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The individual and simultaneous effects of Cu and cypermethrin upon glycosidase, phosphomonoesterase and the total microbial activity of soil.

A dissertation submitted as part of the requirement for MRes Applied Science by research as awarded by Bournemouth University

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February 2019

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Abstract

Enzymatic and microbial assays are frequently used in the determination of the response of soil organisms to external pollutants. Enzymes and soil microorganisms are particularly used as bioindicators to determine the health and quality of soil following a chemical disturbance. This study chose β-glycosidase, phosphomonoesterase and the total microbial activity of soil to establish the chemical disturbance of pyrethroid insecticide cypermethrin and $CuSO_4$, the main chemical component of widely used Bordeaux mixture. Both were applied individually and as a simultaneous mixture. Results show the greatest negative impact of the joint contamination is upon phosphomonoesterase. Little to no effects are detected at higher concentrations to the total microbial activity, and a positive impact to glycosidase is observed. This study indicates that the enzymes most at risk from Cu/cypermethrin toxicity are soil phosphatases, which are enzymes intrinsic to plant growth and development. A depletion of this enzyme in the rhizosphere has serious implications to soil health and subsequent potential damage to agricultural harvests.

Contents

1. Introduction

On a global scale, soil is a fundamental part of the infrastructure for life on our planet and the soil's ability to generate the required processes and services depends on soil fertility. Land and aquatic systems are also connected; improper land utilization is one of the chief factors responsible for atmospheric deposition, drainage and seepage of contaminants, all of which may be damaging to surrounding bodies of water (Niu et al, 2015). Population growth, land degradation, climate change, nutrient depletion, changing diets concurrent with development and urbanization are testing the ability of the Earth's systems to meet growing demand for services supplied by soil. For example, 75 billion tons of fertile soils are lost each year due to human activities that cause soil erosion, deterioration of the physicochemical or biological properties of the soil and long term loss of natural vegetation (UNCCD, 2012).

Soils are a mixture of inorganic / organic solids, gases, water, microorganisms, animals, plant roots and nutrients, all of which influence each other; microorganisms catalyze many soil reactions that release nutrients and plant roots absorb these or release inorganic / organic substances back into the soil (Bohn et al, 2001). Microbes in soil play key roles in a range of ecosystem functions and processes, including nutrient acquisition, nutrient cycling (e.g. N, P, K, S and C cycles; Kibblewhite et al, 2008) and soil structure (Wall, 2004), thereby increasing plant productivity (Bardgett, 2005). The rhizosphere (plant root area) is a chemically complex arena that has an energetic microbiome. This region controls physicochemical properties, plant growth, development and the chemical signals being exchanged between microbial populations and the rhizosphere (Sahu et al, 2018; Lareen et al, 2016). Therefore it is clear soil, the life within it and the complex interactions between them must be protected.

1.1 Soil Health and Soil Microbes

Soil Health is a term widely used in discussions regarding sustainability and is used to describe the general condition and quality of soil, the management of which is fundamental to all farming systems.

Soil quality is difficult to define, but has been described by Karlen et al (1997) as ‘the capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation’. The term ‘soil health’ may be favoured by some as it portrays the Earth and its soil as a living, breathing and dynamic system whose roles are facilitated by diverse populations of living organisms requiring management and conservation (Doran et al, 1996; Doran and Safley, 1997). Soil health, biological diversity and resilience are severely restricted in extreme climates and are more sensitive to man-made disturbances (Freckman and Virginia, 1997). As Doran and Zeiss (2000) summarised, both quality and health of soil governs agricultural sustainability, quality of the environment and as a result, plant, animal and human health.

Microbes in soil play key roles in a range of ecosystem functions and processes, but, represent the unseen majority in soils and encompass a large fraction of genetic diversity on Earth. It is estimated that 1g of soil may contain as many as 10^{10} – 10^{11} bacteria, 6 – 50 000 bacterial species and 200m of fungal hyphae (Kibblewhite et al, 2008). Microbes indirectly and directly influence every aspect of soil health (Table 1.1), productivity and diversity of plant communities, which are critical to agricultural harvests (Horner-Devine et al, 2003; Curtis et al, 2002). Indeed, the activity of microbial enzymes in soils is taken as a key indicator of soil health (Chae et al, 2016; Elsas, 2007; Sahu et al, 2018). Soil fertility and microbial activity are connected because it is through soil microbes that mineralization of essential organic elements occur (Frankenberger and Dick, 1983).

Table 1.1 Four major ecosystem functions and their relationship with the soil biological community (Kibblewhite et al, 2008)

Aggregate Ecosystem Functions	Functional Bodies
1. C transformations	Decomposers • Fungi • Bacteria • Microbivores • detritivores
2. Nutrient Cycling	Nutrient transformers • decomposers • element transformers • N-fixers • Mycorrhizae
3. Soil structure maintenance	Ecosystem engineers • Megafauna • Macrofauna • Fungi • Bacteria
4. Biological population regulation	Biocontrollers • Predators • Microbivores • hyperparasites

Burger and Kelting (1998) suggested that the health of soil could be judged by its ability to promote root growth; accept, hold and supply water; cycle nutrients; promote gas exchange and encourage all biological activity. A large percentage of soil functions are catalyzed by enzymes, making them suitable indicators of soil health (Alkorta et al, 2003). Enzymes are central to essential soil nutrient cycles including carbon, sulphur, nitrogen and phosphate cycles (Table 1.2). These processes aid in the decontamination of soil through degradation of pollutants, immobilization of trace metals, formation of soil structure and ultimately may have a positive or negative effect upon plant growth (enzyme stimulation or inhibition; Nannipieri et al, 2002). Nutrients digested by microbial communities are given back to the soil in the form of beneficial organic matter embedding minerals, which are released gently to improve soil health and quality (Kaschuk et al, 2010). This is particularly important in agriculture, where continuous nutrient depletion can occur through crop harvest, leaching loss and evaporation, which may result in a

substantial deficit of nitrogen and other important elements that are important for crop production (Henao and Banaante, 1999). Microbial communities, in association with other flora and fauna, restore the balance to nitrogen depleted soil by fixing biological nitrogen, making it available to plant roots and delivering organic nitrogen by breaking down N-rich residues and releasing mineral N (Barrios, 2007; Sahu et al, 2018).

Although the source of enzymes may in part originate from plants and fauna, the main source of soil enzymes are initiated by microorganisms (Kibblewhite, 2008). Furthermore, although certain enzymes may be associated with viable, live cells, others remain catalytic in cell debris, the soil solution and within the soil structure or soil colloids (Tabatabai, 1994). Burns (1982) classified enzymes as;

- Biotic – associated with living, proliferating cells, located intracellularly in cell cytoplasm, periplasmic space or outer cell surfaces.
- Abiotic – Excreted by living cells and attached to cell debris or deceased cells and leaked into soil solution from existent or lysed cells.

Microbial enzymes yield information about soil-ecological processes, much more clearly than physical or chemical parameters and are therefore effective indicators of soil health (Alkorta et al, 2003) and are sensitive indicators to very slight changes in soil properties. For example, the enzymes β-glucosidase and phosphatase were proven to be highly sensitive even to minor changes in the soil environment and were able to differentiate between different land uses and soil amendments when other physicochemical indicators failed to do so (Dose, 2015).

Table 1.2. Soil enzymes as indicators of soil health (Shukla and Varma, 2011)

Soil enzyme	Enzyme reaction	Indicator of microbial activity
Dehydrogenase	Electron transport system	C-cycling
β-glucosidase	Cellobiose hydrolysis	C-cycling
Cellulase	Cellulose hydrolysis	C-cycling
Phenol oxidase	Lignin hydrolysis	C-cycling
Urease	Urea hydrolysis	N-cycling
Amidase	N-mineralisation	N-cycling
Phosphatase	Release of PO_4^-	P-cycling
Arylsulphatase	Release of SO_4^-	S-cycling
Hydrolytic enzymes	Hydrolysis	General organic matter degradative enzyme activities

Numerous studies have discovered that environmental pollution may be affecting metabolic functions of soil microbes, reflecting alterations of the soil microbial population, composition and diversity (Liu et al, 2015; Luo et al, 2018; Pidatala et al; 2016). Soil pollution could potentially decrease the diversity of microorganisms, but may also enrich the more tolerant species (Giller et al, 2009). Increased populations of tolerant microorganisms, could, in turn, affect overall ecosystem functions and upset the natural balance of soil microorganisms. The consequence of microbial reaction to environmental change is a key question still largely unanswered (Liu et al, 2015; Sun et al, 2016). On the other hand, there are studies that suggest that enzymatic coupling is an encouraging method for the decontamination of environments polluted by xenobiotics. For example, the enzyme dependent oxidative coupling involving the loss of a proton and an electron from phenols, resulted

in the transformation of xenobiotics (Bollag, 1992). Enzymes are intrinsically linked to pesticide degradation. Microbes such as *Aspergillus, Pseudomonas, Chlorella,* and *Arthrobacter* are capable of pairing a range of biochemical components for the degradation of toxic pesticides, such as adsorption, hydrolysis of P-O alkyl and aryl bonds and photodegradation (Burrows et al, 2002). Enzymes such as esterase, phosphotriesterase, somanase and parathion hydrolase, amongst others, have been isolated from soil microbes to understand the catabolic pathways involved in the biotransformation of xenobiotic compounds (Kumar et al, 2017). Enzymatic degradation (enzymatic hydrolysis) has gained much interest. For example, an enzyme acquired from a strain of *Pseudomonas diminuta*, named parathion hydrolase, hydrolyses the phosphate ester bond in the organophosphate pesticide molecule, producing as much as a 100-fold decrease in toxicity (Havens and Rase, 1991).

1.2 Glucosidases, phosphatases and hydrolases

The current study is focused upon the enzymes β-glucosidase, phosphomonoesterase and the overall microbial activity of the soil as measured by general hydrolase activity, and how they react to contamination by copper (a component of several fungicides) and a pyrethroid insecticide, cypermethrin, independently and simultaneously in a mixture.

β-Glucosidase

Organic C is a major components of soil organic matter (SOM) and as previously discussed, organic C fuels the metabolic activity of soil microbes (Dick et al, 2013). Glycosidases have an important role in this process. Glycosidases or glycoside hydrolases is the general name used to describe the enzyme group that catalyze the hydrolysis of differing glycosides. The general equation for this reaction can be expressed as:

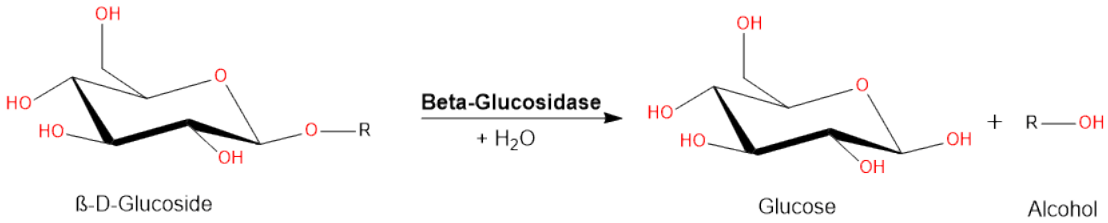

Figure 1.2 Glucosidases catalyze the hydrolysis of glucosides

β-glucosidases catalyze the hydrolysis of the glycosidic bonds to terminal non-reducing residues in β-D-glucosides and oligosaccharides, with the release of glucose. The monosaccharides (e.g. glucose) are important energy sources for microbial poulations (Tabatabai, 1994).

SOM can be enzymatically hydrolyzed into simpler molecules, by fungal and bacterial hydrolyzing enzymes. There are many enzymes involved in the completion of biomass hydrolysis, such as xylanase, ligninase, pectinase etc., included in which, cellulase can be seen as most important as biomass comprises >40% cellulose (McKendry, 2002; Isikgor and Remzi Becer, 2015). Cellulase comprises a tri-enzyme complex; exoglucanase, endoglucanase and β-glucosidase (BGL) all of which work together for complete hydrolysis of cellulose. The action of the complex is as follows:

- Firstly, cellulose fibers are cleaved by endoglucanase releasing small cellulose splinters.
- These fragments are further divided by exoglucanase into oligosaccharides – cellobiose.
- Final hydrolysis into glucose monomers is catalyzed by BGL.

Because the final step of hydrolysis into glucose is completed by BGL, this enzyme may be seen as a rate limiting enzyme (Tabatabai, 1994). Consequently, glycosides play an intrinsic role in the carbon cycle of soil and hence glucosidase activity is regarded as an important biomarker of soil health and quality (Chae et al, 2016).

Phosphomonoesterase

Phosphatase is the general name used to describe a broad range of enzymes that catalyze the hydrolysis of esters and anhydrides of phosphoric acid (H_3PO_4). These phosphatases have been classified into five major groups. These include phosphoric monoester hydrolases, phosphoric diester hydrolases, triphosphoric monoester hydrolases, enzymes that act upon phosphoryl-containing anhydrides and P-N bonds (Tabatabai, 1994). The general equation of the catalysis reaction of alkaline phosphatses is:

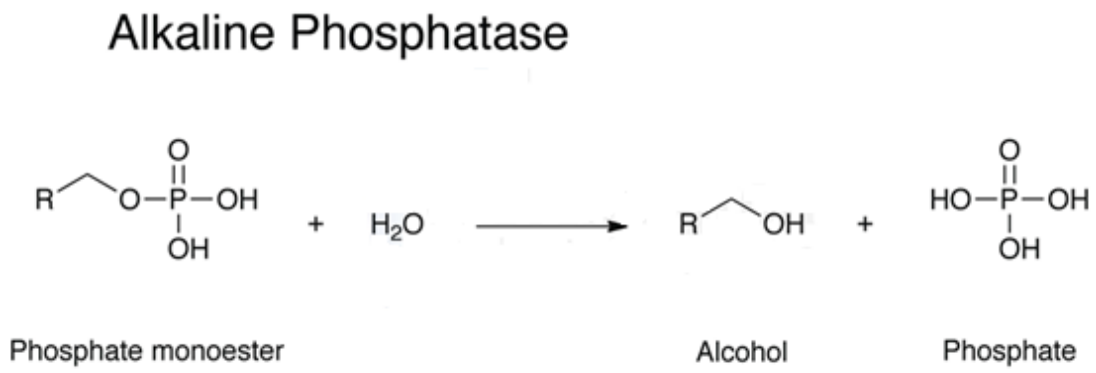

Figure 1.2 Catalysis reaction of alkaline phosphatase

Phosphorous (P) is a limiting nutrient and supplying P to plants is crucial to sustaining crop profits in agro-ecological areas around the globe (Sun et al, 2018). Since some P is bound in organic material as organic P and inorganic P can be bound with Ca^{2+}, Fe^{3+}, and Al^{3+}, forming precipitates, P is largely inaccessible to plants (Huang et al, 2012). Farmers will normally add organic manure and P fertilizers as a remedy for P deficient soils to encourage plant growth. Organic manure has been seen to improve chemical and biological properties of farmland (Liu et al, 2013). Swine manure, generally has a high organic P content although its availability is low (Eghball et al, 2004). Long-term application provides the increase in P precipitation in topsoil (Atia and Mallarino, 2002).

Most P is located in organic forms, such as phosphoinositide, phosphorous esters, and nucleotides (Wang et al, 2011). However, microorganisms and plant-life cannot assimilate P in these states, only mineral orthophosphate P is

available (Redel et al, 2011). Most organic P must be hydrolyzed via phosphatases, originating from microbes and plant roots, into more available forms (Albrecht et al, 2010; Li et al, 2014). Phosphatase enzyme activities are adaptive and the levels increase around plant roots experiencing P deficiency (Wang et al, 2011). Phosphatases play an intrinsic role in the mineralization of organic P, with phosphomonoesterase (PME) considered the predominant phosphatase in most soil (Tabatabai, 1994). PME can be subdivided according to their optimum pH into acid phosphomonoesterase (ACP) (pH 4-6) and alkali phosphomonoesterase (ALP) (pH 8-10) (Tabatabai, 1994; Speir and Ross, 1978).

Phosphatases are known to play a critical role in P-cycling as evidence has shown that they are connected to P stress and plant growth (Tabatabai, 1994). Thus, phosphatases play a fundamental part in soil systems and are thus a good indicator of soil health (Eivazi and Tabatabai, 1977). For example, when there is a signal indicating a deficiency of P content in soils, acid phosphatase secretion from plant roots is increased to enhance the solubility and mobility of phosphate, enabling the plant to cope with a P stressed environment (Shukla and Varma, 2011; Karthikeyan et al, 2002).

In terms of agriculture, the high hydrolytic activity of composts is beneficial because of enzymatic nutrient release of P and C among others (Table 2.) from SOM into a form that plants and microbes can utilize; these enzymes also sustain the release of low molecular weight organic molecules which can be taken up by soil microbes (Vuorinen, 2000). Phosphomonoesterase (PME) enzymes are a prime example, catalyzing the hydrolysis of P esters to orthophosphate (Speir and Ross, 1978).

Perhaps the most appealing property of phosphatases is their specificities. These enzymes are known to hydrolyze a range of phosphomonoesters, phosphodiesters and phosphotriesters. Hydrolysis in soils of β-glycerophosphate, phenylphosphate, β-naphthyl phosphate and *p*-nitrophenyl phosphate has been reported (Tabatabai, 1994; Graciani et al, 1995), and

alkaline phosphatase activity seems to derive completely from soil microorganisms (Tabatabai, 1994).

