II)) and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

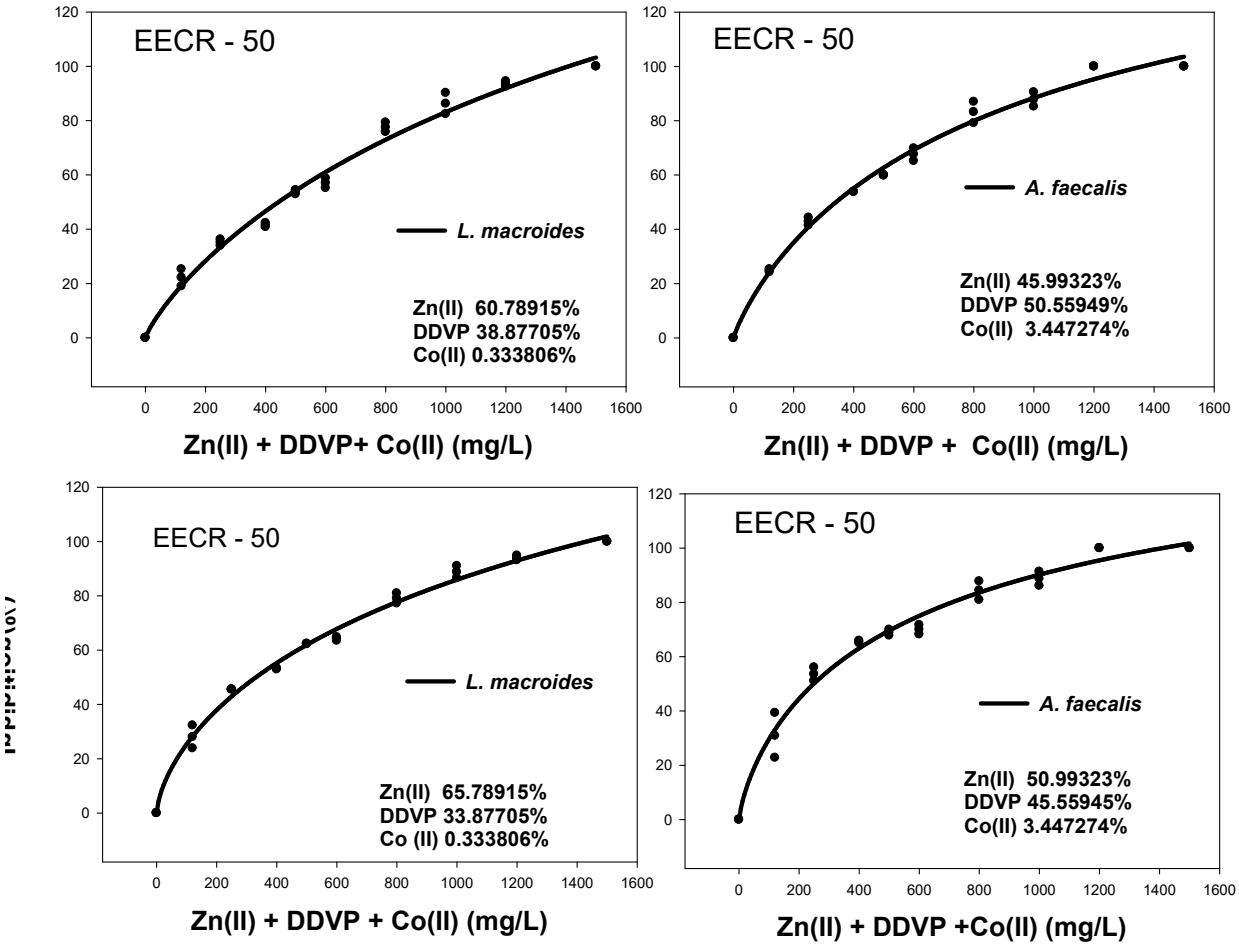


Figure 4.52: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Zn (II) + Co (II)) and pesticides (DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

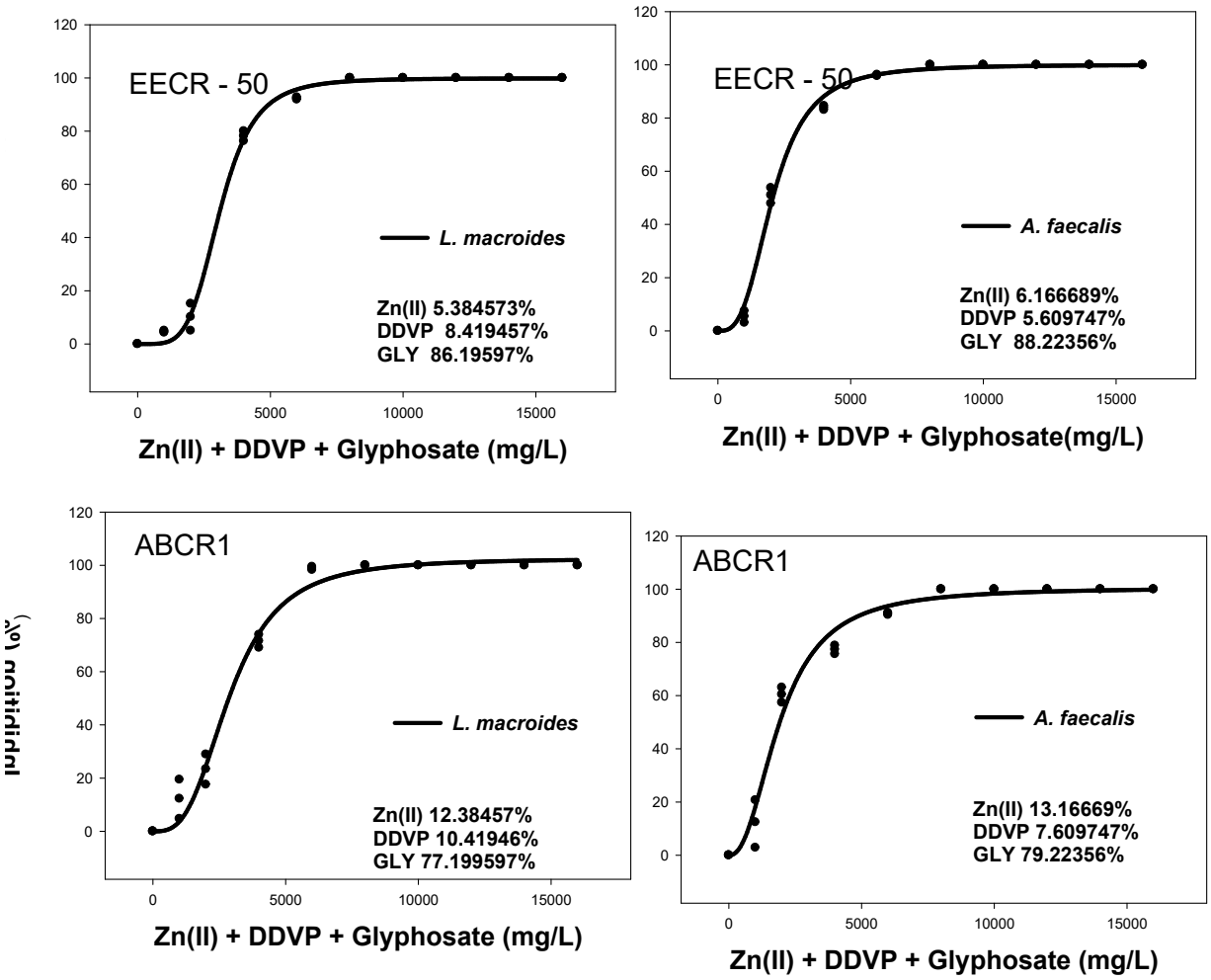


Figure 4.53: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ion (Zn(II))) and pesticides (DDVP+ GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

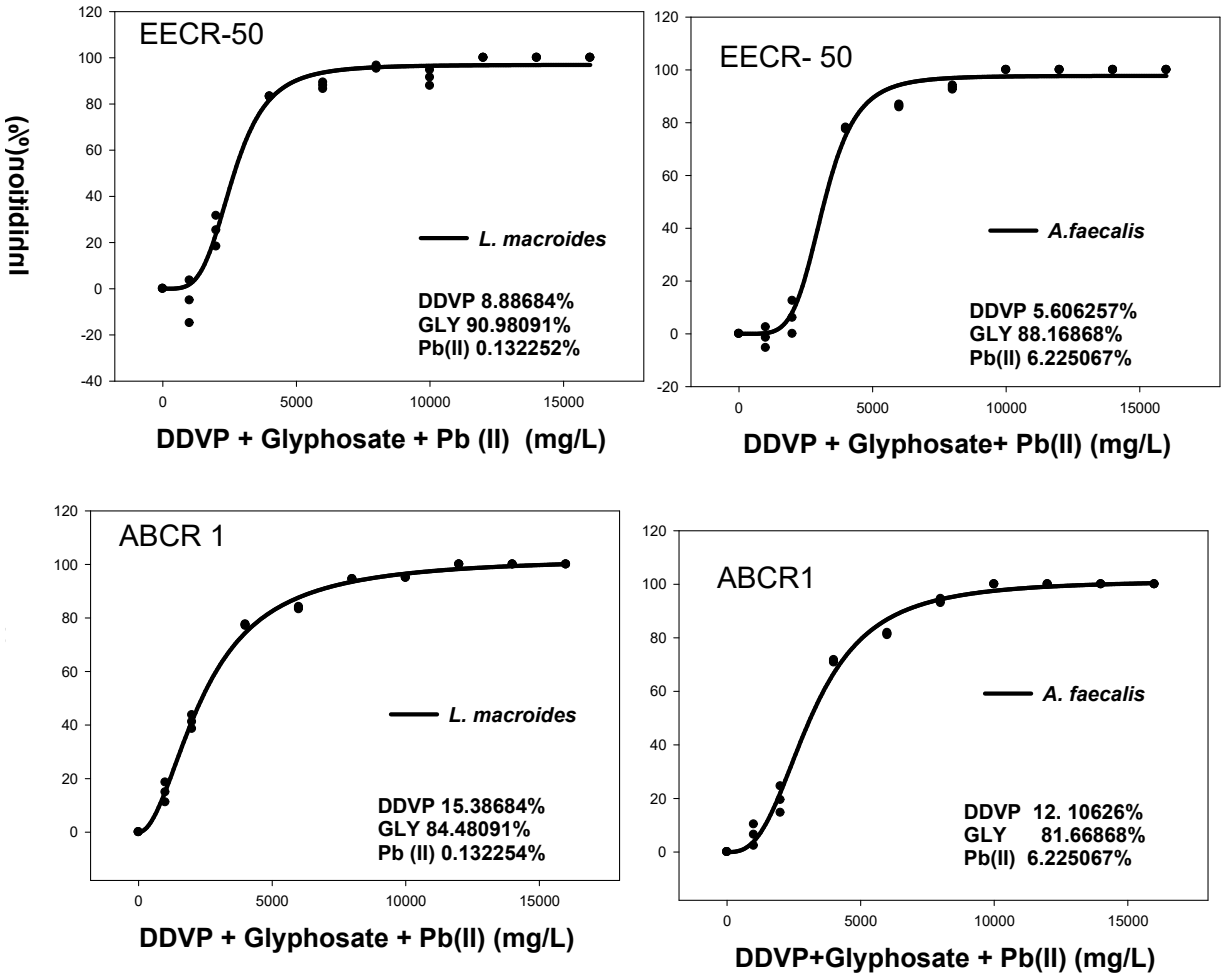


Figure 4.54: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ion (Pb(II)))and pesticides (DDVP+ GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

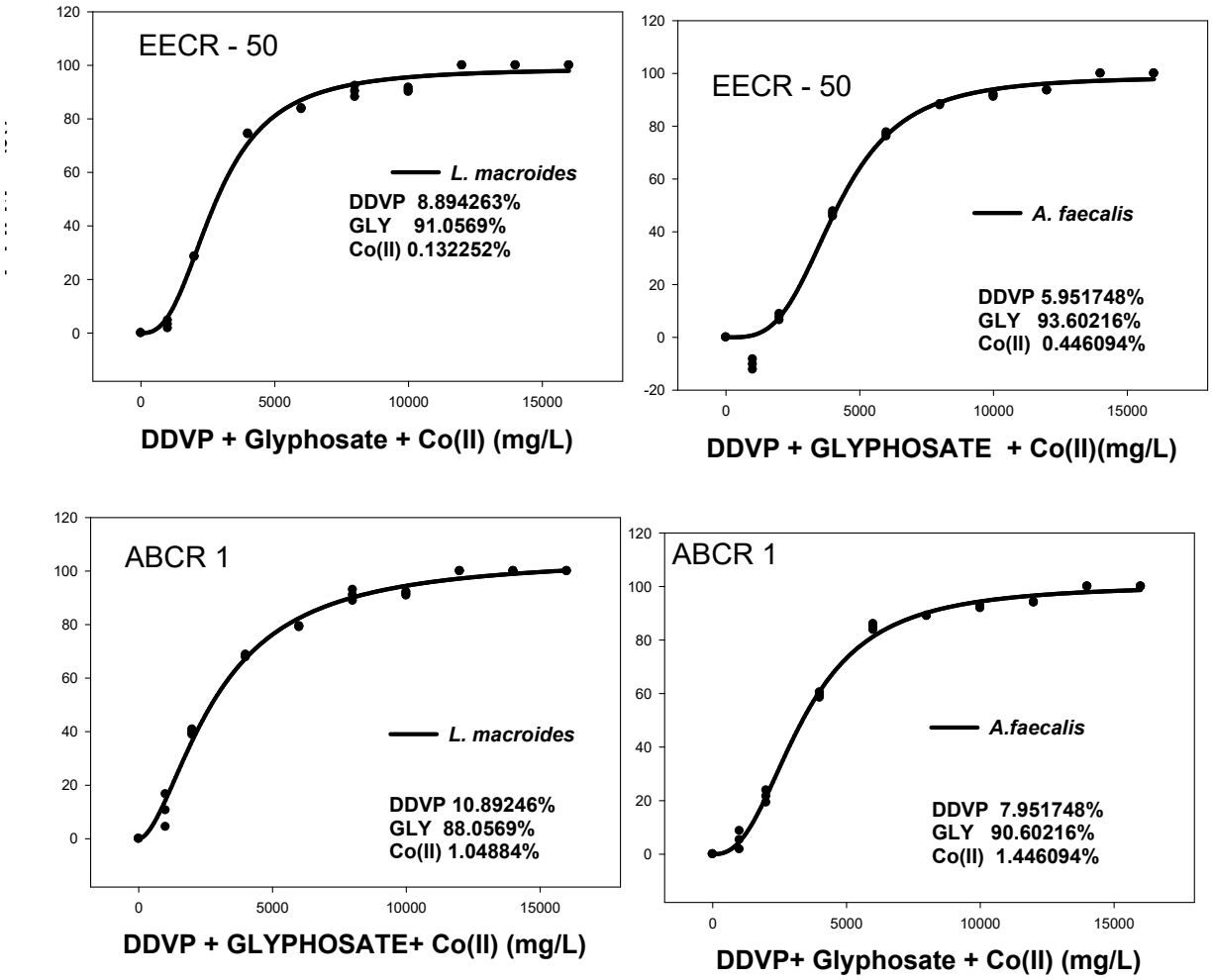


Figure 4.55: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Co (II)))and pesticides (DDVP + GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

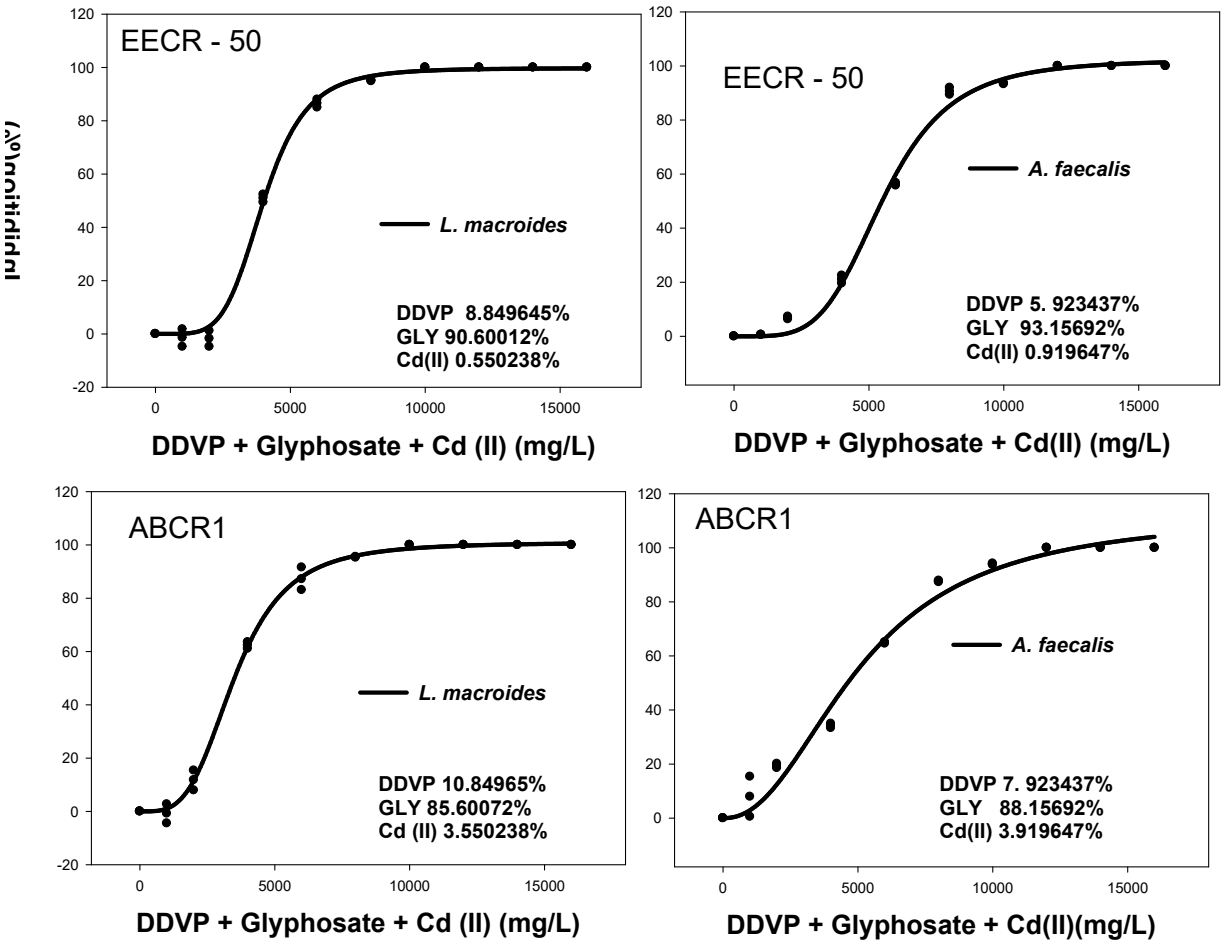


Figure 4.56: Response of *Lysinibacillus macroides*(OK298881) and *Alcaligenes faecalis* (KX302624)total dehydrogenase activity to Ternary Mixtures of metal ion (Cd (II))and pesticides (DDVP+ GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