1.3 Soil and Pollution

Contamination of agricultural soil can be observed internationally, owing to the use of pesticides, fertilizers, plastic film, waste water irrigation and sewage application amongst other activities (Kourous, 2018). Because of the long term applications, the adverse impacts to soil are liable to be chronic, and the cumulative application of pollutants to soil may cast a significant risk to the environmental and ecological function of soil, plant growth and subsequent human health (Sun et al, 2018).

International use and subsequent adverse effects of organic pollution is causing a growing public concern. Organic contaminants (OCs), such as organochlorine pesticides (OCPs, now banned), polychlorinated biphenyls (PCBs), phthalate esters (PAEs) and PAHs are characteristically defined by high toxicity, persistence and bioaccumulation in the environment (Pies et al, 2007; Sun et al, 2016). However, soil microbes also play an important role in the remediation of contaminated soil through an effective involvement in the degradation and transformation of organic compounds (Sun et al, 2018).

Human activity has had a major impact upon the global cycles of elements and as every trace metal has a potential for toxicity above certain levels, this is a source of concern (Nriagu and Pacyna, 1988). Additions of any element to soil or water, in some cases even in trace amounts, can have serious consequences to biological organisms (Deboudt et al, 2003). Obvious extreme metal contamination has been observed at locations in close proximity to smelters; deep accumulated layers of organic matter can be observed, due to inhibition of soil microorganism and soil fauna activities (Giller et al, 1997). Soil microbial biomass can be negatively affected by raised metal levels and has been closely correlated to metal stress (Das et al, 1998). Enzyme activities in soil have also been observed to be greatly depressed at sites of metal contamination (Wang et al, 2007), demonstrating

the correlation between enzyme activity and the impact of pollution on the microbial community.

Supplying soil with essential macro (and trace) elements is the key aim of any fertilizer to produce a high-quality, healthy harvest. However, many fertilizers contain less beneficial elements, in particular, heavy metals (Nicholson et al, 2003). Recently, potentially harmful trace elements and metals have come into focus in Europe, including uranium (U) and boron (B) (Chetellat and Gaillardet, 2005). A recent study has reported that on average, organo-mineral fertilizers contain Cu above the maximum concentration and mineral fertilizers quite often exceed the limit values for Cd. In Germany, the unwanted element content of fertilizers is regulated by the German Fertilizer Ordinance (2008), however Cu is viewed as an essential element and no longer regulated (Kratz et al, 2016). Both of these trace metals are known to be damaging to soil and its biomass (Das et al, 1998).

The pollution of soil and the effects caused to its microbial communities is well known (Dose et al, 2015; Hinojosa et al, 2002; Tian et al, 2017; Chaperon et al, 2007). For example, in 1998 a failed dam in southern Spain released a substantial amount of heavy metal contaminated pyrite sludge and acidic water, consequently flooding fertile farmland. Soil extracellular enzyme activities and general microbiological rates were severely reduced; five-fold decreases were reported (Hinojosa et al, 2004). It was concluded that normal enzymatic functions and important nutrient cycling processes mediated by soil microbes were significantly damaged by the spill (Grimalt, 1999; Alastuey et al, 1999; Lopez-Pamo et al, 1999).

Soil contamination may also have a major and potentially fatal impact to the human food chain. Cd in the environment can enter biogeochemical cycles, become bioaccumulated in produce and may then threaten human health. Cd is explained to be one of the most toxic elements a human being can be exposed to (Aoshima, 2012). In Japan, Cd contaminated rice caused itai-itai disease. The outbreak of itai-itai (the most severe stage of Cd poisoning) took place in the Jinzu River basin of Toyama. The kidney is the organ primarily

and critically affected by chronic (long-term) exposure to Cd. Proximal tubular dysfunction (RTD) was discovered among the population around the Jinzu River. The clinical course of 13 victims presented RTD that had deteriorated concluding in end stage renal failure (Kasuya, 1999; Inaba et al, 2005). The cause was clarified as environmental Cd pollution originating from effluent from a zinc mine located locally upriver and ultimately contaminating the rice paddy fields, entering the food chain with fatal consequences.

Trace metals exist in soil in different forms; dissolved ions (Cu^{2+}, Cd^{2+}, CrO_4^{2-}), organic complexes and exchangeable ions adsorbed on solid soil particles are three extensive forms that maintain a thermodynamic equilibrium in activity and concentration with each other, with insoluble particles as the predominant group (Roberts et al, 2005). Research has demonstrated that it is not the overall concentration, but the reactive component of metals in soil that dictates their toxicity to microbes, plants and human beings (Pendias, 2010; Gobran et al, 2001). Water soluble and exchangeable ionic forms of metals are more reactive and bioavailable than precipitated forms (Roberts et al, 2005, Kim et al, 2015). Distribution of trace metal species are influenced by a number of environmental factors, in particular, soil pH, redox potential, clay and organic matter content, Fe/Mn oxide content and other ionic trace metal forms in the soil solution (Pietrzykowski et al, 2014). To precisely predict the bioavailability of trace metals in soil, these influencing factors would need examination.

1.3.1 Copper Contamination in Soil

Copper is claimed to be one of the most common trace metal contaminants in agricultural systems (Alloway, 2008). Its antimicrobial property has made this metal an intrinsic component in broad spectrum bacterial and fungicidal agricultural pesticides, nutritional feed supplements for animals and widely used fertilizers (Seiler and Berendonk, 2012; Alloway, 2008). Consequently, there is a serious potential for Cu contamination to accumulate with subsequent damage to soil health.

Copper is an essential element that only becomes hazardous when it accumulates to toxic concentrations in soil following repeated use of Cu containing products, such as Cu rich composts, pesticides and fertilizers (Flemming and Trevors, 1989). Brunetto et al (2016) conducted a study reviewing the effects of Cu based fungicides and their repeated application to vineyard soils. Regular use of Cu based fungicides (e.g. Cu sulphate, Cu oxychloride) aimed to protect grapevines and other plants from unwanted pests has led to a continuing accumulation of Cu in vineyard soils, attaining levels that are detrimental to the health of soil and subsequent plant growth (Pietrzak and McPhail, 2004). Aside from Cu-containing fungicidal and bactericidal sprays, considerable Cu addition to soil can ensue from further addition of mineral and organic fertilizers such as pig and poultry manures and organic composts (Couto et al, 2015).

Yruela (2005) emphasized that Cu is an essential elemental nutrient for plants, and necessary for an organism to properly function since Cu is involved in key roles in numerous biochemical and physiological processes linked to plant growth and development. However, depending on the concentration and bioavailability, Cu can produce toxic effects towards microbial populations and subsequently plant - crop productivity impairment and may ultimately lead to the degradation of the quality and nutritional value of the products (Yruela, 2005 ; Brunetto, 2016).

It's well known that Cu availability for plants depend strongly on mineral and organic forms and biogeochemical cycles in soil (Besnard et al, 1999; Alloway, 2008). In the rhizosphere, these cycles are heavily influenced by interactions between roots and microorganisms. Trace amounts of Cu exist in soil solution in its ionic form Cu^{2+}. Microbes facilitate Cu transport across root membranes, where if accumulated in toxic concentrations, it may affect metabolic processes such as respiration, photyosynthesis, CO_2 fixation and gas exchange (Mocquot et al, 1996). The amount of Cu absorbed by plants depends on both availability in the rhizosphere and the protein systems dedicated to the acquisition of Cu, and to further allocate it into shoot tissues (Chaignon et al, 2003). Hence, Cu forms are strongly dependent upon

enzymes and other proteins which facilitate nutrient transport across plasma membranes (Brunetto, 2016; McBride, 1995). Studies show that as Cu concentrations increase, net photosynthesis, efficiency of PSII (photosystems II) photochemistry and electron transport, in turn, decrease. As a consequence, shoot and root biomass also declines with increase of toxic Cu accumulation (Baldi et al, 2018).

Cu based fungicides

Cu based fungicides, such as Bordeaux Mixture, for example, have been extensively used in Europe since the end of the 19th century to protect crops from fungal disease. European Union legislation has restricted its use in order to reduce and control Cu related pollution (European Commission, 2002). Despite this effort, the prolonged and intensive application of the fungicide has led to an accumulation of Cu in soil exceeding background values by up to 300 times, with a negative effect on soil flora, soil fauna and human health. (Chaignon et al, 2003). The high concentration of Cu in the top soil of vineyards may lead to phytotoxicity, yield loss and impairment of wine quality, leading to international concerns (Garcia-Esparza et al, 2006). The main Cu induced phytotoxic symptoms on plants include stunted growth, chlorosis, leaf senescence, reduction of root growth and root elongation (Michaud et al, 2008; Kopittke and Menzies, 2006; Chopin et al, 2008).

The current study examines copper sulphate, which is the main component of Bordeaux Mixture. Bordeaux Mixture is used to control pests such as downy mildew, damping-off (fungal infection caused by damp conditions), powdery mildew, leaf curl and blackspot. It is applied to particular crops including grapevines, apples, pears, olives, peaches, nectarines, walnuts and roses (PPDB, 2017). The control of grapevine fungal diseases has been a continuous problem for wine-growers worldwide, downy mildew being one of the most threatening diseases in European viticulture (Loureiro et al, 2012). Bordeaux Mixtures' two main components are copper sulphate ($CuSO_4$) and lime. Discovered by accident in 1882 in France, it is still applied to this day mainly in organic farming, as copper is broadly recognized as a non-synthetic natural element which, has been proven to be more effectual than numerous

other fungicidal treatments, especially against downy mildew (Pereira et al, 2001; La Torre et al, 2008). The broad-spectrum fungicide has been demonstrated to be more effective than its alternatives.

Numerous studies provide evidence that indicate long-term use of copper based compounds are resulting in increased copper concentrations; France: 100-1500mg kg^{-1} (Besnard et al, 1999), India: 29-131 mg kg^{-1} (Prasad et al, 1984) Australia: 11-320 mg kg^{-1} (Whitewick et al, 2008). The risk of heavy metal accumulation may be considered in the application of animal manures to farmland. Previous studies have provided evidence that continuous application of animal manure containing high levels of metals can result of their excessive accumulation in soil with subsequent adverse effect on soil quality (Paradelo et al, 2011; Wang et al, 2013).

1.3.2 Pesticides

Pesticide is the main umbrella term for a plant protection product (PPP) and covers fungicides (fungi), insecticides (insects), herbicides (weeds), miticides and rodenticides (rodents/vermin) (Matthews, 2006). PPPs play an important role in farming systems, providing a defense against unwanted insects, fungi, weeds and plant diseases. 85% of the worlds' pesticide production is consumed by agricultural practice where they are used to improve crop production and food security (Como et al., 2017). Pesticides are designed and intended to operate with a certainty of minimal risk to human health (Lee et al, 2018). Exposure, however, is linked to an extensive list of health complications. They are one of the few toxic substances released upon the environment with the intention of killing living organisms. There are a wide range of pesticides currently in use, varying in chemical class and with a broad range of chemical properties. These properties will invariably decide the behavior of the particular pesticide in soil and its chemical/ biological activities, and it should be safe to assume that there will always be fractions of bound molecules that will always persist in soil, even after exhaustive extraction techniques (Gevao et al, 2000). Molecular size, ionisation, water solubility, lipophilicity, polarity and volatility are significant factors to governing pesticide behavior towards plants and in soil (Gevao et al., 2000). A

distinction should also be made between bioavailable and non-bioavailable fractions. Calderbank (1989) described the bioavailable pesticide residue as the fraction that is available for plant-root or microorganism uptake, while the non-available fraction is not.

The most important mode of interaction between a pesticide and soil to consider is adsorption. The adsorption process may vary from reversible to completely irreversible (Senesi, 1992) and may also be physical (van der Waals forces) or chemical in nature (electrostatic interactions). The binding of an agrochemical compound, from a toxicological perspective may lead to: (1) a reduction of available compound to interact with microbial communities; (2) a decrease in the toxicity of the pesticide; (3) immobilization of the toxicant with subsequent reduction of leaching and transport capabilities (Calderbank, 1989; Berry and Boyd, 1985; Bollag, 1992).

Pesticides may be classed according to the intended target pest, chemical structure of the compound or the type of health hazard that may be caused. Figure 1 is a classification system as systemized by Gevao et al (2000) and is based on their major properties in soil and water.

Unintended exposure can be hazardous to humans as pesticides are fundamentally and specifically designed to be poisonous (Sarwar, 2015). As well as being linked to cancer, hormone interference, asthma and allergies, there are studies that provide evidence of pesticide exposure linked to birth defects, reduced birth weight, leukaemia and fetal death (Van Maele-Fabry et al., 2010; Wickerham et al., 2012). According to Carter and Blizard (2016), in the UK there arose evidence in the early 1990s of a five-fold increase in autism, which was seen to plateau from 2000-2010. The cause of this is thought to be partly due to environmental consequences, which include the environmental co-contaminants pesticides and trace metals (Carter and Blizard., 2016; Sealey et al., 2016). At sub-cytotoxic levels, fungicides (pyraclostrobin, trifloxystrobin, famoxadone or fenamidone) produce transcriptional mutations in mice cortical cultures that are comparable to those seen in brain samples from humans with autism (Pearson et al., 2016). Furthermore, residential proximity to agricultural pyrethroids, neonicotinoids

and manganese-based fungicides are linked to imperfect neurodevelopment in children (Gunier et al., 2016).

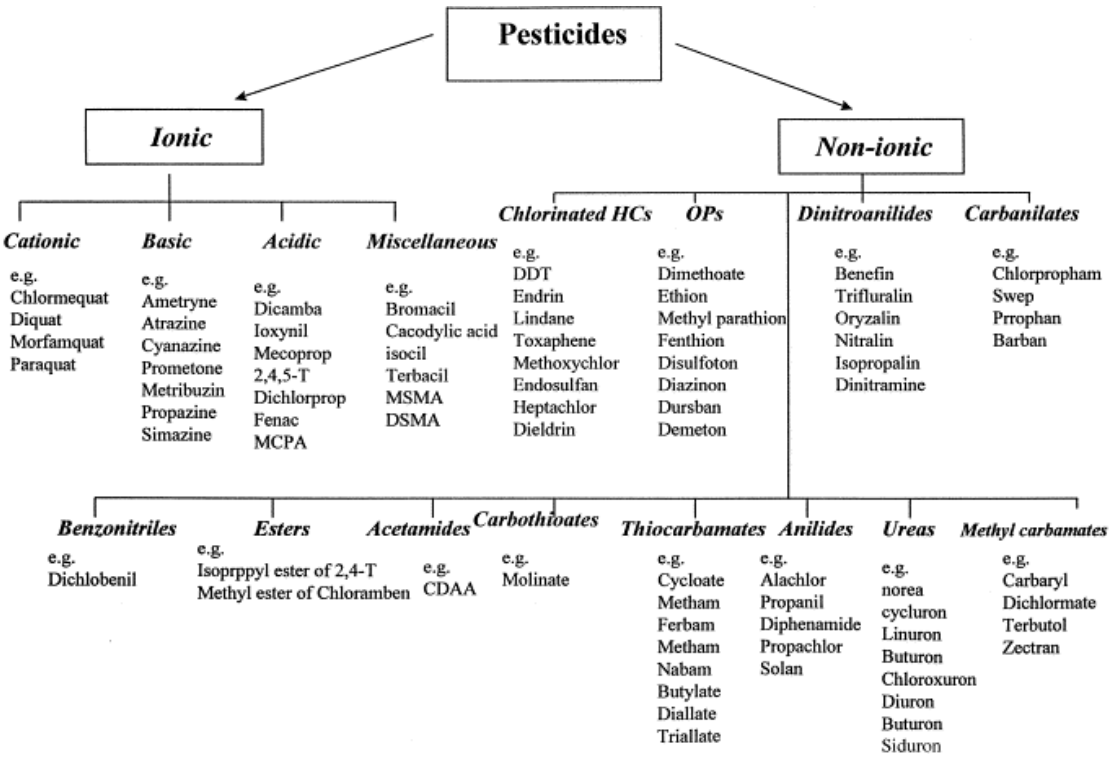

Figure 1.3 Classification of pesticides (Gevao et al, 2000)

By targeting unwanted pests that may be threatening a crop, beneficial species are simultaneously endangered; genetic mutations to biological organisms may be induced causing a resistance to pesticide action; the soil microorganisms that play key roles in nutrient cycles and overall health of the soil may be destroyed; water bodies could be polluted and general harm to the ecosystem can occur. Due to such non-target effects, many PPPs have been made illegal for use. For example, dichlorodiphenyltrichloroethane (DDT), beta-hexachlorocyclohexane (HCH), Aldrin and Dieldrin are but a few compounds now banned for agricultural use (Diaconu et al., 2016).

The repercussions of neonicotinoid insecticides upon bee populations has been a subject of some intensity and considerable research in recent years. Bees perform vital ecosystem services such as the pollination of wild plants and other important crops, but whilst carrying out their operations are exposed

to a wide range of natural and synthetic xenobiotic pesticides. Certain neonicotinoids, i.e, *N*-nitroguanidine compounds, imidacloprid and thiamethoxam are fundamentally as toxic as the pests they are intended to target (Manjon et al, 2018). The reduction and deterioration of bee poopulations would have serious consequences upon global ecosystems and agricultural production (Gill et al, 2012; Moreira et al, 2017). Gill et al (2012) discovered that the predisposition of colonies to fail, increased where different pesticide compounds were combined. This illustrates that, despite rigorous testing modern pesticides must undergo, unexpected environmental side effects can occur.

There are studies to suggest analysis of enzyme activity in soil can be used to evaluate soil functionality after pesticide disturbances. Enzyme activities are used for analysis of soil because of their essential roles in nutrient cycles. Floch et al (2011) concluded that glucosidase was most efficient as an indicator of pesticide contamination.

A study conducted by Singh and Singh (2005) on phosphomonoesterase (PME) and dehydrogenase activity after soil exposure to the insecticides diazinon, imidacloprid and lindane discovered an increase in enzymatic activity, and an observation was made towards an adaptability of the soil biota toward the compounds. Adaptions by microbes towards insecticides have been observed by other authors (e.g. Schuster and Schroder, 1990). It was suggested by Floch et al (2011) that future studies may usefully evaluate how appropriate these enzymes are as indicators of pesticide resilience. Furthermore there is an interest in the investigation of repeated pesticide applications as soil resilience can be noticeably different after a previous exposure (Schaeffer et al, 2016).

It is therefore not surprising that the presence of pesticides can affect the population and functions of soil microbes. For example, the properties of pesticides have been shown to be adverse to microbial growth, restricting usual metabolic functions (Pandey and Singh, 2006). Chen et al (2015) explored the dynamics of microorganism communities within pesticide and chlorophenol (PCP) contaminated soils, discovering microbial abundance is

inhibited by PCP stress. Soil microflora experienced difficulties in recovering from PCP induced toxicity, indicating the microbial community had low resilience due to interference, by the pesticide, of normal metabolic functions (Chen et al, 2005).

From the current evidence the action of a pesticide is very difficult to predict, which is compounded when the history of a soil, and therefore the adaptation of the microbial community, is unknown. Even with the knowledge of which chemicals have been applied to soil and for the period of time and the concentrations which they have been utilised, it is still a challenge to hypothesize correctly how the enzymatic or microbial content of soil will react to a chemical applied at potentially toxic levels.

Cypermethrin

Cypermethrin is a synthetic insecticide belonging to the pyrethroid family and has the chemical formula of $C_{19}H_{19}Cl_2NO_3$ (Fig 2). It currently has approval for use in the EU. It has low aqueous solubility and is volatile. It is regarded a serious marine pollutant and moderately persistent in soil. It is considered moderately toxic to mammals with some concern regarding the potential to bioaccumulate (IPCS, 1989). It is highly toxic to honeybees (Manjon et al, 2018) and moderately toxic to earthworms (Zhou, 2011). The chief target pests are aphids, weevils, caterpillars, flies, beetles and midges. It is applied to cereal crops (wheat, barley and oats among others), vegetables, potatoes, apples and pears (PPDB, 2018).

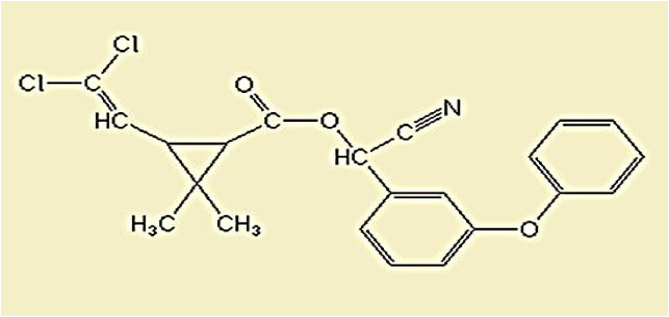

Figure 1.4 Molecular structure of cypermethrin (University of Hertfordshire, 2017)

Despite the beneficial application of cypermethrin, uncontrolled and repeated employment of the insecticide may lead to unintended effects upon non-target organisms (Antwi and Reddy, 2015). Increasing use of this compound has caused concern over ecological non-target effects. Aquatic insects are highly susceptible to pyrethroid toxicity, often at extremely low concentrations (less than 1 ppb) (Antwi and Reddy, 2015).

Cypermethrin is a class II synthetic insecticide, which crosses the blood brain barrier and causes neurotoxicity in the central nervous system (CNS). Cypermethrin increases the opening of sodium channels, subsequently leading to hypo-polarization and hyper-excitation of neurons (Singh et al, 2012). The action of cypermethrin as a neurotoxin is similar to that of DDT. Pyrethroids have been demonstrated to act upon membrane proteins, and there is evidence that ATP affiliated enzymes and ion pumps may be associated in the neurotoxic action of cypermethrin and DDT (Kakko et al, 2003). Soil and plant microorganisms may also be considered as non-target organisms, as the health of soil and plants relies intrinsically and dependently upon biological microbe communities.

The effects of cypermethrin on microbes are dependent on its chemical properties, the concentration at which it is applied, the types of microorganisms present and the environment in which the microbes populate (Zhuang, 2011). The application of pesticides may decrease soil microbial biomass, due to chronic or acute toxicity, or it may increase soil biomass because of the pesticide supplying a nutrient source, or due to indirect effects such as the termination of microbial predators, or in some cases, no observed effect at all (Chinalia and Killham, 2006; Pandey and Chauhan, 2007).

As previously discussed, adsorption is an important chemical pathway for a pesticide, and is a key process to controlling the environmental behaviour of pyrethroid insecticides (Liu et al, 2009). Different adsorption affinities may be attributed to differences in regards to their physical and chemical properties. The FTIR spectra in a study conducted by Liu et al (2009), suggested that competitive adsorption occurs between CYP and copper for the same adsorption sites, leading to suppression of CYP adsorption by Cu. This would

indicate an interaction between Cu and CYP resulting in an increase of CYP bioavailability and hence toxicity. This toxicity may be associated with a subsequent suppression of soil microbial community functions (Calderbank, 1989). Furthermore, research by Jun et al (2013) provides evidence that an increase of Cu concentration not only inhibits CYP adsorption rates, but subsequently stimulates pyrethroid transport. In previous research it was suggested by Liu et al (2008) that Cu may penetrate readily into cells and affect microbial functions in CYP contaminated soil.

Humic substances, with several oxygen and hydroxyl containing functional groups, form H-bonds with similar complementary groups on pesticide molecules. Pesticides compete with water for these binding sites. It is suggested that H-bonding plays a crucial role in the absorption of many non-ionic polar pesticides (Senesi and Testini, 1980; Senesi and Testini, 1983). Cypermethrin, however, is a non-polar, hydrophobic molecule. It is readily adsorbed and bound onto the soil surface. Because of this, very little CYP insecticide is able to move through the soil profile (Kaufman et al, 1981), although its major metabolites, 3-phenoxybenzoic acid (PBA) and cyclopropanecarboxylic acid derivatives are very polar and transport more easily through soil (Jones, 1995). The behaviour of pesticides in soil depends largely on adsorption – desorption mechanisms. The knowledge and understanding of these processes is important to enable a prediction of mobility and bioavailability of pesticides in soil, and therefore limit the impact of these potentially toxic compounds on non-target organisms and surrounding ecosystems (Olvera-Velona et al, 2008).

1.3.3 Trace Metal/Pesticide Interactions

Excessive release of trace metals through fertilizers, composts and industrial effluents paired with an overuse of pesticides are limiting crop production by polluting the environment and reducing food quality (Shahzad, 2018). A wide variety of pesticides are used in agriculture to advance annual crop productivity. However, their residues contaminate the soil which may cause harm to soil organisms either directly or indirectly (Choung et al, 2012). In past assessments of environmental contaminants, the main focus has been

on individual compounds. However, assessment of the joint effects of chemical contaminants by analysing data obtained from the toxicity of single substances, may under or overestimate the level of joint toxicity (Schnug et al, 2014). The joint toxicity of potentially toxic substances can be additive (combined), synergistic (greater than additive) or antagonistic (less than additive), depending upon the sum of effects from separate exposures. Most studies suggest that most metal mixtures produce synergistic effects (Warne and Hawker, 1995; Cedergreen, 2014).

Chachada et al (2016) acknowledged that there is a great need to improve our understanding about the potentially harmful effects of chemical mixture exposures, but also that soil is widely understood to be a complex terrestrial system containing a substantial amount of organisms, minerals and organic matter, and therefore the variables of predicting the interactions of two potential contaminants are, in turn, complex. Uwizeyimana et al (2017) indicated that beside pesticides, heavy metal pollution in soil biospheres has become a global challenge due to the increase of geologic and anthropogenic activities. Uwizeyimana et al (2017) also reported indications that pesticide and metal mixtures at all organization levels has a negative effect upon soil biota. Therefore studies that evaluate individual and joint toxicity of chemical mixtures are of great importance to the agricultural, ecological and environmental society.

The joint interactions of pesticides and metals and their effects on earthworm species have been widely studied, as earthworms are regarded as important bioindicators of soil pollution. Although the combination of pesticide and metal generally have been observed to produce synergistic interactions (60%), Uwizeyimana (2017) found many mixtures may produce dual reactions where synergistic and antagonistic effects are seen in the same mixture, depending upon the dose levels. It is recognized that combinations of mixtures of pesticides and metal ions may produce adverse effects to all biotic life habituating the soil. Pesticide and metal mixtures have been observed to cause alterations of DNA/gene expression, oxidative stress, deceleration of sexual development, causes mortality and reduces earthworm populations

(Zhou, 2011). Clearly, there is evidence of detrimental effects of chemical mixtures to soil macrofauna (Yang et al, 2015; Uwizeyimana, 2017; Booth and O'Halloran, 2001).

Wuana et al (2014) recognised the threat that complex chemical mixtures of heavy metals (HMs) and organic contaminants (OCs) poses on microbial components of soil and its health, describing this as an issue of great concern affecting both the health of human beings and ecosystems. Wuana et al (2014) found mixed interactions of HMs and OCs may also synergistically or antagonistically promote their accumulation in the soil biota. Furthermore, knowledge of mixed chemical interactions and their synergistic and antagonistic mechanisms in soil is needed to advance soil ecotoxicological literature which, appears to be dominated by studies of single toxicant exposure (Naidu et al, 2010).

Interactions between toxicants may occur in the toxicokinetic phase (uptake, distribution, metabolism and excretion) or the toxicodynamic phase (effect of compounds on receptor, cellular target or organ) (IGHRC, 2009). In co-contaminated soil, HM uptake may be magnified in the presence of OCs due to (i) facilitated transport caused by metal association (ii) development of metal – organic and inorganic complexes that do not adsorb to soil surfaces (iii) competition with other inorganic and organic waste fractions for sorption sites (Puls et al, 1991).

Cedergreen (2014) agrees that the synergistic interactions of pesticide and metal ions would occur in the toxicokinetic phase. With regards to bioavailability, interactions between chemicals can occur outside of an organism, where one chemical affects the availability of another chemical. This takes place more commonly for metal ions, where speciation and competition for binding sites to soil organic matter changes ion availability and composition (Meyer et al, 2013).

One chemical can affect the uptake rate of another chemical by competition for biological ligands or competitive inhibition of transport proteins, which is often recognized in metal ion uptake (Elliot et al, 1986). Interactions of

affected uptake rates have also been measured for organic contaminant combinations (Broderius et al, 1995). Belden and Lydy (2009) examined the synergistic interactions between herbicide atrazine and organophosphate insecticide chlorpyriphos, and found addition of atrazine increased chlorpyriphos uptake by 40%. It was proposed that this was due to an increase in oxygen consumption. Many contaminants will increase respiration rates when microorganisms spend the energy to metabolize them (Belden and Lydy, 2000).

With OCs, microorganisms will utilise them as a source of carbon or transform them into nontoxic products, assisted by enzymes and extracellular products. However, HMs interfere with microbial physical and metabolic processes and inhibit the degradation of OCs leading to the persistence of the compound in soil (Thavamani et al, 2011) resulting in potential subsequent toxicity.

Recent studies have provided evidence to suggest that pesticides have a clear, detrimental effect upon microbial populations and inhibit the enzymatic systems of agricultural soil, in some instances reducing activity by 50% (Singh and Singh, 2005; Pandey and Singh, 2006), therefore potentially having a harmful effect upon the microbial functions and subsequent nutrient cycles of soil. Elevated concentrations of heavy metals in soil introduced by a range of fertilizers may also be potentially damaging to soil processes. Interactive toxic effects to organisms exposed to more than one toxin simultaneously have been widely reported and it has been suggested that interactions between pesticides and heavy metals may have an important impact on soil processes (Mansour, 2009).

The Current Study

It is of environmental, ecological and agricultural importance to understand the combined effects that heavy metals and pesticides are having on agricultural soils, especially when there is evidence to suggest that both substances are damaging to the microbial processes that the quality and health of soil, and subsequent plant life, depend upon. Specifically, there is little information about the combined effects of both cypermethrin and Cu

contaminants on microbial processes within soils, despite the widespread use of both in agricultural systems. This study aims to expand on the current paucity of knowledge of the combined toxicity of pesticides and trace metal to microbial and enzymatic functions of soil. The specific aim of this study is to investigate the interactive effects that the pyrethroid pesticide cypermethrin and trace metal Cu, have upon the activity of soil enzymes. Enzyme activity may be easily measured and is a direct reflection upon microbial abundance, soil quality and health. Inhibition or a lack of enzymatic activity may indicate toxic effects on key soil processes.

The current research will seek to answer the following objectives:

- To determine concentration at which the pesticide and Cu negatively impact soil enzyme activity.

- To determine if there is a toxic interaction between cypermethrin and Cu.

- To investigate if one soil process is most vulnerable.

Rationale

There is a great deal of scientific data and knowledge concerning pesticides, and their potential hazards towards human health and especially their teratogenic impacts, understandably. Consequently, much is known about pyrethroids and their effect upon the environment and persistence in soil. Research and knowledge begins to become limited regarding their effects upon microbial populations and enzymatic systems. There is virtually no information regarding the joint toxicity of $CuSO_4$, cypermethrin and their actions when applied simultaneously. Liu et al (2008) investigated the joint effect of Cu and cypermethrin upon catalase in an attempt to provide more information concerning the potential harm of the simultaneous use of compounds upon the soil ecosystem. Catalase is an essential enzyme for almost all aerobic and anaerobic microorganisms. The enzyme splits hydrogen peroxide into oxygen and water, thus protecting cells from damage by reactive oxygen species. If Catalase is inhibited, the metabolic activity of

microorganisms and the health of soil may be compromised (Stepniewska et al, 2009). The study by Stepniewska et al (2009) is one of many that reveal i) how sensitive enzymes can be in response to an external pollutant, and ii) how important enzymes are to all soil processes, agriculture and the environment.

By answering the stated objectives, the knowledge gained by the current study will help inform the wider community of the appropriate use of cypermethrin and Cu in circumstances where they may be used simultaneously. New information will be provided of their potential danger at certain concentrations and which particular nutrient cycle of the soil environment is at risk. The findings of the current study will also shed light on the need to investigate the combination of other PPPs with trace metals and their threat to essential nutrient cycles, the subsequent health of the soil ecosystem and the environment at large.

2.0 Materials and Methods

2.1 Introduction

The current study provides a novel approach by utilizing a sterilized, lime and chalk free, composite soil obtained from a commercial supplier (Wickes Building Supplies Limited, Northampton UK). This method eliminates the historical application and persistence of chemicals that would apply to the vast majority of agricultural soil samples. By testing this soil type, substances being investigated have the benefit of acting upon a soil that has not previously been exposed to them. Therefore the soil microbial community under analysis has no previous exposure to the substances. There no evidence in the literature concerning the joint toxicity of cypermethrin and copper sulphate or their effect upon the particular microbial and enzyme systems employed in the current research.

The enzymatic assays chosen were due to their being good representatives of the overall health of soil. Glycosidase is intrinsic to the carbon cycle, and

plays an essential role as an energy provider to soil microorganisms (Shewale, 1982; Dick et al, 2013; Tabatabai, 1994).Phosphomonoesterase is an essential enzyme in the catalysis of phosphates to forms that are more efficient for root uptake. Phosphate is an essential element / nutrient for plant growth. Phosphatases maintain a key role in soil systems and are thus, a good indicator of soil health (Shukla and Varma, 2011; Karthikeyan et al, 2002; Tabatabai, 1994). Fluorescein diacetate (FDA) hydrolysis is generally accepted as a precise and straightforward method for the measurement of the total microbial activity in a range of differing soil types. Fluorescein diacetate is colourless until hydrolyzed by free and membrane bound enzymes, releasing fluorescein, a coloured end product, measured easily by spectrophotometry (Adam and Duncan, 2001). Total microbial activity produces a general measure of SOM turnover as approximately 90% of energy in the soil biosphere emanates through microbial decomposers (Green et al, 2006).

2.2 Soil Preparation

The 60% water holding capacity of the soil was calculated. This is the optimal water content for the movement of organisms, nutrients, amino acids and transport of essential elements throughout the soil environment (Kaye and Hart, 1997). The determination of optimal water capacity for the measurement of microbial parameters and activity is of essential importance (Mansson et al, 2014). Soil moisture is described as an important factor because it changes the environment in which microbial activity can occur. A water holding capacity (WHC) of approximately 60% has been suggested as an adequate water percentage suitable for experimental work as this is thought to be the optimal for maximum microbial capacity (Tibbet et al, 2004; Linn and Doran, 1984).

2.2.1 Determining Water Holding Capacity (WHC)

Approximately 25 g of lime and chalk free topsoil was thoroughly soaked in water for 24 h. Filter paper placed in a ceramic funnel were both weighed to 3 decimal places. The funnel was placed in a 250 mL conical flask and the soaked soil placed in the ceramic funnel. Excess water was left to drain from the soil for 2 h, or until the drips had completely ceased. At this point the soil is at 100% WHC. The soil and funnel were weighed and placed in a drying oven set at 105^{o}C for 24 h. Water holding capacity was determined using equation 1.