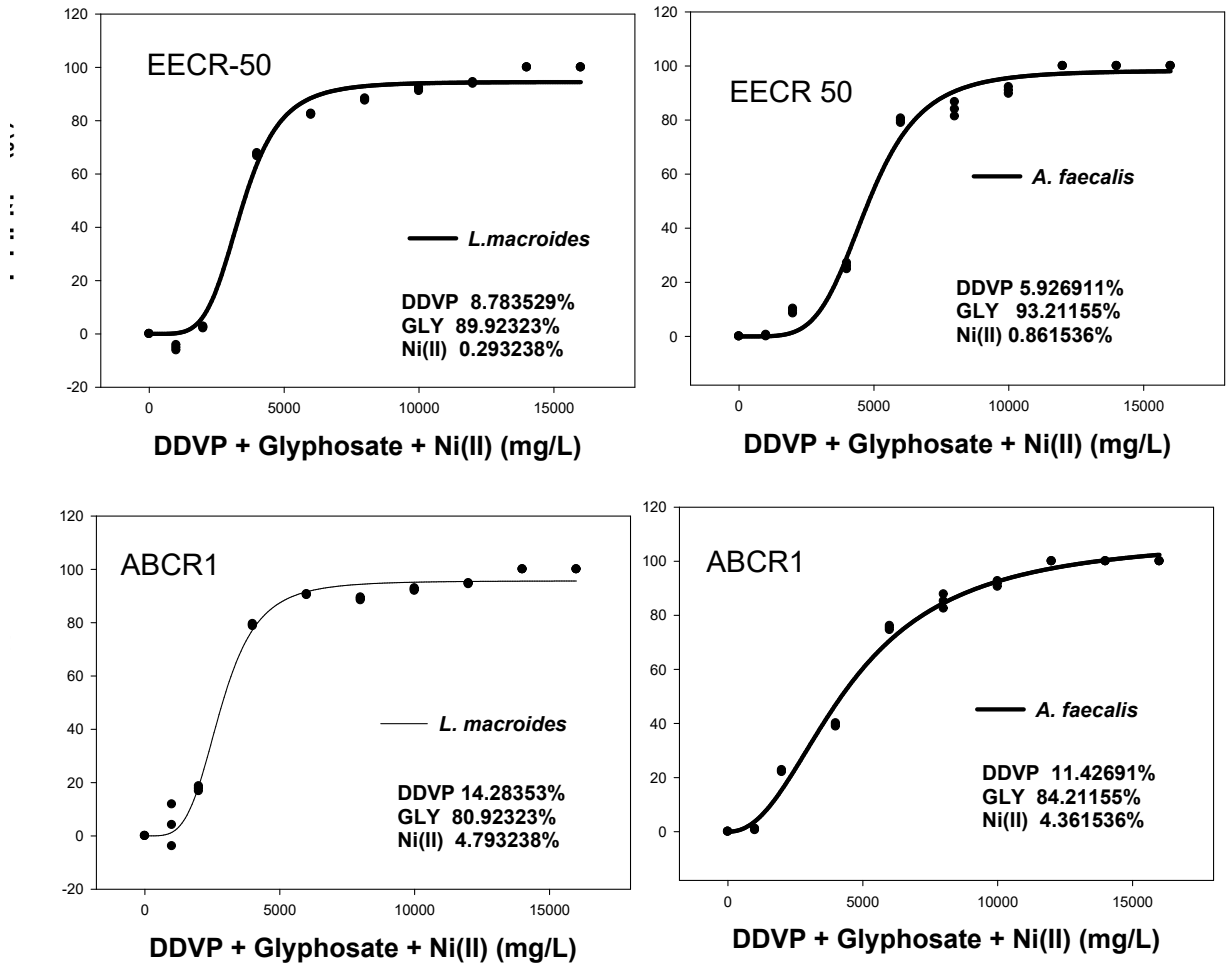


Figure 4.57: Response of *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Ni(II))and pesticides (DDVP+GLY) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

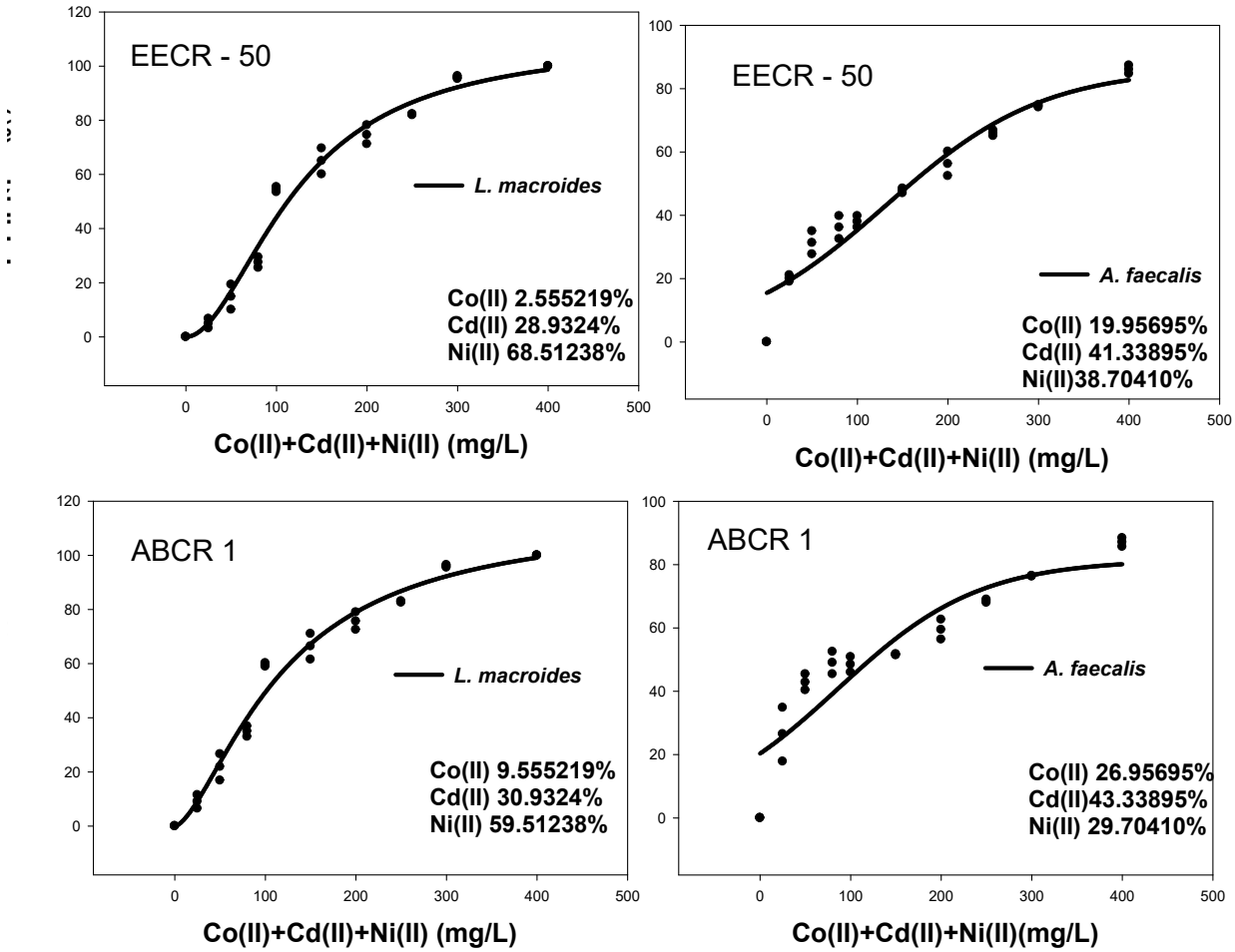


Figure 4.58: Response of Lysinibacillus macroides(OK298881) and Alcaligenes faecalis(KX302624) total dehydrogenase activity to Ternary Mixtures of metal ions (Co (II)+ Cd(II)+ Ni(II)) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

4.1.9 Toxicity threshold of Septenary Mixtures of Metal ions and Pesticides:

4.1.9.1 Toxicity of septenary mixtures of metal (Pb (II), Co (II), Ni (II) Cd (II) and Zn (II)) ions and pesticides (Glyphosate and DDVP) *to Lysinibacillus macroides* (OK298881) total dehydrogenase activity.

The result presented in table 4.13 shows the experimental toxicity thresholds of five metal ions and two pesticides to the total dehydrogenase activity of *L. macrolides* (OK298881) *isolated* from Otamiri river water. The experiment was done at six different mixture ratios. The EC_{50S} of different ratios of the toxicants on *L. macroides* (OK298881) ranged from 190.79 ± 9.54 - 1244.59 ± 62.22 mg/L. The order of increasing toxicity on *L. macroides* (OK298881) was mixture 3 < mixture 6 < mixture 4 < mixture 1 < mixture 2 < mixture 5.

Mixture 1 showed a dose dependent inhibition of total dehydrogenase activity of *L. macroides* closely fitted into a logistic three parameter equation (R^2=0.993) with EC_{50} value of 954.09mg/L. Mixture 2 showed a dose dependent inhibition of total dehydrogenase activity fitted into a logistic three parameter equation with R^2 values of 0.988 and EC_{50} value of 1043.74mg/L.

The mixture 3 and 4 showed inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) closely fitted into a logistic four and sigmoidal three parameter equation with R^2 values of 0.993 and 0.924. The EC_{50} values of both isolates were190.79mg/L. and 643.73mg/l, respectively.

The toxicity effect of mixture 5 on the total dehydrogenase activity of *L. macrolides* (OK298881) progressively increased with concentration of the metal ion, the inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) was describable by Sigmoid, 3 parameter equations, with R^2 values of 0.949, and EC_{50} values of 1244.59mg/l.

Mixture 6 equally showed a dose dependent inhibition of total dehydrogenase activity of *L. macroides* (OK298881) isolate closely fitted into a sigmoidal three parameter equation with R^2 values of 0.967 and EC_{50} values of 420.10 mg/l as shown in figure 4.59.

Table 4.13 Septenaryy mixtures of Metal ions and pesticides on *L. macroides*(OK298881) at different ratios.

Mixtures	Toxicants septenary mixture	R^{2-} value	EC_{50} on *L. macrolides* (mg/L)
Mixture 1	Pb(II)0.12% +Co(II)0.05% +Cd(II) 0.51% + Ni(II) 1.22% + Zn(II) 5.28% + DDVP 8.28% + Glyphosate 84.66%	0.9933	954.09 ± 47.70
Mixture 2	Pb(II)0.25% +Co(II)0.09% +Cd(II) 1.03% + Ni(II) 2.43% + Zn(II) 10.56% + DDVP 16.52% + Glyphosate 69.12%	0.9875	1043.74 ± 52.19
Mixture 3	Pb(II)0.37% +Co(II)0.14% +Cd(II) 1.54% + Ni(II) 3.65% + Zn(II) 15.85% + DDVP 24.75% + Glyphosate 53.68%	0.9931	190.79 ± 9.54
Mixture 4	Pb(II)0.49% +Co(II)0.18% +Cd(II) 2.05% + Ni(II) 4.86% + Zn(II) 21.13% + DDVP 33.04% + Glyphosate 38.24%	0.9244	646.73 ± 32.33
Mixture 5	Pb(II)50.00% +Co(II)0.23% +Cd(II) 2.57% + Ni(II) 6.08% + Zn(II) 5.28% + DDVP 8.26% + Glyphosate 27.58%	0.9492	1244.59 ± 62.22
Mixture 6	Pb(II)1.23% +Co(II)0.45% +Cd(II) 5.14% + Ni(II) 12.16% + Zn(II) 5.28% + DDVP 8.26% + Glyphosate 67.48%	0.9611	420.10±21.005

TABLE 4.14. Mean EC50, Toxic Index and Toxic Effects of Metals and Pesticides Septenary Mixtures on *L. macroides*(OK298881)

TOXICANT MIXTURES	MEAN EC_{50}	TOXIC INDEX	TOXIC EFFECT
Pb(II)+Co(II)+Cd(II)+Ni(II)+Zn(II)+DDVP+GLY			
Pb(II) 0.12%+ Co(II) 0.05% + Cd(II) 0.51% +Ni(II) 1.22%+ Zn(II) 5.28%+ DDVP 8.28%+GLY 84.66%(EECR-50)	954.0955 ±66.78669	0.344752 ±0.020685	Synergistic
Pb(II) 0.25%+ Co(II) 0.09% + Cd(II) 1.03% +Ni(II) 2.43%+ Zn(II) 10.56%+ DDVP 16.52%+GLY 69.12%(ABCR 1)	1043.746 ±73.06221	0.785907 ±0.047154	Synergistic
Pb(II) 0.37%+ Co(II) 0.14% + Cd(II) 1.54% +Ni(II) 3.67%+ Zn(II) 15. 85%+ DDVP 24.75%+GLY 53.68%(ABCR 2)	190.7955 ±13.35569	0.205695 ±0.012342	Synergistic
Pb(II) 0.49%+ Co(II) 0.18% + Cd(II) 2.05% +Ni(II) 4.86%+ Zn(II) 21.13%+ DDVP 33.04%+GLY 38.24%(ABCR 3)	646.7321 ±45.27125	1.175932 ±0.070556	Antagonistic
Pb(II) 50%+ Co(II) 0.23% + Cd(II) 2.57% +Ni(II) 6.08%+ Zn(II) 5.28%+ DDVP 8.26%+GLY 27.58%(ABCR 4)	1244.593 ±87.12149	2.573944 ±0.154437	Antagonistic
Pb(II) 1.23%+ Co(II) 0.45% + Cd(II)5.14% +Ni(II) 12.16%+ Zn(II) 5.28%+ DDVP 8.26%+GLY 67.48%(ABCR 5)	420.1027 ±29.40719	0.831974 ±0.049918	Synergistic

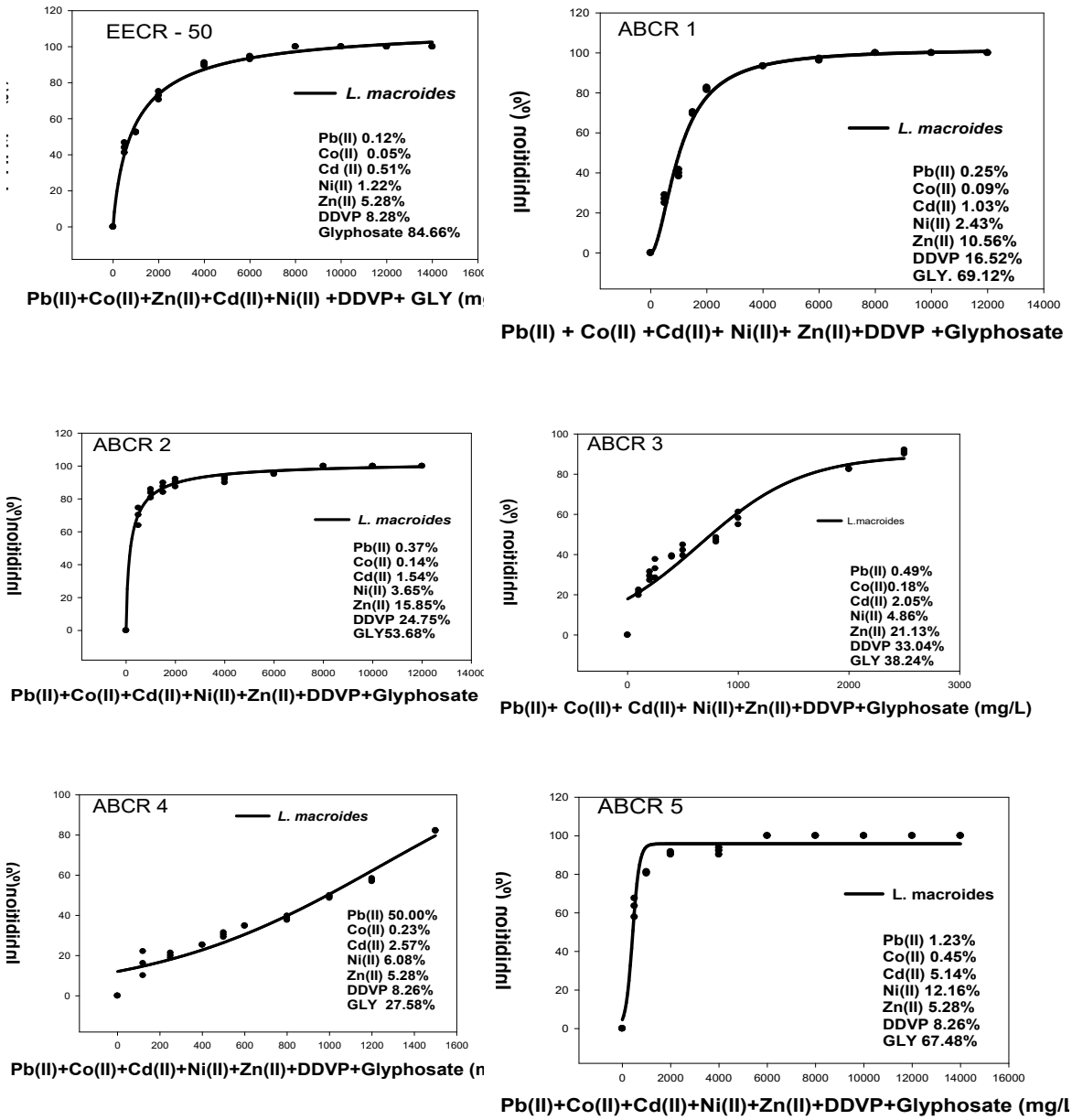


Figure 4.59: Response of *Lysinibacillus macroides*(OK298881) total dehydrogenase activity to septenary mixtures of metal ions (Pb(II), Co(II), Cd(II), Ni(II) and Zn(II)) and pesticides (Glyphosate and DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

4.1.8.1 Toxicity of septenary mixtures of metal (Pb (II), Co(II), Ni(II) Cd(II) and Zn (II)) ions and pesticides (Glyphosate and DDVP) to Alcaligenes faecalis (KX302624)total dehydrogenase activity.

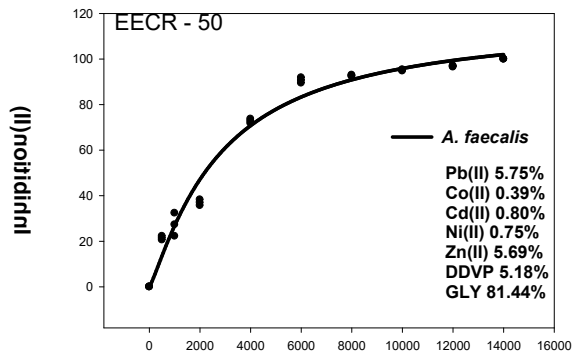
The result presented in table 4.15 shows the experimental toxicity thresholds of five metal ions and two pesticides to the total dehydrogenase activity of *A. faecalis* (KX302624) isolated from Otamiri river. The experiment was done at six different mixture ratios. The EC_{50S} of different ratios of the toxicants on *Alcaligenes faecalis* (KX302624) ranged from 351.14±21.0684 - 2800.30 ± 168.018mg/L. From the table 4.16, the Experimental concentration ratio (EECR) showed an antagonistic toxic effect at toxic index of 1.526863 ±0.1068, the ABCR.equally shows antagonistic toxic effect on four of the mixture ratios but mixture 5 was synergistic, the order of increasing toxicity was mixture 5 < mixture 6 < mixture 3 < mixture 4 < mixture 2 < mixture 1. The experimental (mixture 1) R^2 value was 0.9801 while the ABCRs were range from 0.8132 to 0.9926, it was closely fitted in three parameters logistic model using sigmaplot 10.0 as shown in figure 4.60. The interaction showed that the combination of all the seven toxicants had a very high inhibitory effect to the total dehydrogenase activity of *A. faecalis.*

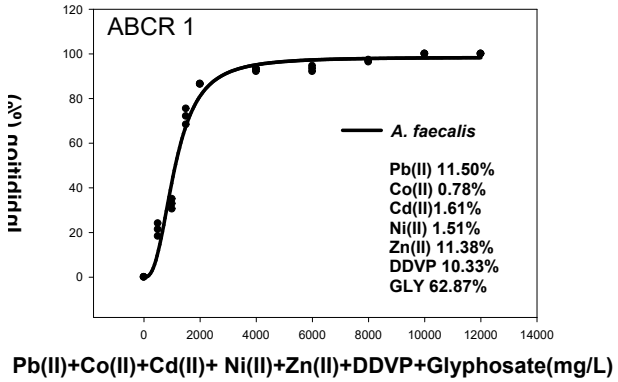
Table 4.15: Experimental Toxicity (EC_{50}) Thresholds of septenary mixtures of Metal ions and pesticides on *A. faecalis* (KX302624) at different ratios.

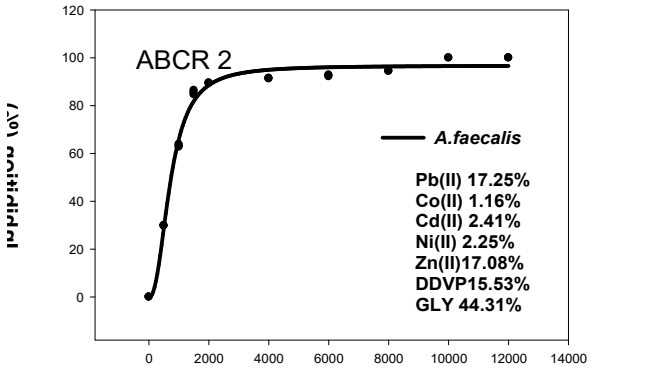
Mixtures	Toxicants septenary mixture	R^{2-} value	EC_{50} on *A.faecalis* (mg/L)
Mixture 1	Pb(II) 5.75% +Co(II) 0.39% +Cd(II) 0.80% + Ni(II) 0.75% + Zn(II) 5.69% + DDVP 5.18 % + Glyphosate 81.44%	0.9801	2800.30 ±168.018
Mixture 2	Pb(II) 11.50% +Co(II) 0.78% +Cd(II) 1.61% + Ni(II) 5.51% + Zn(II) 11.38% + DDVP 10.36% + Glyphosate 62.87%	0.9763	1103.21±66.1926
Mixture 3	Pb(II)17.25% +Co(II)1.16% +Cd(II) 2.41% + Ni(II) 2.25% + Zn(II) 17.08% + DDVP 15.53% + Glyphosate 44.31%	0.9926	719.26±43.1556
Mixture 4	Pb(II)23% +Co(II)1.55% +Cd(II) 3.22% + Ni(II) 3.01% + Zn(II) 22.75% + DDVP 20.71% + Glyphosate 25.74%	0.9782	727.47±43.6482
Mixture 5	Pb(II)50% +Co(II)1.94% +Cd(II) 4.02% + Ni(II) 3.76% + Zn(II) 5.69% + DDVP 5.18% + Glyphosate 29.41%	0.8132	351.14±21.0684
Mixture 6	Pb(II)57.50% +Co(II)3.88% +Cd(II) 8.04% + Ni(II) 7.53% + Zn(II) 5.69% + DDVP 5.18% + Glyphosate 12.19%	0.8132	368.90±22.134

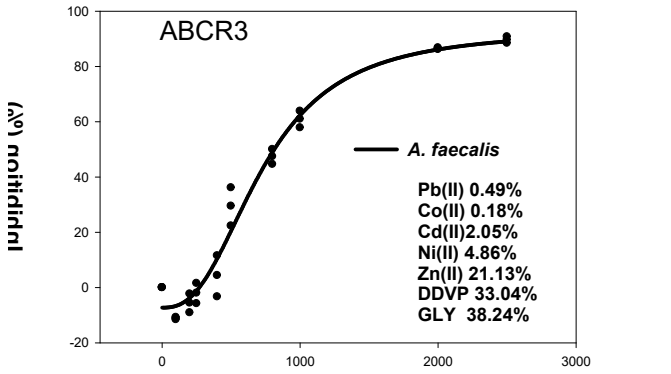
TABLE 4. 16 Mean EC50, Toxic Index and Toxic Effects of Metals and Pesticides Septenary Mixtures on *A. faecalis*(KX302624)

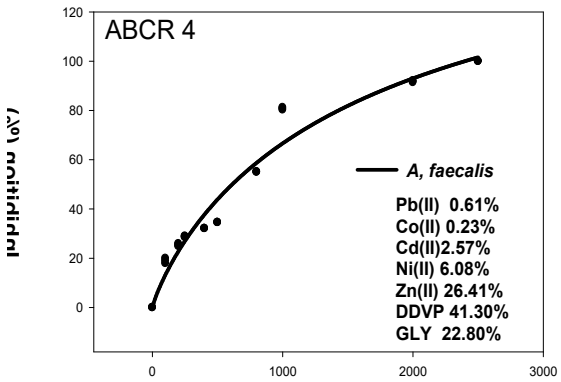
TOXICANT MIXTURES	MEAN EC_{50}	TOXIC INDEX	TOXIC EFFECT
Pb(II)+Co(II)+Cd(II)+Ni(II)+Zn(II)+DDVP+GLY			
Pb(II) 5.75%+ Co(II) 0.39% + Cd(II)0.80% +Ni(II) 0.75%+ Zn(II) 5.69%+ DDVP 5.18%+GLY 81.44%(EECR-50)	2800.305±140.0153	1.526863±0.10688	Antagonistic
Pb(II) 11.50%+ Co(II) 0.78% + Cd(II) 1.61% +Ni(II) 1.51%+ Zn(II) 11.38%+ DDVP 10.36%+GLY 62.87% (ABCR 1)	1103.217 ±55.1608	1.29 ±0.090303	Antagonistic
Pb(II) 17.25%+ Co(II) 1.16% + Cd(II) 2.41% +Ni(II) 2.25%+ Zn(II) 17.08%+ DDVP 15.53%+GLY 44.31%(ABCR 2)	719.2658±35.96329	1.216463±0.085152	Antagonistic
Pb(II) 23%+ Co(II) 1.55% + Cd(II) 3.22% +Ni(II) 3.01%+ Zn(II) 22.77%+ DDVP 20.71%+GLY 25.74%(ABCR 3)	727.4743±36.37372	1.610019±0.112701	Antagonistic
Pb(II) 50%+ Co(II) 1.94% + Cd(II)4.02% +Ni(II) 3.76%+ Zn(II) 5.69%+ DDVP 5.18%+GLY 29.41%(ABCR 4)	351.1424±17.55712	0.866104±0.060627	Synergistic
Pb(II) 57.50%+ Co(II)3.88% + Cd(II)8.04% +Ni(II) 7.53%+ Zn(II) 5.69%+ DDVP 5.18%+GLY 12.19% (ABCR 5)	368.9012±18.44506	1.424843±0.099739	Antagonistic











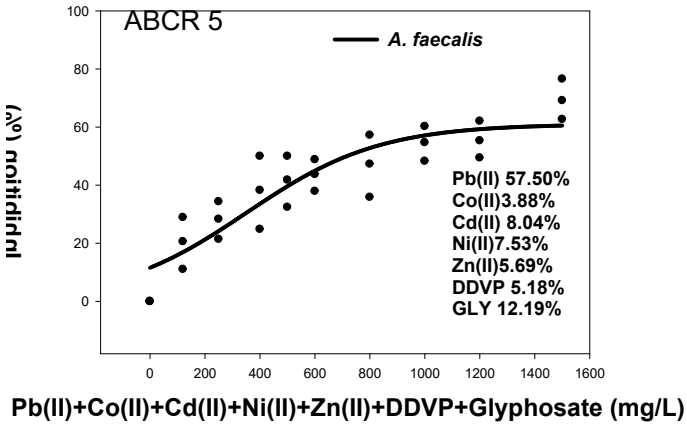


Figure 4.60: Response of *A. faecalis*(KX302624) total dehydrogenase activity to septenary mixtures of metal ions (Pb(II), Co(II), Cd(II), Ni(II) and Zn(II))and pesticides (Glyphosate and DDVP) toxicity. The data points represent the experimental dose–response data while the lines represent toxicities obtained by fitting experimental data to logistic model

4.2 Discussion

4.2.1 Physicochemical Parameters of Otamiri River and Soil

The physiochemical parameters as shown in tables 4.1 and 4.2 showed that pH ranges for river water was 5.22 - 6.4 ; while that of soil was 5.2 - 6.6. The pH ranges are slightly acidic. Similar finding was recorded by Ekhaise & Anyasi, (2005) and Eze *et al*., (2021b) in their various studies in an aquatic environment. It is equally in agreement with Okechi & Chukwura, (2020) who reported slightly acidic pH in their study on Otamiri River and sediment. The pH of the surface water is important to aquatic life because it affects their ability to regulate basic life sustaining processes, primarily the exchange of respiratory gases as opined by Okechi &Chukwura (2020). The pH of Otamiri as at the time of this study is within the WHO (2017) recommended range for drinking water.

The temperature ranges from 23^{o}C to 27^{o}C which is below the WHO, (20117) recommended range for drinking water. Temperature is known to affect the dissolved oxygen level in aquatic ecosystem (Njoku- Tony, et al., 2016). Temperature is considered as a critical parameter with many impacts such as the rate of disinfectant decay by-product formation. As temperature increases, decay by-product formation, nitrification, microbial activity, algal growth, lead copper solubility increases as well as calcium carbonate ($CaCO_3$) precipitation (Njoku- Tony, et al., 2016). Similarly, (Howard et al*.,* (2013) in their study also recorded temperature of 25^{o}C- 26.5^{o}C in a river water during wet seasons of their sampling.

Dissolved Oxygen (DO) reflects the physical and biological processes prevailing in the water. According to WHO, (2017), the ideal standard for dissolved oxygen is <10 mg/l. The DO of all water samples of the Otamiri River fell below the ideal standard ranging from 4.9 mg/l to 7.3 mg/l. Dissolved oxygen is an important element in water because

most aquatic organisms use it for respiration. The values obtained from the results showed that the stretches of the Otamiri River is very low in DO and may affect aquatic respiration.

The biochemical oxygen demand (BOD) ranges from 2.2mg/l to 3.5mg/l. The BOD is used as an approximate measure of the amount of biochemically degradable organic matter present in a sample (Ademoroti, 2016). The low level of BOD recorded was equally observed by Eze et, al., (2021a) in their work on the Impact of municipal solid waste on the water quality of Otamiri River. This is to say that anthropogenic activities impact negatively on the oxygen level of aquatic ecosystem.

The concentration of chloride in the river water ranged from 5.911 mg/l - 8.921 mg/l which is above WHO, (2017) standard, 5 mg/l. High concentration of chloride makes water unpalatable and unfit for drinking and livestock watering. Soil porosity and permeability plays a key role in building up the chloride concentration.

Total hardness ranged from 45mg/l – 98mg/l, which is far below maximum permissible limit of 500 mg/l by WHO (2017). This shows that there is low level of concentration of alkaline earth metals (Calcium and magnesium ions) in the river.

Turbidity level in all the sampling stations were not above WHO permissible limits of 50NTU. This high level of turbidity is due to suspended matter such as clay, silts, finely divided organic and inorganic matter, soluble coloured organic compounds, planktons, other microscopic organisms in the water body. Runoffs, mining and other anthropogenic activity could be among the contributing factors.

Conductivity level was above the permissible limits 100 mg/l except for station as Mgbirichi which had 99 mg/l, Inland Bridge 98mg/l and Mechanic Village 99mg/l.

Electrical conductivity is the capacity of water to conduct current. It is caused by the presence of salts, acids bases, called electrolytes, capable of producing cations and anions. The major ions present in water causing EC are chlorides, sulphates and magnesium.

Total suspended solid (TSS) recorded in the samples were high, and it contributed to the greatest variability among the physical, chemical and biological parameters studied. Otamiri River contains varying levels of inorganic elements such as phosphate 0.119mg/l - 0.196 mg/l, calcium 30 mg/l - 63.9mg/l, magnesium 15mg/l - 43.1 mg/l.

Excessive phosphate contents in the river water are an indication of the presence of pollution largely responsible for eutrophic conditions as opined by Venkatesharaju et al., (2010) in their study. Low levels of phosphate in this study have been reported by other workers (Dike et al., 2016 & Okoro et al*.,* 2016) who has done similar work on this site. The hardness of water depends on the presence of dissolved calcium and magnesium ions as stated by Amadi et al*.,* (2010). The concentration of calcium and magnessium are below the recommended permissible limit of 200.00 mg/l^{-1} for both calcium and magnesium (NSDWQ, 2007).

Sulphate range from 1.19mg/l -2.2mg/l. The high levels of sulphate could be due to depletion of oxygen as a result of anthropogenic activities of man and water inhabitants' usage for respiration.

4.2.2 Heavy metal content of Otamiri river and soil

Heavy metal in aquatic ecosystem according to Onyekuru, Nwankwoala, & Uzor, (2017) is as a result of weathering of rock coupled with other anthropogenic activities. Man's activities such as sand mining, agricultural practices, and indiscriminate refuse disposal among others have been a contributing factor to that effect. Anthropogenic activities

have impacted on the environment severely. Many studies have shown that there are deposition and accumulation of heavy metal in Otamiri River (Nweke, 2013; Okechi &Chukwura, 2020; Dike et al., 2016; Okeke *et al*., 2019; Onyekuru *et al*., 2017). From all the heavy metals analyzed, Zinc (Zn), Nickel (Ni), Cadmium (Cd), mercury (Hg), Lead (Pb) and Cobalt (Co) have raised a very huge concern because they exceeded the threshold as indicated by WHO, (2017). Some of the aforementioned metals are not essential in metabolic activity of the organisms and does not support their growth as such are detrimental even at a very low dose. Cadmium in all the twelve sites analyzed range from 0.036-0.096 mg/L for water and 0.071-0.128mg/L for soil samples. This is far above the WHO 0.003 mg/L recommendation of 2017. Lead also exceeded the 0.01mg/L stipulated threshold as it ranges from 0.06-0.261. The implication of these rises in heavy metal content of the river body is that the aquatic lives will be adversely affected and the tendency of bioaccumulation and uptake to the food chain cannot be ruled out. The work of Qishlaqi &Farid (2007) showed that heavy metals in soil have the tendency of magnifying to the food chain and affect human. Microorganism responds differently to the toxicity of heavy metals. These responses are due to the levels and accumulation of the heavy metals in the environment.

4.2.3 The pesticides content of Otamiri River and soil:

The pesticide content analysis of Otamiri river showed that most of the pesticides found to be present in the river were above the permissible limit as recommended by WHO. The WHO, (2017) guideline for river water stated that Athrazine should not be more than 0.1mg/l in a river but the findings from this study showed that most of the sampled stations recorded higher. Akachi bridge and Mechanic village recorded 2.2mg/l and 1.2mg/l respectively while Nekede, mgbirichi and Umuagwo recorded 0.2mg/l. Similarly, Erhunmwunse *et al*. (2012) in their study on Implications of Pesticide usage in Nigeria

eqally recorded high Athrazine deposite in the river sample analyzed. The adverse effects of pesticides contamination are not limited to the environment but, extended to human health. Butachlor and Alachlor equally exceeded the permissible limits. Glyphosate deposite was recorded higher in Mgbirichi and Umuagwo. There was no standard set for DDVP. This result justifies the findings of Michael, & Stephen, (2016) who also recorded high pesticides deposite on the aquatic environment they analyzed. The high deposite of pesticides in these study environment could be attributed to the increase / rise in agricultural practices in these environment. Mgbirichi and Umuagwo is known to be an Agricultural hub in Owerri west local government area of the state, indiscriminant production and use of these product formulation can never be ruled out.