WHC (%) = (W/F) X 100 Eq. (1)

Where:

W = The weight of the soil at saturation – weight of the soil after drying

F = weight of the soil after drying

By multiplying WHC by 0.6, the moisture content at 60% WHC was found

2.2.2 Determining Soil Moisture Content of Fresh Soil

4-5 g of soil was weighed into a 100 mL beaker. The weight of the beaker and the soil was then taken. The soil was then dried in a drying oven at 105^{o}C for 24 h. To calculate the % of water in soil, equation 2 was used:

% water = (B-C / B-A) X 100. Eq. (2)

Where:

A = weight of the empty beaker

B = weight of the beaker and fresh soil

C = weight of the beaker and dry soil

2.2.3 Adjusting Soil to 60% WHC

The amount of water in mL that must be added to 100 g soil to bring the soil to 60% WHC was calculated from equation 3.

Water required (mL) = (D*H) – W

Eq. (3)

Where:

D = Dry weight of soil in 100 g fresh soil (= 100% water content from 2.2.2 above expressed as decimal)

H = % water content at 60% WHC, determined from 2.2.1 above and expressed as a decimal

W = % water determined from 2.2.2 above and expressed as a percentage

2.3 Pot Trials

Two pot trials were conducted to investigate the effect of $CuSO_4$ and CYP on the activity of selected soil enzymes. The first experiment used doses of CYP and $CuSO_4$ applied singly and the second trial compared the effects of single and mixed applications of the two substances. Soil was spiked with increasing concentrations of $CuSO_4$ and CYP. Separate pots were arranged for each concentration, .00, 35, 70, 140, 240, 480, 720 and 960 mg kg^{-1} $CuSO_4$. .00, 10, 20, 40, 80, 160, 320, 640 mg kg^{-1} CYP. The activity of two enzymes and the total microbial activity were measured for each concentration. In the second trial the lower, medium and higher doses were combined and their effects were compared to doses applied individually.

2.3.1 Substances Applied Singularly

The soil control was prepared by weighing 2000g of fresh soil and adding distilled water in the amount to reach 60% water holding capacity (WHC). The soil was homogenized and split among four replicate 1L pots (500g per pot).

The soil $CuSO_4$ contaminations were prepared by weighing 2000g of soil and adding an appropriate amount of 10,000 mg mL^{-1} $CuSO_4$ solution. Distilled water was added in the appropriate amounts to bring the total volume of water added to bring the soil to 60% WHC (Table 2.1). Spiking treatments were calculated to raise soil Cu concentrations by 35, 70, 140, 240, 720 and 960 mg kg^{-1}. After the addition of water and $CuSO_4$ solution, the soil was homogenized by thorough mixing and separated into four replicate 1 L pots.

The soil cypermethrin spiking was conducted in a similar way to that for Cu Samples of 2000g of soil were weighed and cypermethrin solution of 10,000 mg mL^{-1} and distilled water were added in the appropriate amounts to maintain soil WHC at 60% whilst reaching soil cypermethrin concentrations of 10, 20, 40, 80, 160, 320 and 640 mg kg^{-1} (Table 2.2). Again, spiked soil was distributed amongst four 1 L pots.

Soil samples were kept frozen before analysis. The most commonly employed methods for soil storage before microbiological analysis are refrigeration and freezing. There are studies that demonstrate that the effects of freezing are generally smaller than refrigeration. For example, Stenberg et al (1998) conclude that storage at -20^{o}C for up to 13 months does not affect microflora in frozen soils. Hence, freezing was the chosen method of storage.

2.3.2 Substances Applied as Mixtures

Results from the first trial were used to inform doses used in the second pot trial, which investigated the effect of the two substances combined in a mixture. The same basic method was used when spiking soil with a single substance, except now the two substances are combined. Concentrations of 70 mg kg^{-1} $CuSO_4$ and 80 mg kg^{-1} CYP were applied to 2000 g of soil and distilled water was added to ensure soil was maintained at 60% water holding capacity. The same procedure was applied to mixed concentrations of 140 mg kg^{-1} $CuSO_4$ and 160 mg kg^{-1} CYP and mixed concentrations of 960 mg kg^{-1} $CuSO_4$ and 640 mg kg^{-1} CYP. These concentrations were chosen, because

when applied individually the data determined the doses to be statistically significantly different to other concentration groups.

2.4 Determination of Soil Enzyme Activity

2.4.1 Soil Glycosidase Assay

The assay was performed as described by Tabatabai (1994). 1g of soil was weighed and placed in a 50 mL conical flask. 0.25 mL of toluene, 4 mL of modified universal buffer (pH6) and 1 mL of p-Nitrophenyl-β-glucoside (PNG) were added to the soil sample. The flask was swirled and incubated at 37^{o}C for 1 h in an orbital incubator (1000 rev min^{-1}). The enzyme activity was then halted by the addition of 1 mL $CaCl_2$, 4 mL Tris(hydroxymethyl)aminomethan (THAM) buffer (pH 12) and swirled for a few seconds. The soil suspension was filtered through a Whatman no.2 filter paper into a 30 mL sterilin tube. This was replicated twice for each sample, along with a blank with soil but without the addition of PNG. This blank is used to account for any background absorbance arising from fine suspended matter and dissolved solids from the soil that passed through the filter paper and absorb light. The absorbance of all filtrates were measured at 405 nm using a UV/vis spectrophotometer (Varian Cary 50, Varian inc., California).

A calculation was performed on all values to calculate for µg p-nitrophenol released g^{-1} soil h^{-1}.

$$\mu g\ p\text{-nitrophenol released } g^{-1} \text{ soil } h^{-1} = \frac{Fs*10.25}{Wts*Sd} - \frac{Fb*10.25}{Wtb*Sd} \qquad \text{Eq. (4)}$$

Where:

F_s = concentration of *p*-nitrophenol in sample

F_b = concentration of recorded in the blank

Wt_s = weight of soil sample

Wt_b = weight of blank soil

S_d = dry matter content of soil expressed as a decimal, e.g 85% = 0.85

2.4.2 Soil Phosphomonoesterase Assay

Again the assay was performed as described by Tabatabai (1994). 1 g of soil was weighed and placed in a 50 mL conical flask. 0.2 mL of toluene, 4 mL of modified universal buffer (pH 11 for alkaline phosphatase), and 1 mL of sodium *p*-nitrophenol phosphate (NPP) were added to the soil sample. The flask was swirled and orbital incubated at 37^{o}C for 1 h. The enzyme activity was halted with the addition of 0.5 M $CaCl_2$, 4 mL of 0.5 M NaOH and swirled for a few seconds. The soil suspension was filtered through a Whatman No.2 filter paper into a 30 mL sterilin tube. This was replicated twice and NPP blank used to account for any background absorbance arising from suspended matter from the soil that passed through the filter paper. The absorbance of all filtrates were measured at 405 nm using a UV/vis spectrophotometer.

A calculation was performed on all values to determine µg p-nitrophenol released g^{-1} soil h^{-1}.

µg *p*-nitrophenol released g^{-1} soil h^{-1} = $\frac{Fs*10.25}{Wts*Sd} - \frac{Fb*10.25}{Wtb*Sd}$ Eq. (5)

Where:

F_s = concentration of *p*-nitrophenol in sample

F_b = concentration of recorded in the blank

Wt_s = weight of soil sample

Wt_b = weight of blank soil

S_d = dry matter content of soil expressed as a decimal, e.g 85% = 0.85

2.4.3 Total Microbial Activity Assay

The assay was performed as described by Adam and Duncan (2001). 2 g of soil was placed in a 50 mL conical flask. 15 mL of 60 mM of potassium phosphate buffer (pH 6) and 0.2 mL of fluorescein diacetate (FDA) stock solution were added to start the reaction. The flasks were stoppered and shaken by hand. The flasks were placed in an orbital incubator at 30^{o}C for 20

min. The flasks were removed from the shaker and 15 mL of chloroform / methanol (2:1 v/v) were added to terminate the reaction. The stoppers were replaced and the flask was shaken for a few seconds. The contents of the flask was transferred to a 50 mL centrifuge tube and centrifuged at 2000 rev min^{-1} for 3 min. The supernatant was filtered through a Whatman no.2 filter paper collecting the filtrate in a 30 mL sterilin tube. The procedure was replicated twice and a blank without the addition of the FDA stock solution was used to account for any background absorbance arising from suspended matter from the soil that passed through the filter paper. The absorbance of all filtrates were measured at 490 nm on a UV/vis spectrophotometer.

A calculation was performed on all values to calculate for μg fluorescein g $(d.w.)^{-1}$ h^{-1}.

$$\left(\frac{Fs*20}{Wts*Sd} - \frac{Fb*20}{Wtb*Sd}\right) \text{ X 3 = μg fluorescein g } (d.w)^{-1} h^{-1}$$

Where:

F_s = concentration of fluorescein in sample

F_b = concentration of fluorescein in the blank

Wt_s = weight of soil sample

Wt_b = weight of blank soil

S_d = dry matter content of soil expressed as a decimal, e.g 85% = 0.85

2.5 Data Analysis

All data analysis was performed using the SPSS statistical analysis program. In the context of this research, one way ANOVA was used to investigate the significance between the doses and enzyme / microbial activity. Tukey HSD or Games Howell *post hoc* tests were employed to test for differences between treatments, which test was used depended upon the outcome of the Test of Homogeneity of Variances (Levene's Statistic); Tukey's test was used were Levene's test indicated homogeneity of variance, otherwise Games Howell's test was used.

3. Results

3.1 Effect of CuSO4 on Enzyme Activity

3.1.1 Impact of CuSO4 Concentration on Glycosidase Activity

A rise in glycosidase activity was proceeded by a slight fall as $CuSO_4$ increased (Fig.3.1). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used to explore the relationship between the dose of $CuSO_4$ (35, 70, 140, 240, 480, 720, 960 mg kg^{-1}) and glycosidase activity. This showed a significant difference existed among the treatments ($F_{(7, 9.6)} = 7.18$, $p = .004$). Using the Games-Howell *post hoc* procedure, doses of 35 and 960 mg kg^{-1} were found to differ significantly ($M = .89$, $SD = .16$) as were as 70 and 960 mg kg^{-1} ($M = .94$, $SD = .14$).

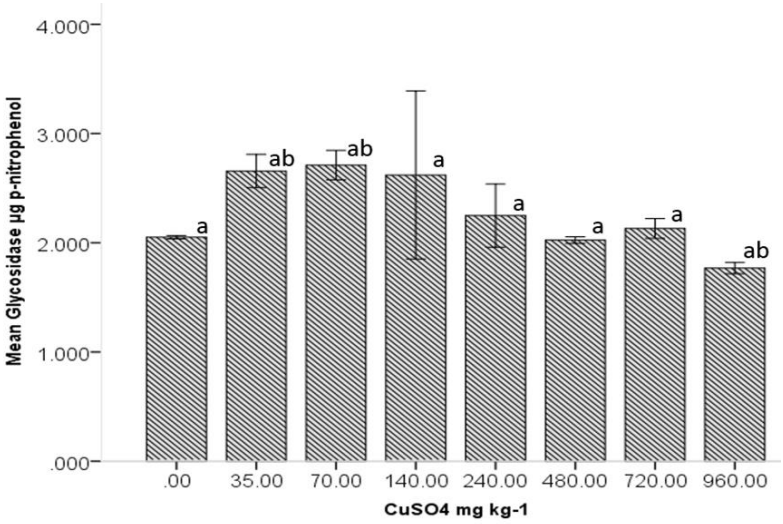

Figure 3.1 The effect of rising concentration of $CuSO_4$ upon the activity of glycosidase in soil. Mean ± 1SE, letters indicate significant differences between treatments.

3.1.2 Impact of CuSO4 on PME activity

PME activity showed a complex change with increasing $CuSO_4$, but a drop of activity was seen towards the higher doses of $CuSO_4$ (Fig 3.2). A one way ANOVA was used to explore the relationship between the dose of CuSO4 and PME activity. Again, preliminary testing demonstrated that the data failed Levene's test – ($F_{(7, 24)} = 2.75$, $p = .030$). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, which found significant differences among the treatments ($F_{(7, 10.13)}$

= 34.09, *p* = 0.00). Using the Games-Howell *post hoc* procedure, significant differences were found between the control and 70mg kg^{-1} treatment, (*M* = 1.29, *SD* = .22), control and 140 mg kg^{-1} (*M* = 1.11, *SD* = .21), control and 720 mg kg^{-1} , (*M* = 2.96, *SD* = .29), control and 960 mg kg^{-1}, (*M* = 2.99, *SD* = 1.20), 70 and 720 mg kg^{-1}, (*M* = 1.67, *SD* = .28), 70 and 960 mg kg^{-1}, (*M* = 1.70, *SD* = .17), 140 and 720 mg kg^{-1}, (*M* = 1.85, *SD* = .28), 140 and 960 mg kg^{-1}, (*M* = 1.89, *SD* = .17), 240 and 720 mg kg^{-1}, (*M* = 3.06, *SD* = .50), 240 and 960 mg kg^{-1}, (*M* = 3.09, *SD* = .44).

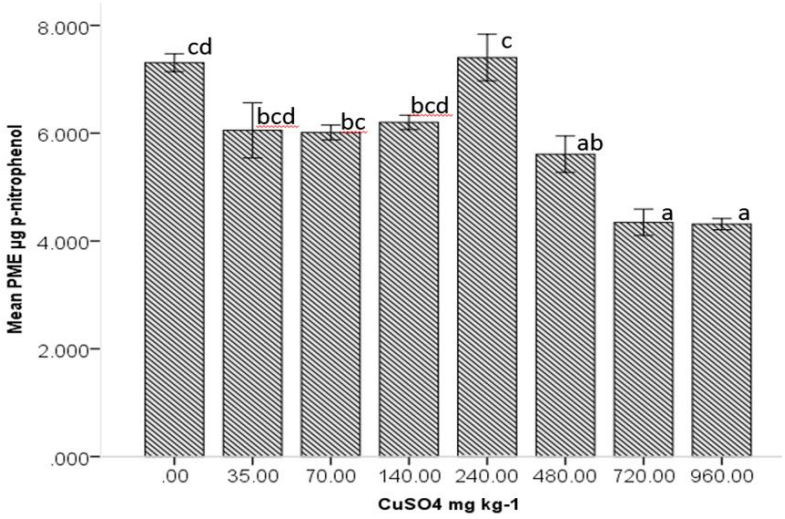

Figure 3.2 The effect of rising concentration of CuSO4 upon the activity of phosphomonoesterase in soil. Mean ± 1SE, letters indicate significant differences between treatments.

Impact of CuSO4 on Total Microbial Activity

Total microbial activity demonstrated an initial increase as $CuSO_4$ concentrations increased, but little effect was observed until a sudden drop in activity occured at the highest dose (Fig 3.3). A one way ANOVA was used to explore the relationship between the dose of $CuSO_4$ and the total microbial activity. The relationship passed Levene's test ($F_{(7, 24)}$ = .86, *p* = .550). Subsequent use of Tukey's HSD *post hoc* procedure showed significant differences between the control and 960 mg kg^{-1}, (*M* = 6.85, SD = 2.05), 35 and 240 mg kg^{-1}, (M = 7.05, SD = 2.05), 35 and 960 mg kg (M = 12.62, SD = 2.05), 70 and 960 mg kg^{-1}, (M = 8.17, SD = 2.05), 140 and 960 mg kg^{-1}, (M = 7.77, SD = 2.05).

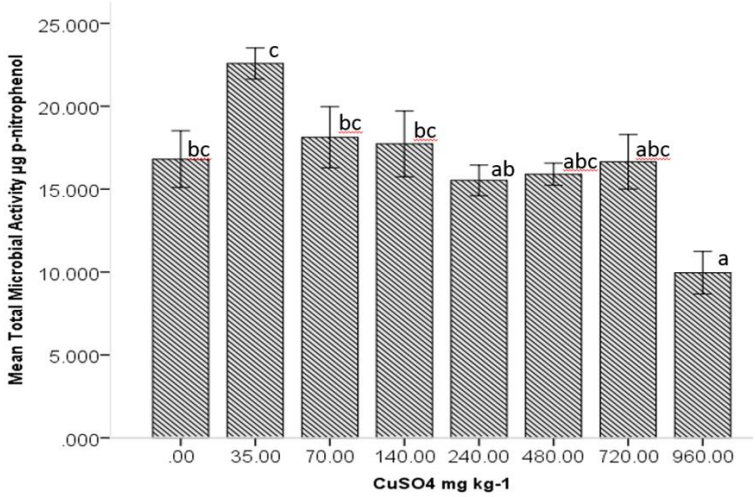

Figure 3.3 The effect of rising concentration of CuSO4 upon the total microbial activity in soil. Mean ± 1SE, letters indicate significant differences between treatments.

3.2 Effect of Cypermethrin on Enzyme Activity

3.2.1 Impact of Cypermethrin on Glycosidase activity

Complex effects on glycosidae activity appear to be occurring due to $CuSO_4$ treatment (Fig 3.4), leading to with fluctuations of enzyme activity as doose increased. A one way ANOVA was used to explore the relationship between the dose of cypermethrin and glycosidase activity. The data passed Levene's test, ($F_{(7, 24)}$ = 2.09, p = .084), hence Tukey's HSD *post hoc* procedure was used. This test found significant differences between the control and 320 mg kg^{-1} treatment, (M = 1.36, SD = .31), 10 and 320 mg kg^{-1}, (M = 1.22, SD = .31), 20 and 320 mg kg^{-1}, (M = 1.77, SD = .31), 20 and 640 mg kg^{-1}, (M = 1.08, SD = .31), 80 and 320 mg kg^{-1}, (M = 1.54, SD = .31), 160 and 320 mg kg^{-1}, (M = 1.24, SD = .31).