4.2.4 Bacterial isolates from Otamiri River water and soil

This present study has revealed the bacterial diversity in Otamiri River. From the study total bacteria counts recorded from the sampling stations were high. This could be as a result of introduction of waste and waste water into the river and anthropogenic activities in these sampling stations. Egbu abattoir sampling station recorded the highest total heterotrophic count. This is in agreement with the work of Victor et al*.,* (2019) who reported that Egbu abattoir had the highest heterotrophic bacteria count in their study. The high indicator and fecal indicator bacteria counts recorded in this present study, *Escherichia coli, Klebsiella, Enterobacter* could be as a result of disposal of untreated sewage and waste water into the river. However there were high counts of indicator bacteria as reported in this study across all sampling stations. The counts obtained were in agreements with other researchers who reported high indicator bacteria counts. Rheinheimer, (1991) and Victor et al*.,* (2019) reported high indicator bacteria counts in their study. It was also observed that high counts of bacterial load reflected the level of

water pollution as it gives indication of the amount of organic matter present as described by Adieze et al*.,* (2016).

With respect to soil, the high values for bacteria bio load is as a result of the conditions as stated above for river water. The bacteria isolated from Otamiri river water and soil includes; *Klebsiella* sp*., Klebsiella* sp*., , Eschericia coli, Enterobacter* sp*., , Enterococcus* sp*., Alcaligens* sp. *Staphylococcus* sp*., Vibrio cholera, Bacillus* sp*., Salmonella* sp*, Shigella* sp*., Citrobacter* sp*, Pseudomonas* sp*, Bacillus* sp*, Serratia* sp*, Proteus* sp, *Clostridium* sp*, Lysinibacillus* sp*, Streptococcus* sp*, Micrococcus* sp*., Lactobacillus* sp. Amongst these bacteria, *Alcaligens* sp and *Lysinibacillus* sp. had the highest percentage occurrence in river water and soil of Otamiri. The bacteria isolated are pathogenic in nature. *Serratia marcescens* is known to cause hospital acquired infections, particularly catheter-associated bacteriaemia, urinary and respiratory tract, as well as wound infections. The presence of indicator bacteria in water can cause cholera, hepatitis, dysentery, typhoid plus other gastroenteritis (Ahiarakwem, 2013). The presence of indicator bacteria indicates that the water is polluted with fecal materials. Faecal *streptococci*, particularly *Enterococcus Spp* and *Clostridium spp* also indicates faecal contamination, the former indicating recent contamination as they do not multiply in the environment, and the latter representing more ancient contamination (Payment & Franco, 1993). Since the water body (Otamiri River) is constantly used for domestic purposes, agriculture and aquaculture by the communities living along its banks, there is possibility of transferring these organisms to human beings and eventual outbreak of their related diseases (Tita *et al*., 2013). The presence of hospitals and other medical facilities, abattoir, sand dredging, channels of waste water, anthropogenic activities of man may have contributed to the high occurrences of these human pathogens in the river and its sediment. The highest occurring organisms where further subjected to molecular

identification. The organisms were identified to be *Lysinibacillus macrolides* (OK298881), *Pseudomonas aeruginosa* (CP058331) *Klebsiella pneumonia* (MK641337), *Alcaligenes faecalis* (KX302624), *Proteus mirabilis* (MZ067158), these results showed that harmful bacteria were isolated from the Otamiri River. There are different cases of sickness that are brought about by these germs. Pneumonia, thrombophlebitis, urinary tract infection, cholecystitis, diarrhea, upper respiratory infection, wound infection, osteomyelitis, meningitis, bacteremia, and sepsis are among the clinical illnesses brought on by *Klebsiella pneumonia*. Typically, *Pseudomonas aeruginosa* causes blood infections as well as infections of the airways, urinary tract, burns, and wounds. Most typically, *Proteus mirabilis* is linked to urinary tract infections, particularly in difficult or infections of the urinary tract brought on by catheters. Endocarditis, bacteremia, meningitis, endophthalmitis, skin and soft tissue infections, urinary tract infections, otitis media, peritonitis, and pneumonia have all been linked to *Alcaligenes faecalis*. In people with impaired immune systems, *Lysinibacillus* can cause sepsis. Other workers have isolated and reported these bacteria throughout their exploration of the Otamiri River. *Pseudomonas, Klebsiella,* and *Proteus* have been isolated by Mgbemena, Nnokwe, Adjeroh, & Onyemekara, (2012) in their study. However, prior studies did not disclose that *Alcaligenes faecalis* and *Lysinibacillus macroides* had been identified from the Otamiri

4.2.4 Toxicity of individual toxicants:

4.2.4.1: Toxicity of single metal ions (Pb (II), Co (II), Ni (II) Cd (II) and Zn (II)) to *Lysinibacillus macrolides* (OK298881) and *Alcaligenes faecalis*(KX302624) total dehydrogenase activity:

Microorganisms are vital for the efficient functioning of any ecosystem; hence factors that affect their metabolism, composition and abundance are of great concern. Heavy

metals and pesticides contamination of aquatic environment is a serious issue due to their persistence and toxicity. Environmental pollution from hazardous metals and minerals can arise from natural as well as anthropogenic sources. Natural sources are: seepage from rocks into water, volcanic activity, forest fires etc. Some of the pollutants like lead (Pb), nickel (Ni), cadmium (Cd), cobalt (Co), pesticides, etc are very harmful, toxic and poisonous even in ppb (parts per billion) range (Rashmi & Pratima 2013), there are some minerals which are useful for human and animal health in small doses beyond which they are toxic; Zinc (Zn). Microbes encounter metals and metalloids of various kinds in the environment and it is, therefore, not surprising that they should interact with them, sometimes to their benefit, at other times to their detriment. The pollution of Otamiri River is due to anthropogenic activities along its bank. Enzymatic activities of microbes can be a very functional tool in determination of toxicity effects of heavy metals and pesticides to microorganisms hence it's common to them. This form one of the justification/ reason for the choice of total dehydrogenases in this assay.

From this present study, Co (II) and Cd (II) proved to be more toxic than other toxicants under study to *L. macrolides* while *A. faecalis* was more sensitive to Ni (II). The order of increasing toxicity on *L. macroides* (OK298881) is Co (II) < Cd (II) < Ni (II) < Pb (II) < Zn (II) while that of *A. faecalis is* Ni (II) < Cd (II) < Co (II) < Pb (II) < Zn (II). This finding is in agreement with the work of other researchers who discovered that Ni (II), Cd (II) and Co (II) deters microbial activities in aquatic ecosystem. (Okechi, *et al*., 2020b, Nweke et al 2007 and Widenfalk, *et al*., 2004.).

Lead (Pb) showed a very strong relationship to both organisms as it exhibited a high inhibition of total dehydrogenase activity of both isolate. The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) when fitted into a logistic four parameter equation had an R^2 value of 0.978 with EC_{50} value of 493.38 mg/l while the

inhibition of the total dehydrogenase activity of *A faecalis* (KX302624) when fitted into a logistic three parameter equation is R^2= 0.985 with EC_{50} value of 509.75mg/L, this is an indication that they have high positive correlation as R^2 tends to 1.

Nickel (Ni) according to Gikas, (2007), Gikas, (2008), Okechi et al, (2020b) & Nweke, *et al*., (2012) has low dose positive effect on microorganism as they have been proven to stimulate microbial growth. This justifies the finding of this research where Ni(II) showed some stimulatory effect at low dose before toxicity sets in when dose was elevated to a higher concentration.

Cadmium (Cd) ion showed inhibitory response to *L. macrolides* between the concentration range of 0 – 82mg/L. Beyond this concentration, there was a rapid inhibition of total dehydrogenase activity of both isolates. The inhibition of the total dehydrogenase activity of *L. macroides* (OK298881) and *A. faecalis* (KX302624) *closely* fitted into a sigmoidal three parameter equation showed R^2 values of 0.981 and 0.971. The R^2 value indicates that the toxicants have a very high positive correlation. This finding clears the doubt of Okechi et al (2020b), where *S. marcescens was* relatively tolerance to Co and Cd. as they inhibited dehydrogenace activity even at low concentration.

Zinc (Zn) is a trace element that supports the growth and metabolism of microbe but can elicit toxicity at high or elevated dose. Beard *et al*., (1995) in their study found out that, though zinc is an essential element, it is an inhibitor of respiratory activities in microorganisms. According to the work of Nweke *et al.* (2007) on the effects of Zinc (Zn^{2+}) on the dehydrogenase activity of heterotrophic bacteria from tropical river sediment, they discovered that Bacillus *sp*. SED1 dehydrogenase activity decreased with increasing concentration of Zn^{2+}. The outcome of this research showed that Zinc (II) ion

equally showed a dose dependent inhibition of total dehydrogenase activity of both isolates. The inhibition of the total dehydrogenase activity of *L. macroides*(OK298881) and *A. faecalis*(KX302624) isolates was experienced at EC_{50} values of 713.27 mg/L. and 624.41 mg/L respectively

4.2.4.2: Toxicity of pesticides formulations (Glyphosate and 2, 2- dichlorovinyl dimethyl phosphate (DDVP)) to *Lysinibacillus macroides* (OK298881) and *Alcaligenes faecalis* (KX302624) total dehydrogenase activity

Glyphosate and 2,2- dichlorovinyl dimethyl phosphate (DDVP showed hormetic response to *L. macrolides* between the concentration ranges of 0 - 4000 and 0 – 400mg/L respectively. At elevated concentrations, there was rapid inhibition of total dehydrogenase activity of both isolates; *L. macroides* (OK298881) *and A. faecalis* (KX302624). This finding justifies the report of WHO, (2017) standards on guidelines for drinking water were they did not consider DDVP a public health issue, as they said its occurrence in drinking water or drinking water source is at concentration well below those of health concern. Continuous use of this insecticide in an uncontrolled manner can pose a lot of treat to the microbial load of Otamiri water and soil (River banks).

4.2.5 Toxicity of Binary mixtures of toxicants to *L. macroides* (OK298881) *and A. faecalis* (KX302624).

The aquatic environment is continuously exposed to varied loads of chemicals from natural and anthropogenic sources. Release of these chemicals from anthropogenic sources continues to be on the ascendency due to rapid industrialization and urbanization in many regions of the world. Microorganisms in these ecosystems are exposed to these chemicals not as a single rather in arrays of mixtures. Incidentally, the toxic effects of these interactions is quite different from their individual component as they modulate the toxicity of each other. This was exposed in this analysis, for instance, the reactions

observed in the combination of cobalt and zinc which are trace elements with lead; a non physiological important element showed that there was a tremendous increase in toxicity effect of zinc and lead as well as cobalt and lead compared with their individual actions. In the combination of lead (Pb (II)) ion with other toxicants, it was observed that their combined toxic effect was synergistic and antagonistic.

In the mixture of lead (Pb(II)) ion and cobalt(CO(II)) ion, it was observed that the experimental equieffect concentration ratio (EECR- 50) at the mixture ratio of (Pb(II)73.05% + Co(II) 26.95%), the mean EC_{50} was 37.36 ± 1.86 which was highly toxic compare to their singles (Pb(II) = 493.38 ± 29.60 and Co(II) =20.49 ± 1.22).This findings was in agreement with the work of Nweke et.al., (2014) in their work on toxicity if binary mixtures of formulated glyphosate and phenol to Rhizobium species dehydrogenase activity. In their study, it was observed that glyphosate compliments the activity of phenol when combined together. In the mixtures of heavy metals and pesticides, it was also obseverved that DDVP modulated the actions and activities of Pb to produce a more toxic and synergistic effect. At a trace concentration ratio as low as 1.47%, Pb was able to exert a very high toxic effect when combined with DDVP (98.53%). This concurs with the report of Samuel et.al. (2016) in their review of toxicity and mechanism of individual and mixtures of heavy metals in the environment where they stated that most toxicity tests are done using individual metals in the environment, neglecting the potential effect of metal mixtures especially at very low concentrations. The experimental toxicity threshold (EC_{50}) of the binary mixtures showed that at all effective concentration/ ratio, organisms under study *(L. macrolides* and *A. faecalis*) were generally sensitive to the toxicants, apart from the mixtures of DDVP and Cadmium in *A. faecalis* where some level of additivity was withnessed and recorded.

The Toxic Index (TI) and isoblographic analysis used to analyse the binary mixture toxicity showed that the toxic effects of most binary mixtures were either synergistic or antagonistic. According to Boillot and Perrodim (2008), TI =1, indicates additive interaction, TI > 1 describes antagonistic while TI < describe synergistic. All the TI values of the binary mixtures in *L. macrolides* showed either synergistic or antagonistic effects apart from DDVP and Cadmiun where some level of additivity was recorded while the TI values recorded with *A. faecalis* showed strictly synergistc and antagonistic without any form of additivity. Synergistic interactions have been reported by (Okechi et al., (2021); Nweke *et al*., (2007) and Nweke et, al. (2014) for the toxicity of binary mixtures of heavy metals and organic compounds to microbial species. The isobolograph was used to translate this report where the reaction effects are either above or beneth the additivity line.

4.2.6 Toxicity of Ternary mixtures of toxicants to *L. macroides* (OK298881) *and A. faecalis* (KX302624)

Microbes encounter metals of various kinds in the environment and it is, therefore, not surprising that they should interact with them, sometimes to their benefitt, at other times to their detriment (Marie et.al 2000). They also opined that aquatic environments receive direct and indirect pesticide inputs, inevitably exposing microorganisms to pesticides. While pesticides elicit a variety of acute and chronic toxicity effects in microorganisms, microorganisms also have the capability to accumulate, detoxify, or metabolize pesticides to some extent. Heavy metals and pesticides are common aquatic pollutants; their impacts and detrimental effects should not be neglected. There are records and information about impacts of heavy metals exposure to aquatic lives so is that of

pesticides to aquatic ecosystem (Rashmi & Pratima (2013) and Nweke *et al.,* (2007)) but limited of such information about the combined effects of heavy metals and pesticides.

The mixtures of Pb(II) ions and Co(II) with DDVP and Glyphosate as shown in Table 4.11 (interaction with *L. macroides*) and Tablen4.12 (interaction with *A. faecalis*) exhibited an antagonistic effect as the toxic index(TI) was greater than one (TI >1) in both the EECR-50 and ABCRs of all the mixtures.When the same mixtures of Pb (II) and Co(II) was combined with Ni(II) the resultant effect was synergistic.. The action of the mixtures of lead, cadmium and glyphosate (Pb (II) + Cd (II) + GLY) on total dehydrogenase activity of *L. macrolides* showed an additive effect on EECR-50 and an antagonistic effect on the ABCRs. When the same Pb(II) and Cd(II) interacts with DDVP, the resultant effects was synergistic. The ternary mixtures exhibited more toxic effect than their binary counterparts. This result conforms with the findings of Reuben et al., (2020) on Invitro Interactive Toxicities of Quaternary and Quinary Mixtures of SDS and Metal Ions to *Serratia marcescens* (SerEW01) where they justified that the interactive effects of quaternary mixtures was higher than the binary effects. All the ternary mixture that contain cadmium (Cd(II)) ions showed to be more toxic than other mixtures. This was equally observed in our single and binary mixtures.

The toxic index (TI) analyses of combinations of DDVP and Glyphosate with the metal ions as expressed on tables 4.11 and 4.12 indicated synergistic effects of the two toxicants with lead (Pb) and cobalt (Co) on *L. macrolides* and *A. faecalis*. When combined with Cd and Ni, the resultant effect was antagonistic.

The responses of the dehydrogenase activities of microbial species to the stress of glyphosate, DDVP, and metal ions (Pb (II), Cd (II), Ni (II), Co (II) and Zn (II)) as individual substances and the mixtures showed that some substances had biphasic effect on the enzyme activity. Dehydrogenase were stimulated at low doses (hormesis) and

inhibited at high doses. As individual substances, glyphosate stimulated the enzyme activity at concentrations up to 2500 mg/L and 250 mg/l, for DDVP on both organisms. At concentrations above the hormetic range, glyphosate and DDVP progressively inhibited their dehydrogenase activities. Equally as ternary, mixtures of glyphosate and DDVP exhibited such effects. The Figure 4.32 showed the interactions of the two test organisms when exposed to the mixtures of Cadmium (Cd (II)) + Nickel (Ni (II)) + Glyphosate at different concenteration ratios. The EECR -50 (at ratios of Cd = 0.96 + Ni=0.90 + Gly= 98.12) the mixture showed a stimulatory effect upto 2500 mg/L and it was more at ABCR 1. This result is in conformity with the findings of Nweke et al., (2016) in their work on "Toxicity of quaternary mixtures of phenolic compounds and formulated glyphosate to microbial community of river water" where glyphosate exhibited such attributes when combine with other toxicants.

In Figiure 4.40, there was equally a case of stimulated dehydrogenase activity at concentrations ranging from 0-20 mg/l to 0 -50 mg/l for DDVP, Pb and glyphosate mixtures on *Lysinibacillus maroides* and *Alcaligenes faecalis* respectively. Similar reaction was experienced when DDVP and glyphosate were combines with Cobalt. At concentrations above the hormetic range, the mixtures also progressively inhibited dehydrogenase activities. This justifys the findings of Nweke et al., (2014), on their study on "Toxicity of Binary Mixtures of Formulated Glyphosate and Phenols to *Rhizobium* Species Dehydrogenase Activity" where hormetic effects of glyphosate and phenolic compounds on microorganisms were recorded.

4.2. 7Toxicity of Septenary mixtures of toxicants to *L. macroides* (OK298881) *and A. faecalis* (KX302624)

The seven mixtures of all the toxicants under study showed that the interactions of the mixtures with *L macrolides* indicated high level of tolerance as almost all the mixture ratios were synergistic while in *A. faecalis* the effects were antagonistic.

In table 4.14, the EECR-50 (Mixture 1), at low concentration of metals and high concentration glyphosate (Pb(II) 0.13%+ Co(II) 0.045% + Cd(II) 0.51% +Ni(II) 1.22%+ Zn(II) 5.28%+ DDVP 8.26%+GLY 84.56%), the effect was synergistic but when the metals were elevated on ABCR 3 and ABCR 4 the resultant effect was antagonistic (Pb(II) 0.49%+ Co(II) 0.18% +Cd(II) 2.05% +Ni(II) 4.86%+ Zn(II) 21.13%+ DDVP 33.04%+GLY 38.24%(ABCR 3) and Pb(II) 50%+ Co(II) 0.23% +Cd(II) 2.57% +Ni(II) 6.08%+ Zn(II) 5.28%+ DDVP 8.26%+GLY 27.58%(ABCR 4)).

CHAPTER FIVE

SUMMARY, CONCLUSION AND RECOMMENDATIONS

5.1 Summary

1. This is a study on pesticides and heavy metals toxicity effect on bacterial isolate from river using Otamiri as a case study.
2. It investigated and determined the pesticides and heavy metal content of Otamiri (water and soil) as well as the physicochemical parameters of the river.
3. It captured the bacteriological diversity of the sample sites then the preponderant organism was identified molecularly to specie level.
4. It highlighted the toxic effect of pesticides and heavy metals to bacteria, both in singles and mixtures. The dose responses were also monitored.
5. It pointed out the need to study the effects of toxicants in their mixtures.
6. From the study it was found out that *L. macrolides* (OK298881) was highly resistant to pesticides and heavy metals under study compared to *A. faecalis*(KX302624). Thus this is a very serious public health concern.

5.2: Conclusion

In conclusion, the study has shown that indiscriminate use of pesticides, improper disposal of waste and poor waste management, sand mining activities among other anthropogenic activities have impacted negatively on the health of aquatic ecosystem. The heavy metal and pesticides content of the river was at the increase which exceeded the World Health Organisation (WHO) standards for river water (WHO, 2017). The physical and some chemical conditions were also impaired as reported in the physicochemical analysis., the physiochemical parameters assayed for had values higher than the World Health Organization (WHO) recommended quality standards for drinking water, thus making the water unfit for human consumption. The study also revealed that the bacteriological diversity of Otamiri river and its environs (soil) varies from one sampling station to the other. The results showed the presence of pathogenic bacteria including the indicator bacteria in both river water and soil, suggesting the possibility of fecal contamination of Otamiri River. Hence, the populace within Owerri municipality that depend on the river water especially for domestic purposes should endeavor to subject the water to thorough purification processes such as chlorination, boiling, ozonization, etc, before usage. Conclusively, this study has shown that exposure of these toxicants in their mixtures pose a very high risk both singly and in combination. The interactive effects were mostly synergistic thus suggesting possible detrimental and co-contamination effects on microbial populations of the ecosystem.

5.3 Recommendations

After the review of the findings of this research, the following recommendations are made to profer solution to the impact of human activities on Otamiri river.

1. Agricultural activities along the banks should be monitored to ensure that indiscriminate use of pesticides is prohibited. The Governing bodies should enact laws in that regard.
2. All sand mining activities should be under strict monitoring and supervision.
3. There is a need for constant monitoring of the microbial population of the river.
4. In addition, communities along the river bank need to ensure that untreated wastes are not disposed improperly on land where it can be washed into the river plus unacceptable anthropogenic activities of man in the river environment should be regulated and properly checked.
5. Government should ensure that companies do not discharge untreated effluent into the river / channeling of drainage system into the river by the government by providing alternative routes for such. Hence, those populace within Owerri municipality that depend on the river water especially for domestic purposes should endeavor to subject the water to thorough purification processes such as chlorination, boiling ,ozonization, etc, before usage.
6. The state ministry of water resources should ensure that natural bodies of water in the state are well protected from pollution via several laws with strict sanctions and punishment for the offenders.
7. There should be a waste treatment facility to curb the menace of indiscriminate disposal of waste into the river.

Therefore, it is necessary to preserve a peaceful environment in order to safeguard human living standards, particularly with regard to water, on which all living creatures significantly depend.

5.4 Contributions to Knowledge

1. The observations from this study has filled the knowledge gap on toxicity of septenary mixture of metal ions and pesticides by providing data for their mixture toxicity in the environment.
2. It also serve as a tool in any future risk assessment protocol of similar mixture by relevant government and regulatory agencies.
3. From this study, I found out that *L. macrolides* is highly resistant to pesticides and heavy metals compared to *A. faecalis*.
4. Finally, this research finding will serve as a tool in environmental laws and regulations formulations

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APPENDIX 1

COMPOSITION AND PREPARATION OF MEDIA AND REAGENTS

Nutrient agar, MacConkey agar, Mannitol salt Agar, Eosin methylene blue Agar and salmonella shigella Agar was procured from a commercial vendor(Steve kens Nig. Ltd)

TCBS agar and peptone were procured from Oxoid

1. PEPTONE WATER

The medium was used as the basis of carbohydrate fermentation. It was also used to test for indole formation.

Formulation	gram/litre
Peptone	10g
Nacl	5g
Distilled water	1 litre

The peptone and the salt was dissolved in the water. 3ml of which was dispensed into bijou bottles, sterilized by autoclaving at 121^{o}c for 15mins.

2. LUGOLS IODINE

Formulation	gram/litre
Potassium	20g
Iodine	10g
Distilled water	1 litre

The potassium was weighed as stated and transferred into a brown bottle, about 250ml of water was added into the bottle. It was shaken until it is dissolved completely. Iodine was added the 750ml of water to make up the one litre

3. CRYSTAL VIOLET

Formulation	gram/litre
Crystal violet	20g
Ammonium oxalate	9g
Absolute Ethanol	95ml
Distilled water	1 litre

Crystal violet was weighed into a bottle, ethanol was added and mixed until dye was completely dissolved then dissolved Ammonium oxalate was put into 100ml of distilled water and added to the mixture. 900ml of water was added finally to make up the litre.

4. NORMAL SALINE

Formulation	gram/litr
Nacl	8.5g
Distilled water	1litre

Sodium chloride (Nacl) was added into a bottle and 1litre water was added to make up the 1000ml mark. It was shaken until the mixture was completely dissolved

5. SIMMON CITRATE AGAR

This is a biochemical test medium used to test for organisms that can utilize citrate as its sole source of carbon and energy.

Formulation	gram/litre
Magnesium sulphate	0.2g
Ammonium dihydrogen phosphate	1.0g
Dipotassium phosphate	1.0g
Sodium citrate	2.0g
Bromothymol blue	0.08g
Sodium chloride	5.0g
Distilled water	1litre

6. CATALASE REAGENT

Formulation	ml
Hydrogen peroxide	3ml
Distilled water	100ml

7. SUGAR FERMENTATION MEDIUM

The sugars used were glucose, sucrose, lactose, galactose, and mannitol

Formulation	gram/litre
Bacteriological peptone	10g
Sodium chloride	5g
Sugar	1g
Deionized water	1 litre
Bromo crysol purple	3ml
pH	7.1

The reagents were weighed and mixed as stated above, then dispensed into test tubes. sterilized by autoclaving at 1231^{o}c in 15psi pressure for 15min.

8. MR VP MEDIUM

The medium used to carry out methyl red and voges proskauer test.

Formulation	gram/litre
Peptone	5.0g
Glucose	5.0g
K_2HO_4	5.0g
Distilled water	1litre

Medium was dissolved as stated and dispensed 10ml into a screw capped tube and autoclaved at 121^{o}c for 15mins.

9. KOVAC REAGENT

Formulation	ml
Amyl alcohol	25ml
Concentrated HCL	25ml
Deionised water	1000ml
Paradimethyl amino Benzaldehyde	50ml

APPENDIX 2a

APPENDIX 2ai **TOTAL HETEROTROPHIC BACTERIAL COUNT (THBC)**

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	**MEAN**	9.70E+06	4.60E+06	1.53E+07	2.10E+06	7.20E+06	8.50E+06	1.60E+06	3.20E+06	3.10E+06	9.70E+06	7.00E+06	2.20E+07
	SDV	1.27E+06	2.83E+05	6.36E+06	7.07E+05	4.53E+06	2.97E+06	8.49E+05	1.41E+06	1.84E+06	2.12E+06	2.83E+05	3.04E+06
MID/S	**MEAN**	1.20E+07	4.00E+06	1.94E+07	1.60E+07	4.50E+05	7.70E+06	1.20E+06	3.00E+06	5.39E+06	7.75E+06	1.00E+06	1.95E+07
	SDV	1.13E+06	8.49E+05	1.63E+06	6.22E+06	2.12E+05	3.54E+06	5.66E+05	2.69E+06	7.09E+06	1.63E+06	2.83E+05	1.77E+06
DOWN/S	**MEAN**	1.27E+07	5.50E+06	1.95E+07	7.30E+06	1.85E+06	8.05E+06	2.00E+06	2.30E+06	1.85E+06	8.00E+06	8.90E+06	7.25E+06
	SDV	1.41E+05	4.24E+05	1.27E+06	6.36E+06	6.36E+05	4.17E+06	5.66E+05	7.07E+05	4.95E+05	2.55E+06	2.40E+06	3.61E+06
SOIL	**MEAN**	4.40E+06	9.05E+06	1.37E+07	1.37E+07	1.90E+06	7.60E+06	4.50E+06	5.40E+06	2.85E+06	1.47E+07	9.45E+06	1.23E+07
	SDV	3.11E+06	2.47E+06	4.10E+06	1.06E+07	7.07E+05	5.09E+06	1.27E+06	8.49E+05	1.63E+06	2.69E+06	2.33E+06	1.07E+07

APPENDIX 2aii **TOTAL COUNT ON EMB (TCEMB)**

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	**MEAN**	4.95E+06	2.50E+06	3.50E+05	9.00E+06	2.80E+06	1.90E+06	2.00E+05	1.40E+06	2.00E+06	1.70E+06	1.00E+06	9.00E+06
	SDV	1.20E+06	9.90E+05	1.06E+05	8.49E+05	1.98E+06	7.07E+05	1.41E+05	2.83E+05	5.66E+05	1.41E+05	5.66E+05	2.55E+06
MID/S	**MEAN**	9.00E+05	3.15E+06	4.45E+06	7.90E+06	6.90E+06	9.00E+06	1.10E+06	2.50E+05	1.00E+06	4.00E+06	1.25E+06	8.20E+06
	SDV	2.83E+05	3.46E+06	7.07E+04	2.40E+06	4.95E+06	3.11E+06	2.83E+05	2.12E+05	2.83E+05	1.13E+06	7.78E+05	1.41E+06
DOWN/S	**MEAN**	7.05E+06	5.00E+06	3.50E+06	2.46E+07	5.10E+06	1.15E+07	1.30E+06	3.00E+05	1.20E+06	3.10E+06	3.00E+05	1.80E+06
	SDV	8.13E+06	4.24E+06	9.90E+05	5.16E+06	1.84E+06	1.84E+06	1.41E+05	1.41E+05	8.49E+05	4.24E+05	1.41E+05	8.49E+05
SOIL	**MEAN**	1.50E+06	5.35E+06	4.50E+05	1.01E+07	2.44E+07	5.00E+06	1.80E+06	3.40E+07	1.45E+06	8.50E+05	1.50E+06	4.20E+06
	SDV	1.41E+05	1.63E+06	2.12E+05	1.63E+06	5.44E+06	1.70E+06	5.66E+05	3.68E+07	7.07E+04	3.54E+05	1.41E+05	2.83E+06

APPENDIX 2aiii

TOTAL SALMONELLA SHIGELLA COUNT (TSSC)

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	**MEAN**	4.70E+06	1.14E+07	3.20E+06	1.70E+06	5.00E+05	3.65E+06	1.50E+06	1.20E+06	2.40E+06	7.10E+06	7.50E+05	6.00E+05
	SDV	1.70E+06	9.62E+06	2.40E+06	1.41E+05	1.41E+05	9.19E+05	4.24E+05	1.41E+05	5.66E+05	5.23E+06	7.07E+04	2.83E+05
MID/S	**MEAN**	1.18E+07	6.75E+06	2.00E+06	2.45E+06	7.50E+05	6.10E+06	3.50E+05	9.50E+05	1.20E+06	2.20E+06	5.50E+05	1.35E+06
	SDV	1.34E+06	2.19E+06	2.83E+05	3.54E+05	4.95E+05	2.97E+06	2.12E+05	1.06E+06	0.00E+00	1.98E+06	7.07E+04	6.36E+05
DOWN/S	**MEAN**	1.52E+07	6.00E+06	1.65E+06	1.10E+06	3.40E+06	9.50E+05	3.50E+05	2.00E+06	2.50E+05	1.28E+07	1.45E+06	1.80E+06
	SDV	7.78E+06	5.09E+06	2.12E+05	8.49E+05	2.83E+05	4.95E+05	3.54E+05	1.41E+05	2.12E+05	1.98E+06	4.95E+05	7.07E+05
SOIL	**MEAN**	2.40E+06	5.50E+06	5.30E+06	7.80E+06	6.50E+05	2.00E+06	7.00E+06	3.00E+06	3.10E+06	1.01E+07	6.50E+06	2.70E+06
	SDV	2.83E+06	4.10E+06	9.90E+05	5.09E+06	6.36E+05	8.49E+05	6.22E+06	1.56E+06	4.24E+05	1.48E+06	2.12E+06	7.07E+05

APPENDIX 2aiv

TOTAL STAPHYLOCOCCAL COUNT (TSC)

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	**MEAN**	9.00E+06	2.40E+06	1.90E+06	1.60E+06	8.00E+05	3.90E+06	8.50E+05	2.30E+06	9.00E+05	9.50E+05	2.00E+05	5.80E+06
	SDV	3.11E+06	0.00E+00	7.07E+05	2.83E+05	5.66E+05	4.24E+05	3.54E+05	1.70E+06	4.24E+05	3.54E+05	1.41E+05	1.41E+06
MID/S	**MEAN**	8.50E+05	3.30E+06	2.60E+06	9.20E+06	7.50E+05	8.40E+06	9.00E+05	3.50E+05	7.50E+05	2.00E+05	2.50E+05	5.40E+06
	SDV	7.78E+05	1.41E+05	2.83E+05	2.83E+06	4.95E+05	3.11E+06	4.24E+05	7.07E+04	7.07E+04	1.41E+05	2.12E+05	1.70E+06
DOWN/S	**MEAN**	1.79E+07	4.70E+06	5.25E+06	1.06E+07	1.60E+06	1.16E+07	1.60E+06	2.00E+05	2.55E+06	2.40E+06	1.95E+06	3.05E+06
	SDV	1.43E+07	1.56E+06	6.15E+06	8.98E+06	2.83E+05	4.95E+05	7.07E+05	1.41E+05	1.06E+06	1.41E+06	9.19E+05	7.07E+04
SOIL	**MEAN**	1.09E+07	7.00E+05	2.45E+06	1.50E+06	1.05E+06	8.50E+06	9.20E+06	1.70E+07	1.60E+06	3.50E+05	4.30E+06	8.30E+06
	SDV	1.41E+05	7.07E+05	1.63E+06	1.41E+05	6.36E+05	2.40E+06	1.41E+06	1.84E+07	2.83E+05	7.07E+04	4.24E+06	2.12E+06

APPENDIX 2av

TOTAL COLIFORM COUNT (TCC)

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	**MEAN**	5.10E+06	1.00E+07	1.10E+06	4.20E+06	9.00E+05	9.00E+06	2.00E+06	3.50E+05	2.35E+06	7.20E+05	4.25E+06	2.50E+06
	SDV	1.84E+06	2.83E+05	0.00E+00	2.83E+06	7.07E+05	3.68E+06	1.70E+06	3.54E+05	1.63E+06	9.62E+05	2.90E+06	8.49E+05
MID/S	**MEAN**	1.22E+07	4.35E+06	2.14E+07	8.40E+06	1.05E+06	2.90E+06	9.00E+05	1.05E+06	3.10E+06	5.45E+06	1.75E+06	9.50E+05
	SDV	2.83E+06	3.54E+05	1.13E+06	2.55E+06	1.20E+06	4.24E+05	7.07E+05	3.54E+05	2.26E+06	6.86E+06	6.36E+05	2.12E+05
DOWN/S	**MEAN**	1.55E+07	1.00E+07	2.03E+07	1.52E+07	7.00E+05	1.50E+06	9.50E+05	9.50E+05	2.00E+05	4.80E+06	2.30E+06	3.04E+06
	SDV	6.08E+06	2.83E+05	8.56E+06	7.07E+06	7.07E+05	4.24E+05	2.12E+05	1.06E+05	1.41E+05	2.26E+06	8.49E+05	1.41E+05
SOIL	**MEAN**	6.80E+06	1.05E+07	1.83E+07	1.52E+07	2.40E+06	1.35E+06	2.35E+06	4.59E+08	1.65E+06	1.70E+06	8.40E+06	2.30E+06
	SDV	5.37E+06	9.90E+05	1.40E+07	7.07E+06	4.24E+05	2.12E+05	7.78E+05	5.53E+08	7.78E+05	1.56E+06	4.10E+06	1.41E+05

APPENDIX 2avi

TOTAL VIBRO COUNT (TVC)

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	**MEAN**	1.58E+06	5.25E+05	3.30E+06	1.20E+06	9.00E+05	1.50E+06	1.75E+06	1.70E+06	3.52E+07	1.70E+06	4.50E+05	1.15E+06
	SDV	1.73E+06	1.77E+05	7.07E+05	1.41E+05	4.24E+05	4.24E+05	9.19E+05	1.41E+05	4.50E+07	1.41E+05	2.12E+05	1.48E+06
MID/S	**MEAN**	1.49E+06	7.15E+06	2.30E+06	1.05E+06	1.50E+06	2.50E+06	1.00E+06	1.50E+05	2.50E+06	5.10E+06	9.50E+05	2.00E+05
	SDV	1.43E+06	9.19E+05	7.07E+05	2.12E+05	1.41E+05	9.90E+05	1.41E+05	7.07E+04	2.12E+06	5.52E+06	7.78E+05	1.41E+05
DOWN/S	**MEAN**	1.86E+06	5.35E+06	1.50E+06	1.25E+06	1.25E+06	1.70E+06	2.10E+06	7.00E+05	2.00E+06	5.20E+06	9.90E+06	9.00E+05
	SDV	1.33E+06	2.76E+06	4.24E+05	2.12E+05	7.07E+04	7.07E+05	4.24E+05	5.66E+05	1.41E+06	3.96E+06	2.12E+06	2.83E+05
SOIL	**MEAN**	4.55E+06	9.00E+06	1.86E+06	3.00E+06	2.30E+06	5.20E+06	3.00E+05	9.00E+07	7.50E+06	1.70E+06	2.70E+06	1.05E+06
	SDV	1.48E+06	3.11E+06	2.18E+06	2.83E+05	1.41E+05	5.66E+06	1.41E+05	9.90E+07	2.12E+06	9.90E+05	1.84E+06	3.54E+05