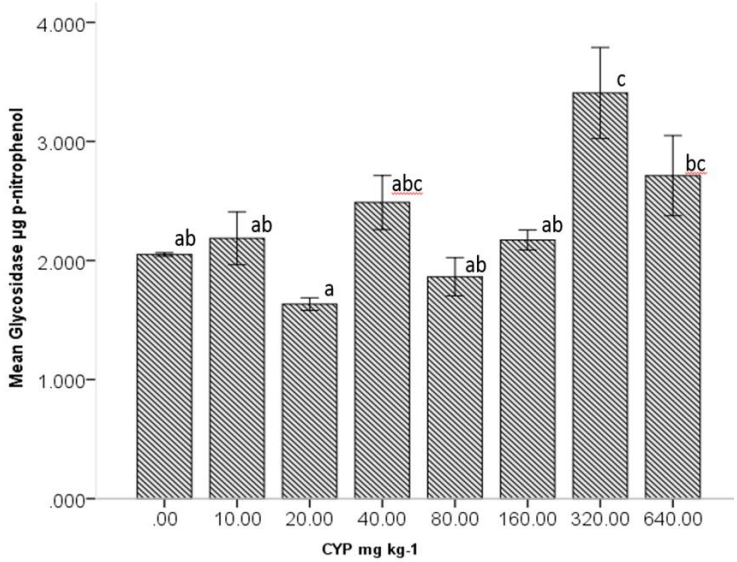

Figure 3.4 The effect of rising concentration of cypermethrin upon the activity of glycosidase in soil. Mean ± 1SE, letters indicate significant differences between treatments.

3.2.2 Impact of Cypermethrin on PME Activity

A gradual rise in activity can be observed (Fig 3.5) with the highest level of activity taking place at the highest dose. A one way ANOVA was used to explore the relationship between the dose of cypermethrin and phosphomonoesterase activity. The relationship passed Levene's test, ($F_{(7, 24)}$ = 1.91, p = 0.11). Using Tukey's HSD *post hoc* procedure, significant differences were found between the control and 80 mg kg^{-1}, (M = 3.92, SD = .78), control and 160 mg kg^{-1}, (M = 4.65, SD = .78), control and 320 mg kg^{-1}treatment, (M = 3.08, SD = .78), control and 640, (M = 7.92, SD = .78), 10 and 80 mg kg^{-1}, (M = 4.69, SD = .78), 10 and 160 mg kg^{-1}, (M = 5.42, SD = .78), 10 and 320 mg kg^{-1}, (M = 3.85, SD = .78), 10 and 640 mg kg^{-1}, (M = 8.69, SD = .78), 20 and 80 mg kg^{-1}, (M = 3.25, SD = .78), 20 and 160 mg kg^{-1}, (M = -3.98, SD = .78), 20 and 640 (M = 7.25, SD = .78), 40 and 160 mg kg^{-1}, (M = 2.91, SD = .78), 40 and 640 mg kg (M = 6.18, SD = .78), 80 and 640 mg kg^{-1}, (M = 4.00, SD = .78), 160 and 640 mg kg^{-1} (M = 3.27, SD = .78), 320 and 640 mg kg^{-1}, (M = 4.84, SD = .78). Thus, the 640 mg kg^{-1} treatment showed an enzyme activity higher than all other treatments.

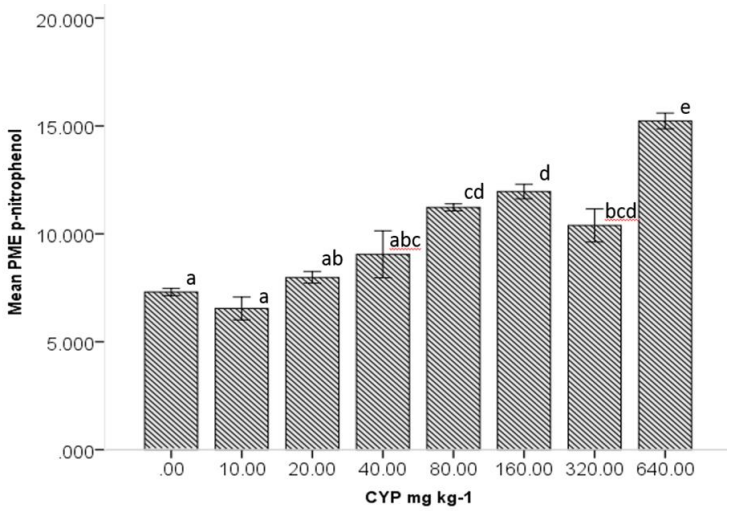

Figure 3.5 The effect of rising concentration of cypermethrin upon the activity of phosphomonoesterase in soil. Mean ± 1SE, letters indicate significant differences between treatments.

3.2.3 Impact of Cypermethrin on Total Microbial activity

A gradual increase of total microbial activity was observed, with the highest activity occurring at the highest dose. A one way ANOVA was used to explore the significance of differences between the dose of cypermethrin and total microbial activity. The relationship passed Levene's test, ($F_{(7, 24)}$ = 1.03, p = .438). Using Tukey's *post hoc* procedure, significant differences were found between the control and 320 mg kg^{-1}, (M = -12.67, SD = 3.12), the control and 640, (M = -13 80, SD = 3.12), 10 and 640 mg kg^{-1}, (M = -10.92, SD = 3.12), 20 and 320 mg kg^{-1}, (M = -11.46, SD = 3.12), 20 and 640 mg kg^{-1}, (M = 12.59, SD = 3.12).

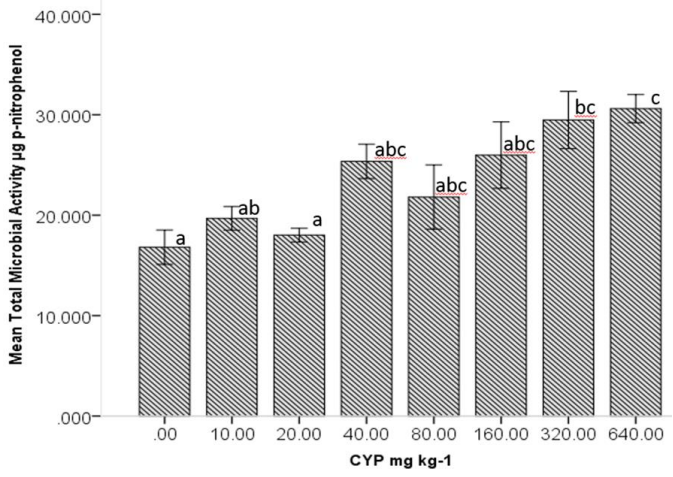

Figure 3.6 The effect of rising concentration of cypermethrin upon the total microbial activity in soil. Mean ± 1SE, letters indicate significant differences between treatments.

3.3 Interactions between CuSO4 and cypermethrin

3.3.1 Interactive effects between CuSO4 and cypermethrin on glycosidase activity

Interaction Between Low/Low Dose Mixture On Glycosidase

A markedly higher level of activity was seen in the low dose mixture when compared to the control and low dose of $CuSO_4$ only(Fig 3.7). A one way ANOVA was used to explore the relationship between the lowest dose of $CuSO_4$ (70 mg kg^{-1}) and the lowest dose mixture of $CuSO_4$ and cypermethrin combined (70 / 80 mg kg^{-1}) on glycosidase activity. The relationship failed Levene's test, ($F_{(2, 17)}$ = 8.11, p =.003), therefore Welch's adjusted F ratio was used, which indicated a significant difference amongst treatments ($F_{(2, 9.02)}$ = 54.12, p = .001). Using the Games-Howell *post hoc* procedure, significant differences were found between the control and 70/80 mg kg^{-1}, (M = 2.45, SD = .44), and between 70 and 70/80 mg kg^{-1}, (M = 2.66, SD = .25).

A significantly higher rate of activity was also seen in the lower dose Cu/CYP mixture when compared to the control and individual dose of CYP (Fig 3.8). A one way ANOVA was used to explore the relationship between the lowest dose of cypermethrin (80 mg kg^{-1}) and the lowest dose mixture of CuSO4 and cypermethrin combined (70 / 80 mg kg-1) on glycosidase activity. The relationship failed the test for Levene's, ($F_{(2, 17)}$ = 7.84, p =.004). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, which showed that there were significant differences amongst treatments ($F_{(2, 9.40)}$ = 82.55, p = .001). Using the Games-Howell procedure significant differences were found between the control and 70/80 mg kg^{-1}, (M = -2.45, SD = .44), 80 and 70/80 mg kg^{-1} (M = -3.51, SD = .26). Consequentlly, at lower doses, Cu combined with cypermethrin had a significant stimulatory rather than toxic effect upon glycosidase activity.

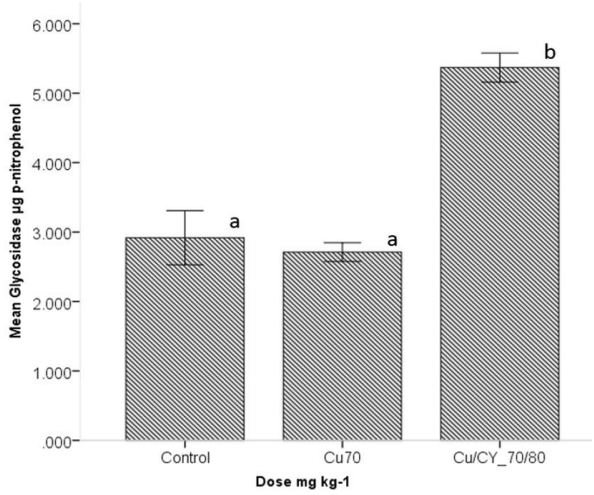

Figure 3.7 Comparison of low Cu dose to low Cu/CYP mixture upon glycosidase activity. Mean ± 1SE, letters indicate significant differences between treatments.

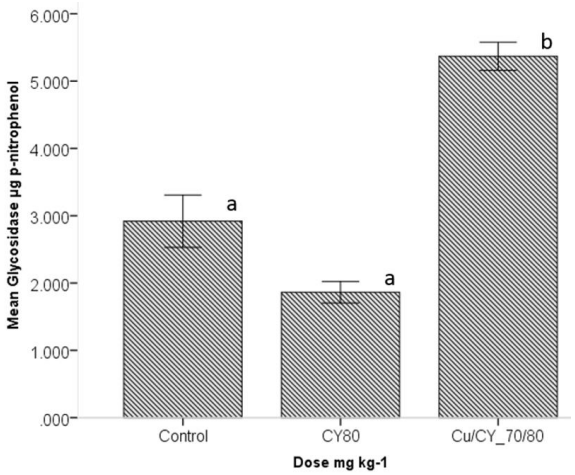

Figure 3.8 Comparison of low CYP dose to low Cu/CYP mixture upon glycosidase activity. Mean ± 1SE, letters indicate significant differences between treatments.

Relationship Between Medium/Medium Dose Mixture On Glycosidase

A markedly higher activity was witnessed in the mixture of Cu and CYP when compared to the control and individual dose of Cu (Fig 3.9). A one way ANOVA was used to explore the relationship between the medium dose of $CuSO_4$ (140 mg kg^{-1}) and the medium dose mixture of $CuSO_4$ and cypermethrin combined (140/160 mg kg^{-1}) on glycosidase activity. The relationship passed Levene's test ($F_{(2, 17)} = .367$, $p = .698$). Using Tukey's HSD *post hoc* procedure, significant differences were found between the

control and 140/160 mg kg^{-1} (*M* = 3.85, SD = .90), 140 and 140/160 (*M* = 4.15, *SD* = 1.10).

Markedly higher activity can be observed in the mixture when compared with the control and individual dose of CYP (Fig 4.0). A one way ANOVA was used to explore the relationship between the medium dose of cypermethrin (160 mg kg^{-1}) and the medium dose mixture of $CuSO_4$ and cypermethrin combined (140/160 mg kg^{-1}) on glycosidase activity. The relationship failed the Levene's test, ($F_{(2, 17)}$ = 4.30, *p* = .31) therefore since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, ($F_{(2, 6.5)}$ = 9.51, *p* = 0.01). Using the Games-Howell procedure, a significant difference was found between 160 and 140/160 mg kg^{-1} (*M* = 4.60, *SD* = 1.09), indicating that at medium dosing rates, Cu has a significant antagonistic effect when combined with cypermethrin upon glycosidase activities.

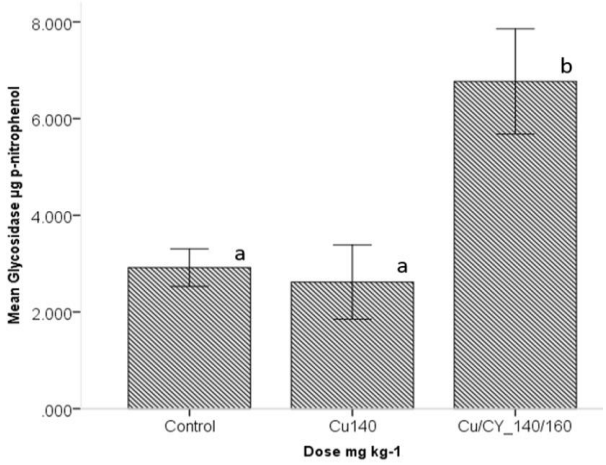

Figure 3.9 Comparison of medium Cu dose to medium Cu/CYP mixture upon glycosidase activity. Mean ± 1SE, letters indicate significant differences between treatments.

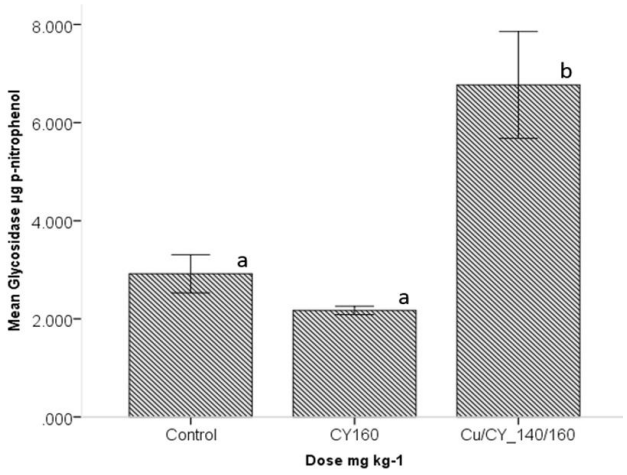

Figure 4.0 Comparison of medium CYP dose to medium Cu/CYP mixture upon glycosidase activity. Mean ± 1SE, letters indicate significant differences between treatments.

Relationship Between High/High Dose Mixture on Glycosidase

A markedly activity can be observed in the mixture when compared to the control and individual dose of Cu (Fig 4.1). A one way ANOVA was used to explore the relationship between the high dose of $CuSO_4$ (960 mg kg^{-1}) and the high dose mixture of $CuSO_4$ and cypermethrin (960/640 mg kg^{-1}) on glycosidase activity. The relationship failed Levene's test, ($F_{(2, 17)}$ = 6.51, p = .008), and since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, ($F_{(2, 7.7)}$ = 170.25, p = .001). Using the Games-Howell procedure, significant differences were found between the control and 960 mg kg^{-1} (M = 1.33, SD = .411) control and 960/640 mg kg^{-1} (M = 5.71, SD = .52) 960 and 960/640 mg kg^{-1} (M = 7.04, SD = .37).

Markedly higher activity is seen with the mixture compared to the control and individual dose of CYP (Fig 4.2). A one way ANOVA was used to explore the relationship between the high dose of cypermethrin (640 mg kg^{-1}) and the high dose mixture of $CuSO_4$ and cypermethrin 960/640 mg kg^{-1}) on glycosidase activity. The relationship failed Levene's test, ($F_{(2, 17)}$ = 3.82, p = 0.43), therefore since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, ($F_{(2, 8.36)}$ = 86.76, p = .001). Using the Games-Howell procedure, significant differences were found between the control and 960/640 (M = 5.71, SD = .52), 640 and 960/640 mg

kg^{-1}, which indicates that at low, medium and high dosing rates, Cu has a significant antagonistic effect when combined with cypermethrin upon glycosidase activities. This antagonistic effect increases as the dosing concentration also increases.

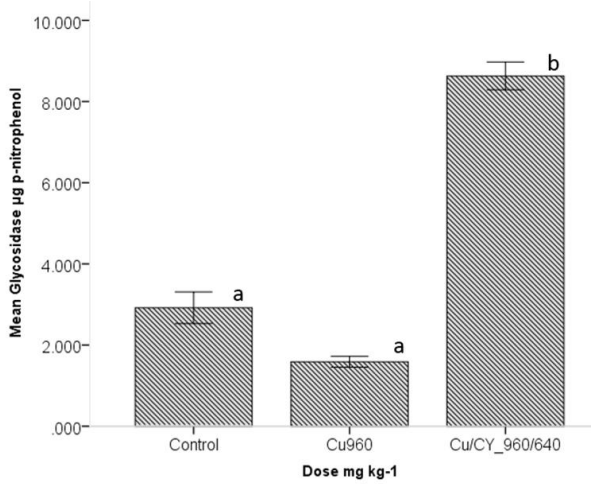

Figure 4.1 Comparison of high Cu dose to high Cu/CYP mixture upon glycosidase activity. Mean ± 1SE, letters indicate significant differences between treatments.