APPENDIX 2avii

TOTAL ANEROBIC BACTERIAL COUNT

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	MEAN	4.50E+05	1.25E+06	1.35E+06	1.90E+06	1.25E+06	1.45E+06	1.30E+06	6.00E+05	6.50E+05	2.00E+05	1.00E+06	7.40E+05
	SDV	3.54E+05	7.07E+04	6.36E+05	7.07E+05	2.12E+05	2.12E+05	4.24E+05	4.24E+05	3.54E+05	1.41E+05	5.66E+05	9.33E+05
MID/S	MEAN	7.50E+05	7.50E+05	1.04E+06	5.60E+06	8.00E+05	1.10E+06	1.35E+06	9.00E+05	9.00E+05	1.10E+06	7.50E+05	1.05E+06
	SDV	4.95E+05	2.12E+05	3.75E+05	1.13E+06	8.49E+05	7.07E+05	2.12E+05	2.83E+05	2.83E+05	4.24E+05	7.07E+04	7.07E+04
DOWN/S	MEAN	2.75E+06	1.95E+06	1.95E+06	1.50E+06	1.50E+06	3.90E+06	1.40E+06	2.10E+06	1.70E+06	4.50E+05	1.60E+06	1.95E+06
	SDV	2.05E+06	2.12E+05	1.77E+06	1.27E+06	1.41E+05	3.25E+06	5.66E+05	4.24E+05	8.49E+05	4.95E+05	8.49E+05	1.20E+06
SOIL	MEAN	2.85E+06	2.55E+06	2.20E+06	2.30E+06	1.00E+06	2.80E+06	1.55E+06	1.50E+06	1.70E+06	7.50E+05	1.35E+06	1.50E+06
	SDV	2.47E+06	1.06E+06	8.49E+05	1.41E+05	2.83E+05	1.98E+06	3.54E+05	4.24E+05	8.49E+05	7.07E+04	6.36E+05	2.83E+05

MEMBRANE FILTERATION TECHNIQUE

APPENDIX 2aviii

TCC

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	MEAN	2.60E+01	1.10E+01	1.00E+01	2.55E+01	5.00E+00	1.20E+01	2.40E+01	2.15E+01	2.90E+01	1.70E+01	2.15E+01	1.20E+01
	SDV	2.83E+00	2.83E+00	2.83E+00	1.91E+01	4.24E+00	2.83E+00	1.56E+01	7.07E-01	8.49E+00	7.07E+00	7.07E-01	1.41E+01
MID/S	MEAN	1.70E+01	3.35E+01	5.05E+01	2.05E+01	9.50E+00	2.35E+01	2.55E+01	1.50E+01	1.70E+01	3.95E+01	1.15E+01	3.70E+01
	SDV	7.07E+00	1.34E+01	2.05E+01	2.12E+00	7.78E+00	1.06E+01	3.54E+00	8.49E+00	8.49E+00	7.78E+00	7.07E-01	1.98E+01
DOWN/S	MEAN	6.65E+01	4.10E+01	5.00E+01	4.75E+01	1.15E+01	2.90E+01	6.35E+01	4.05E+01	3.85E+01	8.45E+01	8.85E+01	8.85E+01
	SDV	1.48E+01	4.24E+00	3.11E+01	7.78E+00	4.95E+00	4.24E+00	7.07E-01	2.12E+00	2.19E+01	3.89E+01	1.91E+01	2.76E+01
SOIL	MEAN	4.35E+01	1.60E+01	4.35E+01	9.00E+00	2.40E+01	4.50E+01	3.00E+01	6.40E+01	3.90E+01	1.45E+02	9.20E+01	1.84E+02
	SDV	1.77E+01	1.13E+01	2.12E+00	7.07E+00	2.83E+00	1.56E+01	4.24E+00	2.83E+00	7.07E+00	1.56E+01	5.66E+00	3.89E+01

APPENDIX 2aix

MEMBRANE FILTERATION TECHNIQUE

TEC

		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	INLAND BRIDGE	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
LOCATION													
UPS	MEAN	3.20E+01	3.45E+01	3.05E+01	2.55E+01	2.70E+01	3.30E+01	1.00E+01	1.20E+01	1.70E+01	2.20E+01	4.30E+01	2.50E+01
	SDV	5.66E+00	4.31E+01	3.54E+00	6.36E+00	4.24E+00	1.70E+01	2.83E+00	1.56E+01	8.49E+00	1.41E+00	1.56E+01	1.13E+01
MID/S	MEAN	3.65E+01	7.15E+01	2.30E+01	1.85E+01	1.95E+01	2.05E+01	2.20E+01	3.25E+01	1.65E+01	6.35E+01	3.85E+01	3.90E+01
	SDV	1.63E+01	9.19E+00	7.07E+00	4.95E+00	6.36E+00	3.54E+00	1.41E+00	2.12E+00	6.36E+00	1.06E+01	9.19E+00	8.49E+00
DOWN/S	MEAN	6.00E+01	5.35E+01	1.50E+01	3.10E+01	1.45E+01	5.85E+01	3.35E+01	3.45E+01	4.50E+01	1.01E+02	6.15E+01	9.00E+01
	SDV	4.53E+01	2.76E+01	4.24E+00	4.24E+00	4.95E+00	6.36E+00	1.48E+01	2.12E+00	3.11E+01	4.67E+01	3.61E+01	1.41E+00
SOIL	MEAN	4.55E+01	9.00E+01	3.30E+01	4.00E+01	2.95E+01	6.15E+01	3.95E+01	3.80E+01	9.70E+01	1.68E+02	3.95E+01	7.05E+01
	SDV	1.48E+01	3.11E+01	1.41E+00	1.56E+01	9.19E+00	6.36E+00	7.78E+00	5.66E+00	1.70E+01	5.02E+01	7.78E+00	6.58E+01

APPENDIX 2b

APPENDIX 2bi

The mean bacterial count and Biochemical characteristics of isolates from Otamiri river water and soil.

MEAN BACTERIAL COUNT OF OTAMIRI RIVER AND SEDIMENT.

SITE	LOCATION	THBC	TCEMB	TSSC	TSC	TCC	TVC
E/A	WATER	1.15×10^{7}	4.30×10^{6}	1.06×10^{7}	9.25×10^{6}	1.09×10^{7}	1.64×10^{6}
	SED	4.40×10^{6}	1.50×10^{6}	2.40×06	1.09×10^{7}	6.80×10^{6}	4.55×10^{6}
A/B	WATER	4.70×10^{6}	3.55×10^{6}	8.05×10^{6}	3.47×10^{6}	8.12×10^{6}	4.34×10^{6}
	SED	9.05×10^{6}	5.35×10^{6}	5.50×10^{6}	7.00×10^{5}	1.05×10^{7}	9.00×10^{6}
M/V	WATER	1.81×10^{7}	2.77×10^{6}	2.28×10^{6}	3.25×10^{6}	1.43×10^{7}	2.37×10^{6}
	SED	1.37×10^{7}	4.50×10^{5}	5.30×10^{6}	2.45×10^{6}	1.83×10^{7}	1.86×10^{6}
A/R	WATER	8.47×10^{6}	1.38×10^{7}	1.75×10^{6}	7.12×10^{6}	9.27×10^{6}	1.17×10^{6}
	SED	1.37×10^{7}	1.01×10^{7}	7.80×10^{6}	1.50×10^{6}	1.52×10^{7}	3.00×10^{6}
W/B	WATER	3.17×10^{6}	4.93×10^{6}	1.55×10^{6}	1.05×10^{6}	8.83×10^{5}	1.22×10^{6}
	SED	1.90×10^{6}	2.44×10^{7}	6.50×10^{5}	1.0×10^{6}	2.40×10^{6}	2.30×10^{6}
UME	WATER	8.08×10^{6}	7.47×10^{6}	3.57×10^{6}	7.95×10^{6}	4.47×10^{6}	1.90×10^{6}
	SED	7.60×10^{6}	5.00×10^{6}	2.00×10^{6}	8.50×10^{6}	1.35×10^{6}	5.20×10^{6}
I/B	WATER	1.60×10^{6}	8.67×10^{5}	7.33×10^{5}	1.12×10^{6}	1.28×10^{6}	1.62×10^{6}
	SED	4.50×10^{6}	1.80×10^{6}	7.00×10^{6}	9.20×10^{6}	2.35×10^{6}	3.00×10^{5}
N/B	WATER	2.83×10^{6}	6.50×10^{5}	1.38×10^{6}	9.50×10^{5}	7.83×10^{5}	8.50×10^{5}
	SED	5.40×10^{6}	3.40×10^{7}	3.00×10^{6}	1.70×10^{7}	4.59×10^{8}	9.00×10^{7}
FUTO	WATER	3.45×10^{6}	1.40×10^{6}	1.28×10^{6}	1.40×10^{6}	1.88×10^{6}	1.32×10^{7}
	SED	2.85×10^{6}	1.45×10^{6}	3.10×10^{6}	1.60×10^{6}	1.65×10^{6}	7.50×10^{6}
I/B	WATER	8.48×10^{6}	2.93×10^{6}	7.37×10^{6}	1.18×10^{6}	3.66×10^{6}	4.00×10^{6}
	SED	9.45×10^{6}	8.50×10^{5}	1.01×10^{7}	3.50×10^{5}	1.70×10^{6}	1.70×10^{6}
MGB	WATER	5.63×10^{6}	8.50×10^{5}	9.17×1	8.00×10^{5}	2.77×10^{6}	3.77×10^{6}
	SED	9.45×10^{6}	1.50×10^{6}	6.50×10^{6}	4.30×10^{6}	8.40×10^{6}	2.70×10^{6}
UMU	WATER	1.62×10^{7}	6.33×10^{6}	1.25×10^{6}	4.75×10^{6}	7.63×10^{6}	7.50×10^{5}
	SED	1.23×10^{7}	4.20×10^{6}	2.70×10^{6}	8.30×10^{6}	2.30×10^{6}	1.05×10^{6}

E/A= EGBU ABATTIOR, A/B = AKACHI BRIDGE, M/V =MECHANIC VILLAGE, A/R = ABA ROAD, W/E = WESTEND BRIDGE, UME = UMEZURUIKE HOSPITAL, I/B = INLAND BRIDGE, N/B = NEKEDE BRIDGE, I/B = IHIAGWA BRIDGE, MGB = MGBIRICHI, UMU = UMUAGWO , SED = SEDIMENT**THBC**= TOTAL HETEROTROPHIC BACTERIAL COUNT, **TCEMB**= TOTAL COUNT ON EIOSEN METHYLENE BLUE, **TSC**= TOTAL STAPHYLOCOCCAL COUNT, **TSSC**= TOTAL SALMONELLA SHIGELLA COUNT, **TCC**= TOTAL COLIFORM COUNT. **TVC** = TOTAL VIBRIO COUNT

APPENDIX
2bii

MEAN ANEROBIC BACTERIAL COUNT

LOCATION		EGBU/A	AKACHI	MV	ABA/RD	WEST END	UMZ	ALVAN	NEKEDE	FUTO	IHIAGWA	MGBIRICHI	UMUAGWO
WATER	MEAN	1.32×10^{6}	1.32×10^{6}	1.45×10^{6}	3.00×10^{6}	1.18×10^{6}	2.15×10^{6}	1.35×10^{6}	1.20×10^{6}	1.08×10^{6}	5.83×10^{5}	1.12×10^{6}	1.25×10^{6}
SED	MEAN	2.85×10^{6}	2.55×10^{6}	2.20×10^{6}	2.30×10^{6}	1.00×10^{6}	2.80×10^{6}	1.55×10^{6}	1.50×10^{6}	1.70×10^{6}	7.50×0^{5}	1.35×10^{6}	1.50×10^{6}

APPENDIX 2biii

MEAN TCC COUN BY MEMBRANE FILTERATION TECHNIQUE

LOCATION		**EGBU/A**	**AKACHI**	**MV**	**ABA/RD**	**WEST END**	**UMZ**	**ALVAN**	**NEKEDE**	**FUTO**	**IHIAGWA**	**MGBIRICHI**	**UMUAGWO**
WATER	MEAN	1.70×10^{1}	3.35×10^{1}	5.05×10^{1}	2.05×10^{1}	9.50×10	2.35×10^{1}	2.55×10^{1}	1.50×10^{1}	1.70×10^{1}	3.95×10^{1}	1.15×10^{1}	3.70×10^{1}
SED	MEAN	4.35×10^{1}	1.60×10^{1}	4.35×10^{1}	9.00×10	2.40×10^{1}	4.50×10^{1}	3.00×10^{1}	6.40×10^{1}	3.90×10^{1}	1.45×10^{2}	9.20×10^{1}	1.84×10^{2}

APPENDIX 2biv

MEAN TEC BY MEMBRANE FILTERATION TECHNIQUE

LOCATION		**EGBU/A**	**AKACHI**	**MV**	**ABA/RD**	**WEST END**	**UMZ**	**ALVAN**	**NEKEDE**	**FUTO**	**IHIAGWA**	**MGBIRICHI**	**UMUAGWO**
WATER	MEAN	4.28×10^{1}	5.32×10^{1}	2.28×10^{1}	2.50×10^{1}	2.03×10^{1}	3.73×10^{1}	2.18×10^{1}	2.63×10^{1}	2.62×0^{1}	6.22×101	4.77×10^{1}	5.13×10^{1}
SED	MEAN	4.55×10^{1}	9.00×101	3.30×10^{1}	4.00×10^{1}	2.95×10^{1}	6.15×10^{1}	3.95×10^{1}	3.80×10^{1}	9.70×10^{1}	1.68×10^{2}	3.95×10^{1}	7.05×10^{1}

APPENDIX 3

APPENDIX3i

BIOCHEMICAL CHARACTERISTICS OF ISOLATES FROM OTAMIRI WATER

Morphology	Gram Stain	Spore	Capsule	Oxidase	Catalase	indole	Motility	MR	VP	Urease	Coagulase	Nitrate	Citrate	Lactose	Sucrose	Maltose	Glucose	Mannitol	Gas	slope	Butt	H_2S	Organism
Thick mucoid, pink on MacConkey colonies circular, convex with an entire margin.	- rods	-	+	-	+	-	-	-	+	+	+	+	+	+	+	+	+	+	+	Y	Y	-	*Klebsiella aerogenes*
2mm colony, green metallic sheen on EMB, regular with entire margin	-rods	-	-	-	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	Y	Y	-	*Eschericia coli*
4-5mm large ,round colonies, muccoid, pink on EMB without sheen, tiny transpereny on TCBS, raised, tending to confluent.	-rods	-	+	-	+	-	+	-	+	-	-	+	+	+	+	+	+	+	+	Y	Y	-	*Enterobacter aeruginosa*

Small, 1-2mm round, regular yellow haemolytic colonies on MSA with entire margin.	+cocci	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	+	+	-	Y	Y	-	*Staphylococcus aureaus*
Small round glistening yellow colonies on TCBS with entire edge.	- curve rodwith a single flagellum	-	-	+	+	+	+	-	+	-	+	+	+	-	+	+	+		-	R	Y	-	*Vibrio cholerae*
wn with black center nies, opaque on SSA, urless transluscent on B	- rods	-		+		-	+	+	-	-	-	+	-	-	-		+	+	-	R	Y	+	*Salmonella Typhi*
Colourless with black center colonies,	-rods	-	+	-	+	-	+	+	-	-	-	+	-	-	-		+	+	+	R	Y	-	*Salmonella paratyphi*
Cream with pink center on SSA, Colourless on MacConkey	-rods	-	-	-	+	+	-	+	-	-	-	+	-	-	-		+	+	-	R	Y	-	*Shigella Spp.*
Round, small glistening with dark center on SSA, dry pink on macConkey,	-rods	-	-	+	-	-	+	+	-	-	-	+	+	+	+	+	+	+	+	R	Y	+	*Citrobacter spp*
Red colonies on nutrient Agar(NA), round and regular colonies. Rose red on MacConkey	-rods	-	-	-	+	-	+	+	+	+	+	+	+	-	+	+	+		- / +	R	Y	-	*Serratia marcescens*

Swarming colonies on NA, round on MacConkey Fishy odour,	- rods	-	-	-	+	-	+	+	-	+	+	+	+	-	-	-	+	-	+	R	Y	+	*Proteus mirabilis*
Round, raised, pink on NA	+cocci	-	-	+	+	-	-	+	-		-	+	+	+	+	-	+			R	R		*Micrococcus roseus*

+ = positive, - = negative, R= no fermentation, Y= fermentation, spp= species, MR = , VP =, H_2S =Hydrogen sulphide

APPENDIX3ii

BIOCHEMICAL CHARACTERISTICS OF ISOLATES SOIL

Morphology	**Gram Stain**	**Spore**	**Capsule**	**Oxidase**	**Catalase**	**indole**	**Motility**	**MR**	**VP**	**Urease**	**Coagulase**	**Nitrate**	**Citrate**	**Lactose**	**Sucrose**	**Maltose**	**Glucose**	**Mannitol**	**Gas**	**slope**	**Butt**	**H_2S**	**Organism**
Thick mucoid, opaque cream colonies on SSA, circular, convex with an entire margin.	-rods	-	+	-	+	-	-	-	-	-	-	+	+	+	+	+	+	+	+	Y	Y	-	*Klebsiella peumonia*
2mm colony, green metallic sheen on EMB, regular with entire margin	-rods	-	-	-	+	+	+	+	-	-	-	+	-	+	+	+	+	+	+	Y	Y	-	*Eschericia coli*
Punctiform, convex with entire margin, red on MacConkey,small round	+cocci in pairs	-	-	-	-	-	-	-	+	-	-	+	-	+	+	+	+	+	-	R	Y	-	*Enterococcus faecalis*

colonies and non muccoid																							
Small,2-3mm round, regular pink colonies without haemolysis on MSA	+ cocci	-	+	-	+	-	-	+	+	+	-	-	+	+	+	+	+	-	+	Y	Y	+	*Staphylococcus epidermidis*
Small round green with black center colonies on TCBS	- curve rod with a single flagellum	-	-	-	+	+	+	-	-/+	-	+	+	+	-	-	+	+	+	-	R	Y	-	*Vibrio parahaemolyticus*
Pale colour fluorescent colonies on MAC, Colourlss irregular colonies on EMB. Large,opaque.flat irregular with fruity smell	-rods	-	-	+	+	-	+	-	-	-	-	+	+	-	-	-	-	+	+	R	R	-	*Pseudomonas aeruginosa*
Dry, raised, irregular large grey- white colonies.	+rods	+	+	-	+	-	+	-	+	-	-	-	+	-	+	-	+	-	-	Y	R	+	*Bacillus anthracis*
Swarming colonies on NA, round on MacConkey Fishy odour,muccoid	-rods	-	-	-	-	+	+	+	-	+	+	+	-	-	+	+	+	+	+	R	Y	+	*Proteus vulgaris*
Large, cream and irregular colony with zone haemolysis	+rod	+	+	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	Y	Y		*Clostridium spp*
Large, white, lumniscent colony, that grow in chain on NA	+ rod	+		-	+	-	-	-	+	-	-	+	+	-	-	+	+	+	-	Y	R	+	*Bacillus subtilis*
Round, cream, raised,regular colony with zone of haemolysis	+cocci	-	+	-	-	-	-	+	-	-	-	-	-	-	+	+	+	-					*Streptococcus spp*
Round, cream	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	Y	Y	-	

	Coccobacilli																						*Lactobacillus*
Round, raised, entire edge, smooth surface, tranluscent, white colonies	-ROD	-	-	-	-	+	+		+	-		+	+	- / +	+	+	+	+	+	R	Y	+	*Aerococcus*

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APPENDIX 4

APPENDIX4i

B1= *Klebsiella pneumonia* (MK641337)

GAGAGCYYGCTCTCGGGTGACGAGCGGCGGACGGGTGAGAATAGKCTGGGAAACTGCCTG
ATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAG
TGGGGGACCTTCGGGCCTCATGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGG
GGTAAYGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTG
GAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGCAAGCCTGATGCAGCCATGCCGCGTGTRTGAAGAAGGCCTTCGGGTTGTAAAGYACTT
TCAGCGGGGAGGAAGGCGATRAGGTTAATAACCTYGYCGATTGACGTTACCCGCAGAAGA
AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGG
AATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCAAGTCGGATGTGAAATCCCCGGGC
TCAACCTGGGAACTGCATTCGAAACTGGCAGGCTAGAGTCTTGTAGAGGGGGGTAGAATT
CCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCC
CTGGACAAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCT
GGTAGTCCACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCG
GAGCTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGA
ATTGACGGGGGCCCGCACAASCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAA
CCTTACCTGGTCTTGACATCCACAGAACTTRC

B2 = *Lysinibacillus macrolides* (OK298881)

ARGAGCTTGCTCCTTTGACGTTAGCGGCGGACGGCTGAGTAAYRCGTGGGCAACCTACCC

TATAGTTTGGGATAACTCCGGGAAACYGGGGGYTAATACCGAATAATCTCTTTTGCTTCAT

GGKGAAAGACTGAAAGACGGTTTCGGCTGWCGCTATAGGATGGGCCCGCGGCGCATTAGC

TAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATC

GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTT

CCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGAGTGAAGAAGGTTTTCGGATCG

TAAAACTCTGTTGTARGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTAC

CTTATTAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG

CGTTGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTCCTTTAAGTCTGATGTGAA

AGCCCACGGCTCAWCCGTGGAGGGTCATTGGAAACTGGGGGACTTGAGTGCAGAAGAGGA

AAGTGGAATTCCAAGTGTAGCGGTGAAATGCGTAGAGATTTGGAGGAACACCAGKGGCGA

AGGCGACTTTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGGAT

TAGATACCCTGSTAGTCCACGCCGTAAACGATGAGTGCTAASTGTTAGGGGGT

B3= *Alcaligenes faecalis* (KX302624)

ACGGCAGCGCGAGAGAGCTTGCTCTCTTGGCGGCGAGTGGCGGACGGGTGAGTAATATAT
CGGAACGTGCCCAGTAGCGGGGGATAACTACTCGAAAGAGTGGCTAATACCGCATACGCC
CTACGGGGGAAAGGGGGGGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATT
AGCTAGTTGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGGACG
ACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
TTTGGACAATGGGGGAAACCCTGATCCAGCCATCCCGCGTGTATGATGAAGGCCTTCGGG
TTGTAAAGTACTTTTGGCAGAGAAGAAAAGGTATCYCCTAATACGRGATACTGCTGACGG
TATCTGCAGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGC
AAGCGTTAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAGATGT
GAAATCCCAGGGCTCAACCTTGGAACTGCATTTTTAACTGCCGAGCTAGAGTATGTCAGA
GGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTAGATATGTGGAGGAATACCGATGG
CGAAGGCAGCCCCCTGGGATAATACTGACGCTCAGACACGAAAGCGTGGGGAGCAAACAG
GATTAGATACCCTGGTAGTCCACGCCCTAAACGATGTCAACTAGCTGTTGGGGCCGTTAG
GCCTTAGTAGCGCAGCTAACGCGTGAAGTTGACCGCCTGGGGAGTACGGTCGCAAGATTA
AAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGATGATGTGGATTAATTCGATG
CAACGCGAAAAACCTTACCTACCCTTGACATGTCTGGAAAGCCGAAGA

B4= *Proteus mirabilis* (MZ067158)

KTTACAGCGTGGACTACCAGGGKATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTGA

GCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTCCACATCTCTACGCA

TTTCACCGCTACACATGGAATTCTACCCCCCTCTACAAGACTCTAGCCAACCAGTTTCRG

ATGCAATTCCCAAGTTAAGCTCGGGGCTTTCACATCTGACTTAATTGACCGCCTGCGTGC

GCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGGC

ACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGACAAGGGWATTAACCTTAT

CACCCTTCCTCCCCCGCTGAAAAGTACTTTACAACCCCTAAGGCCTTCTTYCATACACGS

CGGCATGGGCTGCATCAGGGCTTGCGCCCCATTGGKGCAATATTCCCCACTGCTGCCTCC

CGGAGGAGTCTGGGCCGGTGTCTCAGGTCCCAGKGTGGYTGATCATCCTCTCAGACCAGC

TAGAGATCGTCGCCCTAGGGGAGCCTTTACCCCACCTACTAGCTAAATCCCATATGGGGT

TCATCCGATAGTGCAAGGKCCGAAGAGCCCCTGCTTTKGGCCSGTAGACATTATGCGGAT

TAGCCACCGTTTCCAGTAGTWATCCCCCTCTATCGGGCAGATCCCCCATACATTACTCACCCC

B5= *Pseudomonas aeruginosa* (CP058331)

TTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCCCCACGCTTTCGCACCTSAG

TGTCAGTATYWGTCCAGGKGGTCGCCTTCGCCACYGGTGTTCCTTCCTATATCTACGCAT

TTCACCGCTACACAKGRAATTCYACCACCCTCTACCGTACTCTAGCTGRRTAGTTTTGGA

TGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCAACTTGYTGAACCACCTRCGCGCG

CTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTTCGTATTACCGCGGCTGCTGGCA

CGAAGTTAGCCGGTGCTTATTCTGYKRGTAACGTCAAAACARCAAGGTATTAACTWACKG

CCCTTCCTCCCAACTKAAAGTGCTTTACAAYCCGAAGACCTTCTTCACACACGCGGCATG

GCTGSATCAGGCTTTCSCCCATTGTCCAATATTCCCCACTGCTGCCTCCCGTAGGAGTCT

GGACCGTGTCTCAGTTCCAGTGTGACTGATCATCCTCTCAGACCAGYTACGGATCGTCGC

CTTGGTRRGCCTTTACCYCACCAACTAGCTAATCCGACCTRGGCTCATCTRATAGCGTRA

GGYCCGAAGATCCCCYRCTTTCTCCCTMAGGACGTATGCGGTATTAGCKMCCGTTTCCRG

GACGTTATCCCCCTCTACYRGGCAGATYCCTAGGCATTACTCACCCGTCCGCCGCTGAAT

APPENXIX 5

The Equieffect Concentration Ratio (EECR) and Arbitrary Concentration Ratio (ABCR) of mixtures for *Lysinibacillus macrolides* and *Alcaligenes faecalis.*

APPENDIX5i

The Equieffect Concentration Ratio (EECR) and Arbitrary Concentration Ratio (ABCR) of Binary mixtures for *Lysinibacillus macrolides* and *Alcaligenes faecalis.*

Lysinibacillus macroides

S/No	Binary mixtures of Toxicants	EECR-50(%) Toxicant 1	EECR-50(%) Toxicant 2	ABCR 1 Toxicant 1	ABCR 1 Toxicant 2	ABCR 2 Toxicant 1	ABCR 2 Toxicant 2
1	Pb(II)+ Co(II)	73.04656	26.95344	75	25	70	30
2	Pb(II) + Ni(II)	9.179682	90.82032	10	90	5	95
3	Pb(II) + Cd(II)	19.31239	80.68761	20	80	25	75
4	Pb(II) + Zn(II)	2.274033	97.72597	5	95	10	90
5	Pb(II) + DDVP	1.466353	98.53365	2	98	3	97
6	Co(II) + Ni(II)	3.595476	96.40452	5	95	10	90
7	Co(II) + Cd(II)	8.114995	91.885	10	90	5	95
8	Co(II) + Zn(II)	0.851311	99.14869	0.5	99.5	2	98
9	Co(II) + DDVP	0.546123	99.45388	1	99	0.25	99.75
10	Ni(II)+ Cd(II)	70.30893	29.69107	75	25	80	20
11	Ni(II) + Zn(II)	18.7137	81.2863	20	80	15	85
12	Ni(II) + DDVP	12.83386	87.16614	15	85	10	90
13	Cd(II) + Zn(II)	8.860612	91.13939	10	90	5	95
14	Cd(II) + DDVP	5.85367	94.14633	3	97	8	92
15	Zn(II) + DDVP	39.00726	60.99274	35	65	45	55

APPENDIX5ii ***Alcaligenes faecalis***

S/No	EECR-50(%) Binary mixtures of Toxicants	Toxicant 1	Toxicant 2	ABCR 1 Toxicant 1	Toxicant 2	ABCR2 Toxicant 1	Toxicant 2
1	Pb(II)+ Co(II)	93.67672	6.323282	95	5	90	10
2	Pb(II) + Ni(II)	88.42434	11.57566	85	15	90	10
3	Pb(II) + Cd(II)	87.73298	12.26702	90	10	85	15
4	Pb(II) + Zn(II)	50.25111	49.74889	60	40	55	45
5	Pb(II) + DDVP	52.61513	47.38487	50	50	45	55
6	Co(II) + Ni(II)	34.02078	65.97922	25	75	30	70
7	Co(II) + Cd(II)	32.55837	67.44163	30	70	25	75
8	Co(II) + Zn(II)	6.383041	93.61696	5	95	10	90
9	Co(II) + DDVP	6.97257	93.02743	10	90	5	95
10	Ni(II)+ Cd(II)	48.35411	51.64589	50	50	45	55
11	Ni(II) + Zn(II)	11.67887	88.32113	10	90	15	85
12	Ni(II) + DDVP	12.6912	87.3088	15	85	10	90
13	Cd(II) + Zn(II)	12.37553	87.62447	15	85	10	90
14	Cd(II) + DDVP	13.43907	86.56093	10	90	15	85
15	Zn(II) + DDVP	52.36465	47.63535	50	50	55	45

APPENDIX5iii

The Equieffect Concentration Ratio (EECR) and Arbitrary Concentration Ratio (ABCR) of Tenary mixtures for *Lysinibacillus macrolides and Alcaligenes faecalis*

			Lysinibacillus macroides					
			EECR-50(%)			ABCR1		
S/ No		Toxicant mixtures	Toxicant 1	Toxicant 2	Toxicant 3	Toxicant 1	Toxicant 2	Toxicant 3
1	ABC	Pb(II) + Co(II) + Cd(II)	18.02772	6.652046	75.32023	20.030	9.650	70.32
2	ABD	Pb(II) + Co (II) +Ni(II)	8.878934	3.276236	87.84483	2.260	0.830	96.90
3	ABE	Pb(II) + Co(II) + Zn(II)	2.255111	0.832113	96.91278	46.950	6.560	36.480
4	ABF	Pb(II) + Co(II) + DDVP	1.458462	0.538158	98.00338	8.460	2.540	80.00
5	ABG	Pb(II) + Co(II) + Gly	0.145073	0.053531	99.8014	2.560	28.930	68.510
6	BCD	Co(II) + Cd(II)+Ni(II)	2.555219	28.9324	68.51238	7.600	3.400	89.00
7	ACE	Pb(II) + Cd(II) + Zn(II)	2.076725	8.676601	89.24667	46.950	6.560	36.480
8	ACF	Pb(II) + Cd(II) + DDVp	1.381704	5.77279	92.84551	8.380	7.770	83.850
9	ACG	Pb(II) + Cd(II) +Gly	0.144276	0.602789	99.25293	7.150	2.600	90.250
10	ADF	Pb(II) + Ni(II) +DDVP	1.280574	12.66951	86.04991	8.280	14.670	77.08
11	ADG	Pb(II) + Ni(II) Gly	0.143096	1.415739	98.44116	7.140	3.420	89.44
12	AEG	Pb(II) + Zn(II) +GLY	0.136628	5.871571	93.9918	6.190	6.130	87.68
13	BCE	Co(II) + Cd(II) +Zn	0.776465	8.791812	90.43172	7.770	10.790	81.43
14	BCF	Co(II) + Cd(II) +DDVP	0.514319	5.823563	93.66212	7.510	7,82	84.66
15	BCG	Co(II) + Cd(II) + GLY	0.053285	0.603338	99.34338	6.190	6.130	87.68
16	BDA	Co(II) + Ni(II)+ Pb(II)	3.276236	87.84483	8.878934	6.280	85.840	7.87
17	BDF	Co(II) + Ni(II) +DDVP	0.476368	12.77272	86.75091	7.480	14.770	77.75
18	BDG	Co(II) + Ni(II) +GLY	0.052849	1.417019	98.53013	6.190	6.130	87.68
19	CDA	Cd(II) + Ni(II) + Pb(II)	3.276236	87.84483	8.878934	13.370	85.580	1.05
20	CDB	Cd(II) + Ni(II) + Co(II)	28.9324	68.51238	2.555219	31.930	66.510	1.56
21	CDE	Cd(II) + Ni(II) + Zn(II)	7.323905	75.33297	17.34312	14.320	77.330	8.34
22	CDF	Cd(II) + Ni(II) +DDVP	12.17407	5.14104	82.68489	19.170	7.140	73.69
23	CDG	Cd(II) + Ni(II) + GLY	0.595152	1.40933	97.99552	7.600	3.400	89.00
24	DEA	Ni(II) + Zn(II) + Pb(II)	18.3663	79.77732	1.856378	13.370	85.580	1.05
25	DEB	Ni(II) + Zn(II) + Co(II)	80.7229	18.58399	0.693103	80.730	18.580	0.69
26	DEF	Ni(II) + Zn(II) +DDVp	55.96679	35.79296	8.240241	55.970	35.790	8.24
27	DEG	Ni(II) + Zn(II) +Gly	1.335522	5.801081	92.8634	12.38	10.42	77.20
28	EFA	Zn(II) + DDVP + Pb(II)	38.65638	60.44411	0.899514	45.660	53.440	0.99
29	EFB	Zn(II) + DDVP + Co(II)	60.78915	38.87705	0.333806	65.790	33.880	0.33
30	EFC	Zn(II) + DDVP+ Cd(II)	37.58203	58.76423	3.653742	37.580	60.760	1.65
31	EFG	Zn(II) + DDVP+ Gly	5.384573	8.419457	86.19597	12.38	10.42	77.20
32	FGA	DDVP+ GLY+ Pb(II)	8.88684	90.98091	0.132252	15.39	84.48	0.13
33	FGB	DDVP + GLY + Co(II)	8.894263	91.0569	0.04884	10.89	88.06	1.04
34	FGC	DDVP+ GLY + Cd(II)	8.849645	90.60012	0.550238	10.85	86.60	3.55
35	FGD	DDVP+ GLY + Ni(II)	8.783529	89.92323	1.293238	55.970	35.790	8.24