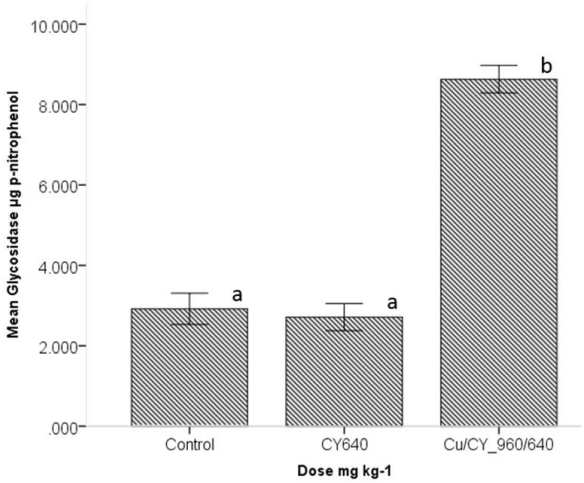

Figure 4.2 Comparison of high CYP dose to high Cu/CYP mixture upon glycosidase activity. Mean ± 1SE, letters indicate significant differences between treatments.

3.3.2 Interactive Effects Between CuSO4 and Cypermethrin on PME activity

Relationship Between Low / Low Dose Mixture on PME

Very little difference is observed for all three treatments. A one way ANOVA was used to explore the relationship between low dose of $CuSO_4$ (70 mg kg^{-1}) and the low dose mixture of $CuSO_4$ and cypermethrin (70/80 mg kg^{-1}) on PME activity (Fig 4.3). The relationship failed Levene's test, ($F_{(2, 17)} = 6.05$, $p = 0.10$). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, which confirmed there was no difference among treatments ($F_{(2, 7.75)} = .05$, $p = .951$).

A different effect was observed when the CYP treatment alone was compared with the CYP / $CuSO_4$ mixture, resulting in the highest level of PME activity in the individual dose of CYP (Fig 4.4). The mixture did not produce any significant difference to the control, suggesting that $CuSO_4$ negated the positive effect of CYP alone. A one way ANOVA was used to explore the relationship between low dose of cypermethrin (80 mg kg^{-1}) and the low dose mixture of $CuSO_4$ and cypermethrin (70/80 mg kg^{-1}) on PME activity. The data failed Levene's test, ($F_{(2, 17)} = 5.65$, $p = 0.13$). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, which confirmed that the observed differences among treatments was significant ($F_{(2, 8.15)} = 113.28$, $p = .001$). Using the Games-Howell procedure, significant differences were found between the control and 80 mg kg^{-1} ($M = 5.04$, $SD = .54$), 80 and 70/80 mg kg^{-1} ($M = 5.20$, $SD = .40$).

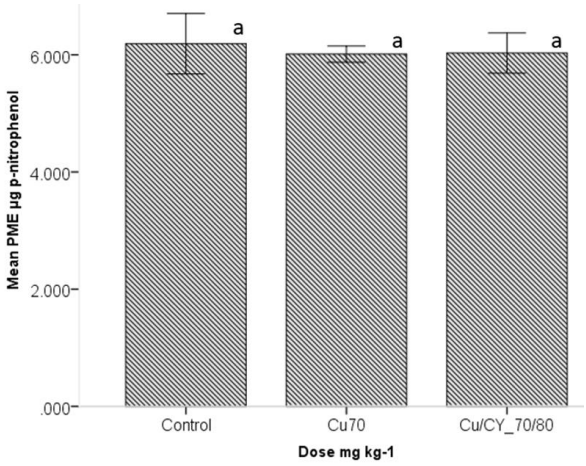

Figure 4.3 Comparison of low dose Cu to low Cu/CYP mixture upon PME activity. Mean ± 1SE, letters indicate significant differences between treatments.

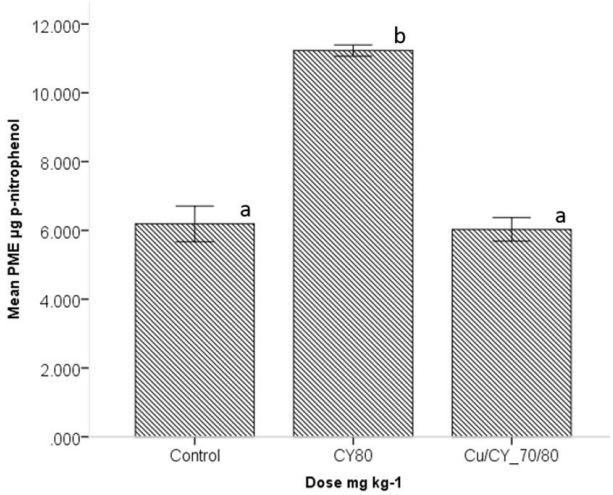

Figure 4.4 Comparison of low dose CYP to low Cu/CYP mixture upon PME activity. Mean ± 1SE, letters indicate significant differences between treatments.

Relationship Between Medium / Medium Dose Mixture on PME

It is of interest that almost the same PME activity is observed for both low / low dose mixture and medium / medium dose mixture (Fig 4.5); no significance is observed between treatments in the low or medium doses. A one way ANOVA was used to explore the relationship between medium dose of CuSO4 (140 mg kg^{-1}) and the medium dose mixture of $CuSO_4$ and cypermethrin (140/160 mg kg^{-1}) on PME activity. The relationship failed Levene's test, ($F_{(2, 17)}$ = 6.05, p = 0.10). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, ($F_{(2,}$

$_{7.75)}$ = .05, p = .951). Using the Games-Howell procedure, no significant differences were found between individual Cu dose and mixture treatments.

A significant difference is seen in the individual dose compared to that of the control and the mixture (Fig 4.6). A one way ANOVA was used to explore the relationship between medium dose of cypermethrin (160 mg kg^{-1}) and the medium dose mixture of $CuSO_4$ and cypermethrin (140/160 mg kg^{-1}) on PME activity. The relationship passed the test for Levene's, $F_{(2, 17)}$ = 3.22, p = 0.65. Using the Tukey HSD procedure, significant differences were found between the control and 160 mg kg^{-1} (M = 5.77, SD = 1.08), 160 and 140/160 mg kg^{-1} (M = 6.90, SD = 1.33).

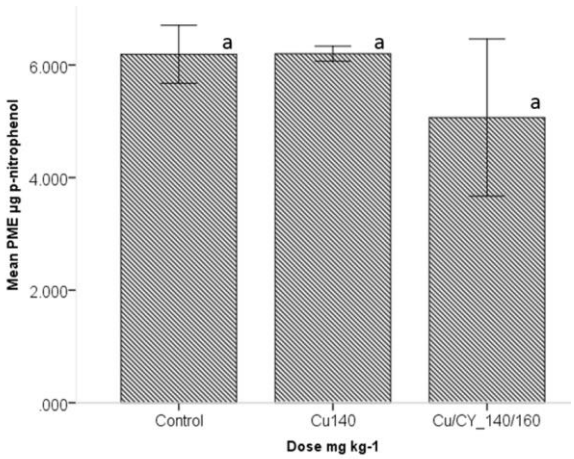

Figure 4.5 Comparison of medium dose Cu to medium Cu/CYP mixture upon PMEactivity. Mean ± 1SE, letters indicate significant differences between treatments.

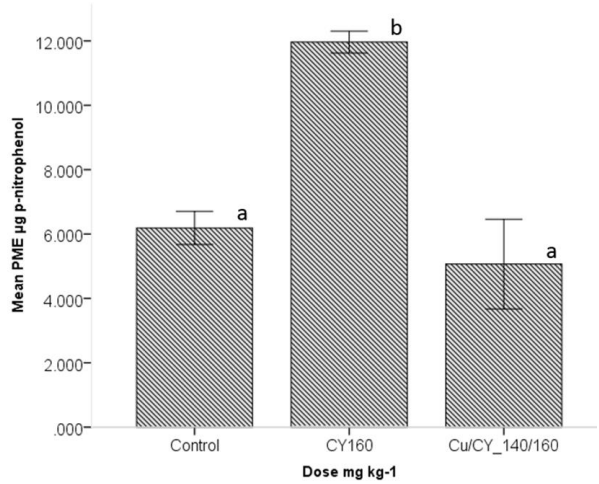

Figure 4.6 Comparison of medium dose CYP to medium Cu/CYP mixture upon PME activity. Mean ± 1SE, letters indicate significant differences between treatments.

Relationship Between High / High Dose Mixture on PME

There is a marked difference at higher dosing when compared to low and medium dosing. A much lower level of activity is observed when comparing the mixture to the control and individual Cu dose (Fig 4.7). Cu significantly suppressed PME activity and the mixture reduced activity significantly further. A one way ANOVA was used to explore the relationship between high dose of $CuSO_4$ (960 mg kg^{-1}) and the high dose mixture of $CuSO_4$ and cypermethrin (960/640 mg kg^{-1}) on PME activity. The data failed Levene's test, ($F_{(2, 17)}$ = 6.65, p = .007). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, ($F_{(2, 7.40)}$ = 12.66, p = .004). Using the Games-Howell procedure, significant differences were found between the control and 960 mg kg^{-1} (M = 1.88, SD = .53), the control and 960/640 mg kg^{-1} (M = 3.04, SD = .60).

A higher level of activity is witnessed with the individual cypermethrin dose compared to the control (Fig 4.8). A significantly lower level of activity is seen in the mixture compared to that of the control. A one way ANOVA was used to explore the relationship between high dose of cypermethrin (640 mg kg^{-1}) and the high dose mixture of $CuSO_4$ and cypermethrin (960/640 mg kg^{-1}) on PME activity. The relationship failed Levene's test, ($F_{(2, 17)}$ = 4.34, p = .030). Since the assumption of homogeneity of variance was not met for this data, Welch's adjusted F ratio was used, ($F_{(2, 9.04)}$ = 304.1, p = .000). Using the Games-Howell procedure, significant differences were found between the control and 640 mg kg^{-1} (M = 9.04, SD = .634), the control and 960/640 mg kg^{-1} (M = 3.04, SD = .60), 640 and 960/640 mg kg^{-1} (M = 12.08, SD = .477).

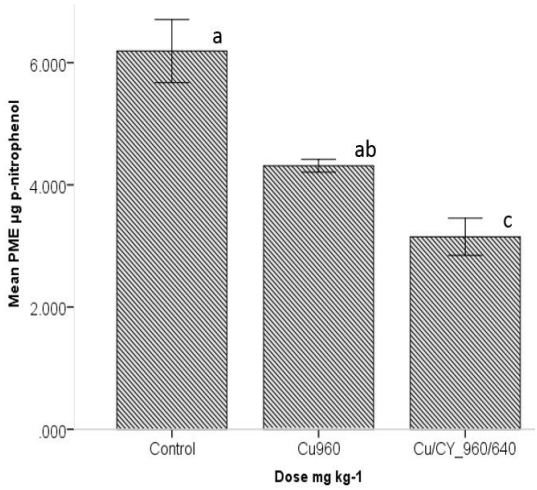

Figure 4.7 Comparison of high dose Cu to high Cu/CYP mixture upon PME activity. Mean ± 1SE, letters indicate significant differences between treatments.

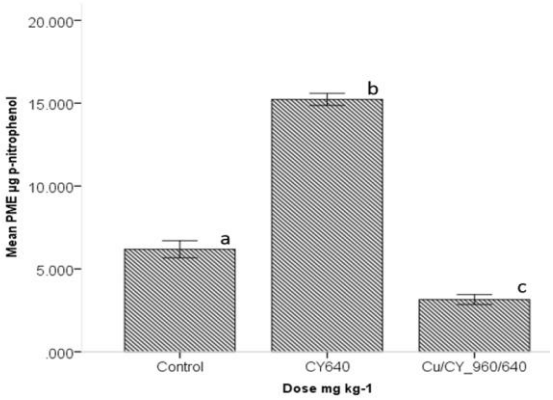

Figure 4.8 Comparison of high dose CYP to high Cu/CYP mixture effects upon PME activity. Mean ± 1SE, letters indicate significant differences between treatments.

3.3.3 Interactive Effects Between CuSO4 and Cypermethrin on Total Microbial Activity

Relationship Between Low / Low Dose Mixture on Total Microbial Activity

At low doses, the mixture causes a significantly higher rate of total microbial activity when compared to both Cu and CYP. A significant difference is observed in the Cu/CYP mixture compared to both the control and individual Cu dose (Fig 4.9). A one way ANOVA was used to explore the relationship between low dose of CuSO4 (70 mg kg-1) and the low dose mixture of CuSO4 and cypermethrin (70/80 mg kg-1) on the total microbial activity. The relationship passed Levene's test, ($F_{(2, 17)}$ = 1.74, p = .205). Using Tukey's

HSD procedure, significant differences were found between the control and 70/80 mg kg^{-1}, (*M* = 16.83, *SD* = 1.98), 70 and 70/80mg kg^{-1}, (*M* = 15.06, *SD*= 2.42).

The highest concentration of activity is seen in the Cu/CYP mixed dose compared to the control and individual dose of CYP (Fig 5.0). A one way ANOVA was used to explore the relationship between low dose of cypermethrin (80 mg kg^{-1}) and the low dose mixture of CuSO4 and cypermethrin (70/80 mg kg^{-1}) on the total microbial activity. The relationship passed Levene's test, ($F_{(2, 17)}$ = 1.89, *p* = .182). Using the *post hoc* Tukey HSD procedure, significant differences were found between the control and 70/80, (*M* = 16.83, *SD* = 2.36), 80 and 70/80, (*M* = 11.56, *SD* = 2.89).

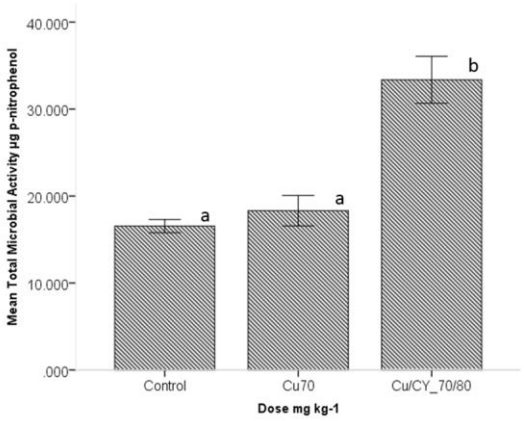

Figure 4.9 Comparison of low dose Cu to Cu/CYP mixture effect upon the total microbial activity. Mean ± 1SE, letters indicate significant differences between treatments.

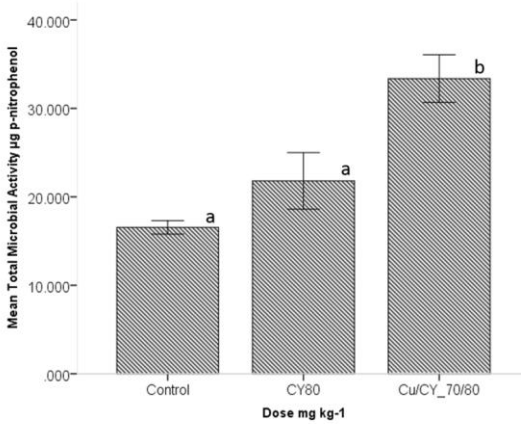

Figure 5.0 Comparison of low dose CYP to Cu/CYP mixture effect upon the total microbial activity. Mean ± 1SE, letters indicate significant differences between treatments.

Relationship Between Medium Dose and Medium Dose Mixture on Total Microbial Activity

The highest concentration of activity is seen in the mixed dose of Cu/CYP compared to the control and individual dose of Cu (Fig 5.1). A one way ANOVA was used to explore the relationship between medium dose of $CuSO_4$ (140 mg kg-$^{-1}$) and the medium dose mixture of $CuSO_4$ and cypermethrin (140/160 mg kg^{-1}) on the total microbial activity. The relationship passed Levene's test, ($F_{(2, 17)}$ = 1.35, p = .285). Using Tukey's HSD procedure, significant differences were found between the control and 140/160, (M = 9.36, SD = 1.95), 140 and 140/160, (M = 8.18, SD = 2.39).

Both the individual dose of CYP and the mixed dose of Cu/CYP display a significant difference when compared to the control (Fig 5.2). A one way ANOVA was used to explore the relationship between medium dose of cypermethrin (160 mg kg^{-1}) and the medium dose mixture of $CuSO_4$ and cypermethrin (140/160 mg kg^{-1}) on the total microbial activity. The relationship passed Levene's test, ($F_{(2, 17)}$ = 1.91, p = .178). Using Tukey's HSD procedure, significant differences were found between the control and 160 mg kg^{-1}, (M = 9.43, SD = 2.34), the control and 140/160 mg kg^{-1}, (M = 9.36, SD = 2.34). The mixed dose displays a significant difference when compared to the individual Cu dose, but no significant difference when compared with CYP.

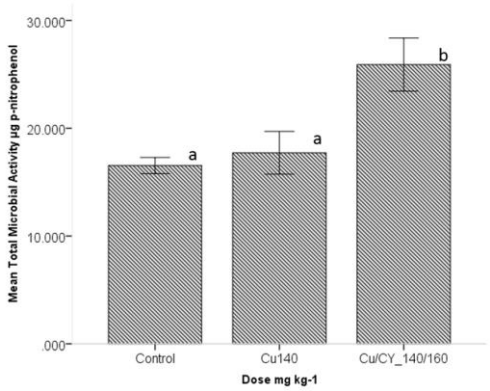

Figure 5.1 Comparison of medium dose Cu to Cu/CYP mixture effect upon the total microbial activity. Mean ± 1SE, letters indicate significant differences between treatments.