APPENDIX5iv			Alcaligenes faecalis					
			EECR-50(%)			ABCR1		
S/ No		Toxicant mixtures	Toxicant 1	Toxicant 2	Toxicant 3	Toxicant 1	Toxicant 2	Toxicant 3
1	ABC	Pb(II) + Co(II) + Cd(II)	82.82785	5.590971	11.58117	84.83	8.59	6.58
2	ABD	Pb(II) + Co (II) +Ni(II)	83.4438	5.632548	10.92365	90.44	7.63	1.92
3	ABE	Pb(II) + Co(II) + Zn(II)	48.60251	3.280723	48.11677	55.6	5.58	39.12
4	ABF	Pb(II) + Co(II) + DDVP	50.81056	3.429769	45.75968	57.81	5.43	37.76
5	ABG	Pb(II) + Co(II) + Gly	6.56556	0.443183	92.99126	13.56	2.44	84
6	BCD	Co(II) + Cd(II)+Ni(II)	19.95695	41.33895	38.7041	26.96	43.34	29.79
7	ACE	Pb(II) + Cd(II) + Zn(II)	46.95215	6.564953	46.4829	53.95	6.56	46.48
8	ACF	Pb(II) + Cd(II) + DDVp	49.00961	6.852633	44.13776	56	8.85	35.15
9	ACG	Pb(II) + Cd(II) +Gly	6.534532	0.913673	92.55179	13.53	2.98	83.55
10	ADF	Pb(II) + Ni(II) +DDVP	49.22461	6.444008	44.33138	56.22	8.44	35.33
11	ADG	Pb(II) + Ni(II) Gly	6.53834	0.855936	92.60572	13.54	2.86	83.6
12	AEG	Pb(II) + Zn(II) +GLY	6.19061	6.12874	87.68065	13.19	8.13	78.68
13	BCE	Co(II) + Cd(II) +Zn	5.637639	11.67784	82.68452	12.64	13.68	73.68
14	BCF	Co(II) + Cd(II) +DDVP	6.092613	12.62028	81.28711	13.69	14.62	72.29
15	BCG	Co(II) + Cd(II) + GLY	0.46971	0.97296	98.55733	13.53	2.98	83.55
16	BDA	Co(II) + Ni(II)+ Pb(II)	5.632548	10.92365	83.4438	12.43	7.12	80.44
17	BDF	Co(II) + Ni(II) +DDVP	6.142018	11.91171	81.94627	13.14	13.91	72.94
18	BDG	Co(II) + Ni(II) +GLY	0.470001	0.911511	98.61849	7.47	2.91	89.61
19	CDA	Cd(II) + Ni(II) + Pb(II)	5.632548	10.92365	83.4438	8.63	8.92	82.44
20	CDB	Cd(II) + Ni(II) + Co(II)	41.33895	38.7041	19.95695	44.34	36.7	18.96
21	CDE	Cd(II) + Ni(II) + Zn(II)	11.0905	78.52588	10.38362	18.1	80.52	1.38
22	CDF	Cd(II) + Ni(II) +DDVP	11.17624	11.93708	76.88667	18.18	13.94	67.88
23	CDG	Cd(II) + Ni(II) + GLY	0.968685	0.906944	98.12437	7.47	2.91	89.61
24	DEA	Ni(II) + Zn(II) + Pb(II)	6.172346	46.67822	47.14943	1.17	52.48	46.35
25	DEB	Ni(II) + Zn(II) + Co(II)	83.30457	11.01552	5.679916	77.8	16.51	5.68
26	DEF	Ni(II) + Zn(II) +DDVp	44.55055	48.97358	6.475867	49.55	43.97	6.48
27	DEG	Ni(II) + Zn(II) +Gly	0.856496	6.477228	92.66628	13.53	2.98	83.55
28	EFA	Zn(II) + DDVP + Pb(II)	34.24915	31.15595	34.5949	41.24	24.16	34.6
29	EFB	Zn(II) + DDVP + Co(II)	45.99323	50.55949	3.447274	50.99	45.56	3.45
30	EFC	Zn(II) + DDVP+ Cd(II)	48.75863	44.35501	6.886363	18.18	13.94	67.88
31	EFG	Zn(II) + DDVP+ Gly	6.166689	5.609747	88.22356	13.17	7.61	79.22
32	FGA	DDVP+ GLY+ Pb(II)	5.606257	88.16868	6.225067	12.11	81.67	6.23
33	FGB	DDVP + GLY + Co(II)	5.951748	93.60216	0.446094	7.95	90.6	1.45
34	FGC	DDVP+ GLY + Cd(II)	5.923437	93.15692	0.919647	7.92	88.16	3.92
35	FGD	DDVP+ GLY + Ni(II)	5.926911	93.21155	0.861536	11.42	84.21	4.36

APPENDIX5v

The Equieffect Concentration Ratio (EECR) and Arbitrary Concentration Ratio (ABCR) of Septenary mixtures for *Lysinibacillus macrolides and Alcaligenes faecalis*

	Lysinibacillus macroides						
	Toxicant 1 Pb	Toxicant 2 Co	Toxicant 3 Cd	Toxicant 4 Ni	Toxicant 5 Zn	Toxicant 6 DDVP	Toxicant 7 GLY
EECR-50(%)	0.1200	0.045356	0.513555093	1.216107	5.282378	8.2596606	84.56002
ABC R 1	0.24583	0.09071	1.02711018	2.43221	10.5647	16.51932	69.12005
ABC R 2	0.36875	0.13606	1.54066528	3.64832	15.8471	24.77898	53.68008
ABC R 3	0.49167	0.18142	2.05422037	4.86442	21.1295	33.03864	38.2401
ABC R 4	0.61459	0.22677	2.56777546	6.08053	26.4118	41.29830	22.80013
ABC R 5	50	0.22677	2.56777546	6.08053	5.282378	8.2596606	27.58287
ABC R 6	1.22918	0.45355	5.13555093	12.1610	5.282378	8.2596606	67.47861

	Alcaligenes faecalis						
	Toxicant 1 Pb	Toxicant 2 Co	Toxicant 3 Cd	Toxicant 4 Ni	Toxicant 5 Zn	Toxicant 6 DDVP	Toxicant 7 GLY
EECR50(%)	5.75	0.39	0.80	0.75	5.69	5.18	81.44
ABCR 1	11.50	0.78	1.61	1.51	11.38	10.36	62.87
ABCR 2	17.25	1.16	2.41	2.26	17.08	15.53	44.31
ABCR 3	23.00	1.55	3.22	3.01	22.77	20.71	25.74
ABCR 4	28.75	1.94	4.02	3.76	28.46	25.89	7.18
ABCR 5	50.00	1.94	4.02	3.76	5.69	5.18	29.41
ABCR 6	57.50	3.88	8.04	7.53	5.69	5.18	12.19

APPENDIX 6

PREPARATION OF STOCK SOLUTIONS OF HEAVY METALS:

1) Zinc nitrate hexahydrate Zn $NO_3.6H_2O$ (Molecular weight (MW)= 297.49g/mol)

1M	=	297.49g/L
1000 m M	=	297.49g/L
10 m M	=	2.9749g/L
10 m M	=	0.297g/100ml
10 m M	=	0.744g/250ml
	=	744mg/250ml
	=	2.976g/L
	=	2976mg/L
	≈	3000mg/L

2) Cadmium sulphate hydrate = $CdSO_4.8H_2O$ (MW= 256.5g/mol)

1M	=	256.5g/L
1000 m M	=	256.5g/L
10 m M	=	2.565g/L
10 m M	=	0.257g/100ml
10 m M	=	0.641g/250ml
	=	641mg/250ml
	=	2.65g//L
	=	2565mg/L
	≈	2600mg/L

3) Lead (II) nitrate = Pb $(NO_3)_2$ (MW= 331.21g/mol)

1M	=	331.21g/L
1000 m M	=	331.21g/L
10 m M	=	3.3312 g/L
10 m M	=	0.331g/100ml
10 m M	=	0.828g/250ml
	=	828mg/250ml
	=	3.312g/L
	=	3312.1mg/L
	≈	3320mg/L

4) Nickel sulphate hexahydrate = $NiSO_4.6H_2O$ (MW= 262.86g/mol)

1M	=	262.86g/L
1000 m M	=	262.86g/L
10 m M	=	2.6286g/L
10 m M	=	0.26286g/100ml
10 m M	=	0.657g/250ml
	=	657mg/250ml
	=	2.6286g/L
	=	2629mg/L
	≈	2650mg/L

5) Cobalt (II) nitrate hexahydrate = Co $(NO_3)_2$. $6H_2O$ (MW= 291.03g/mol)

1M	=	291.03g/L
1000 m M	=	291.03g/L
10 m M	=	2.9103g/L
10 m M	=	0.291g/100ml
10 m M	=	0.7275g/250ml
	=	7275mg/250ml
	=	2.9103g/L
	=	2910.3mg/L
	≈	3000mg/L

6) GLYPHOSATE = 360g/L
= 360000mg/L

7) DDVP = 100g/L
= 100,000mg/

APPENDIX 7

WORKING PROTOCOL FOR SINGLE METALS:

GLYPHOSATE:

								Stock	20000mg/L		
					Glyphosate						
S/No	1	2	3	4	5	6	7	8	9	10	
Conc (mg/L)	0	200	400	600	800	1000	2000	4000	8000	10000	
Water (µl)	550	540	530	520	510	500	450	350	150	50	
Toxicant (µl)	0	10	20	30	40	50	100	200	400	500	2700.00
Nutrient broth (µl)x4	250	250	250	250	250	250	250	250	250	250	
Strenght organism (µl)	100	100	100	100	100	100	100	100	100	100	
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100	
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000	

DDVP:

				DDVP		**Stock =6000mg/L**				
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	50	100	250	500	1000	2000	2500	3000	5000
Water (µl)	550	542	533	517	508	467	383	217	133	50
Toxicant (µl)	0	8	17	33	42	83	167	333	417	500
Nutrient broth (µl)x4	250	250	250	250	250	250	250	250	250	250
Strenght Organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

Protocol for toxicity of Lead							Stock = 5mM	1655	1655mg/L		
S/No	1	2	3	4	5	6	7	8	9	10	
Conc (mg/L)	0.00	16.55	33.10	66.20	99.30	165.50	264.80	331.00	662.00	827.50	
Water (µl)	550	540	530	510	490	450	390	351	151	52	
Toxicant (µl)	0	10	20	40	60	100	160	199	399	498	2971.02
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	

Protocol for toxicity of Cadmium							Stock = 5mM	1282.5	1282.5mg/L		
S/No	1	2	3	4	5	6	7	8	9	10	
Conc (mg/L)	0.00	12.83	25.65	51.30	64.13	76.95	102.60	128.25	205.20	256.50	
Water (µl)	550.00	540.00	530.00	510.00	500.00	490.00	470.00	450.00	390.00	350.00	
Toxicant (µl)	0.00	10.00	20.00	40.00	50.00	60.00	80.00	100.00	160.00	200.00	1440.00
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	

Protocol for toxicity of Zinc							Stock = 5mM	1488	1488mg/L		
S/No	1	2	3	4	5	6	7	8	9	10	
Conc (mg/L)	0.00	29.76	59.52	119.04	148.80	238.08	297.60	357.12	446.40	595.20	
Water (µl)	550.00	530.00	510.00	470.00	450.00	390.00	350.00	310.00	250.00	150.00	
Toxicant (µl)	0.00	20.00	40.00	80.00	100.00	160.00	200.00	240.00	300.00	400.00	3080.00
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	

Protocol for toxicity of Nickel							Stock = 5mM	1314.5	1314.5mg/L		
S/No	1	2	3	4	5	6	7	8	9	10	
Conc (mg/L)	0.00	13.15	26.29	52.58	65.73	78.87	105.16	131.45	262.90	315.48	
Water (µl)	550.00	540.00	530.00	510.00	500.00	490.00	470.00	450.00	350.00	310.00	
Toxicant (µl)	0.00	10.00	20.00	40.00	50.00	60.00	80.00	100.00	200.00	240.00	1600.00
Nutrient broth (µl))x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	

	Protocol for toxicity of Cobalt						Stock = 5mM	1455.15	1455.15mg/L		
S/No	1	2	3	4	5	6	7	8	9	10	
Conc (mg/L)	0.00	14.55	23.28	29.10	58.21	72.76	145.52	232.82	291.03	349.24	
Water (µl)	550.00	540.00	534.00	530.00	510.00	500.00	450.00	390.00	350.00	310.00	
Toxicant (µl)	0.00	10.00	16.00	20.00	40.00	50.00	100.00	160.00	200.00	240.00	1672.00
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	

NO. 2: WORKING PROTOCOL FOR BINARY MIXTURES OF METALS:

Lysinibacillus macroides

Protocol for toxicity of Binary mixtures										
	Stock =1000mg/L					Stock =3000mg/L				
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0.00	16.55	26.48	33.10	66.20	99.30	165.50	264.80	331.00	397.20
Water (µl)	550	533	524	517	528	517	495	462	440	418
Toxicant (ul)	0	17	26	33	22	33	55	88	110	132
Nutrient broth (µl)x4	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
Strenght organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (ul)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

Alcaligenes faecalis

Protocol for toxicity of binary mixtures										
	Stock =1000mg/L					Stock =3000mg/L				
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0.00	14.55	23.28	29.10	58.21	72.76	145.52	232.82	291.03	349.24
Water (µl)	550	535	527	521	531	526	501	472	453	434
Toxicant (µl)	0	15	23	29	19	24	49	78	97	116
Nutrient broth (µl)x4	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
Strenght organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

Protocol for toxicity of Binary mixtures Pb/Zn										
	Stock =1000mg/L					Stock =3000mg/L				
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0.00	16.55	26.48	33.10	66.20	99.30	165.50	264.80	331.00	397.20
Water (µl)	550	533	524	517	528	517	495	462	440	418
Toxicant (µl)	0	17	26	33	22	33	55	88	110	132
Nutrient broth (µl))x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

	Protocol for toxicity of Binary Mixtures with Zinc						**Stock = 3000mg/L**			
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	132	263	394	526	657	789	921	1051	1315
Water (µl)	550	506	462	419	375	331	287	243	200	112
Toxicant (µl)	0	44	88	131	175	219	263	307	350	438
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

NO. 3: WORKING PROTOCOL FOR TENARY MIXTURES OF METALS:

	Tenary mixtures of Metal +DDVP				Stock =10000mg/L					
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	100	200	250	400	500	800	1000	2000	2500
Water (µl)	550	540	530	525	510	500	470	450	350	300
Toxicant (ul)	0	10	20	25	40	50	80	100	200	250
Nutrient broth (µl))x4 Strenght	250	250	250	250	250	250	250	250	250	250
Organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (ul)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

Tenary mixtures of metal with Glyphosate										
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	1000	2000	3000	4000	5000	6000	8000	10000	11000
Water (µl)	550	500	450	400	350	300	250	150	50	0
Toxicant (µl)	0	50	100	150	200	250	300	400	500	550
Nutrient broth (µl)x4 Strenght	250	250	250	250	250	250	250	250	250	250
organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

	Protocol for toxicity of Tenary Mixtures with Zinc						Stock = 3000mg/L			3000mg/L
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	120	250	400	500	600	800	1000	1200	1500
Water (µl)	550	510	467	417	383	350	283	217	150	50
Toxicant (µl)	0	40	83	133	167	200	267	333	400	500
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

	Protocol for toxicity of Tenary Mixtures with Ni/Cd						Stock = 3000mg/L	1314.5	3000mg/L	
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0.0	25.0	50.0	80.0	100.0	150.0	200.0	250.0	300.0	400.0
Water (µl)	550	542	533	523	517	500	483	467	450	417
Toxicant (µl)	0	8	17	27	33	50	67	83	100	133
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

	Tenary mixtures of Metal +DDVP				Stock =10000mg/L					
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	100	200	250	400	500	800	1000	2000	2500
Water (ul)	550	540	530	525	510	500	470	450	350	300
Toxicant (ul)	0	10	20	25	40	50	80	100	200	250
Nutrient broth (ml)x4 Strenght	250	250	250	250	250	250	250	250	250	250
Organism (ul)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (ul)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

Tenary mixtures of metal with Glyphosate										
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	1000	2000	4000	6000	8000	10000	12000	14000	16000
Water (µl)	550	525	500	450	400	350	300	250	200	150
Toxicant (µl)	0	25	50	100	150	200	250	300	350	400
Nutrient broth (µl))x4 Strenght	250	250	250	250	250	250	250	250	250	250
organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

	Protocol for toxicity of Tenary Mixtures with Zinc						Stock = 3000mg/L		3000mg/L	
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	120	250	400	500	600	800	1000	1200	1500
Water (µl)	550	510	467	417	383	350	283	217	150	50
Toxicant (µl)	0	40	83	133	167	200	267	333	400	500
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

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	Protocol for toxicity of Tenary Mixtures with Ni/Cd						Stock = 3000mg/L	1314.5	3000mg/L	
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0.0	50.0	80.0	100.0	200.0	250.0	300.0	400.0	500.0	600.0
Water (µl)	550	533	523	517	483	467	450	417	383	350
Toxicant (µl)	0	17	27	33	67	83	100	133	167	200
Nutrient broth (ul)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (ul)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

	Tenary mixtures of Metal +DDVP					Stock =10000mg/L				
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	100	200	250	400	500	800	1000	2000	2500
Water (µl)	550	540	530	525	510	500	470	450	350	300
Toxicant (µl)	0	10	20	25	40	50	80	100	200	250
Nutrient broth (µl)x4 Strenght	250	250	250	250	250	250	250	250	250	250
Organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

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	Tenary mixtures of metal with Glyphosate									
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	1000	2000	4000	6000	8000	10000	12000	14000	16000
Water (µl)	550	525	500	450	400	350	300	250	200	150
Toxicant (µl)	0	25	50	100	150	200	250	300	350	400
Nutrient broth (ul)x4 Strenght	250	250	250	250	250	250	250	250	250	250
organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

	Protocol for toxicity of Tenary Mixtures with Zinc						Stock = 3000mg/L		3000mg/L	
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	120	250	400	500	600	800	1000	1200	1500
Water (µl)	550	510	467	417	383	350	283	217	150	50
Toxicant (ul)	0	40	83	133	167	200	267	333	400	500
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

	Protocol for toxicity of Tenary Mixtures with Ni/Cd						Stock = 3000mg/L	1314.5	3000mg/L	
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0.0	50.0	80.0	100.0	200.0	250.0	300.0	400.0	500.0	600.0
Water (µl)	550	533	523	517	483	467	450	417	383	350
Toxicant (µl)	0	17	27	33	67	83	100	133	167	200
Nutrient broth (µl)x4 Strenght	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00

NO. 3: WORKING PROTOCOL FOR SEPTENARY MIXTURES OF METALS:

			Septenary mixtures of Metal +DDVP			**Stock =10000mg/L**				
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	100	200	250	400	500	800	1000	2000	2500
Water (µl)	550	540	530	525	510	500	470	450	350	300
Toxicant (ul)	0	10	20	25	40	50	80	100	200	250
Nutrient broth (µl)x4 Strenght	250	250	250	250	250	250	250	250	250	250
Organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100
Total Volume (µl)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

		Septenary mixtures of metal with Glyphosate								
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	1000	2000	4000	6000	8000	10000	12000	14000	16000
Water (µl)	550	525	500	450	400	350	300	250	200	150
Toxicant (µl)	0	25	50	100	150	200	250	300	350	400
Nutrient broth (µl)x4 Strenght	250	250	250	250	250	250	250	250	250	250
organism (µl)	100	100	100	100	100	100	100	100	100	100
0.02% MTT (µl)	100	100	100	100	100	100	100	100	100	100
Total Volume (ul)	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

	Protocol for toxicity of Septenary mixtures Pb						Stock = 3000mg/L		3000mg/L	
S/No	1	2	3	4	5	6	7	8	9	10
Conc (mg/L)	0	120	250	400	500	600	800	1000	1200	1500
Water (µl)	550	510	467	417	383	350	283	217	150	50
Toxicant (µl)	0	40	83	133	167	200	267	333	400	500
Nutrient broth (µl)x4	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00	250.00
Strenght organism (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.02% MTT (µl)	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Total Volume (µl)	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00