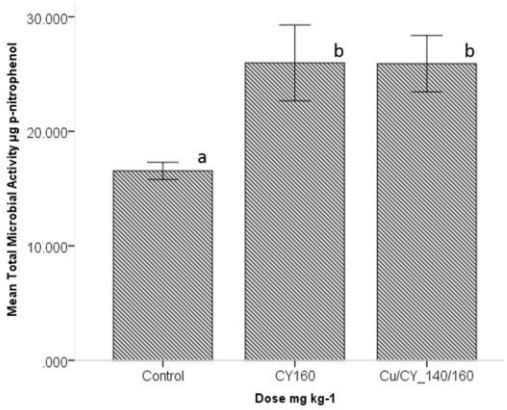

Figure 5.2 Comparison of medium dose CYP to Cu/CYP mixture effect upon the total microbial activity. Mean ± 1SE, letters indicate significant differences between treatments.

Relationship Between High Dose and High Dose Mixture on Total Microbial Activity

No significant difference was observed between the mixed dose of Cu/CYP and the control, but significantly lower activity can be observed with the individual dose of Cu when compared to the control and mixed dose (Fig 5.3). A one way ANOVA was used to explore the relationship between high dose of $CuSO_4$ (960 mg kg^{-1}) and the high dose mixture of $CuSO_4$ and cypermethrin (960/640 mg kg^{-1}) on the total microbial activity. The relationship passed Levene's test, ($F_{(2, 17)}$ = .730, p = .496). Using Tukey's HSD procedure, significant differences were found between the control and 960 mg kg^{-1}, (M = 6.49, SD = 1.42), 960 and 960/640 mg kg^{-1}, (M = 7.19, SD = 1.74).

No significant difference is observed between the mixed dose of Cu/CYP and the control, but significantly higher activity can be observed with the individual dose of CYP when compared to the control and mixed dose (Fig 5.4). A one way ANOVA was used to explore the relationship between high dose of cypermethrin (640mg kg^{-1}) and the high dose mixture of $CuSO_4$ and cypermethrin (960/640 mg kg^{-1}) on total microbial activity. The relationship passed Levene's test, ($F_{(2,17)}$ = 1.76, p = .202). Using Tukey's HSD procedure, significant differences were found between the control and 640 mg kg^{-1} (M = 11.57, SD = 1.59), 640 and 960/640 mg kg^{-1}, (M = 10.87, SD = 1.94). At higher dosing rates, the Cu/CYP mixture produced no significant differences compared to controls.

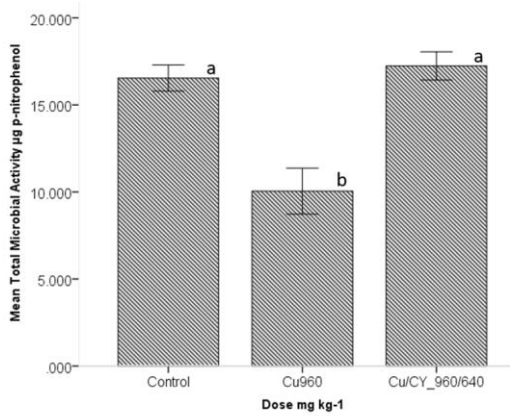

Figure 5.3 Comparison of high dose Cu to high dose Cu/CYP mixture effect upon the total microbial activity. Mean ± 1SE, letters indicate significant differences between treatments.

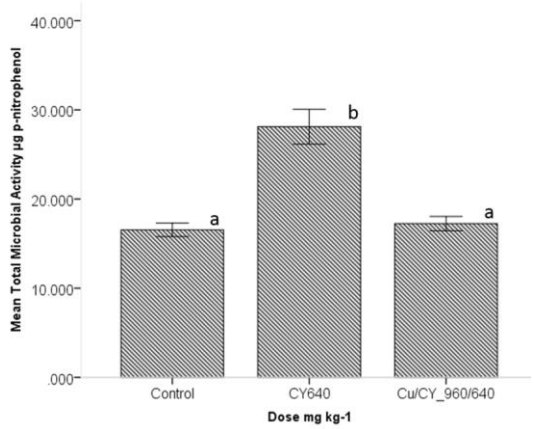

Figure 5.4 Comparison of high dose Cu to high dose Cu/CYP mixture effect upon the total microbial activity. Mean ± 1SE, letters indicate significant differences between treatments.

4. Discussion

4.1 Effects of copper

Cu is an essential element for the survival of all biological organisms. In the human body, Cu is a fundamental component of ~1% of proteins, including fundamental enzymes. Cu plays an assisting role in the transformation of melanin for skin pigmentation, and maintenance of connective tissues, especially in the heart and arteries (Angelova et al, 2011; Uauy, 1998). Adult bodies require 1.2 mg of copper per day (Parsons and Dixon, 2013). However, Cu contamination is also a serious issue. Anthropogenic activities are largely responsible for metal contamination in soil (Wang et al, 2007; Hinojosa, 2002), with principle sources of Cu contamination of soil deriving from slurries, sludges and fungicides. In high concentrations Cu may cause depletion of microbial diversity, biomass size and activities (Alloway, 2008).

These changes can be used as bio-indicators in the assessment of heavy metal ecotoxicity (Wang et al, 2007). The most likely mechanism of toxicity has been linked to the bioavailable fraction of Cu; significant negative correlations have been observed between microbial activities and the bioavailable form of Cu. The inhibition of enzymatic activities are thought to be due to the complexation or combination of trace metal ions with the substrate or active protein faction of enzymes (Eivazi and Tabatabai, 1990; Hansda et al, 2017). Trace metals affect growth and metabolic rates of soil microorganisms through functional disturbance, denaturation of protein structure or destruction of cell membrane integrity (Leita et al, 1995). It is hypothesized that it is by these mechanisms that Cu is affecting the activity of the enzymes included in the current study.

It is widely accepted that Cu is also an essential nutrient for soil microbes and plants, but above certain concentrations, can also be toxic (Flemming and Trevors, 1989; Yruela, 2005). An excess of copper can inhibit plant growth and impair important cellular processes (e.g. photosynthetic mechanisms), that begin to occur at the cellular level in contaminated soil (Flemming and Trevors, 1989; Brunetto et al, 2016; Pietrzak and McPhail, 2004; Yruela, 2005; McBride, 1995). The current study develops the knowledge already presented in the literature and demonstrates both an increase and decline of both enzymatic and microbial activity in response to elevated copper concentrations, with all tested enzymes displaying their lowest activity at the highest dosage of 960 mg kg-1 CuSO4.

However, results demonstrated significant differences in glycosidase activity between 35 and 960 mg kg-1 and 70 and 960 mg kg-1. The highest activity is seen at doses 35 and 70 mg kg-1, and a gradual decrease in activity was then displayed until the lowest activity occurred at a dose of 960 mg kg-1, but this was not significantly lower to the control. This demonstrated a stimulatory effect of Cu upon glycosidase activity at lower doses, but no clear evidence of inhibition. A study by Wyszkowska (2010) reported glycosidase activity to have a certain resistance to copper concentrations of 150 mg to 450 mg kg-1. This appeared to be dependent upon soil type and cultivated plant species. In

sandy loam soils and planted with oat, β-glucosidase activity, in both cases, was the most resistant to copper contamination out of four tested enzymes. Yet in treatments containing spring rape, β-glucosidase activity was least resistant (Wyszkowska, 2010). There is also evidence to show β-glucosidase activity resistance to copper in a study conducted by Aoyama et al (1993) who concluded the presence of Cu did not appreciably affect the activity of β-glucosidase in soil unamended with orchard grass and increased activity in soil. Effron et al (2004) Also reported little to no effect of Cu upon the activity of β-glucosidase at doses of up to 2,500 mg kg-1.

The effect of metal toxicity to soil ecosystems depends not only on total metal concentration and soil physicochemical properties, but perhaps more essentially the biochemical speciation and bioavailability of the metal (Singh, 2002). Clearly, Cu has caused little effect upon glycosidase activity except stimulation at lower doses. This would make sense considering Cu has long been established as an essential nutrient for microbial life and subsequent plant growth (Yruela, 2005; Brunetto, 2016; Besnard et al, 1999; Alloway, 2008). At cellular levels, Cu plays an important role in transcription signalling, protein transport, oxidative phosphorylation and iron mobilization (Yruela, 2005), therefore, this would explain an increase in glycosidase activities through nutritional effects. It may also be the case that through its negative effect on carbon availability, glycosidase increases in activity. Aoyama et al (1993) discovered mineralization of organic C and amount C biomass was reduced by increasing levels of enriched Cu. Due to the soil type that was employed for the current research, there was no history of agricultural chemical use within the soil and therefore no elevated levels of trace metals or agricultural pesticides. This is important because this provides an unobstructed view of how Cu behaves when there are no chemical elements or compounds to interact with. There are no background levels of any trace metals or pesticide over-use that may interfere with the current findings. The findings of this study go some way to prove that in moderation, the application of Cu-based fungicides do not acutely harm the activity of soil biota, however, a chronic build-up of this essential element leading to toxicity is becoming a

global concern. (Flemming and Trevors, 1989; Fernandez-Calvino et al, 2010; Brunetto et al, 2016; Ju et al, 2019).

PME activity, displayed significant differences between the control and 70, 140, 720 and 960 mg kg-1 treatments. These data showed a significant inhibition of PME activity by Cu, but no clear evidence of stimulatory effects, a contrast with the effects of Cu upon glycosidase. PME is directly involved in the cleavage of soil phosphates to forms more readily available P for plant uptake and is therefore an essential enzyme associated with plant growth. Consequently, it is of significant importance that Cu has a negative impact upon processes fundamental to plant growth and development. Cu performs a structural role in regulatory proteins and engages in photosynthetic electron transport, mitochondrial respiration, oxidative stress responses and hormone signalling (Marschner, 1995; Raven, 1999). Cu ions act as cofactors in a wide range of enzymes. Aoyama et al (1993) concluded that urease and acid phosphatase activities were depressed by Cu addition, but results confirmed the findings of the present study that β-glucosidase is not sensitive to Cu pollution, but significantly enhanced (Aoyama et al, 1993). Similar enzymatic activities were discovered by Fernandez-Calvino et al (2010). It was found that phosphatase was the enzyme most affected by Cu, displaying significant reduction of activity and was the most sensitive in evaluating pollution, whereas β-glucosidase was less affected by metal stress.

Phosphatases are important for plant growth and development and phosphate solubilising bacteria have been employed to promote the improvement of plant growth. These bacterium have increased root and shoot elongation in lettuce and tomato and have aided in an increase of crop yields of up to 30% in a wide range of fruit and vegetables (Rodriguez and Fraga, 1999). Copper is known to cause inhibitory effects upon alkaline phosphomonoesterase. This was demonstrated in a study by Wang (2018) that confirmed that Cu inhibited ALP activity and as such is considered a possible tool for the measurement of Cu induced toxicity. The current study confirms the inhibitory effects of Cu upon alkaline PME activity. This suggests impairment of phosphate cycling in soils with high concentrations of Cu (≥480 mg kg-1). The consequences of

this would mean impaired development and growth processes in plants due to the resulting decreased soil fertility. This may also have consequences for crop production and harvest yields in Cu contaminated soil.

The overall total microbial activity of the soil samples displayed a significant reduction between the control and 960 mg kg-1. Other significant differences were between 35 and 240 mg kg-1, 35 and 960 mg kg-1, 70 and 960 mg kg-1 and 140 and 960 mg kg-1. Although there appears to be a definite spike in activity at the 35 mg kg-1 dosing level, it was not statistically different to the control. These results indicate that at higher dosing, microbial activity is significantly suppressed when compared to the lower doses. The total microbial activity is measured by fluorescein diacetate (FDA) hydrolysis. Fluorescein diacetate, which is colourless, is hydrolysed by free and membrane bound enzymes, releasing fluorescein, a coloured end product, which can be subsequently measured by spectrophotometry (Adam and Duncan, 2001). The results could mean that the total microbial activity is resistant to Cu toxicity up to high concentrations. The challenge to soil ecologists is to quantify the role of microorganisms in facilitating the soil to cope with anthropogenic stress as the underlying mechanisms of this are still poorly understood (Griffiths and Philippot, 2013). Definitions of resistance, resilience and stability have been previously reviewed; Resistance is generally defined as the capacity of a system to cope with a disturbance. Resilience describes the behaviour of a system that will show displacement and readjustment to its pre-disturbed state or new stable state (Botton et al, 2006; Brand and Jax, 2007). Resistance to a pollutant and the rate of return (resilience) are the main components of ecosystem stability (Loreau et al, 2002). The results of this study show that microbial activity is resistant and resilient to Cu perturbation.

Soil microorganisms are responsible for a range of soil functions such as nitrogen fixation, assimilation and degradation of organic residues for nutrient release. When metals are introduced and are retained in the soil through repeated and unmanaged additions they interfere with these essential biochemical functions, which subsequently alter ecological balance (Nwuche,

2008). Toxicity to microorganisms could cause decrease of OM decomposition and nitrogen fixation, impairment to nutrient cycles and the inhibition of enzyme synthesis and activity, which are essential for fertile, healthy soil (Lipman and Burgess, 1914; Giller et al, 1998; Freedman and Hutchinson, 1998). The current study provides evidence that, without historical and background anthropogenic chemical disturbances, the total microbial activity is quite resistant to Cu toxicity except at higher doses, providing protection to the range of important functions they are responsible for.

Effects of Cypermethrin

In the current study, cypermethrin produced stimulatory effects upon PME and total microbial activity. In the case of glycosidase the results produce some evidence of stimulatory effects, but with more complex behaviours. Cypermethrin displayed results that were not consistent with the literature, which indicates an unpredictable effect of insecticides (Filimon, 2015; Xie, 2009; Goswami et al, 2012). The behaviour of any chemical compound in soil is difficult to predict due to the influence of multiple factors connected with soil physio-chemistry. Das (2016), provided evidence of a range of effects displayed by pyrethroids on soil microbes, although the total microbial content of the soil were mostly suppressed. For example, the effects of fenalverate displayed both increases and decreases in microbial populations (Das, 2016). Permethrin decreased ammonifying bacteria by 9.4% but also increased N2-fixation by 7.8%. Cypermethrin had a neutral effect on microorganisms and N2-fixation rates (Das, 2016). This is in direct contrast to a study by Filimon et al (2015) which demonstrated up to a 75% decrease in numbers of nitrifying bacteria, and that cypermethrin and thiomethoxam displayed an inhibitory effect upon metabolic processes in soil. By contrast, a study by Zhuang et al (2011) concluded that beta-cypermethrin had no significant effect on soil microbial activity at a range of 10-80 µg g-1.

In the current study, cypermethrin clearly demonstrated stimulatory effects upon enzyme activity. This characteristic has been reported in other studies. For example Maddaka et al (2017), discovered that the pesticides profenofos

and cypermethrin enhanced soil microbial and enzyme activity significantly. Two agricultural samples of black clay soil and red sandy loam displayed 6-85% increase in protease and invertase activity with pesticides applied individually (Maddaka et al, 2017).

The current study provides evidence of gradual stimulation of PME activity by cypermethrin compared to controls with significant differences commencing at 80 mg kg-1, and the highest activity seen at 640 mg kg-1. Very similar behaviour is displayed in the total microbial activity, although significant differences to the control are only seen at concentrations of 320 mg kg-1 and 640 mg kg-1. Stimulatory effects were again observed in glycosidase activity, most significantly at 320 mg kg-1 when compared with controls, but inhibitory behaviours were also observed at 20 and 80 mg kg-1. Evidence of both stimulatory and inhibitory effects upon glycosidase suggests cypermethrin exhibited a complex influence upon glycosidase activity with subsequent potential perturbation to the C-cycle. The disruption to this cycle would have consequences for C-cycle processes that include fixation of CO2, degradation of chitin, transformation of C compounds in the rhizosphere, methane production and degradation of organopollutants. The latter would have the potential for increasing the persistence of these pollutants in soils potentially increasing the harm to organisms, rhizosphere processes and subsequent plant health (Elsas et al, 2007).

Pesticides are thought to exert stimulatory effects through serving as nutrient and energy sources for the soil microbiota (Patnaik et al, 1994). The population density of microbial pesticide utilizers expands, provided the nutrient and energy source is there alongside a complementary reduction of active pesticide residues (Debnath, 2002; Samanta et al, 2005). An increase in enzymatic and microbial activity, is widely viewed as having a positive consequence toward the health of soil and subsequent plant growth (Van Elsas et al, 2007; Bandick and Dick, 1999).

A rise in PME activity can be regarded as evidence of stimulation to the P-cycle in the soil and potential increase of mineralization mechanisms creating more readily available phosphates for root uptake (Van Der Heijden et al,

2007). Furthermore, since higher plants are known to be destitute of phosphatase activity (Dick et al, 1983; Juma and Tabatabai, 1988; Tabatabai, 1994). PME enzymes derive completely from soil microbes. Consequently, it may be postulated that the increase of PME activity may explain the mirrored characteristics that the total microbial activity of the soil exhibited under cypermethrin influence.

In a study conducted by Das et al (2012), it was discovered that the stimulation of phosphate solubilising microbes due to the application of herbicides had significantly increased the organic carbon content of the soil. This indicated that during the metabolism of the herbicide by microorganisms, an unquantifiable amount of the pesticide was converted and secured in the soil as oxidizable carbon (Nongthombam et al, 2008). This hypothesis may explain the erratic behaviour of glycosidase as found in the current study. If the increase of PME and its link between the total microbial activity of soil is due to the metabolism and degradation of CYP and subsequent release of its carbon atoms (C22 per molecule) as oxidizable carbon, this may account for a suppression of glycosidase activity. To elaborate, carbon released by CYP degradation into the soil may impact the C-cycle, reducing the need of microorganisms to utilise glycosidase to access C resulting in a decrease of glycosidase. As Zhang et al (2015) concurs, glycosidases are essential in terrestrial C-cycling for providing the necessary energy for soil microorganisms and the two components are intrinsically linked. Glycosidases are valuable indicators in the evaluation of bioavailable soil carbon. Das et al (2012) reported a delicate balance between carbon content and microorganisms under pyrethroid stress. Oxidizable C content was greatly influenced by application of pyrethroids. Organic carbon content in soils treated with insecticides reached maximum levels in the early stages of the experiment. Latter, a greater concentration of insecticide degrading microbes result in a significant decrease of soil C content (Das et al, 2012). Nongthongbam et al (2009) also reported a significant carbon increase following application of pesticides indicating that microorganisms were degrading the pollutants to a greater extent and accumulating carbon to their cellular components during the course of metabolism. It is agreed that the

decrease of soil C is due to degradation of organic matter by autochthonous soil microorganisms (Nongthongbam et al, 2009; Das and Mukhergee, 1989; Mukhergee et al, 1991). During the application of pesticides, a large proportion of the compound accumulates or adsorbs to the top layer of the soil (0-15 cm). Most microbiological activities occur at this level and where degradation of carbon based substances take place for energy and nutrient release for microbial processes and release of CO2 (Debnath et al, 2002).

Cypermethrin is known to rapidly degrade in soil and microbes play a significant role in that degradation. Kaufman et al (1981), provided evidence that approximately 96-97% of carbon content remained in 0-2.5 cm layer of soil after pyrethroid degradation. It is suggested that cypermethrin is immobile in soil and cannot be readily leached. The extent of its mobility is mainly governed by soil pH and degradation rate (Kaufman et al, 1981). In natural soils, hydrolysis at the ester linkage is the primary and foremost degradative route of cypermethrin, leading to formation of the intermediate metabolite 3-phenoxy phenyl hydroxyacetonitrile, which is oxidised to 3-phenoxybenzaldehyde and further to 3-phenoxybenzoic acid. Microbial degradation is widely believed to be the main contributing factor to the attenuation of CYP in the natural environment, contributing to the release of oxidised carbon and enrichment of heavier isotopes (e.g., 13C) into the soil environment (Xu et al, 2015; Chen et al, 2012; Zhao et al, 2013). Thus, in the current study, CYP may be displaying evidence of a disruption to the soil C-cycle and the enzyme glycosidase that is intricately involved in this particular soil process.

4.3 Effects of Cu and Cypermethrin Mixtures

For this second stage, both Cu and cypermethrin were applied to the soil in combination, and comparisons were made to the compounds and their effect upon soil enzymes when they were applied individually. As previously reviewed, there are three main outcomes that may be expected when toxic substances are applied together - the joint toxicant effects may be similar (additive), stronger (synergistic) or weaker (antagonistic).

Glycosidase demonstrates a clear stimulatory reaction to the co-contamination of Cu/pesticide. In the cases of low, medium and high dosing rates the simultaneous combination of Cu and CYP produces significant differences between the control and the individual dose compared to the mixture where there is evidence of much higher activity of glycosidase. Clearly, the joint effect of CuSO4 and CYP was not synergistic or additive, but benefited, rather than had a toxic effect towards the soil C-cycle to which glycosidase is intrinsically connected. Liu et al (2009) discovered similar behaviours. When applied individually, Cu2+ and CYP both reduced the germination rates of Pak choi (Brassica rapa subsp. chinensis), but when applied as a mixture produced germination rates ranging from 70-90%, higher than exposure to Cu2+ and CYP alone. It was determined that with an increase of CYP, the Pak choi roots could endure increased concentrations of Cu, indicating the complexities of joint Cu2+ / CYP relationship. It was demonstrated that CYP changed the surface structure characteristics of soil by distribution/absorption, meaning the soil could absorb more Cu, reducing its bioavailability and its subsequent toxic effects (Liu et al, 2009). This may certainly be the case in the current study. At all dosing rates, Cu had no significant effect upon glycosidase, but when applied simultaneously, glycosidase activity is markedly higher than both the controls and the substances applied individually.

Cedergreen (2014) described cocktail effects, or synergistic interactions of chemicals as being an area of concern to both authorities and to the general public. Synergy, in most basic terms, is a chemical enhancing the effect of another chemical, so that they jointly exert an effect larger than predicted. Research by Cedergreen (2014) indicated that pyrethroids displayed tendencies to enter synergistic relationships with particular fungicides and that combinations were not random; 95% of cases occurred when mixed with azole fungicides. Cu based Bordeaux Mixture is not an azole fungicide, but is a popular and widely used fungicide nonetheless. The current data strongly suggests that CuSO4 and CYP does not have a toxic effect upon glycosidase activity. Of the three assays employed in the current study, the data suggests that glycosidase is least affected by joint toxicity of Cu and cypermethrin,

which has been observed in other studies (An and Kim, 2009; Zhuang et al, 2011). Due to its intrinsic role in soil C cycle it may be suggested that the carbon cycle may also be relatively undisturbed after pyrethroid application. However, the soil type used in the current research must be taken into account. The unpolluted composite top soil has no history of agricultural fertilizers, pesticides or any other chemical stress that would interfere or exacerbate further toxicity of Cu and cypermethrin. Hence, caution should be exercised when applying an insecticide to crops, but the effect of cypermethrin to glycosidase may not be serious.

The joint toxicity effects of Cu and CYP upon PME activity were slightly more varied and complex. There were no significant differences between treatments at lower dosage when compared to Cu treatment alone. CYP displayed a stimulatory effect on its own, but the mixture presented no significant difference to the control. Hence, Cu suppressed the stimulatory action of CYP. These behaviours are almost exactly mirrored when observing the medium dosing comparisons. The joint toxicity of Cu and CYP had no clear observable effects at low to medium dosing compared with controls. At higher dosing, there is a clear indication of increased negative effects when Cu and CYP are applied simultaneously. PME activity is markedly decreased to both control and Cu applied alone, and the seemingly stimulatory effects of CYP are greatly suppressed. This provides evidence that Cu may be more dangerous to soil microbes than cypermethrin when applied alone. At higher dosing the inhibitory effects of Cu are amplified by CYP in a potentiating manner.

When compared to each other, CYP, mixture and controls presented significant differences to each other. PME displayed activity significantly higher to controls in the CYP alone treatment, but the joint contamination was significantly lower. This is a clear indication of Cu causing a detrimental effect to PME activity, acting to negate the otherwise stimulatory effect of CYP applied individually. To summarise, at low to medium concentrations, Cu/CYP co-mixture exhibits little differences when compared with individual dosing and controls. CYP when applied individually produced stimulatory effects

towards PME, but when applied simultaneously with Cu produces a significant negative effect towards soil PME activity. Christen et al (2014), recognised that the joint toxicity of pesticides was underexplored in research and regulatory toxicology. The current research proves the importance of taking joint toxicity of substances into account when considering the environmental consequences of pesticide use.

The toxicity of insecticides, such as pyrethroids, are quite often acutely enhanced when combined with Cu based fungicides (Cedergreen, 2014; Walker, 2009; Johnson et al, 2013). At higher doses Cu caused inhibitory effects towards PME activity and this effect is increased when combined with CYP resulting in much lower PME activity when compared to both control and individual application of Cu. However, even at higher doses, where CYP is having a stimulatory effect upon PME activities, when combined together, Cu has proven to be the chief factor towards a marked enzymatic decrease of PME activity. With regards to the apparent interactive effects at higher doses, the mechanisms involved in the current research may be affecting metabolic enzyme activity. Cu fungicides are known to cause inhibition to a wide range of P450 monooxygenases, enzymes involved in the phase 1 metabolization of pyrethroid compounds (Cedergreen, 2014). If Cu is causing inhibition of P450 enzymes, the key enzyme involved in the metabolization of cypermethrin within cells, an accumulation of toxic pesticide residue may increase to levels causing toxicity.

The effect on the total microbial activity at lower doses by co-contamination, appear to be stimulatory. The mixture displays significantly higher microbial activity compared to both Cu and CYP acting independently. Similar behaviour was found for the medium dose when the mixture is compared with Cu individually, i.e. significantly higher total microbial activity was found, but when compared with CYP at the medium dose, the mixture produced a level of microbial activity that is similar to the stimulatory effect of CYP alone. At higher concentrations, the total microbial activity is showing signs of resistance to simultaneous application of CYP/Cu. Cu displays evidence of inhibiting the stimulatory effects of CYP when both Cu and CYP are jointly

applied and CYP appears to reverse the inhibitory effect of Cu. The study by Goswami et al (2012) indicated that when applied individually, CYP had a weak toxic effect on soil microorganisms. Tejada et al (2015), discovered microbial populations grew tolerant and eventually increased after initial toxic inhibition after CYP application. Adverse effects of Cu upon soil microbial communities have been reported (Fernandez-Calvino et al, 2010; Dewey et al, 2012) however, there is no evident work in the literature on the joint toxicity of CYP and Cu to the total soil microbial activity. Future studies may further investigate the findings of the current study and the mechanisms behind the actions produced by the contaminants towards the enzymatic and microbial activities.

The current research has provided clear evidence of the complexities that the enzymatic activity and microbial population within soil display under toxic stress caused by two compounds acting both individually and simultaneously. This is also reflected in the literature, where any given individual contaminant or joint contamination may display a range of reactions and provoke a range of behaviours ranging from stimulation or synergism (Xie et al, 2009), inhibition or antagonism (Sasirekha et al, 2012), no effect or additive interactions (Zhuang, 2011). These, sometimes, convoluted biochemical reactions are dependent upon the immediate soil environment upon which, the particular chemical compound is placed. 1g of soil may comprise 1010-1011 bacteria, 6-50,000 bacterial species and 200m fungal hyphae (Kibblewhite et al, 2008). Soil will contain organic and inorganic fractions. There are three major groups to which soil inorganic particles are classified according to size: sand, silt and clay; the proportion of these determines soil texture. The physicochemical environment will determine the efficiency of trace metal ion and chemical compound transport and degradation rates as well as pH, temperature and water holding capacity (Elsas et al, 2007; Chachada et al, 2016; Uwizeyimana et al, 2017). Therefore, it is a major challenge to fully understand, intrinsically, the physicochemical composition of the soil under analysis and the subsequent behaviour of an external chemical will have upon the biological content of soil.

The consequences and wider implications of the current findings are chiefly where the compounds occur as co-contaminants. The effects of Cu as an individual toxicant have been well established. The current study provides new evidence of enzymatic and microbial resistance to toxicity of Cu in a previously undisturbed soil. The effects of CYP when applied individually, although unexpected, were that of a potential benefit to microorganisms in a previously undisturbed, uncontaminated soil. However, the findings of the current research imply mixtures of the two contaminants may effect nutrient cycles. For example in the case of glycosidase, a stimulatory response is observed with a clear increase of enzymatic activity, indicating an effect on the C cycle. The molecular interactions involved are not clear, and may be the scope for future studies. It is thought that CYP alters soil surface structure characteristics through adsorption affinities, which attributes to its chemical – physical properties, and through competitive adsorption occurring between CYP and Cu for the same adsorption sites.

Where CYP acting alone produces stimulatory effects upon the activities of PME, this activity is greatly reduced when applied simultaneously with Cu at high dose. This is a clear indication of a negative interaction whereby the activity of PME is greatly reduced by the mixture when compared to individual applications. Cu based fungicides are known to be disruptive to a range of P450 monooxygenases that are involved in the metabolization of pyrethroid compounds. This is thought to be a cause of the inhibition of PME activity seen in the current study, especially as toxicity of pyrethroids may be acutely amplified when combined with Cu based fungicides (Johnson et al, 2013; Cedergreen, 2014). With PME playing an intrinsic part of cleaving P molecules to more readily available forms for root uptake, and P cycles being critical for plant growth and development, there are implications for agricultural harvests and maximum yield of crops. The mixture of compounds that are meant to aid in the production of essential fruit and vegetables, have the potential to cause detrimental effects to these vital agricultural crops.

It is known that the effects of soil pollutants on the activity of soil enzymes are complex, especially in field conditions where numerous interactive factors that

affect potential enzyme activity can influence the results. Several workers have provided evidence that the response of different soil enzymes to the same pollutant may vary greatly (Fernandez-Calvino et al, 2010; He et al, 2003; Trasar-Cepeda et al, 2000). This has been displayed in the current study. Potentiation, antagonistic and additive effects have been demonstrated. This is indicative of complex interactions in a soil that has no agricultural history.

Cypermethrin is a synthetic pyrethroid insecticide, created with the specific purpose of targeting unwanted pests that may be detrimental to crops and crop production. Non-target organisms can be the microbial communities within soil that a healthy crop production depends upon. Although beneficial effects may be implicated in the case of glycosidase and the total microbial activity, the current study has provided evidence that simultaneous use of Cypermethrin and Cu based fungicides can seriously inhibit the activities of PME enzymes that are intrinsically and centrally involved in plant growth and development. Thus, the inhibition of PME may affect agricultural yields. Therefore, there are serious implications in the use of pyrethroids on soils with high Cu concentrations and other metals that may cause inhibition to microbial populations. The soil employed in the research was previously unexposed to Cu and CYP and has provided an unobstructed view to the effects of dual toxicants. The negative effects of cypermethrin and Cu when used simultaneously may be enhanced in an agricultural setting where multiple contaminants, including elevated levels of trace metals caused by manures, fertilizers, insecticides, or Cu based fungicides may be present.

Conclusion

There appears to be a balance attributed with the current study, whereby the Cu/CYP mixture produced stimulatory, no effect and inhibitory results. Glycosidase showed antagonistic results when confronted with the joint effects of Cu and CYP, i.e, an increase in glycosidase activity is produced. In contrast, PME activity is significantly reduced when under toxic stress of the combined compounds by a potentiation reaction. Therefore, it is unsurprising

the overall microbial activity in the soil was resistant to the toxic interactions of Cu and CYP at higher doses. With the soil C and P cycles being affected it is possible that microbial populations are resisting toxicity in defence of its own functions. Although there are signs of stimulation in the lower doses, no significant differences are seen between the higher doses of the mixture and the control of the total microbial activity. The individual application of Cu is causing inhibition, and CYP is causing stimulation to soil enzymes. It is proposed that the joint contamination of the two compounds ultimately leads to little effect to the overall microbial population of the soil. However, the current study provides evidence that caution must be taken when applying cypermethrin to a soil that has elevated levels of Cu. The application may be harmful to phosphatase enzymes that play an intrinsic role in plant growth and development.

PME was the only enzyme in the current study to be inhibited by simultaneous application of Cu and CYP. Evidence was found of a marked decrease in PME activity due to a potentiation of the two pollutants in high concentrations. At higher dosing rates the Cu/CYP mixture inhibits PME more so than Cu when applied individually. There seems to be an indication of Cu effecting a suppressive action upon CYP. The stimulatory effect that was initially exerted by CYP is markedly inhibited by the addition of Cu at higher doses. Antagonistic results were reflected by stimulation to glycosidase activity at all dosing levels implying a direct effect to the soil C cycle. Initial stimulation to the total microbial activity of soil led to additive effects at higher dosing concentrations. It is suggested that this may be due to microbial resistance to toxicity, as previous studies have provided evidence of microbial resistance to external pollutants.

In answer to the aims and objectives set out in the current study, the determined concentration at which the pesticide and Cu negatively impacted soil enzyme activity was at the highest dosing rates causing significant inhibition to PME activity. Because of this it is determined that there is a toxic interaction of Cu and cypermethrin towards soil PME. Therefore the enzyme that was most vulnerable in this investigation is phosphomonoesterase,

placing the soil P cycle most at risk. This is followed by the total microbial activity and glycosidase in that respective order.

Future Studies

The negative action of Cu in this study is worth investigating further and to examine the molecular mechanisms behind the suppressive action of copper to cypermethrin, especially as it is negatively affecting an enzyme so chiefly fundamental to the growth and development of plants. This initial finding is of significance to farming, agriculture and our food industry.

Future studies may also benefit from investigating why CYP has these stimulatory effects on these particular soil enzymes in an undisturbed soil compared with samples from a worked, agricultural soil that is known to have had a history of pesticide applications. The current study implicates that it is likely to be molecular interactions between CYP and other soil contaminants. The molecular action of copper in soil and its effects on plant cells are well documented. Cypermethrin is designed to specifically induce neurotoxic effects in target species, with its primary action related to prolonged opening of voltage-gated sodium channels in central nervous system. It is highly likely that molecular mechanisms are responsible for the range of behaviours exhibited in the current study.

The current research has produced important information that may be of interest to cultivators of farm produce and agriculture in general. The use of cypermethrin on target organisms may be of some benefit to certain soil enzymes when applied individually on land that has historically been completely undisturbed by anthropogenic activities. When applied to a soil that has a high copper content, there may be unfavourable consequences for soil phosphatases, the enzymes that are intrinsic to the growth and development of crops that are vital for an ever increasing human consumption.

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